Extracellular vesicles: Multi-signal messengers in the gametes/embryo-oviduct cross-talk

Almiñana, Carmen ; Bauersachs, Stefan

Abstract: Extracellular vesicles (EVs) have emerged as novel cell-to-cell communication mediators in physiological and pathological scenarios. Their ability to transfer their molecular cargo (RNAs, proteins and lipids) from one cell to another, in the vicinity or far from the cell of origin, together with their capacity of exerting a functional impact on the target cell make them valuable diagnostic tools as well as therapeutic vectors in a variety of diseases. In the reproductive field, there is a growing interest in the role of EVs in gamete/embryo-maternal communication and their potential implications in the reproductive success. In this review, we provide current knowledge of EVs secreted by the oviduct (oEVs) and embryos (eEVs), since both have been proposed as key players in the crucial two-way dialogue between the oviduct (lining epithelium and secretions) and the embryo that ensures successful pregnancy. Both oEVs and eEVs molecular cargos and their potential role as multi-signal messengers in the gametes/embryo-oviduct cross-talk and in the embryo-to-embryo communication in different species are also addressed. Eventually, a comparative analysis between oEVs and eEVs has been performed to shed some light on common and specific cargos responsible for their functions supporting the early reproductive events and as prime candidate molecules for improving fertility and assisted reproductive technologies outcomes.

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Extracellular vesicles: Multi-signal messengers in the gametes/embryo-oviduct cross-talk

Carmen Almiñana [a, b, *], Stefan Bauersachs [a]

[a] University of Zurich, Genetics and Functional Genomics Group, Clinic of Reproductive Medicine, VetSuisse Faculty, Zurich, Switzerland
[b] UMR85 PRC, INRA, CNRS 7247, Université de Tours, IFCE, 37380, Nouzilly, France

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ABSTRACT

Extracellular vesicles (EVs) have emerged as novel cell-to-cell communication mediators in physiological and pathological scenarios. Their ability to transfer their molecular cargo (RNAs, proteins and lipids) from one cell to another, in the vicinity or far from the cell of origin, together with their capacity of exerting a functional impact on the target cell make them valuable diagnostic tools as well as therapeutic vectors in a variety of diseases. In the reproductive field, there is a growing interest in the role of EVs in gamete/embryo-maternal communication and their potential implications in the reproductive success. In this review, we provide current knowledge of EVs secreted by the oviduct (oEVs) and embryos (eEVs), since both have been proposed as key players in the crucial two-way dialogue between the oviduct (lining epithelium and secretions) and the embryo that ensures successful pregnancy. Both oEVs and eEVs molecular cargos and their potential role as multi-signal messengers in the gametes/embryo-oviduct cross-talk and in the embryo-to-embryo communication in different species are also addressed. Eventually, a comparative analysis between oEVs and eEVs has been performed to shed some light on common and specific cargos responsible for their functions supporting the early reproductive events and as prime candidate molecules for improving fertility and assisted reproductive technologies outcomes.

1. Introduction

The oviduct is the tubular organ connecting the ovary and the uterus and holding crucial events for successful pregnancy [44]. Gamete maturation, sperm transport and capacitation as well as fertilization and early embryo development are supported by the oviduct. These events take place in different anatomic regions of the oviduct: i) the funnel-shaped infundibulum, nearest to the ovary, captures the released oocyte(s) after ovulation; ii) the ampulla, the wide part of the tube, the fertilization site; and iii) the isthmus, the narrow part of the tube connecting to the uterus at the utero-tubal junction, where the sperm reservoir is formed. For many years, the oviduct epithelium, composed of ciliated and secretory cells [30] and the oviductal secretions have been pointed as the main responsible for providing the optimal environment for sperm cells, oocytes and embryos during the early reproductive events [11,55].

Recently, extracellular vesicles (EVs) have been identified as one of the main components of the oviductal fluid in different species and proposed as mediators of gamete/embryo interactions leading to successful pregnancy [70]. Extracellular vesicles (EVs), referred to as exosomes and microvesicles, are membrane-enveloped vesicles released in an evolutionally conserved manner by most cells from yeast to mammals [23]. The importance given to EVs lies in their capacity to transfer their molecular cargo (proteins, RNAs, genomic DNA, lipids and metabolites) to other cells while exerting a functional effect in the target cell. Extracellular vesicles confer cells with protection of their message-cargo and the option of simultaneous delivery of multiple different signal-messengers in the vicinity or even to remote sites in the organism/body [95]. These characteristics make oEVs unique multi signal-messengers in gamete/embryo-oviduct interactions. If fertilization occurs, EVs present in the oviduct might be composed by a mixture of EVs from the oviduct and the early embryo, since it has been shown that embryos also secreted EVs in vitro (eEVs) [67]. Embryo-derived EVs (eEVs) have been pointed as modulators of embryo-to-embryo communication in vitro [82], and possibly in vivo in polytocus species, and with the maternal environment [83].

In this review, we gathered data from oEVs and eEVs studies regarding their molecular cargo and functions, to highlight their role as multi-signal messengers in the gamete/embryo-oviduct

* Corresponding author. University of Zurich, Genetics and Functional Genomics Group, Clinic of Reproductive Medicine, VetSuisse Faculty, Zurich, Switzerland.
E-mail addresses: carmen.alminanabrines@uzh.ch (C. Almiñana), stefan.bauersachs@uzh.ch (S. Bauersachs).

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cross-talk and embryo-to-embryo communication. This review charts recent progress in EVs research, challenges and future directions, with the aim of increasing our understanding about the key role of EVs in the early reproductive events and their potential implications to optimize assistant reproductive technologies (ARTs) protocols.

2. Extracellular vesicles derived from the oviduct

The presence of EVs in the oviductal fluid was described for the first time in the mouse by Al-Dossary et al. [4], who referred them as "oviductosomes"; using similar terminology to epididymosomes or prostasomes discovered decades before [1]. Since then, several studies have identified EVs in the oviduct of different species: cattle, pig, dog, cat, birds, turtle, mouse and human. Table 1 gathers the main findings of these studies. In this review, we focus mainly on oEVs molecular cargo and their potential functions since isolation methods and characterization of oEV were recently reviewed by Almiñana and Bauersachs [6].

2.1. Oviductal extracellular vesicles and their source of origin

One of the main characteristics of EVs is that their cargo reflects the cells of origin. This led researchers to question if oEVs secretion may differ among anatomical regions of the oviduct, different cell source (in vivo versus in vitro origin) or even between different species.

In bovine oviduct, Lopera-Vazquez et al. [59] showed that oEVs released by the isthmus had a higher concentration of smaller vesicles mainly recovered in the 100 K pellet, whereas in the ampulla, vesicles were more or less equally divided in the 10 K and the 100 K pellet. Neither the oEVs supplementation from ampulla or isthmus had an effect on blastocyst yield, however the isthmus-derived oEVs improved the blastocyst quality in terms of embryo cryosurvival. In porcine oviduct, a higher concentration of oEVs was found in the ampulla than in the isthmus, with no differences regarding their size across regions [46]. Moreover, these authors showed that oEVs from ampulla and isthmus had significantly different zeta potential measurements, which may be related to different surface characteristics and membrane composition among regions. Comparisons among studies are difficult since they have been performed in different species, with oEVs from in vivo and in vitro sources, and with different EV isolation methodologies. However, both studies point at distinct oEVs secretion patterns and probably different functionality, according to the anatomic regions of the oviduct which have different functions. Further studies are needed to fully elucidate if these differences are also reflected in the EVs molecular cargo. The development of new isolation techniques and methodologies to characterize EVs cargo using small amounts of sample will help in this matter.

On the other side, considering the fact that many EVs studies are based on EVs derived from cell lines or primary culture and extrapolated to in vivo EVs biology and function, the question arises if the cell source of origin could affect the EVs secretion and molecular content. In this regard, we performed a study examining the protein cargo of in vivo and in vitro oEVs [7]. Our study compared oEVs collected from bovine OF oviducts obtained from the abattoir versus bovine oviductal epithelial cells primary culture (bOECs) and clearly showed a differential protein profile between in vivo and in vitro oEVs. Studies on EVs from mesenchymal stem cells also showed that the cell passage, the frequency of EVs collection and the cell seeding density can also impact the production of EVs [74]. Furthermore, 3D cell culture systems increased secretion of EVs enriched with miRNA and protein content that were similar to the in vivo ones, when compared to 2D cell culture [80,89], which might have an effect on uptake and functional effects on recipient cells. Although novel oviductal cells cultures based on air-liquid interphase system [22] or 3D-printing oviduct device [33] have been proposed to generate oviduct fluid surrogates more similar to the in vivo ones, the oEVs production by these new models, has not been examined yet. Altogether, these studies highlight the need for careful consideration of the in vitro models/systems used to produce EVs for research purposes or for the production of therapeutic EVs at a laboratory scale.

Oviductal EVs may also differ in secretion and molecular cargo among species. Several reasons support this speculation: 1) The time that the embryo spends in the oviduct is different among species (from 2 to 6 days depending on the species); and 2) the oviduct might provide different environment to spermatozoa, oocytes and embryos from different species to adapt to their physiological needs. In the bitch for example, the oocyte maturation occurs in the oviduct, thus the oviductal milieu has unique features to support oocyte maturation. In the mare, the ampullary-isthmus junction acts as a regulatory checkpoint allowing only fertilized oocytes to pass any further along the oviduct and into the uterus. Moreover, monotocous and polytocous species have differences in gamete/embryo maternal interactions and thus, these differences might be also reflected in oEVs.

2.2. Oviductal extracellular vesicles and their molecular content: comparison among species

Studies in cattle and mouse clearly showed that the oEVs content is very dynamic and is under the influence of the ovarian hormones [7,32], controlling packing and release of mRNA, proteins, small ncRNAs and even metabolites in EVs to the oviductal lumen. Similar results have been found by Greening et al., in EVs released by a human endometrial cell line in vitro [38]. Moreover, exogenous treatment of progesterone modulates uterine EVs (uEVS) secretion and RNA cargos in the sheep [18]. The presence of the conceptus (day 14) in the uterus also affects the secretion and molecular cargo of uEVs, as shown by the differential protein profiles of uEVs from pregnant and cyclic ewes. Therefore, it can be expected that the oEVs secretion and cargo might change in the presence of spermatozoa, oocytes or early embryos or even in the presence of a non-competent embryo, pointing at an early embryo-maternal recognition system.

Here, we attempt to compare the oEVs molecular cargo from different species based on our own data and data available in the literature. Up to date, only three studies are published that systematically analysed the protein cargo of oEVs, one study in the cat and two studies in cattle [7,9,32]. In the study of feline oEVs, oviducts were collected during the follicular phase. In the first bovine study [7], a pool of oviduct fluid samples was used for isolation of in vivo oEVs which were mainly derived from peri-ovulation stages. The in vitro oEVs were isolated from the supernatant of bOECs primary cell culture. In the second bovine study, the samples were derived from 4 different stages of the estrous cycle [9]. A comparison of these 4 data sets performed on the basis of the official gene symbols revealed a good overlap of the oEVs protein cargo between the studies (Fig. 1A). Whereas in feline oEVs more than 2000 different proteins were identified, the bovine studies identified altogether 459 different proteins. Although, the number of identified proteins in the feline oEVs study was much higher probably due to the more sensitive mass spectrometry technique, 134 proteins were identified in the bovine oEVs but not in feline oEVs. Furthermore, the studies used different EVs isolation methods, classical serial ultracentrifugation in the bovine studies and a simple isolation technique using an EVs precipitation reagent in the study of feline oEVs. The latter probably resulted in considerable

Table 1
Summary of published studies related to oviductal extracellular vesicles (oEVs).

Species	Year	Citation	oEVs content Analysed	Findings
	2016	Lopera-Vásquez [58]	–	Oviductal EVs from in vitro origin improve embryo quality and cryosurvival.
Bovine	2017	Almiñana et al. [7],	Proteins	Differential protein cargo between oEVs from in vivo and in vitro; .Oviductal EVs can be taken up by embryos in vitro, improving their development and quality.
	2017	Lopera-Vásquez [59]	–	Characterization of oEVs from ampulla and isthmus and their impact on in vitro embryo development and quality.
	2018	Almiñana et al. [9],	Proteins, mRNAs Small ncRNA	Dynamic profile of mRNAs, small ncRNAs and proteins from oEVs cargo across the estrous cycle
Avian	2017	Huang et al. [43],	–	Identification of hen oEVs from culture medium of Utero-vaginal junction and vagina cells; effects on sperm viability and motility.
Turtle	2017	Waqas et al. [92],	–	Identification turtle oEVs: oEVs contact with cilia and with the sperm membrane give the turtle a unique secretory morphology.
Canine	2017	Lange-Consiglio et al. [54]	Specific miRNAs	Identification of oEVs from in vitro origin; containing miRNAs related to roles oocyte maturation and improving canine IVM
Felid	2019	Ferraz et al. [32],	Proteins	Identification of oEVs in domestic cat, protein cargo and their interactions with spermatozoa and roles in sperm functions
Porcine	2019	Alcàntara-Neto et al. [98]	Specific Proteins:	Characterization of oEVs from in vivo origin: revealing proteins related to reproductive events and effects in regulating polyspermy
	2019	Jamaludin et al. [46],	Specific miRNAs	Characterization of oEVs secreted by in vitro origin (ampulla and isthmus); comparison with human endometrial and non-reproductive immortalized cell lines;
	2013	Al-Dossary et al. [4],	Protein PMCA4a	Identification of oEVs for first time; Expression of PMCA4a in oEVs during estrous cycle; PMCA4a in sperm function via oEVs uptake
Murine	2015	Al-Dossary et al. [3]	No	Oviductosome-Sperm Membrane Interaction in Cargo Delivery: Fusion and Underlying Molecular Players Using SR-SIM
	2018	Fereshteh et al. [31],	MiRNAs	Identification of 272 miRNAs in murine oEVs across the estrous cycle; Delivery of miRNA contained in oEVs to sperm
	2017	Nakano et al. [69],	Specific mRNA	Oviductal EVs from mesenchymal cells modulate oviductal ciliogenesis.
	2019	Qu et al. [78],	No	Differences in oEVs from donor and recipient: donor has higher amounts of oEVs and improve efficiency of embryo transfer
Human	2018	Bathala et al. [12],	Proteins (PMCA1-4)	Identification of oEVs in the woman fallopian tubes for first time; oEVs contain fertility-modulating proteins conserved in humans.

MS: Mass Spectrometry. IVM: in vitro oocyte maturation.

contaminations from co-precipitating proteins and cellular particles other than EVs. This is indicated by the nature of proteins solely found in the study of feline oEVs, e.g., typical plasma proteins (albumins, hemoglobins, alpha-fetoprotein, apolipoproteins), antibody peptides, and other atypical proteins such as histones, collagens, nucleolar proteins, and 30 different keratins.

Regarding the analysis of the miRNA content of oEVs, there is only two studies that performed a systematic investigation (small RNA-seq) while other studies just focused on a few selected miRNAs. Fig. 1B shows a comparison of the miRNAs detected in oEVs in 4 different studies in the dog, pig, mouse, and cattle [9,31,46,54]. Small RNA-seq has been performed for murine [31] and bovine [9]

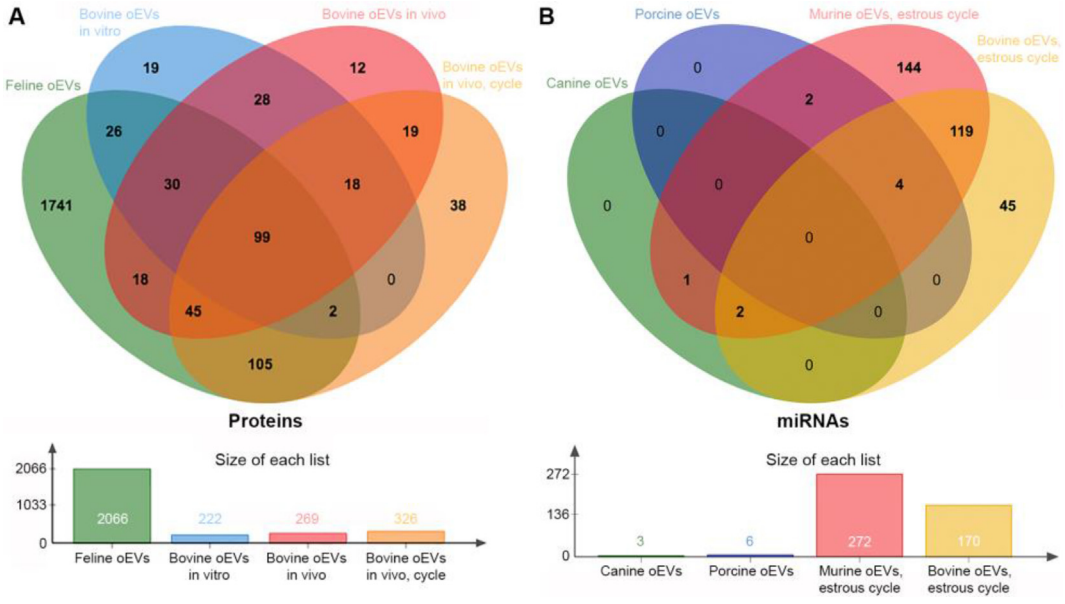

Fig. 1. Venn Diagram showing overlap of identified proteins (A) and miRNAs (B) in oviductal extracellular vesicles (oEVs) in different species.

oEVs. The study of murine oEVs revealed the highest number of detected miRNAs (n = 272). More than two third of the miRNAs found in bovine oEVs were also present in murine oEVs indicating a conservation of oEV miRNA content between species. Whereas in the mouse study proestrus/estrus was compared to metestrus/diestrus, 4 stages were compared on the bovine study. In both studies, most of the miRNAs showed similar levels throughout the estrous cycle and only a few miRNAs showed differences between peri-ovulation and luteal phase. All miRNAs potentially differentially expressed (DE) in bovine oEVs during the cycle were also detectable in murine oEVs. However, a comparison of the results regarding DE miRNAs among studies is difficult since incomplete information is available for DE miRNAs in the study of Fereshteh et al. [31].

2.3. Oviductal extracellular vesicles: multi-signal messengers in the early embryo-oviduct crosstalk

Since embryo-maternal communication is a two-way traffic of molecules [63] and EVs have been proposed as nanoshuttles packing and transferring this cocktail of molecules between the oviduct and the embryo [5], studies have been directed to prove this EVs traffic. To date, it has been demonstrated that oEVs can be taken up by embryos at the blastocyst stage in vitro [7] and also by oviductal epithelial cells [75]. However, the uptake of eEVs by the maternal side has been only shown by endometrial epithelial cells in vitro (eEVs of day 3 and day 5 embryo) [36] but not by oviductal cells (to the best of our knowledge). On the embryo side, eEVs can be taken up by both, cleavage embryos and blastocysts [76,82].

Functionally, a few studies have demonstrated that oEVs can exert a beneficial effect on embryo development [7,78], quality [7,58] and cryoresistence [58,59] and even improve implantation and birth rates after embryo transfer [78].

Analyses of oEVs molecular cargo at different levels have been performed looking for the molecules responsible for such effects. Fereshteh et al. [31], showed that oEVs contained miR-143-3p, miR-22-3p and miR-34c-5p with a role in the first cleavage of mouse embryos and implantation [42,57]. In bovine, oEVs were enriched in mRNAs associated to embryo development, cell proliferation and epigenetic regulation [8] (chromatin modification including histone methyltransferases: *EHMT1, EHMT2, EZH1, KMT2A, KMT2B, KMT2C, PHF1, PHF2, PRMT5, SETD1A, SETD2*; histone demethylases: *ARID5B, JMJD1C, KDM2A, KDM3B, KDM5B, KDM5C* and *KDM6B* as well as DNA methyltransferase gene (*DNMT1*). Furthermore, *GJA1* and *GAPDH* mRNAs in oEVs have been associated with better quality embryos and cryotolerance [40]. MiR-449a, upregulated in oEVs during post-ovulatory period compared to the rest of stages, has been associated to different causes of infertility in men and women. Target analysis of miR-449a showed a network of genes involved in embryo development, angiogenesis, response to oxidative stress and chemotaxis, suggesting implications in successful pregnancy [8]. MiR-30d identified in bovine oEVs and in human uEVs, was taken up by mouse embryos leading to alterations of the embryonic transcriptome related to embryo adhesion [8,90].

2.4. Oviductal extracellular vesicles: multi-signals messengers in sperm/oocyte-oviduct crosstalk

In the mouse, Al-Dossary et al. [4], identified PMCA4 protein in oEVs, associated to regulation of progressive and hyperactivated sperm motility. Bathala et al. [12], showed the presence of tyrosine phosphorylated sperm proteins, which play a key function in sperm capacitation. Fereshteh et al. [31], showed that miR-34c-5p was localized in specific sperm head compartments, being highly concentrated in the centrosome after sperm-oEVs co-incubation. In Bovine, annexin family proteins were identified in oEVs in high abundance, which has been suggested to hold sperm cells in the oviductal reservoir (ANXA2). Other proteins identified were: OVGP1, HSPA90, HSP70, gelsolin and ezrin, which have been associated with key roles in gamete-oviduct interactions [7]. Functional experiments have shown that HSPA8 is involved in enhancing sperm survival and improving sperm fertilizing ability [29,68]. Different mRNAs for CATSPER units were identified in oEVs, which has been associated to sperm Ca^{2+} channels and related to male fertility. Dysregulation of miR-449a, found in oEVs, has been associated with defective cilia in the oviduct leading to female infertility [94]. In the pig, oEVs supplementation during IVF (sperm-oocyte co-incubation) improved monospermy rates and total efficiency of IVF system [98]. Such improvement was associated to proteins identified in oEVs with known roles in sperm viability, sperm motility, sperm capacitation, sperm-binding, polyspermy regulation and fertilization [24] such as OVGP1, MYH9, ANXA1,4 and 5. In the domestic cat, oEVs contained proteins essential for fertilization such as OVGP1, HSP70, HSP90, CD9, CCT8, CCT7, which has been suggested to be responsible for increasing the fertilization rates when oEVs where pre-incubated with spermatozoa before IVF. In canine, it has been shown that oEVs contained miR-30b, miR-375 and miR-503 with key roles in follicular growth and oocyte maturation, which could explain the increased in vitro maturation rates of oocytes when oEVs were used as a supplement.

3. Extracellular vesicles derived from the early embryo

In the last couple of years, there has been a great increase in the number of studies on eEVs, suggesting them as: vectors of communication in the early embryo-maternal dialogue, in embryo-to-embryo communication and regulators of further embryo development. Here, we summarized the main findings of eEVs, their molecular cargo and potential associated functions (Table 2).

3.1. Embryo-derived extracellular vesicles and their source of origin

Most studies performed up to date have used the spent embryo culture medium (SEM) after culturing embryos from different origins or embryo stages as a source to obtain eEVs. However, the use of SEM as eEVs source has some limitations, since EVs as well as miRNAs could also be derived from: components of the IVC media (protein supplements and serum) [2,49,91]; the cumulus cells remaining in the culture drop or attached to the embryo [10,60]; or the spermatozoa still in the media or attached to the Zona Pellucida (ZP).

Recently, Battaglia et al. [14], identified and characterized EVs in human blastocoel fluid (BF), proposing this source of eEVs as a better option to overcome the limitations of the SEM. Given the tiny amount of BF sample/embryo, miRNA profiles of the BF were analysed instead of eEVs isolated from BF. Therefore, although BF could be a better source of EVs, the very low volume of the BF can limit characterization studies and/or analysis by different omics technologies after isolation of EVs. Moreover, we raise the question if eEVs released into the culture media might be the same as the eEVs secreted and kept inside the BF. It can be hypothesized that they represent two different sources of eEVs, with different molecular cargo, and bringing different information regarding the competence of the embryo. Both together might provide a more complete picture of embryo-maternal communication [36] and embryo-embryo communication [76]; and even between different embryonic cells types (inner cell mass (ICM)-trophoblast cells communication) [25].

Interestingly, eEVs secretion varies according to the

Table 2
Summary of published studies related to embryo-derived extracellular vesicles (eEVs).

Species	Year	Citation	eEVs content Analysed	Findings
Bovine	2017	Mellisho et al. [67]	–	Identification of eEVs from IVP blastocysts by IVF or PA
	2017	Qu et al. [77]	–	Identification of eEVs derived from SCNT embryos
	2019	Andrade et al. [10]	miRNAs	Oxygen tension modulates EVs secretion and their miRNA content in IVP bovine embryo-cumulus during in vitro
	2018	Pavani et al. [76]	–	BSA is not a contaminating EV source in IVC media; eEVs were internalized by blastocysts cells
	2019	Taqi et al. [88]	Specific mRNA:	EVs secretion into IVC media vary according to the IVP embryo sex and the oxygen tension (5 vs 20%), with different mRNA cargo
	2019	Mellisho et al. [99]	Specific miRNAs:	Embryo classification as a competent blastocyst based on secreted eEVs and miRNA cargo: non-invasive tool for embryo selection
Murine	2018	Kim et al. [49]	–	The outgrowth embryo (day 7.5) derived eEVs that enhance embryo developmental competence and implantation potential
	2018	Pallinger et al. [72]	PIBF protein (by IEM)	Mouse produce eEVs that contain PIBF, which plays an immunological role at the feto maternal maternal
	2019	Bognar et al. [17]	–	The effect of light exposure increased release of eEVs compared to embryos in darkness: measured by PI-EVs and FC.
Equine	2012	Bemis et al. [15]	Specific Proteins and MiRNAs	EVs secreted by day 8 embryos can modulate the oviductal epithelium through the transfer of HSP10 and miRNAs. (in SEM, not in EVs)
Porcine	2014	Saadeldin et al. [82]	Specific mRNAs	First evidence IVP embryos can secrete EVs in their IVC media as mediators of the embryo microenvironment and embryo-to embryo cross-talk. (PA and SCNT embryos)
	2018	Nawaz et al.,[a]	miRNAs	Identification of eEVs and their miRNA cargo: Comparative analysis between embryonic cells, embryonic EVs and semen EVs. No description of specific miRNAs
	2013	Gardiner et at.,[b]	–	Increasing EV size strongly associated with decreasing embryo quality; EVs concentration increase with developmental stage
Human	2017	Abu-Halima et al. [2]	miRNAs	Differential EVs concentration and miRNA cargo between pregnant and non-pregnant (pregnant less EVs; less miRNAs) (in SEM, not in EVs)
	2017	Pallinger et al. [71]	–	Characterization of human eEVs by measuring only those EVs positive for DNA staining using Flow cytometry (PI + EVs)
	2018	Giacomini et al. [36]	Specific Protein and mRNAs	Secretome of IVC human embryos contains eEVs, eEVs are taken up by maternal side (in vitro endometrial epithelial cells)
	2018	Gunnala et al.,[c]	Proteins	EVs in SEM from blastocyst contained higher EVs concentration and higher protein concentration than day 3 or control samples. Mass Spectrometry identified 30 proteins with higher expression in SEM from embryos compared to control. (in SEM, not in EVs)
	2018	Tankov et al.,[d]		Isolation of eEVs from single human embryo IVC by SEC; EVs Concentration higher in embryos day 5 compared to day 3
	2018	Fraikin et al.,[e] 2018 ISEV	–	Microfluidic resistive pulse sensing (MRPS): novel system to predict embryo implantation by quantifying EVs in SEM
	2019	Vyas et al. [91]	–	Ultrastructural analysis showed that human embryos at different stages secrete eEVs, transported across ZP into IVC media
	2019	Battaglia et al. [14]	miRNAs	Identification of eEVs in BF of human embryos; miRNA profile of BF (not in EVs): potential tool for assessing embryo competence
	2019	Godakumara et al.,[f]	–	Differential respond of endometrial cell line (RL95-2) to eEVs from normal and degraded embryos based on ZNF81expression

#All sources of eEVs were based on spend embryo culture media (SEM) except Battaglia et al. [14], that used Blastocoel fluid; In vitro production of embryos (IVP); In vitro embryo culture (IVC); In vitro fertilization (IVF): Parthenogenetic activation (PA); Somatic cell nuclear transfer (SCNT).
[a] Nawaz et al., 2016 Personal communication ISEV.
[b] Gardiner et at., 2013 Personal communication ISEV.
[c] Gunnala et al., 2018 Fertility and Sterility Personal Communication.
[d] Tankov et al., 2018 ISEV Personal Communication.
[e] Fraikin et al., 2018 ISEV Personal communication.
[f] Godakumara et al., 2019 ISEV Personal communication.

biotechnology used to produce the embryos. Mellisho et al. [67] showed differences in eEVs size and concentration between parthenogenetic (PA) and in vitro fertilization (IVF) embryos. Other technologies such as intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT) may also provide different eEVs. We believe that these differences may also be reflected in the eEVs molecular cargo, although further studies are needed to confirm it. Additionally, the use of in vivo embryos cultured in vitro

for some days or embryos produced completely in vitro (IVP) has associated consequences due to the in vitro conditions, as we have seen in oEVs from in vivo and in vitro origins [7]. Regardless the efforts directed to improve IVP conditions, the quality of IVP embryos remains lower compared to in vivo embryos [79]. However, obtaining eEVs from in vivo sources is difficult, given that oEVs and eEVs are mixed in the oviductal fluid. Recently, a transgenic dual color Cre-reporter mouse model was developed to investigate the fetal-maternal EVs dialogue, being able to isolate and characterize eEVs from maternal plasma [85]. Similar approaches, such us using embryo- and oviduct-specific EVs biomarkers during the early reproductive events could help to discriminate in vivo oEVs and eEVs in the oviductal fluid. Nevertheless, regardless the source of the embryo, all secrete eEVs support embryo development in vitro in different species and even enhance implantation potential (porcine: [82]; bovine [77]: murine [49]).

3.2. Embryo-derived extracellular vesicles secretion depends on different factors

Considering the current literature, it seems there is a consensus -that eEVs concentration increases with the developmental stage (Table 1). Several studies in human embryos have shown that early cleavage embryos (day 3) release lower amounts of EVs compared to the blastocyst stage (day 5) [36,91]. In the pig, small size EVs (<40 nm) were identified in SEM of embryos from two-cell stage until blastocyst stage and greater size (<120 nm) from blastocyst to hatched stage, which was associated to the pore size of the zona pellucida [65,82].

However, recent studies have shown that the concentration not only depends on the embryo developmental stage but also the embryo sex and IVC conditions. EVs concentration was found higher for day 7 bovine embryos compared to day 3 under 20% oxygen tension, while under 5% an inverse pattern was observed [10]. Taqi et al. [88] showed that male embryos release a higher concentration and bigger sized EVs under 20% O_2 and female embryos secrete higher concentrations and bigger sized EVs under 5% O_2 (no statistical significance).

An association between the release of higher amounts of eEVs into the SEM with less competent embryos has also been suggested. By using flow cytometry, Pallinger et al. [71], were able to identify EVs in the SEM without isolation and to correlate lower concentration of EVs in the SEM with embryos resulting in “clinical pregnancy”. Since this approach was based on DNA detection in EVs (propidium iodide binding to DNA from EVs), it may be biased by the selection of a subpopulation of EVs in the SEM. Incompetent embryos have more apoptotic cells and may release higher amounts of DNA packed via EVs into the SEM. In the same line, Abu-Halima et al. [2] showed that there was a positive correlation between embryos leading to successful pregnancies and lower concentration of EVs in the SEM compared to embryos with higher concentration of EVs that did not lead to pregnancies. This study also revealed differential miRNA profiles analysed from total SEM between embryos resulting in a pregnancy or not and a smaller number of miRNAs obtained in embryos resulting in a pregnancy. It has to be noted that the obtained miRNA profiles can only be associated in part to embryo-derived EVs, since miRNAs have also been found in the culture media [93] and EVs were not isolated for miRNA analysis. Factors such as light exposure with detrimental effects on embryo development seem to also increase the EVs release by the embryo during culture in mice [17]. By contrast, when microfluidic resistive pulse sensing (MRPS) was used to measure EVs secretion into SEM and to predict embryo implantation potential by quantifying EVs in SEM, higher concentrations of EVs in the media was found for successfully implanted embryos (Fraikin et al., 2019 personal communication). Although the authors mentioned that they have used a small number of samples, they found that MRPS analysis predicted pregnancy outcome with 80% sensitivity and 80% specificity.

Regarding the correlation between the EVs size and embryo competence, only one pilot study using 18 samples has shown that increasing EVs size was strongly associated with decreasing quality (202 nm good, 218 nm average, 222 nm poor and 227 nm arrested embryo development) (Gardiner et al., 2013 personal communication).

3.3. Embryo-derived extracellular vesicles and their molecular cargo

To date, only very few studies have been performed to analyse specific proteins in eEVs (HLA-G “major histocompatibility complex, class I, G” [36] and PIBF1 “progesterone immunomodulatory binding factor 1”, [72]). Regarding mRNA eEVs cargo, Saadeldin et al. [82] showed that porcine eEVs mRNAs were derived from genes related to pluripotency (*OCT4*, *SOX2*, *KLF4*, *MYC* and *NANOG*). After eEVs-embryo co-culture, some of these embryonic mRNAs were significantly increased (*OCT4*, *KLF4*, and *NANOG*), while others (*MYC* and *SOX2*) did not show a significant difference compared to the control group. Similarly, Giacomini et al. [36] showed that *NANOG* and *POU5F1* transcripts were present in human eEVs on day 3 and 5 of embryo development, but not *SOX2* and *KLF4*. Taqi et al. [88] analysed the abundance of mRNAs encoding specific transcription factors (*NFE2L2, NOTCH1, KLFA, E2F*), antioxidant proteins (*SOD1, CAT*) and proteins related to EVs biogenesis (*ALIX, VPS4B, TSAP6*) and EVs secretion (*RAB11FIP1, RAB35 and RAB27A*) in eEVs. Interestingly, these authors showed that the abundance of those mRNAs in eEVs depends on oxygen tension in IVC conditions and the sex of the embryos from which they were released. These findings suggest a selectivity of molecules to be exported to EVs, which may be involved in cell-to-cell communication processes or in maintenance of cellular homeostasis [41]. Besides, it suggests that male and female embryos may face the environmental stress

Table 3
MicroRNAs identified in embryo-derived extracellular vesicles or in culture media of embryos.

miRNAs (eEVs miRNA studies/spent embryo culture media studies)	No. of studies[a]
miR-16-5p (3/4), miR-518e-5p (6/1)	7
miR-518c-5p (4/2)	6
miR-223-3p (3/2)	5
miR-155-5p (3/1), miR-17-5p (2/2), miR-191-5p (1/3), miR-200c-3p (3/1), miR-24-3p (2/2), miR-26a-5p (2/2)	4
miR-10b (2/1), miR-1246 (3/0), miR-150-5p (3/0), miR-204-5p (2/1), miR-20a-5p (2/1), miR-20b-5p (2/1), miR-21-5p (2/1), miR-221-5p (3/0), miR-222-3p (2/1), miR-296-5p (2/1), miR-301a-3p (2/1), miR-30b-5p (1/2), miR-30c-5p (1/2), miR-31-5p (2/1), miR-320a (2/1), miR-331-3p (2/1), miR-371a-3p (2/1), miR-373-3p (1/2), miR-425-5p (2/1), miR-449b-5p (2/1), miR-454-3p (3/0), miR-512-3p (2/1), miR-517a-3p (2/1), miR-517c-3p (2/1), miR-518b (2/1), miR-519d-5p (2/1), miR-520a-5p (3/0), miR-526b-5p (2/1), miR-9-5p (1/2)	3

[a] (2,10,14,15,16,21,28,39,53,56,66,73,81,84)

associated to IVC conditions (e.g., hypoxia) in a different way.

Regarding the miRNA content in eEVs, only two studies have analysed miRNAs in eEVs derived from bovine embryos [10,66]. Studies derived from human and equine embryos have been based on analysing miRNAs present in the whole SEM [2,15] or in the blastocoel fluid [14] without isolating the EVs. MicroRNAs in the SEM can be found packed in EVs but not all miRNA might be associated to EVs. Moreover, miRNA found in SEM can be part of the IVC media formulation and not released by the embryo. Therefore, in the present review we attempt to differentiate studies analysing miRNAs typically secreted by early embryos but found in different embryo sources (SEM, BF and EVs). With this aim, a total of 383 miRNAs were identified in a total of 14 studies in SEM, BF or EVs (Supplementary Table S1). The most frequent miRNAs related to embryos and obtained from SEM or eEVs are shown in Table 3. Unfortunately, about half of the studies did not provide full lists of identified miRNAs, which clearly limited our comparison. Therefore, we strongly encourage researchers to provide not only the number of identified miRNAs per group or treatment, but also the full list of miRNAs and sequences, in supplementary materials or in repository data, which can help other researchers speeding up the findings in this field of research.

Two miRNAs (miR-16-5p and miR-518e-5p) were found in half of the studies (Table 3). The expression of miR-16-5p seems to be important for the whole pregnancy period starting from oocyte maturation since the expression in follicular fluid has been found as upregulated in case of top-quality embryos [62]. Furthermore, an association of miR-16-5p expression with foetal growth has been found [61]. Dysregulation of miR-16-5p, miR-518e-5p, miR-518c-5p, miR-155-5p, and other miRNAs shown in Table 3 has been shown in the context of preeclampsia [35,45,96,97]. Studies in mice revealed that miR-223-3p suppresses leukemia inhibitory factor expression and pinopodes formation resulting in diminished embryo implantation [27]. Further evidence for a regulatory role of miR-223-3p in embryo implantation provided a comparison of embryonic miRNA profiles of normal and ectopic pregnancies where miR-223-3p has been found as upregulated in ectopic pregnancy embryo samples [26]. A comparison of early and hatched bovine blastocysts revealed miR-155 as upregulated in hatched blastocysts [37]. Likewise, miR-200c has been found as upregulated in outgrowth embryos compared with non-outgrowth blastocysts in mice [48]. Furthermore, miR-200c has been identified as embryonic stem cell-specific miRNA [87]. MicroRNA-26a was found as differentially expressed during porcine embryo development and detected in uterine EVs in the pig on days 14 and 16 of pregnancy [51]. The recent identification of miR-17-5p in human blastocoel fluid [14] further supports the results of an earlier study where miR-17-5p was found as differentially expressed in developing mouse embryos and suggested to be involved in the control of stem cell differentiation [34]. In a comparison of in vivo and in vitro-derived porcine embryos, miR-24 has been found with higher levels in IVF embryos [86]. Human embryos derived from IVF secreted specific miRNAs that varied depending on the fertilisation method, the chromosomal state of the embryos and whether or not they successfully implanted [81]. For example, in culture medium of aneuploid human embryos, miRNA-191 was more abundant, whereas miRNA-191, miRNA-372 and miRNA-645 were mostly highly concentrated in the medium from embryos of failed IVF cycles [81]. In bovine, eleven miRNAs were found to be differentially expressed between embryos that successfully developed to the blastocyst stage and the degenerated embryos. Interestingly, all these miRNAs were higher in concentration in the culture medium of degenerating embryos [52] compared to good embryos. In bovine IVF embryos, increased expression of miR-24 and miR-191 has been found in conditioned media of degenerated embryos [52]. Furthermore, addition of a miR-24 mimic miRNA to culture media of morulae embryos resulted in decreased development to the blastocyst stage [52]. In the context of different sources of stress during embryo in vitro culture, the results of the study by Kasper et al. [47] suggested that miR-24 has an important role in the response and resistance of embryos to stress.

All these findings suggest that embryos may secrete different miRNAs depending on their stage of development, the source of the embryo and the embryo competence. These miRNAs maybe secreted directly into the SEM or the maternal environment or packed in EVs. These miRNA-EVs may be taken up by other embryos in the in vitro culture or in vivo by the maternal tract, and may activate or inhibit signaling cascades in the target embryo or maternal reproductive cells. However, the current knowledge about the eEVs secretions (size, concentration) or their content (miRNAs and other molecular cargo) is not good enough to reveal essential information regarding the embryo competence or ability to maintain a proper communication and exchange of molecular signals with the maternal environment as indicators of the embryo competence. Further investigations are needed to support the use of eEVs as potential biomarkers of embryo quality and indicators of embryonic competence [64].

3.4. Embryo-derived extracellular vesicles: multi-signal messengers in embryo-to-embryo communication

Two studies have demonstrated that eEVs can enter other embryos by passing though the zona pellucida (ZP) and in this way, bring new factors to improve their development and playing an important role in inter-embryo communication during embryo culture in groups. Saadeldin and colleagues [82] demonstrated for the first time that IVP embryos secrete EVs and proposed that these embryonic EVs could be a novel mode of communication within their microenvironment and also between embryos in vitro. These results derived from a study using a co-culture system for paracrine communication between different kinds of porcine embryos (parthenogenetic (PA)) and cloned (obtained by SCNT). The study showed that the co-culture of porcine PA embryos significantly improved the in vitro development of cloned embryos and increased significantly the embryonic expression of some mRNAs (*OCT4*, *KLF4*, and *NANOG*) contained in these EVs. Moreover, these authors demonstrated that PKH67-labeled EVs could be internalized by the cloned embryos. Recently, Pavani et al. [76] demonstrated that bovine embryos also secreted EVs into the culture media (size 25–230 nm) and that PKH67-labeled eEVs can be taken up by zona-intact bovine embryos. Furthermore, eEVs used as supplement in the IVC media increased blastocyst rates at days 7 and 8 and improved embryo quality, with significantly decreased apoptotic cells [76].

3.5. Embryo-derived extracellular vesicles: multi-signal messengers in embryo-maternal communication

In sheep, Burns et al. [19], showed that EVs from in vivo elongating conceptuses collected on day 14 of gestation can release EVs in vitro after 24 h of IVC. The molecular cargo of conceptus-derived EVs was analysed in terms of proteins (n = 231) and mRNAs (n = 512). Additionally, using an elegant in vivo model, Burns et al. [19] showed that conceptus-derived EVs could be taken up by the uterine epithelia. Labeled conceptus-derived EVs were found in the cytoplasm of luminal epithelial and some glandular epithelial cells but were not detected in the uterine stroma or myometrium, as well as ovary, corpus luteum, or other analysed organs. These findings demonstrated that the elongating conceptus secretes EVs

that can target the uterine epithelium, serving as a novel form of cell-to-cell communication during the establishment of pregnancy.

In the pig, using an in vitro model, Bidarimath et al. [16], showed that trophectoderm cells EVs are taken up by porcine aortic endothelial cells and have functional effects on endothelial cell proliferation. From these findings, it can be postulated that EVs secreted by trophectoderm can be internalized by the endothelial cells of the developing vasculature of the endometrium to increase vascularization during conceptus implantation.

In humans, a recent study using day 3 and day 5 pre-implantation embryos showed that embryos at those stages release EVs during IVC that can be taken up by the maternal side (in vitro endometrial epithelial cell line) [36]. To the best of our knowledge, this is the only study showing a traffic of EVs from blastocyst stage embryos to the maternal tract. Furthermore, the study showed a higher uptake of EVs derived from day 5 when compared to day 3 EVs. Additionally, the authors used for the uptake studies EVs obtained from control media (without embryos) but containing protein supplements (presence of EVs derived from media) and showed that these EVs were not taken up by the endometrial epithelial cells. Altogether, this suggests that the maternal tract can distinguish either the origin or the molecular cargo of EVs, being able to regulate the uptake of embryo-derived EVs by the endometrium. These findings point at EVs as a part of a maternal-embryo recognition system.

4. Comparative analysis of the molecular content of oviductal and embryo-derived extracellular vesicles: what makes them different?

This review has gathered cumulative evidence that both oEVs and eEVs have the potential to improve in vitro embryo development. These findings raise the question if there are molecules present in both types of EVs responsible for those beneficial effects and if there are molecules that are specific for each type of EVs. To bring some light into these questions, we compared the molecular cargo of oEVs and eEVs at the miRNA level. Our analysis has been limited by the scarce knowledge of their molecular cargo up to date. In depth analysis of their cargo at different levels (proteins, RNAs, lipids and metabolites) will provide valuable information about what makes them different and can be used as biomarkers of oEVs and eEVs.

Fig. 2 shows the overlap of miRNAs identified in murine and bovine oEVs [9,31] and in eEVs [2,10,73] [46,66]. The overlap of only 68 miRNAs seems to be small, but in consideration of the different techniques used for the identification of miRNAs and that the eEVs were collected from in vitro culture, it is reasonable. Furthermore, this finding indicates that there are miRNAs specific for oEVs and eEVs. A search in the literature showed that a good number of these 66 miRNAs have been described in the context of establishment of pregnancy, embryo development, and pregnancy perturbations. For example, the miR-125 family (including miR-125a-5p, miR-125b-5p and miR-351-5p) plays a crucial role in the regulation of maternal effect genes related to the two-cell stage block and zygotic genome activation in oocytes and embryos [50]. The miRNAs let-7b, miR-23a, miR-200c, miR-425, and miR-429 have been found as DE between outgrowth embryos and non-outgrowth blastocysts in the mouse [48]. Particularly, let-7b-5p, miR-200c-3p and miR-23a-3p were upregulated in outgrowth embryos, suggesting an important role in regulation of embryo attachment and interaction between the embryo and endometrium during the implantation process [48]. In a study of EVs-mediated communication at the porcine maternal-foetal interface, miR-126-5P and miR-296-5P have been identified in EVs released by trophoblast-cells and characterized as the most abundant angiogenic miRNAs important

Fig. 2. Venn Diagram showing overlap of identified miRNAs in oviductal extracellular vesicles (oEVs) and extracellular vesicles embryo-derived (eEVs).

for pregnancy success [16]. As mentioned above, miR-223-3p has been shown to be involved in embryo implantation in the mouse [27]. Overall, the known functions of miRNAs found in oEVs and eEVs points to a general function in reproductive processes.

5. Concluding remarks

The present review has summarized evidence provided so far for oEVs and eEVs as multi-signal messengers in the embryo-maternal cross-talk as well as embryo-to-embryo communication. Current knowledge provides a solid basis that oEVs have a crucial role in oocyte maturation, sperm capacitation and fertilizing ability, regulation of fertilization process and supporting early embryo development, first reproductive events leading to pregnancy. On the other hand, studies on eEVs have also shown their role in supporting early embryo development. These features make both EVs great therapeutic vectors, particularly oEVs, as enhancers of sperm function and, both as boosters of in vitro embryo development. Besides, studies on eEVs emphasize their potential as a non-invasive diagnostic tool of embryonic competence. However, several challenges should be addressed before oEVs and eEVs can be used as therapeutic assets or diagnostic tools.

Regarding the oEVs, the precise molecular cargo and their functions under physiological or pathological reproductive conditions, as well as the extend of the oEVs reproductive effects on gametes/embryos to the delivery of healthy offspring and the later impact in adulthood needs to be well understood. The development of more sensitive technologies to quantify and characterize EVs from tiny volumes of samples with robust and reproducible results, will help in this matter. Altogether, it will pave the way for the application of oEVs to improve the diagnosis and treatment of

infertility and pregnancy disorders and increase the success of ARTs in livestock and human.

Regarding the eEVs, progress on EVs technologies to be able of assessing concentrations, size and cargo of EVs from tiny samples, together with a consensus of which EVs parameters define a competent embryo with great possibilities to implant, no matter the technology used, needs yet to be developed and discussed. This will help to standardize procedures in ARTs laboratories, being able to use EVs features combined with patient information to predict the embryo competence and support the decision of embryo transfer. Furthermore, although the DNA content of eEVs has not been the topic of interest of many research studies up to date, it could also bring valuable information regarding the embryo ploidy status. Embryonic DNA derived from eEVs could be used for pre-implantation genetic testing [20,64] avoiding the invasiveness or contamination issues associated with other DNA sources such as blastomeres or blastocoel fluid.

Finally, considering the therapeutic applications, efforts should be directed towards the development of synthetic EVs-like vesicles or their specific cargo for clinical applications combined with functional studies of their effects on gametes/embryos, to reduce the sources of variability and enhance the application across individuals. This will allow to set up directions for the application of EVs as therapeutic vectors in ARTs and increase pregnancy rates.

Author contributions

Conceptualization, investigation, original draft preparation and editing were performed by CA and SB.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.01.077.

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