Oviduct extracellular vesicles protein content and their role during oviduct–embryo cross-talk

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Abstract

Successful pregnancy requires an appropriate communication between the mother and the embryo. Recently, exosomes and microvesicles, both membrane-bound extracellular vesicles (EVs) present in the oviduct fluid have been proposed as key modulators of this unique cross-talk. However, little is known about their content and their role during oviduct–embryo dialog. Given the known differences in secretions by *in vivo* and *in vitro* oviduct epithelial cells (OEC), we aimed at deciphering the oviduct EVs protein content from both sources. Moreover, we analyzed their functional effect on embryo development. Our study demonstrated for the first time the substantial differences between *in vivo* and *in vitro* oviduct EVs secretion/content. Mass spectrometry analysis identified 319 proteins in EVs, from which 186 were differentially expressed when *in vivo* and *in vitro* EVs were compared ($P < 0.01$). Interestingly, 97 were exclusively expressed in *in vivo* EVs, 47 were present only in *in vitro* and 175 were common. Functional analysis revealed key proteins involved in sperm–oocyte binding, fertilization and embryo development, some of them lacking in *in vitro* EVs. Moreover, we showed that *in vitro*-produced embryos were able to internalize *in vivo* EVs during culture with a functional effect in the embryo development. *In vivo* EVs increased blastocyst rate, extended embryo survival over time and improved embryo quality. Our study provides the first characterization of oviduct EVs, increasing our understanding of the role of oviduct EVs as modulators of gamete/embryo–oviduct interactions. Moreover, our results point them as promising tools to improve embryo development and survival under *in vitro* conditions.

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Introduction

Successful pregnancy requires an appropriate communication between the female reproductive tract and the embryo(s). Disturbance in this unique communication system is associated with high rates of early pregnancy loss, and it is becoming increasingly evident that it also influences the developmental potential of the offspring into adulthood (Baker 1998, Mahsoudi *et al.* 2007). Strong evidence exists with respect to the signals exchanged between the early embryo and the oviduct, leading to an appropriate embryo development and successful pregnancy (Lee *et al.* 2002, Alminana *et al.* 2012, Maillo *et al.* 2015). Absence of these oviduct signals in ART have raised the question of how much these techniques can affect the outcomes (Ostrup *et al.* 2011, O'Neill *et al.* 2012). Significant gains

in our understanding of the essential embryotrophic components of the oviduct fluid and their interactions with the embryo have been achieved in the last years (Georgiou *et al.* 2007, Leese *et al.* 2008, Aviles *et al.* 2010, Schmaltz-Panneau *et al.* 2014). However, there is a need for further exploring the contribution of the oviduct to the reproductive success.

Recently, exosomes and microvesicles have been identified as essential components of uterine (Ng *et al.* 2013, Burns *et al.* 2014) and oviduct fluids (Al-Dossary *et al.* 2013, Lopera-Vasquez *et al.* 2017). Exosomes are 30–150 nm vesicles of endocytotic origin released upon fusion of a multi-vesicular body with the cell membrane, while microvesicles are 100–1000 nm in diameter and bud directly from the cell membrane (Colombo *et al.* 2014). Both are collectively known as extracellular vesicles (EVs) and are considered important

tools in cell-to-cell communication (Valadi *et al.* 2007) by transferring their molecular cargo (proteins, mRNA, miRNA) from one cell to another. In the maternal tract, they have been proposed as important tools to regulate gamete/embryo–maternal interactions (Al-Dossary & Martin-Deleon 2016, Burns *et al.* 2016). However, while different studies have evaluated the EVs secretion/content (proteins, miRNA) produced by the uterus from *in vivo* (Ng *et al.* 2013, Burns *et al.* 2016) and *in vitro* origin (Greening *et al.* 2016, Bidarimath *et al.* 2017), none have provided an extensive characterization of oviduct EVs content up to date. An important requisite to decipher the possible role of the EVs in the embryo–oviduct dialog. Only one protein, PMCA4a, which is essential for sperm hyperactivated motility and fertility have been identified in oviduct EVs (Al-Dossary *et al.* 2013). Despite our lack of knowledge about their content, the EVs derived from *in vivo* oviduct fluid and *in vitro* culture of bovine oviduct epithelial cells (BOEC), seem to improve the cryotolerance of *in vitro*-produced embryos (Lopera-Vasquez *et al.* 2016, 2017).

Given the known differences in secretions by BOEC *in vivo* and *in vitro* (Rottmayer *et al.* 2006), and the increasing number of studies based on EVs derived from *in vitro* primary cell culture or cell lines, a comparative study of the EVs content of *in vivo* and *in vitro* origin seems imperative. Thus, we aimed at (1) deciphering the oviduct EVs protein content from *in vivo* and *in vitro* origin; (2) analyzing whether embryos are able to internalize oviduct EVs and (3) investigating their functional effect on embryo development. For this purpose, a bovine model was used, since bovine has been demonstrated to be a valuable experimental model for addressing ART-related questions.

Materials and methods

Collection of bovine oviduct fluid and epithelial cells (BOEC)

Oviducts and ovaries were obtained from cows at local slaughterhouse (Sablé sur Sarthe, France), with the permission of the direction of the slaughterhouse and the agreement of local sanitary services. Oviducts and their attached ovaries were transported to the laboratory at 37°C within 2–3 h after collection. For all experiments, ipsilateral and contralateral oviducts from the same animal at the post-ovulatory stage of the bovine estrous cycle were used. Animals showing recent ovulation sites in the attached ovaries, indicating they were at post-ovulatory stage (1–5 days of estrus cycle), were selected for EVs collection. To minimize the variability, the same oviducts were used for *in vivo* EVs collection by oviduct flushing than for *in vitro* EVs collection, by using the conditioned media after *in vitro* BOEC primary culture. First, oviducts were dissected free from surrounding tissues. Subsequently, to recover the oviduct flushing the lumen of the oviduct was flushed with 500 µL of sterile PBS (Sigma P4417-TAB). Then, BOEC were isolated by mechanical scraping of the oviduct with a

slide for primary *in vitro* BOEC culture as described by Van Langendonck and coworkers (Van Langendonck *et al.* 1995). BOEC was washed three times by sedimentation in 10 mL of tissue culture medium-199-Hepes (TCM-199, Sigma M7528) supplemented with bovine serum albumin (BSA stock fraction V, Sigma A9647) and 8 µL/mL gentamycin (Sigma G1272). The resulting cellular pellet was diluted 100 times in culture medium consisting in TCM-199 (Sigma M4530) supplemented with 10% heat-treated fetal calf serum (FCS, Sigma F9665) and 8 µL/mL gentamycin before seeding. At this point, an aliquot of *in vivo* BOEC (from the day (day 0) of collection) was stored at –20°C for further comparative protein analysis with *in vitro* BOEC and EVs by Western blotting, while the rest of the BOEC were seeded for culture.

Bovine oviduct epithelial cell *in vitro* culture

Our BOEC *in vitro* culture system has already been used to study early oviduct–embryo interactions, demonstrating to be a good oviduct-like environment to support embryo development *in vitro* (Cordova *et al.* 2014, Schmaltz-Panneau *et al.* 2014). BOEC were cultured in 25 cm² flasks (FALCON 25 cm² 353109) with TCM 199 (Sigma M4530) supplemented with 10% fetal bovine serum (Sigma F9665) and gentamycin (Sigma G1272, 10 mg/mL) in a humidified atmosphere with 5% CO₂ at 38.8°C. The medium was completely renewed at day 2. Subsequently, half of the medium was replaced every two days until cells reached confluence (6–8 days). Then, BOEC were washed and cultured in TCM-199 free of serum. After two days, the serum-free medium was completely renewed and the cells were cultured for two additional days before collection of conditioned medium. BOEC viability was determined after collection of conditioned media by using Live/Dead viability assay kit (LIVE/DEAD Cell Viability Assay, Life Technologies, L3224). At this point, an aliquot of *in vitro* BOEC was stored at –20°C for further comparison of protein content with *in vivo* BOEC (day 0) and EVs collected from them by Western blotting.

Isolation of EVs from *in vivo* and *in vitro* origin

Oviduct flushings from different animals were pooled (*n*=3 animals per replicate; in 4 replicates). Conditioned media obtained from different 25 cm² flasks were also pooled (total of 100 mL/replicate; in 4 replicates). EVs were obtained from oviduct flushings and conditioned media by serial centrifugation as described by Théry and coworkers (Théry *et al.* 2006). First, flushing and conditioned media were centrifuged at 300 g for 15 min, followed by 12,000 g for 15 min to remove cells, blood and cell debris and ultracentrifuged twice at 100,000 g for 90 min (BECKMAN L8-M; SW41T1 rotor) to pellet exosomes. The pellets were resuspended in 100 µL of PBS and stored at –20°C for further analysis.

Transmission electron microscopy (TEM)

Vesicle suspensions were diluted in PBS to attain a protein concentration of 0.6 µg per µL. Then, 3 µL of the sample were placed on the formvar carbon-coated grid for 5 min and

washed with distilled water (three times). For negative contrast, the samples were stained with 2% uranyl acetate for 2 min and left to dry. The micrographs were obtained using TEM HITACHI HT 7700 Elxience at 80kV (with a charge-coupled device camera AMT) and JEM 1011 (JEOL, Japan) equipped with a Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, USA) at 100kV. The processing of the photos and exosome size calculation were carried out by ImageJ software.

EVs labeling, EVs-embryo co-incubation and observation

In vivo EVs preparations from oviduct flushings (pool of three animals; 3 replicates) were labeled with a lipophilic green fluorescence dye (PKH67, Sigma) as described by Saadeldin and coworkers (Saadeldin *et al.* 2014). PKH67 is a widely used dye for visualization of exosomes uptake by cells (Burns *et al.* 2014, Saadeldin *et al.* 2014). First, a dilution of the EVs preparation was performed by mixing 25 μ L of the EVs suspension in PBS with 125 μ L of diluent C (Cell mixture). In addition, 25 μ L of PBS were mixed with 125 μ L of diluent C as a negative control. Then, the dye dilution was prepared by adding 1 μ L dye to 250 μ L of diluent C and 125 μ L of this mixture were added to EVs and control mixtures and incubated for 5 min at room temperature (final concentration of dye is 5×10^{-6} M). To stop the labeling reaction 1 mL of free EVs-FBS (previously ultracentrifuged at 100,000g during 16 h at 4°C to remove exosomes) was added for 1 min. To wash the excess of dye from EVs, the tube of EVs suspension was filled with M199 media with 5% FBS (EVs-free) and twice ultracentrifuged at 100,000g, 4°C, for 30 min. The final pellet was resuspended in 100 μ L of embryo culture medium (SOF) as described below with 5% FBS (EVs-free) and used to prepare the drops for embryo development.

In vitro-produced embryos at the blastocyst stage (with intact zona pellucida) and hatched embryos (with total or partial absence of zona pellucida) were *in vitro* cultured with green-labeled EVs or control (dye-PBS) dilution for 18–20 h. Prior to fixation, embryos were washed twice in EVs-free medium to remove any extraneous labeled vesicle not internalized. Then, embryos were fixed with 4% paraformaldehyde with Saponin 0.5%, labeled with Hoechst 33342 and actin red phalloidin and observed by confocal microscope (LSM780 Confocal Zeiss Observer Z1 with ZEN 2011 software). For this experiment, 4 replicates were performed, with 15–20 embryos incubated with green-EVs or control (dye-PBS) for each replicate.

Proteomic analysis

Mass spectrometry (MS) analysis

EVs preparations from *in vivo* and *in vitro* origins were analyzed by SDS-PAGE combined with nanoLC–MS/MS with spectral counting and extracted ion chromatography (XIC) methods of quantification.

Sample preparation for MS analysis

Samples were lysed in 2% SDS pH 6.8 in Tris buffer with protease inhibitors (Sigma P2714) followed by centrifugation

10 min at 15,000g. Protein concentrations in the samples were determined using the Uptima BC Assay kit (Interchim, Montluçon, France) according to manufacturer's instructions and using BSA as a standard. SDS-PAGE electrophoresis was carried out according to Laemmli's method (Laemmli 1970) on 10% gradient polyacrylamide gels. Reduced Laemmli buffer was used for sample preparation followed by vortexing and heating in water bath at 95°C 5 min.

Forty microgram of proteins from *in vivo* and *in vitro* EVs preparations were migrated separately applied on 10% SDS-PAGE 8.3 cm $\times$ 7.3 cm $\times$ 1.5 mm gels (50V, 30 min) (10 μ g EVs preparation/replicate from 4 replicates were pooled). A brief migration was performed until samples were concentrated in a single narrow band. The resulting protein bands from the two pools (*in vivo* and *in vitro* EVs preparations) were stained with Coomassie blue (G-250). Densitometric quantification of Coomassie blue-stained protein bands was performed by transmission acquisition with an ImageScanner (GE Healthcare) and analyzed with TotalLab (Nonlinear Dynamics Limited, Newcastle, UK) to check for the equivalent amount of protein between samples. Then, each lane was cut horizontally in 3 bands for a quantitative proteomic analysis. Gel slices from the two pooled samples were washed in water/acetonitrile (1:1) for 5 min and in acetonitrile for 10 min. Cysteine reduction and alkylation were performed by successive incubations in 10 mM dithiothreitol/50 mM NH_4HCO_3 for 30 min at 56°C and 55 mM iodoacetamide/50 mM NH_4HCO_3 for 20 min at room temperature in the dark. Gel slices were washed by incubation in 50 mM NH_4HCO_3 /acetonitrile (1:1) for 10 min and by incubation in acetonitrile for 15 min. Proteins were digested overnight in 25 mM NH_4HCO_3 with 12.5 ng/ μ L trypsin (Sequencing Grade, Roche). The resulting peptides were extracted from gel by successive incubations in 0.1% formic acid (FA)/acetonitrile (1:1) for 10 min and in acetonitrile for 5 min. The two extracts were pooled, dried, reconstituted with 30 μ L of 0.1% FA, 2% acetonitrile and sonicated for 10 min before MS analysis.

Nano LC–MS/MS analysis

Peptide mixtures were analyzed by nanoflow liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS). All experiments were performed on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The Netherlands) controlled by Chromeleon Software (version 6.8 SR11; Dionex). Five microliters of each sample was loaded on trap column for desalting and separated using nano-column as previously described by Labas and coworkers (Labas *et al.* 2015). The gradient consisted of 4–55% B for 120 min at 300 nL/min flow rate. The eluate was ionized using a Thermo Finnigan Nanospray Ion Source 1 with a SilicaTip emitter of 15 μ m inner diameter (New Objective, Woburn, MA, USA). Standard mass spectrometric conditions for all experiments were spray voltage 1.2 kV, no sheath and auxiliary gas flow; heated capillary temperature, 275°C; predictive automatic gain control (AGC) enabled and an S-lens RF level of 60%.

Data were acquired using Xcalibur software (version 2.1; Thermo Fisher Scientific). The instrument was operated in

positive data-dependent mode. Resolution in the Orbitrap was set to $R=60,000$. In the scan range of m/z 300–1800, the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated (isolation width, 2 m/z ; 1 microscan) and fragmented using collision-induced dissociation (CID). The ion selection threshold was 500 counts for MS/MS, and the maximum allowed ion accumulation times were 200 ms for full scans and 50 ms for CID-MS/MS in the LTQ. Target ion quantity for FT full MS was $1e6$ and for MS/MS it was $1e4$. The resulting fragment ions were scanned at the 'normal scan rate' with $q=0.25$ activation and activation time of 10 ms. Dynamic exclusion was active during 30 s with a repeat count of 1. The lock mass was enabled for accurate mass measurements. Polydimethylcyclsiloxane (m/z , 445.120025, $(Si(CH_3)_2O)_6$) ions were used for internal recalibration of the mass spectra.

Data processing and statistical analysis

Raw data files were converted to 'Mascot Generic File' (MGF) with Proteome Discoverer software (version 1.4; Thermo Fisher Scientific). The peptides and fragment masses obtained were matched automatically against a locally maintained copy of NCBI (8,000,106 entries, download 08/07/2015). MS/MS ion searches were performed using MASCOT Daemon and search engine (version 2.2.2; Matrix Science, London, UK). The parameters used for database searches included trypsin as a protease with two missed cleavages allowed, and carbamidomethylcysteine, oxidation of methionine and N-terminal protein acetylation as variable modifications and peptide charge 2 and 3+. The tolerance of the ions was set at 5 ppm for parent and 0.8 Da for fragment ion matches. Mascot results were incorporated into Scaffold 4 software (Proteome Software, Portland, USA). Peptides identifications were accepted if they could be established at over 95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.* 2002). A false discovery rate was calculated as <1% at the peptide or protein level.

Five nanoLC-MS/MS analyses were performed for each *in vivo* and *in vitro* EVs preparations. Quantifications were based on the label-free quantitative method, extracted ion chromatogram peptide pattern (XIC) (Higgs *et al.* 2005, Wang *et al.* 2006). SIEVE, version 1.3. software (Thermo Fisher Scientific), was used for XIC quantification. With time-aligned chromatograms, the frame m/z and the retention time (RT) were used to perform extracted ion chromatograms (XICs). The framing parameters were set at 0.02 Da for the 300–1800 m/z mass range and 6 min for the RT window for all MS2 data. The autodigested tryptic peptide at m/z 1082.0300 was used to normalize independent samples. The algorithm determined peptide abundance between 2 sample groups, frame-by-frame. A *t*-test was performed to characterize the changes between *in vivo* and *in vitro* EVs preparations. Differences were considered statistically significant at *P* value <0.01. Following the Proteome Discoverer, version 1.3 databank searches (Thermo Fisher Scientific) using the Mascot server, the .msf files were integrated into SIEVE. The results were filtered with protein normalized ratios <0.5 and ratio >2, with Mascot ion

scores >20. Identified frames were accepted manually when peptides were validated by the Protein and Peptide Prophet algorithms used in Scaffold software.

MS data have been deposited to the ProteomeXchange Consortium (Vizcaino *et al.* 2014) via the PRIDE partner repository with the dataset identifier 10.6019/PXD002280.

Data mining and bioinformatics analysis

Gene symbols and Entrez Gene IDs (bovine and putative human orthologs) were mapped for all protein identifications and analyzed using the online bioinformatics tools available via the biological DataBase network bioDBnet (tool db2db) (<http://biodbnet.abcc.ncicrf.gov/>; (Mudunuri *et al.* 2009)) and custom tools integrated in a local Galaxy (Giardine *et al.* 2005) installation (NCBI annotation mapper, Mammalian Ortholog and Annotation database, MOADB; Bick J, ETH Zurich, unpublished results 2016). The background dataset for the analysis was the human genome. To obtain meaningful findings of the EV proteins identified, functional analysis was performed using PANTHER (<http://www.pantherdb.org/>; (Thomas *et al.* 2003)) and DAVID Functional Annotation (<https://david.ncicrf.gov/>; (Huang *et al.* 2007)). To visualize interactions among candidate EV proteins and integration of the different networks, the Cytoscape app ClueGO was used (<http://www.cytoscape.org/>; Cytoscape 3.3.0 and app ClueGO v2.2.5; (Shannon *et al.* 2003)).

Western blotting

Proteins were separated by SDS-PAGE (8–16% gradient polyacrylamide gels using 4 μ g of proteins per lane) and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences Whatman) over 16 h at 30V, 300A. The membranes were washed in distilled water and blocked with Tris buffered saline (TBS) containing Tween 20 (0.5% (w/v)), and supplemented with lyophilized low-fat milk (5% w/v) for 1 h at room temperature. The membranes were incubated with primary antibodies diluted in TBS-Tween containing low-fat milk (1% w/v) for 2 h at 37°C with gentle shaking. The primary antibodies used were: anti-heat-shock protein 70 (HSP70; Stressgen, SPA-810); anti-78 kDa glucose-regulated protein (GRP78; Santa Cruz Biotechnology sc-13968); anti-heat-shock protein A8 (HSPA8; bioss.com, bs-5117R); anti-bovine oviduct glycoprotein (OVGP; kindly donated by P.A. Mavrogianis at University of Illinois in USA); anti-Myosin heavy chain 9 (MYH9 (H40), Santa Cruz Biotechnology, sc-98978); anti-Cluster of Differentiation 109 (CD109, Santa Cruz Biotechnology, sc98793) and anti-lactadherin (PAS6/7, a gift from Dr J T Rasmussen). After primary antibodies incubation, the membranes were washed with TBS with 0.5% Tween 20 and incubated overnight at 4°C under agitation with secondary antibodies. The secondary antibodies used were: horseradish peroxidase (HRP)-anti-mouse (Sigma A4416) or anti-rabbit (Sigma A6154). Blots were developed using a mixture of two chemiluminescence substrates developing kit (GE Healthcare AmershamTH ECL SelectTH Western blotting

detection Reagent RPN2235 and Supersignal West Pico #34087 Chemiluminescent Substrate Thermo Scientific).

In vitro embryo production (IVP) and EVs supplementation to in vitro embryo culture

Bovine embryos were produced *in vitro* as previously described by Cordova and coworkers (Cordova *et al.* 2014). Briefly, bovine ovaries were collected at a local slaughterhouse. Cumulus–oocyte complexes (COC) were aspirated, washed and incubated in maturation media for 22 h. Subsequently, COC were fertilized with semen from a bull with proven fertility. Twenty hours after IVF, presumptive zygotes were vortexed to remove cumulus cells and attached spermatozoa and washed into wells containing 500 μ L of *in vitro* culture medium. The medium used for *in vitro* culture (IVC) was synthetic oviduct fluid (SOF) medium (Holm *et al.* 1999) supplemented with 5% fetal calf serum (FCS, MP Biomedicals, MP5418) (EVs-depleted by ultracentrifugation).

Having in mind the practical application of EVs in the IVP lab, we decided to compare the effect of fresh and frozen *in vivo* EVs supplementation vs control (non-supplementation) in our embryo culture system. Experiments were performed in 4 replicates. EVs were prepared pooling oviducts from 3 animals for each replicate as previously mentioned. For each replicate, EVs preparations were divided in two aliquots (frozen and fresh samples). Frozen samples were kept at -80°C for 3 h, while fresh samples were kept at 4°C until IVC media supplementation. For each replicate, EVs protein concentration was measured ranging from 1.61 to 5.34 mg/mL and EVs supplementation was added to the culture medium at a final concentration of 0.22–0.42 mg/mL. Fresh and frozen EVs were diluted in IVC media and filtered (0.22 μ m). Then 25 μ L drops of IVC medium supplemented with or without EVs were prepared. Subsequently, groups of 25 presumptive zygotes were cultured into these 25 μ L drops of SOF medium with or without EVs supplementation, overlaid with 700 mL of mineral oil. Embryo-EVs co-culture was performed into 38.8°C , 5% O_2 , 5% CO_2 and 90% N_2 conditions during 9 days. Embryos were allocated in 3 groups according to the experimental design: control, fresh EVs and frozen EVs to evaluate embryo development and quality in terms of cleavage (Day 2 post IVF), blastocyst rate (Days 6, 7, 8 and 9), hatching rates (Days 8 and 9) and number of cells (Day 9).

Results

Differential protein profile in oviduct EVs from in vivo and in vitro origin

We validated the presence of EVs in both *in vivo* and *in vitro* samples by using TEM as well as Western blot analysis. Transmission electron microscopy (TEM) observations confirmed the presence of EVs in bovine oviduct flushings (Fig. 1A) and also in the conditioned media from BOEC primary *in vitro* culture (Fig. 1B). All four replicates analyzed from *in vivo* and *in vitro* preparations showed a population of small-EVs (30–100 nm) resembling exosomes and a population of large-EVs (>100 nm-) resembling microvesicles (Fig. 2A and B). In the literature, microvesicles ranged from (>100 up to ~1000 nm) (Raposo & Stoorvogel 2013). Histograms of Fig. 2 showed the distribution of exosomes and microvesicles in the *in vivo* (Fig. 2A) and *in vitro* preparations (Fig. 2B).

Western blotting for exosomal protein markers and oviduct proteins with known reproductive roles were performed in EVs *in vivo* and *in vitro* preparations and their cells of origin (Fig. 3). EVs were positive for HSP70, a recognized exosomal protein (positive control) present in 89% of exosome proteomic studies (Mathivanan *et al.* 2010, Klothontz *et al.* 2016) and negative for Grp78, an endoplasmic reticulum marker detected in BOEC from both origins and not in EVs (negative control) (Fig. 3). Moreover, *in vivo* EVs expressed oviduct glycoprotein (OVGP1), heat-shock protein A8 (HSPA8) and myosin 9 (MYH9), while only HSPA8 and MYH9 were detected in *in vitro* EVs. When the cells of origin were analyzed for these reproductive proteins, OVGP1, MYH9 and HSPA8 were expressed in all *in vivo* samples. In contrast, MYH9 and HSPA8 were expressed in all *in vitro* oviduct cells, whereas OVGP1 was not expressed in any of the samples *in vitro*. Taken together, the Western blot (molecular) data and TEM (biophysical) showed that both *in vivo* and *in vitro* oviduct cells produce EVs but with different qualitative and quantitative characteristics.

To understand the potential roles of EVs in the oviduct environment as modulators of gamete/embryo-oviduct cross-talk, we performed the first proteomic analysis of oviduct EVs using MS. This analysis allowed us to

Figure 1 Transmission electron microscopy observations of bovine oviduct EVs preparations from *in vivo* (A) and *in vitro* (B) origin. Structures pointed by blue arrows with 30–100 nm size resembling to exosomes and structures pointed by red arrows >100 nm size resembling to microvesicles were identified in oviduct flushing (*in vivo*; A) and conditioned media from BOEC culture (*in vitro*; B) preparations.

Figure 2 Histograms showing the size distribution of bovine oviduct EVs in *in vivo* and *in vitro* preparations. Four replicates of EVs from *in vivo* (A) and *in vitro* (B) preparations were analyzed using TEM and measurement of vesicles was performed. Populations of exosomes (30–100 nm) and microvesicles (>100–250 nm) (A and B) were found in both EVs preparations.

compare the *in vivo* and *in vitro* oviduct EVs signature. MS identified a total of 315 proteins, from which 97 were exclusively detected in *in vivo* EVs, 47 were found only in *in vitro* and 175 were in common to both samples (Venn diagram, Fig. 4 and Supplementary data S1, S2, S3, see section on Supplementary data given at the end of this article). Moreover, Fig. 4 represents the total of proteins identified in *in vivo* (270) and *in vitro* (222) EVs samples. While the bar graph below shows the number of proteins in common (175) or exclusive to *in vivo* or *in vitro* EVs (142). From the total of 315 identified proteins, 186 were differentially expressed when *in vivo* and *in vitro* EVs were compared (P value <0.01; ratio <0.5 or ratio >2) (Supplementary data S4).

Figure 3 Western blotting characterization of bovine oviduct EVs from *in vivo* and *in vitro* origin for known exosomal markers and oviduct proteins with known reproductive roles. (i) Both EVs preparations were positive for HSP70, a known exosomal protein marker and negative for Grp78, an endoplasmic reticulum marker detected on BOEC; (ii) *in vivo* EVs expressed oviduct glycoprotein (OVGP), heat-shock protein A8 (HSPA8) and myosin 9 (MYH9), while only HSPA8 and MYH9 were detected in *in vitro* EVs; (iii) when the cells of origin were analyzed for these reproductive proteins, OVGP1, MYH9 and HSPA8 were expressed in all *in vivo* samples, but only MYH9 and HSPA8 were expressed in *in vitro* oviduct cells.

MS results were confirmed by Western blot analysis on candidate proteins present in EVs and associated to reproductive functions (Fig. 5). Western blot analysis was performed in 4 independent biological replicates for CD109 and lactadherin. While lactadherin was expressed only in *in vitro* EVs preparations, CD109 was expressed only in *in vivo* EVs preparations. These results confirmed the different proteomic profile of EVs from *in vivo* and *in vitro* origin.

Figure 4 Venn diagram showing the number of bovine oviduct EVs proteins identified exclusively in *in vivo* and *in vitro* preparations and in common. Venn diagram illustrates that 97 proteins were exclusively detected in *in vivo* EVs, while 47 were found only in *in vitro* and 175 were in common to both samples.

Deciphering the EV proteomic content from *in vivo* and *in vitro* origin

MS data provided the first proteomic signature of oviduct EVs, and identified family of proteins in their cargo characteristic of exosome protein composition. The exosomal cargo included proteins involved in exosome biogenesis and intracellular vesicle trafficking, including tetraspanins (CD46, CD109, CD9) and Rab GTPases (RAB5C, RAB7A, RAB11B, RAB1A; ARF4). The identified tetraspanin and rab family proteins were present in both *in vivo* and *in vitro* EVs. In addition, we found that EVs cargo were enriched in Annexins, another class of proteins commonly seen in exosomes, involved in membrane trafficking and fusion events (ANXA1, ANXA2, ANXA3, ANXA4, ANXA5, ANXA7, ANXA8, ANXA11). Most of them common to both EVs preparations except ANXA7 that was only expressed

in *in vivo* EVs. EVs were also enriched with heat-shock proteins (HSPA1A, HSP60, HSPA4, HSPA5, HSPA8, HSP90AA1, HSP90AB1, HSP90B1) and other proteins related to adhesion such as MFG8/lactadherin and integrins (ITGB1).

Besides the identification of common exosomal proteins, we performed functional analysis of identified EV proteins from *in vivo* and *in vitro* origin to obtain a better understanding of the role of the EV proteins in different biological processes and, particularly, in gamete/embryo-oviduct communication and in supporting embryo development. From the 315 proteins identified in EVs preparations, gene ontology (GO) analysis using PANTHER database revealed that a high number of these proteins was involved in metabolism (24%), cellular process (20.4%), localization (10.2%), developmental processes (7.7%), immune system (5.6%), response to stimulus (7.4%) and in reproductive processes (1.4%) (Fig. 6). Further analysis revealed that more than 58% of EVs proteins involved in cellular process were associated with cell-to-cell communication (Fig. 6). Moreover, DAVID functional annotation clustering for EVs proteins showed that clusters related to 'vesicle', 'cytoplasmic vesicle' and 'membrane-bounded vesicle' had the highest enrichment score (enrichment score 19.9, 62 proteins involved) while clusters related to fertilization were also found with a relative high enrichment score (Table 1). A complete list of clusters is provided in Supplementary data S5.

A deeper analysis combining PANTHER, DAVID and GeneCards databases and the literature, revealed 36 EVs proteins (11.42% from 315) involved in important reproductive functions such as fertilization and embryo development (Table 2).

Furthermore, to obtain a more integrative visualization of the differential proteins identified in *in vivo* and *in vitro* EVs and their biological functions, Cytoscape (app ClueGO) was used. Fig. 7 shows clear differential networks of functional categories for *in vivo* and *in vitro* EVs proteins.

Demonstrating the traffic of oviduct EVs to the early embryo

Here, we demonstrated that IVP embryos were able to internalize *in vivo* EVs. We selected *in vivo* EVs for our experiment, since our proteomic analysis pointed out important differences between *in vivo* and *in vitro* exosomes such as OVGP1 protein, only present in *in vivo* EVs and involved in supporting early embryo development.

EVs were isolated and labeled with green fluorescent dye (PKH67), filtered (0.22 µm) and co-incubated with blastocysts (with intact zona pellucida) and hatching/hatched (H) blastocysts (with partial or total absence of pellucida) produced *in vitro*. Confocal microscopy observations confirmed that *in vivo* EVs

Figure 5 Validation of mass spectrometry analysis of bovine oviduct EVs by Western immunoblotting. Western blot analysis confirmed MS results in the 4 biological replicates for CD109 and Lactadherin. CD109 was only expressed in *in vivo* EVs while Lactadherin was expressed only in *in vitro* EVs.

Figure 6 Functional analysis of bovine oviduct EVs proteins identified by mass spectrometry by PANTHER database. EVs proteins isolated from *in vivo* and *in vitro* preparations were subjected to ontology and pathway analysis using PANTHER and Gene ontology algorithms and subsequently classified based on their biological process.

Table 1 Selected results of DAVID functional annotation clustering for proteins identified in bovine oviduct EVs from *in vivo* and *in vitro* origin.

Representative functional terms of overrepresented annotation clusters	Enrichment score ^a	No. proteins
Annotation cluster for proteins identified in exosomes from <i>in vivo</i> and <i>in vitro</i> origin		
Vesicle (62, 4.3) ^b cytoplasmic vesicle (57, 4.1); membrane-bounded vesicle (54, 4.4)	19.9	62
Ribosome (21, 17.4); translational elongation (25, 11.9); protein biosynthesis (26, 8.3)	14.3	39
Nucleotide binding (78, 2.8); purine ribonucleotide binding (80, 2.1)	10.9	87
Annexin (8, 34.6); Annexin 3; Annexin 4; Annexin 1; Annexin 2	8.5	10
Actin cytoskeleton organization (21, 4.5); actin filament-based process (23, 4.6)	6.8	27
ATP binding (61, 1.9); adenyl ribonucleotide binding (61, 1.9); purine nucleoside binding (61, 1.8)	6.6	62
Glucose catabolic process (11, 9.1); hexose catabolic process (11, 7.7); glycolysis (9, 9.2)	5.0	17
Vesicle lumen (9, 9.1); cytoplasmic membrane-bounded vesicle lumen (8, 8.5)	5.0	12
Regulation of apoptosis (36, 2.1); regulation of programmed cell death (36, 2.1); regulation of cell death (36, 2.1)	4.6	36
Anti-apoptosis (16, 3.7); negative regulation of apoptosis (21, 2.9); negative regulation of programmed cell death (21, 2.8); negative regulation of cell death (21, 2.8)	4.3	21
Phospholipase inhibitor activity (5, 19.9); lipase inhibitor activity (5, 15.9)	4.2	7
Myosin (8, 9.5); myosin complex (8, 5.7)	3.9	8
Heat-shock protein Hsp70 (5, 22.45); chaperone HSP70 (4, 17.2)	3.8	5
Primary lysosome (3, 35); specific granule (3, 20)	2.4	3
Hemostasis (10, 4.5); regulation of body fluid levels (9, 3.7); blood coagulation (9, 4.2); wound healing (11, 2.8)	2.9	13
Cell migration (15, 2.6); localization of cell (15, 2.5); cell motility (15, 2.5)	2.7	16
Heat-shock protein Hsp90 (3, 32.1)	1.7	3
Peroxiredoxin activity (4, 23.9); response to reactive oxygen species (5, 3.2); antioxidant activity (4, 4.07)	1.8	7
Calcium-binding region: 1; low affinity (4, 13.4); calcium-binding region: 2; high affinity (4, 11.5)	2.1	4
Fertilization (5, 20.2); single fertilization (5, 4.0)	2.2	5
Cellular ion homeostasis (16, 2.1); chemical homeostasis (17, 1.6);	1.7	17
Actin capping (4, 12.8); negative regulation of cytoskeleton organization (5, 4.4); negative regulation of organelle organization (5, 2.9)	1.7	6

^aGeometric mean of member's *P* values of the corresponding annotation cluster (in $-\log_{10}$ scale); ^bin brackets: number of genes and fold enrichment of the functional term.

Table 2 Protein identified in bovine oviduct EVs from *in vivo* and *in vitro* origin associated with reproductive roles.

Human gene ID	Symbol	protein name	Reproductive functions	Source
6813	STXBP2	Syntaxin binding protein 2	Gamete generation, fertilization	PANTHER
6812	STXBP1	STXBP1 protein	Gamete generation, fertilization	PANTHER
7348	UPK1B	Uroplakin 1B	Gamete generation	PANTHER
2950	DNAH5	Dynein heavy chain 5, axonemal; DNAH5; ortholog	Gamete generation, fertilization	PANTHER
2771	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	Gamete generation	PANTHER
23303	KIF13B	PREDICTED: kinesin family	Gamete generation	PANTHER
152007	GLIPR2	GLI pathogenesis-related 2	Gamete generation, fertilization	PANTHER
6809	STX3	Syntaxin 3	Gamete generation, fertilization	PANTHER
3336	EMR1	EGF-like module-containing mucin-like hormone receptor-like 1; EMR1; ortholog	Gamete generation	PANTHER
2273	FHL1	Four and a half LIM domains protein 1; FHL1; ortholog	Gamete generation	PANTHER
1397	CRIP2	GLI pathogenesis-related 2	Gamete generation	PANTHER
226	ADARB1	Double-stranded RNA-specific editase 1; ADARB1; ortholog	Gamete generation	PANTHER
216	ADAM9	Disintegrin and metalloproteinase domain-containing protein 9; ADAM9; ortholog	Fertilization	PANTHER
2934	GSN	Gelsolin	Fertilization	PANTHER
6674	SPAG1	TPA: sperm associated antigen 1	Fertilization	DAVID
5016	OVGP1	oviduct glycoprotein 1	Fertilization	DAVID
4179	CD46	CD46 molecule	Fertilization	DAVID
928	CD9	CD9 molecule	Fertilization	DAVID
4240	MFGE8	PREDICTED: lactadherin isoform X1	Fertilization	DAVID
4904	YBX1	Nuclease-sensitive element-binding protein 1 (Oryctolagus cuniculus)	Embryonic development in uterus	GeneCards
2288	FKBP4	FK506 binding protein 4	Embryo implantation	GeneCards
2776	GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	Post-embryonic development	Literature
6194	RPS6	mCG6197 (Mus musculus)	Fertilization, pacentia development	GeneCards
10521	DDX17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, isoform CRA_h (Homo sapiens)	Embryogenesis, spermatogenesis, cell growth division, post-embryonic development	GeneCards
11196	SEC23IP	SEC23-interacting protein (Bos Taurus)	Spermatid development	GeneCards
8566	PDXK	Pyridoxal kinase (Bos Taurus)	Epididymis secretory sperm binding protein	GeneCards
51181	DCXR	L-Xylulose reductase (Bos taurus)	Sperm surface protein	GeneCards
498	ATP5A1	Chain A, the structure of F1-Atpase inhibited by resveratrol	Epididymis secretory sperm binding protein, embryo development	GeneCards
3308	HSPA4	heat-shock protein family A (Hsp70) member 4	Sperm fertilizing ability	Literature
3336	HSPE1	PREDICTED: 10 kDa heat-shock protein, mitochondrial-like (Macaca mulatta)	Early pregnancy factor	Literature
3312	HSPA8	Heat-shock protein family A (Hsp70) member 8	Sperm fertilizing ability	Literature
7184	HSP90B1	Heat-shock protein 90 kDa beta family member 1	Sperm fertilizing ability	Literature
3320	HSP90AA1	Heat-shock protein 90 kDa alpha family class A member 1	Sperm fertilizing ability	Literature
3326	HSP90AB1	Heat-shock protein 90 kDa alpha family class B member 1	Sperm fertilizing ability	Literature
302	ANXA2	Annexin A2	Embryo adhesiveness to endometrium, sperm-oviduct binding	Literature
308	ANXA5	Annexin A5	Formation sperm reservoir, sperm-oviduct interaction	Literature

were internalized by blastocysts (Fig. 8A, B and C) and H-blastocysts (Fig. 8D, E and F) and located around the nucleus. Figure 8I and J shows that EVs were actually present in the cytoplasm and not only attached to the embryo membrane. No green fluorescent EVs were observed in the negative controls of embryo co-cultured with PBS dye (G and H).

Oviduct EVs supplementation improved bovine in vitro blastocyst yield and quality

Having in mind the practical application of EVs in the IVP lab, we decided to compare the effect of fresh and frozen *in vivo* EVs supplementation vs control (non-supplementation) in our embryo culture system (4 replicates). EVs supplementation had no effect

Figure 7 Integrative visualization of the differential proteins identified in *in vivo* (red) and *in vitro* (green) bovine oviduct EVs and their biological functions and protein interactions using Cytoscape app ClueGO. Differential networks of functional categories from *in vivo* (red) and *in vitro* (green) EVs proteins are shown.

on embryo cleavage (day 2) (Fig. 9A) (73.58 ± 3.44 ; 76.57 ± 1.92 and 81.04 ± 3.64 for fresh, frozen and control respectively) but influenced blastocyst rates over time (days 7–9) (Fig. 9B). Interestingly, frozen EVs significantly improved blastocyst rates at days 7 and 8 compared to fresh EVs and control (day 7: 32.99 ± 3.13 ; 30.18 ± 3.99 and 40.91 ± 2.61 and day 8: 34.04 ± 2.67 ;

37.92 ± 2.57 and 45.91 ± 1.12 for control, fresh and frozen respectively) (Fig. 9B). Fresh EVs showed a significant effect on embryo development at day 9 compared to the control (day 9: 23.78 ± 4.01; 35.2 ± 4.86 and 49.36 ± 0.64, Fig. 9). Embryo quality was measured in terms of the hatching ability and number of cell/blastocyst. There were no differences on hatching rates

Figure 8 Uptake of *in vivo* EVs by *in vitro*-produced embryos. EVs preparations from *in vivo* origin were labeled with green fluorescent dye (PKH67), filtered (0.22 μ m) and co-incubated with blastocysts (A, B and C) and hatching/hatched (D, E and F) blastocysts. Embryos co-cultured with green-labeled EVs were fixed and stained with Hoechst 3342 to visualize the nucleus and with actin red phalloidin to visualize the membrane of the cells. Fluorescence (A, B, C, D, E and F) images demonstrating active uptake of green-labeled EVs by embryos *in vitro* during culture. No green fluorescent exosomes/microvesicles were observed in the negative controls of embryo co-cultured with PBS (G and H, Controls). Detail of EVs inside the blastocyst cells confirmed that *in vivo* EVs were not only attached to the embryo membrane but also were actually internalized by these cells and were present in the cytoplasm (I and J). Images were obtained with 20x with an additional zoom factor from 1 to 2.8x. Scale bar = 50 μ m.

Figure 9 Effect of fresh and frozen *in vivo* EVs on the embryo development and the quality of *in vitro*-produced embryos. Embryo were co-incubated with EVs for 9 days after IVF (4 replicates, number of initial oocytes for control $n=353$; fresh EVs $n=332$ and frozen EVs $n=332$). Cleavage (A), blastocyst (B) and hatched blastocyst (C) rates were expressed as percentages (mean $\pm$ S.E.M.) and were calculated on initial oocytes numbers. Number of cells/blastocyst is shown in figure section (D). Different letters in the graphs represent significant differences ($P < 0.05$). Comparison among treatments in blastocyst rates are made on the same day of culture.

among all groups at day 8 while the addition of frozen EVs improved significantly the hatching rate at day 9 (Fig. 9C) from 7.5% to 26%. The total number of cells was also improved by frozen EVs addition (Fig. 9D). Our results showed that oviduct EVs supplementation during *in vitro* embryo development improves blastocyst yield, quality and extends embryo survival overtime.

Discussion

Our study demonstrated that EVs are essential components of oviduct secretions from *in vivo* and *in vitro* origins. Moreover, our results provide with the first oviduct EVs signature. We found differential protein profiles between *in vivo* and *in vitro* EVs, under our experimental conditions. We demonstrated that EVs from *in vivo* origin were up taken by *in vitro*-produced embryos and exert a functional effect by enhancing embryo development and quality during *in vitro* culture.

TEM observations and WB from our study confirmed the presence of EVs in oviduct secretions from *in vivo* and *in vitro* origin. Distribution size by TEM measurements showed different populations of exosomes and microvesicles from both sources of samples ranging from 30 to 250 nm. Previous analysis performed by laboratory using dynamic light scattering analysis (DLS) showed a higher abundance of bigger vesicles than exosomes in EVs conditioned media when compared to oviduct fluid (Alminana *et al.* 2014) in contrast to current measurement by TEM. We believe that DLS results could be due to artifacts, measurements of aggregates of vesicles instead of individual vesicles (Muller *et al.* 2014). Moreover, Van der Pol and coworkers showed that different

techniques can give a different size distribution and a different concentration for the same vesicle sample (van der Pol *et al.* 2014). Nevertheless, our results are in agreement with previous studies isolating exosomes and microvesicles from bovine oviduct fluid following a protocol similar to us (centrifugation at 100,000g) (Lopera-Vasquez *et al.* 2017).

Exosomes and microvesicles were also identified in both oviduct fluid and BOEC-derived conditioned media by Al-Dossary and coworkers (Al-Dossary *et al.* 2013) and Lopera-Vásquez and coworkers (Lopera-Vasquez *et al.* 2016) respectively. Our study differs from those in 2 important aspects: (1) our study used a wide proteomic approach to characterize oviduct EVs content and decipher the role of EVs during early gamete/embryo-maternal interactions, while Al-Dossary and coworkers (Al-Dossary *et al.* 2013) focused their study on a specific protein approach contained in murine oviduct EVs, PMCA4a, which is essential for sperm hyperactivated motility and fertility and (2) we compared *in vitro* and *in vivo* EVs obtained from conditioned media of primary culture of BOEC and oviduct fluid from same oviducts at the early post-ovulatory stage (1–4 after ovulation) to minimize variability. Lopera-Vásquez and coworkers used oviducts at the mid-luteal phase of the estrous cycle (Lopera-Vasquez *et al.* 2016). Greening and coworkers demonstrated that different hormonal environment during the estrous cycle modulate content of human endometrial derived EVs (Greening *et al.* 2016).

Our findings suggest that BOEC *in vivo* might not secrete the same population of exosomes/microvesicles than *in vitro* under our experimental conditions. It is possible that the *in vitro* culture of BOEC may

affect their ability to secrete and release exosome/microvesicles and their content. BOEC in monolayer after several days in culture are less likely to mimic the oviduct environment than BOEC *in vivo*, as reflected by the loss of morphological hallmarks such as cilia and secretory granules (Rottmayer *et al.* 2006). Differences between *in vivo* and *in vitro* exosome populations were also reported by Ostman and coworkers (Ostman *et al.* 2005), when comparing exosomes secreted by *in vitro* propagated tumor cells vs tumor cells grown *in vivo*. Here, we showed quantitative and qualitative proteomic differences between EVs content from *in vivo* and *in vitro* origin by MS analysis. Moreover, our functional analysis of the EVs proteins from both sources showed that they were associated to different biological functions and networks (Fig. 7). Altogether, highlights the distinct functionality of *in vivo* and *in vitro* EVs during oviduct–embryo interactions. Since EVs play a role in cell-to-cell communication through the transfer of their cargo, it can be expected that the different proteomic oviduct EVs composition between *in vivo* and *in vitro* may exert distinct functional effects on embryo(s) and gametes.

It is worthy to mention that the experimental conditions used in the present study (oviduct source, oviduct collection, BOEC collection, *in vitro* cell culture system, period of cell incubation and culture media, etc.) might affect the *in vivo* and/or *in vitro* EVs secretion/content identified here. To obtain the oviductal flushings the conditioned media from BOEC culture, we used a protocol developed in our laboratory that has shown to be a good method to prepare BOEC monolayer to support embryo development but also, to study the embryo-oviduct dialog and analyzing the BOEC gene expression (Schmaltz-Panneau *et al.* 2014). To avoid that the *in vivo* ‘debris’ and ‘apoptotic/dead cells’ contained in the oviduct fluid could affect the *in vivo* EVs, flushings obtained were immediately centrifuged at 300g, 15 min followed by 12,000g 15 min. Then, BOEC cells were isolated, seeded and *in vitro* cultured for 14 days, as in our previous studies. It is possible that EVs collected from oviducts immediately post-mortem or transported under conditions different to those in our study (i.e. ice) could differ in EVs quality or protein abundance, despite EVs content seems well preserved. On the other hand, the use of a different culture system, with distinct media, period of cell culture could also affect EVs production/content. To the best of our knowledge, there is not any available study that has shown to which extent *in vitro* culture can affect the EVs production and content compared to *in vivo* EVs. Even more, no information exists regarding if the days of *in vitro* culture, the media or other *in vitro* factors can change their content. Cell culture models are important tools for revealing specific effects and mechanisms of cell populations and currently are being used for many studies as a source to obtain EVs. However, it is extensively known that it is very difficult to find an *in vitro* cell culture model that allows the cells

to be as much as possible similar to the *in vivo* ones (in regard to gene expression, secretion, EVs, etc.). Recently, two novel culture methods for oviductal cells based on air–liquid interphase culture system (Chen *et al.* 2017) and a 3D-printing oviduct device (Ferraz *et al.* 2017) have been proposed to generate oviduct fluid surrogates more similar to the *in vivo* ones. Therefore, it is possible that using these novel systems, the *in vitro* content of EVs could be more similar to the *in vivo* ones.

In the present study, the proteomic profiling of oviduct EVs showed an exosome signature and confirmed that oviduct exosomes contain basic machinery important for biogenesis, trafficking, fusion and release. However, our TEM observations also revealed the presence of microvesicles. Establish methods that allow to discriminate between exosomes and microvesicles are a major ongoing challenge in the field of EVs (Raposo & Stoorvogel 2013). Until then, this report provides the first protein cargo signature of oviduct EVs and represents the only exosome and microvesicles protein resource to date.

Furthermore, oviduct EVs characterization revealed proteins with important roles in the gamete/embryo–oviduct interactions, such as OVGP1, HSP90, HSPA8, HSP70, Gelsolin and Ezrin in oviduct EVs (Table 2). Some of them were only identified in *in vivo* or *in vitro* EVs (Supplementary data S1 and S2). Interestingly, these proteins were identified previously by Elliott and coworkers in a subset of 70 kDa oviduct surface proteins that bound to spermatozoa (Elliott *et al.* 2009). These proteins enhanced *in vitro* survival of mammalian spermatozoa, particularly HSPA8 (Elliott *et al.* 2009, Moein-Vaziri *et al.* 2014). The mechanism(s) by which HSPA8 or other proteins are released by the oviduct epithelium and are able to enhance sperm survival is still unknown. It has been suggested that heat-shock proteins might be released via exosomes (Campanella *et al.* 2014) or lipid raft (Pralle *et al.* 2000). Our results support the hypothesis that exosomes/microvesicles could be one of the mechanism(s) by which BOEC may release these proteins into the oviduct milieu and transport them to the embryo, allowing successful gamete interactions and subsequently early embryonic development.

Among the different reproductive proteins identified in oviduct EV under our experimental conditions, we would like to highlight the presence of OVGP1, MYH9, HSP90 (in its HSP90B1, HSP90AA1 and HSP90AB1 forms) and lactadherin (PAS6/7 or MFGE8) because of their important functions in gamete/embryo–oviduct cross-talk. OVGP1 is the major non-serum protein present in the oviduct fluid in different species (Sutton *et al.* 1984, Buhi *et al.* 1990). It increases sperm viability and motility (Abe *et al.* 1995); modulates sperm capacitation and fertilization (King *et al.* 1994) and enhances development rates (Kouba *et al.* 2000). Furthermore, OVGP1 seems to bind to both gametes through the interaction of its non-glycosylated N-terminal conserved

region with MYH9 (Kadam *et al.* 2006). Our results imply that both proteins might be secreted via EVs, or at least partly. Since both OVGP1 and MYH9 were detected *in vivo* EVs, while only MYH9 was expressed *in vitro* EVs. The fact that OVGP1 is only expressed in *in vivo* exosomes/microvesicles was not surprising since OVGP1 is expressed in BOEC after collection but its mRNA is strongly decreased after 7–10 days of culture. Considering that OVGP1 is secreted under steroid control, it is possible that the lack of hormonal stimulation in our experiment could explain the absence of OVGP1 in the *in vitro* exosomes/microvesicles. Together with OVGP1, HSP90B1 was also expressed in oviduct EVs and is associated to ZP hardening mechanism (Mondejar *et al.* 2012). HSP90 has been shown on the surface of 25% of the live capacitated sperm population that is capable of interacting with the ZP of the oocyte (Asquith *et al.* 2004). In addition, we identified lactadherin in EVs preparations, a protein common in exosomes studies and involved in ZP binding. It has been previously identified in *in vitro* microvesicles released by endometrial cell cultures under hormonal stimulation (Sarhan *et al.* 2013). However, lactadherin secretion *in vitro* has also been associated to unhealthy cells (Delcayre & Le Pecq 2006), despite in our *in vitro* culture system, 90% of BOECs were viable. It is interesting to note that exosomes are packing many proteins from heat-shock protein 70 and 90 families as well as other proteins involved in free radicals scavenging (peroxiredoxins, thioredoxin, etc.). These contents may bring to the gametes and embryos some additional factors to survive in the *in vivo* and *in vitro* environment.

Considering all the embryotrophic factors contained in the EVs, we evaluated whether EVs could be up taken by the embryo and exert a functional effect on embryos. Our results showed that oviduct EVs were internalized by the embryo being capable of passing through the zona pellucida and being located around the nucleus of most embryonic cells. Our results are in line with other studies showing the uptake of uterine EVs by the embryo/conceptus at later stages (Vilella *et al.* 2015, Burns *et al.* 2016) (Greening *et al.* 2016, Bidarimath *et al.* 2017). Burns and coworkers suggested that the uterine EVs uptake could have an essential role in the elongation of the conceptus (Burns *et al.* 2016). Vilella and coworkers (Vilella *et al.* 2015) demonstrated that Hsa-miR-30d, contained in uterine exosomes, could induce transcriptional and functional modifications in the adhesive competence of the embryo. In our study, we provide strong evidence for the functional effect of oviduct EVs in supporting bovine preimplantation embryo development, since EVs supplementation improved embryo development and embryo quality. Our results are in part in line with Lopera-Vásquez and coworkers (Lopera-Vásquez *et al.* 2016, 2017), reporting that the use of *in vitro* frozen/thawed BOEC-derived EVs improve embryo quality, by increasing

the number of cells and the survival of blastocyst after cryopreservation. However, these authors did not report any improvement in embryo development over time or hatching rates during *in vitro* culture, as our study shows. The distinct effect of EVs on embryo during IVC between studies could be explained by the differences found in the protein content between EVs from *in vivo* and *in vitro* origin or other EVs molecular components (mRNA, miRNA). Moreover, the moment of the estrous cycle or the different parts of the oviduct (ampulla, isthmus) from which they were collected could also have an effect (Greening *et al.* 2016, Lopera-Vásquez *et al.* 2017). Our next studies will be focused in further analyzing the content of EVs at mRNA and miRNA across the estrous cycle and investigating the possible epigenetic effects of EVs on preimplantation embryo development.

Having in mind the practical application of EVs in optimizing IVF systems, we compared the effect of fresh and frozen EVs during *in vitro* embryo development. Despite both fresh and frozen EVs had a positive effect on embryo development, surprisingly the use of frozen EVs showed better results for IVP. To the best of our knowledge, no studies have been performed to evaluate properly the impact of the freezing procedure on the EVs structure and/or content that could explain our results. The information available to date in the literature is controversial with different studies showing conflicting results regarding the resistance of EVs to freezing (Bosch *et al.* 2016). Most of studies are based on fresh or frozen samples (blood, fluid, urine) from which EVs are isolated. A few studies have indicated that storing samples at -80°C do not alter EVs morphology or size (Sokolova *et al.* 2011, Sarker *et al.* 2014). While others have suggested that freezing may induce membrane damage and leakage of EVs content in the absence of perceivable changes of size and concentration. A recent study has found a significant reduction in the bi-layer membrane of frozen vesicles (-80°C) when compared to fresh EVs (Teng *et al.* 2015), which could explain our results. Regarding their content and functionality, studies point out that freezing seem to preserve almost completely EVs associated proteins (Zhou *et al.* Kidney Int. 2006 Apr; 69(8): 1471–1476) and do not impair their functionality (Sokolova *et al.* 2011, Jayachandran *et al.* 2012) as we have observed in our study. The alterations of EVs following freezing remains a matter of debate in the field of extracellular vesicle research. Although further studies are required to elucidate the impact on the freezing process on EVs, our data provide clear evidence of the positive effect of fresh and frozen oviduct EVs on embryo development. Therefore, our study points out that the use of oviduct EVs is a good strategy to optimize *in vitro* embryo production. Further studies will be conducted to evaluate whether EVs can also improve the pregnancy outcomes after transferring embryos co-incubated with EVs, making them potential tools for the application of other biotechnologies.

In summary, our study identified the first oviduct-derived EVs protein signature and reveals a set of proteins with important roles in gamete/embryo–oviduct interactions that have not been previously identified in the oviduct EVs cargo. Moreover, our results highlight the differential protein cargo between *in vivo* and *in vitro* EVs. Information of great importance since most of functional studies of EVs are based on EVs derived from cell lines or primary culture and extrapolated to *in vivo* EVs biology and function. Functionally, we demonstrated that EVs were internalized by the embryo during *in vitro* culture and enhance their ability to reach the blastocyst stage, to hatch and increase their survival overtime. Taken together, this study brings new insights into the contribution of EVs as modulators of the oviduct–gamete/embryo cross-talk and opens up a new avenue for the use of EVs to optimize ART in human and livestock species.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0054>.

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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