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DOI: 10.1071/RD16318
Emerging role of extracellular vesicles in communication of preimplantation embryos in vitro

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Abstract

In vitro, efficient communication between mammalian embryos and between embryos and their environment, e.g. maternal somatic cells, implies that there is a sender, a message and a receiver which is able to decode the message. Embryos are secreting a variety of autocrine and paracrine factors, and among those, extracellular vesicles have recently been implicated as putative messengers in embryo-embryo communication and in communication of the embryo with the maternal tract. Extracellular vesicles (EVs) are membrane-bound vesicles, found in biofluids and in culture media conditioned by the presence of embryos or cells, that carry and transfer regulatory molecules, such as microRNAs (miRNAs), messenger RNAs (mRNA), lipids and proteins.

Here, we conducted a systematic search of the literature to review and present the currently available evidence on the possible roles of EVs in embryo communication and embryo development. It is important to note that many of the biologically plausible functions of EVs in embryo communication have not yet been substantiated by conclusive experimental evidence. However, indirect evidence, such as the use of media conditioned by embryos or by somatic cells with improved embryo development as a result, may indicate that EVs can be an important asset for the development of tailor-made media allowing better embryo development in vitro, even for single embryo culture.

Additional keywords: Extracellular vesicles, embryo communication, embryo-maternal communication, embryo culture
Introduction

Efficient communication between cells and tissues is paramount in many physiological processes, including embryo development. Typically inside the body, mammalian cells communicate with each other either through direct interaction (juxtacrine signalling) or by secreting molecules such as growth factors, hormones and cytokines. These messengers can turn on the cell or embryo itself (autocrine signalling$^1$), or have an effect on both neighboring (paracrine signalling) and distant cells (endocrine signalling). Cell-cell communication is however changing completely when cells are being cultured outside the body, in vitro.

Mammalian preimplantation embryos develop in vivo inside the female genital tract, i.e. the oviduct and the uterus, and communicate with these dynamic and elastic surroundings on which the embryo depends for its development and survival (Fazeli 2011). In the absence of a genital tract, when embryos are being cultured in vitro, the embryo resides in a semi-defined culture medium in which no endocrine or paracrine factors are present, since all communication with the maternal genital tract is cut off. This communication can be restored by embryo co-culture with somatic cells such as cumulus cells (Goto et al. 1988; Goovaerts et al. 2009), oviduct cells (Eyestone et al. 1989; Gandolfi and Moor 1987; Van Soom et al. 1996, 1997; Liu et al. 2001; Lee et al. 2001; Xu et al. 2001; Lee et al. 2004), and medium conditioned by somatic cells (Mermillod et al. 1993; Van Langendonckt et al. 1996; Li et al. 2004a; Li et al. 2004b). This approach was very popular in the late 20th century to mimic the microenvironment conditions associated with the maternal tract. Nevertheless, even without communication with cells from

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$^1$ The term autocrine is here also used to refer to signaling between similar cells, like embryos
the genital tract, preimplantation embryos are able to promote their own development in vitro by the production of autocrine factors (Paria and Dey 1990), and in this way they are able to communicate to each other. Mostly this accumulation of autocrine factors is typically achieved by culturing bovine embryos in large groups, some 10-25 embryos in a 50 µl droplet of medium covered by oil to avoid evaporation (Sagirkaya et al. 2007; Goovaerts et al. 2009; Wydooghe et al. 2013) (Fig. 1a).

The presence of these autocrine factors in the medium when embryos are cultured in group lies at the basis of the embryos’ superior development in group compared to solitary culture (Paria and Dey 1990; O’Neill 2008). Group culture has been adopted by many research groups as a routine procedure for animal embryo culture, leading to superior embryo development (Vajta et al. 2000; Hoelker et al. 2010). By playing with embryo density, expressed as the number of embryos per volume of medium, it has been shown that embryos develop best in groups cultured at an embryo-volume ratio ranging from 1:1 (Ferry et al. 1994) to 1:5 (Fukui et al. 2000) (Table 1). When embryo-volume ratio is being kept at 1:10 or 1:20, and embryos are cultured individually in a droplet of medium (Fig. 1b), development to the blastocyst stage is much lower to even non-existing in a suboptimal medium such as medium containing fetal calf serum (FCS) (Table 1). Both group culture of embryos, and co-culture of embryos with somatic cells can reduce the negative effects of serum during embryo culture (Donnay et al. 1997; O’Doherty et al. 1997; Goovaerts et al. 2009; Goovaerts et al. 2012). Therefore it appears that some factors released by the adjacent embryos or by the co-cultured somatic cells are either affecting the development of the neighboring embryos in a positive way or are removing a detrimental factor associated with the serum. Interestingly, in serum-free medium, the positive
effects of group culture remain present but to a lesser extent, and these so-far unidentified embryotropins have been demonstrated to promote development, with higher blastocyst cell numbers and less apoptosis (Wydooghe et al. 2014b). This inter-embryo communication has only been identified after in vitro culture of embryos became commonplace. What the exact nature of this communication is, is at present not entirely clear, and a vast range of possible autocrine factors have already been implicated to be important in how embryos ‘talk’ to each other (for review see: Wydooghe et al. 2015). Embryos also ‘talk’ to the somatic cells used in various co-culture models (for review see: Lee and Yeung 2006; Ulbrich et al. 2010).

While many studies have been trying to identify the nature of these autocrine factors, or to relate these factors with markers of embryo quality, the main approach so far was to analyze conditioned medium for the presence of proteins, growth factors, or metabolites (Mermillod et al. 1993; Beardsley et al. 2010; Kropp and Khatib 2015; Foresta et al. 2016). This may be useful, but in this way an important means of cell-cell communication is being overlooked. Shedding of extracellular vesicles (EVs) is now a well-recognized, important method of cell-cell communication in a number of different cell types: EVs have been purified from every prokaryotic (Kim et al. 2015) and eukaryotic (Regente et al. 2009; Oliveira et al. 2010a; Mantel and Marti 2014; Cocucci and Meldolesi 2015) cell type that has been studied to date, including stem cells (Ratajczak et al. 2006; Camussi et al. 2011; Lai et al. 2011; Timmers et al. 2011; Chavez-Munoz et al. 2010), primary cells of immune and nervous systems (Chavez-Munoz et al. 2010; Faure et al. 2006; Guescini et al. 2010; Kesimer et al. 2009; Potolicchio et al. 2005) and various cancer cell lines (Ai-Nedawi et al. 2008; Skog et al. 2008; Ai-Nedawi et al. 2009). Extracellular vesicles are vesicles that are being shed by healthy cells, and are often referred to as microvesicles,
exosomes, or microparticles (Raposo and Stoorvogel 2013). They contain as a cargo, amongst
other molecules, proteins, lipids, RNAs and miRNAs, that may serve as messengers between cells.

However, due to lack of knowledge on the molecular mechanisms for EV formation and lack of
methods to interfere with the packaging of cargo or with vesicle release and addressing to
receiving cells, it is still difficult to assess the physiological relevance of EVs *in vivo* (Raposo and
Stoorvogel 2013; Yañez-Mó *et al.* 2016). *In vitro* model systems such as embryo group culture,
and embryo-oviduct co-culture, may become important tools to study these fascinating
structures. Here we review the current literature as to release of EVs by preimplantation embryos
and we will provide evidence that they may be much more important in embryo-to-embryo or
embryo-maternal communication as previously thought. We will also focus on technical aspects
of EVs isolation, in order to instigate more research into this fascinating topic. A better
understanding of the role of EVs in embryo culture and development may lead to improved
knowledge on how embryos communicate with their environment and to the development of
new *in vitro* culture systems for both animal and human embryos.

**Classification and biogenesis of extracellular vesicles**

As reviewed by Machtinger *et al.* (2015), EVs have been pointed out to be essential players in
gamete maturation, fertilization and embryo implantation. The term ‘extracellular vesicle’ is
generally applied to describe different vesicle types, including exosomes, microvesicles,
apoptotic bodies and in pathological situations, necrotic debris.

a) Exosomes
Exosomes are rounded phospholipid bilayer vesicles, and are in general smaller than microvesicles, with a size ranging from 40-150 nm (Table 2). Exosomes are formed in the late endosomal compartment by inward budding of the membrane of late multivesicular bodies (MVBs) (Fig. 2). Formation of intraluminal vesicles in multivesicular bodies has been shown to involve the endosomal sorting complex required for transport (ESCRT); apart from this, studies indicate that these vesicles can develop independently of this complex (Trajkovic et al. 2008). ESCRT has been shown to be involved in inward budding of intraluminal vesicles of MVBs and cleavage of the necks of these vesicles. When the vesicles are present in MVBs they can be released as exosomes by fusion of MVBs with the plasma membrane or alternatively be degraded via lysosomal fusion (Hurley et al. 2010). Emission of exosomes from the endosomal compartment of MVBs through fusion with the plasma membrane is also dependent on intracellular calcium (Théry et al. 1999; Savina et al. 2005). Many cytoplasmic proteins are present in exosomes including cytostructural proteins such as actin, annexins, tubulin and actin-binding proteins as well as signaling proteins such as signal transduction kinases, cytokines, and heterotrimeric G-proteins (for the whole known protein contents of exosomes, see Exocarta: http://www.exocarta.org). β integrins and ICAM-1 are also found on the exosomal surface as are the tetraspanins CD9, CD63, CD81, and CD82, which are considered to be exosomal markers (Heijnen et al. 1999; Théry et al. 2009; Vlassov et al. 2012).

Once released from producing cells, EVs will reach their target cells in the vicinity or in a distant tissue through transit by biological fluid (blood flow or local fluid). They may be uptaken by target cells through different pathways. EVs can bind randomly to cell
membranes and fuse to deliver their contents in the cytoplasm of recipient cell in a non-specific manner. Alternatively, EVs can bind to a cell surface receptor through their surface proteins (integrins, tetraspanins). This pathway requires a specific receptor at the surface of the recipient cell. This binding can end up with activation of the receptor, inducing a signaling cascade in the cell, and/or internalization of the EVs contents by membrane fusion or by phagocytosis of the whole EV. Although these processes are not yet fully elucidated, they are probably all existing together or in different contexts.

b) Microvesicles

Microvesicles are supposed to be formed by outward budding of the plasma membrane (Fig. 2). They are mostly rounded vesicles with a size of around 100-1000 nm (Table 2). They exhibit similar composition in proteins and lipids to plasma membranes (Wolf 1967; Turiák et al. 2011; Dragovic et al. 2011. György et al. 2011a). Microvesicles are released in response to cellular activation or stress: initiated by rises in intracellular calcium, which eventually lead to the activation of scramblase and calpain resulting in microvesicle formation (Cocucci et al. 2009; Yuana et al. 2013).

c) Apoptotic bodies

Apoptotic bodies also belong to EVs (Yáñez-Mó et al. 2015) and are released as the cell is undergoing apoptosis. Apoptotic bodies are consisting of cytoplasm with tightly packed organelles with or without a nuclear fragment. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded
within phagolysosomes (Elmore 2007). They are heterogeneous in size ranging from 1000-5000 nm (Table 2). Intracellular calcium is increased during apoptosis serving as an initiating event for apoptotic body formation (Cocucci et al. 2009; Baj-Krzyworzeka et al. 2006).

In the present review we will focus on exosomes and microvesicles because of their emerging role in inter-embryonic and embryo-maternal communication. For reasons of clarity, we will refer to exosomes and microvesicles as EVs in the further text, and will not discriminate between the different classes, even if this was done in the original papers.

**Isolation and characterization of extracellular vesicles (EVs)**

Different isolation techniques have been described to collect EVs from cells or fluids. Extracellular vesicles can be isolated using three major methods; with variations possible, namely (a) ultracentrifugation; (b) adsorption to micro beads, or (c) size exclusion chromatography.

After isolation, they can be identified based on morphological properties by several imaging methods which include Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) analysis (that allows quantification of EVs by size, differentiating exosomes and microvesicles) and immunostaining of exosomal markers like CD9, CD63 or HSP70. It is still a problem to find good markers to differentiate exosomes from microvesicles and different isolation methods can change the content of the EVs and abundance (Sunkara et al. 2016).
So far no specific marker can be used to distinguish the subtypes of EVs since protein components of the endosomal sorting complexes required for transport (ESCRT complex), such as Alix and TSG101, and membrane proteins such as CD9, CD81 and CD63 are enriched with either exosomes or microvesicles, depending on size and lower relative abundance (Raposo and Stoorvogel 2013). Moreover EV populations are not yet completely defined by researchers, as the EV subtypes released by cells varies from cell to cells.

a) Ultracentrifugation

Differential ultracentrifugation can be used for the isolation of EVs (Théry 2006; Witwer et al. 2013). The fluid of interest is subjected to repeated centrifugations, each time removing the pellet and increasing the centrifugal force. Separation of EVs is based on their size and density, with larger and denser particles, which are not wanted, pelleting at lower centrifugal forces. During the initial steps conditioned medium is subjected to centrifugation at 300× g for 10 min; after which the supernatant is centrifuged at 2000× g for 10 min, followed by 10,000× g for 30 min of centrifugation. These first three steps of centrifugation are meant to remove intact cells, cell debris and dead cells or apoptotic bodies. In some strategies, these centrifugation step(s) have been replaced by 0.1 μm (Ji et al. 2008) or 0.22 μm (Théry et al. 2001) filtration. After the 10,000× g spin, the supernatant is then subjected to final ultracentrifugation at 100,000×g for 70 min. The final outcome of this rather time-consuming centrifugation method is an exosome pellet which can be stored for further analysis. The re-suspended pellets can be used for
checking the presence of microvesicles or exosomes through electron microscopy (Fig. 3a and b), immunofluorescence, or RNA extraction.

It is important to note that the isolation protocol of EVs varies between different cell types, as well as for the targeted population of the EVs is to be extracted. Exosomes (40-100 nm) are usually isolated by centrifugation at 100,000-200,000×g (Théry et al. 2006; Witwer et al. 2011), whereas microvesicles (10-1000 nm) are isolated by centrifugation at 10,000-20,000×g (Witwer et al. 2011; Baran et al. 2010). Apoptotic bodies (50-5000 nm) are obtained with a centrifugation of 2000×g (Jeppesen et al. 2014). It has also been shown that repeated ultracentrifugation steps can reduce the quality of exosome preparations leading to lower exosome yield (Lobb et al. 2015). Using ultrafiltration devices results in increased vesicle isolation when compared to traditional ultracentrifugation protocols (Lobb et al. 2015).

A similar and quicker method is density gradient centrifugation (Tauro et al. 2012; Van Deun et al. 2014). For density centrifugation, for instance a sucrose gradient can be used to isolate EVs. The primary function of density gradient centrifugation is to separate particles, either on the basis of their buoyant density or their rate of sedimentation.

(b) Immuno affinity isolation:

Another promising method used for EVs isolation involves microbeads, normally magnetic, that are coated with an antibody that recognizes certain markers present on
the EV surface. This technique can be used for EVs from cell culture media, or body fluids.

Initially the EVs samples are mixed with the antibody coated microbeads, and a magnetic force is applied to a column of microplate. This retains the EV covered microbeads, while the rest of the sample is discarded (www.systembio.com\exosomes). Further on, the microbeads with attached EVs are eluted using appropriate buffers and used for analysis.

Compared to other techniques this method has the advantage to select a specific EV population based on specific marker expression regardless of size of the EV (Vlassov et al. 2012).

(c) Size Exclusion Chromatography

This method is mostly used for a low speed centrifugation step that allows the removal of larger objects from the samples such as cellular debris, cell organelles etc. This is followed by a filtration step (0.8 and 0.2 µM pore size filter) to concentrate the EVs. The filtered EV samples are then subjected to size exclusion chromatography (normally gel filtration column) where small volume fractions are ultracentrifuged to pellet down the EVs (Müller 2012; Taylor et al. 2002; Böing et al. 2012 ). The major principle of this technique is that particles based on their size move towards the filtration column at different rates. Hence larger particles will elute more rapidly, whereas small ones will move slowly, due to their ability to penetrate the stationary phase (gel) of the column.

However this method has a few limitations, like forcing EVs passage through filter used to per concentrate the samples may lead to EV deformation and eventual rupture into smaller particles (Witwer et al. 2013).
Autocrine communication among embryos in vitro: role of embryo-derived extracellular vesicles

Like somatic cells, preimplantation embryos are able to produce and secrete autocrine factors by several mechanisms including active secretion, passive outflow, binding to a carrier molecule, or transport within extracellular vesicles (Wydooghe et al. 2015). However, unlike somatic cells, a glycoprotein layer is surrounding mammalian embryos, which is called the zona pellucida. This zona pellucida is composed of four glycoproteins (bZP1, bZP2, bZP3, and bZP4) and is typically visualized under the scanning electron microscope as a complex fibrous network with many pores (Vanroose et al. 2000; Van Soom et al. 2010). In bovine embryos, the pores are >50 nm in diameter, with 20–50% >200 nm (Vanroose et al. 2000). When the passage of fluorescent microspheres through and their location in the zona pellucida was assessed, the smallest beads (40-50 nm) were detected halfway through the thickness of the zona, whereas beads with a size of 200 nm were found only within the outer-fourth part of the zona pellucida (Vanroose et al. 2000). Using fluorescently labelled markers, Legge (1995) showed that the zona pellucida of murine oocytes is permeable to markers up to 170 kDa. Microvesicles of 40–150 nm diameter should be able to pass through these pores, since most lipids and lipid-containing molecules pass through the zona pellucida relatively easy (Turner and Horobin 1997). This hypothesis has elegantly been proven by Saadeldin et al. (2014): they derived EVs from medium conditioned by parthenogenetically activated pig embryos by differential centrifugation. Next the EV pellets were subjected to fluorescent labeling using PKH67 dye, a green fluorescent dye that labels the lipid membranes. Cloned embryos were exposed to these labelled EVs and it was shown that the
EVs can pass through the zona pellucida and are internalized by blastomeres. Moreover, analysis of culture media from porcine embryos cultured individually determined the presence of 30–120 nm vesicles differing in size according to the embryo’s age (less than 40 nm in cultures from two-cell embryos and less than 120 nm in cultures from blastocysts). An important aspect from the experimental set-up was that the culture medium used for porcine embryos was serum-free chemically defined PZM-5 medium (Saadeldin et al. 2014). When using serum-containing medium or medium with BSA, EVs derived from serum or BSA could interfere with the results. Gardiner et al. (2013) demonstrated that also human IVF embryos release EVs into the culture medium. Increasing EV size was strongly associated with decreasing embryo quality (202 nm good, 218 nm average, 222 nm poor and 227 nm arrested development).

Now how do these EVs impact embryo development? In the study of Saadeldin, cloned embryos cultured with porcine parthenogenetic embryos showed a significant increase in their developmental competency (i.e. increased number of blastomeres and better blastocyst formation) compared with cloned embryos cultured alone. Paradoxically, the addition of medium conditioned by parthenogenetic embryos on different time points, either along with the developmental course or preceded by 2 days, was not able to affect embryo development. Authors suggested that a continuous supply of EVs is necessary in contrast to an acute transfer, confirming the highly dynamic microenvironment created by embryonic secretions (Saadeldin et al. 2014). The EVs derived from parthenogenetic embryos and conditioned medium contained mRNA of pluripotency transcription factors (OCT4, SOX2, KLF4,CMYC and NANO). These transcription factors were also found in EVs derived from embryonic stem (ES) cells (Ratajczak et al. 2006). Recently it has also been reported that bovine and human pre-
implantation embryos secrete miRNAs into culture medium (Rosenbluth et al. 2014; Kropp et al. 2014). These miRNAs are secreted within EVs into the extracellular environment where these can be taken up by cells and act in autocrine or paracrine manner to impact gene expression. Human embryos cultured for IVF were found to secrete specific miRNAs which are varying depending on the fertilization method, their chromosomal state and whether or not they successfully implanted (Rosenbluth et al. 2014). In conditioned medium of aneuploid human embryos, miRNA-191 was more abundant, while miRNA-191, 372 and 645 were mostly highly concentrated in medium from embryos of failed IVF cycles. In horses, an in vitro study (Bemis et al. 2012) suggested that EVs can be secreted by Day 8 embryos, which can modulate the functions of the oviduct epithelium through transfer of early pregnancy factor (HSP 10) and miRNAs. Kropp et al. (2014) examined miRNA secretion in day 5-8 in vitro cultured bovine embryos, and observed a clear differentiation of miRNAs expression between the embryos that successfully developed to the blastocyst stage and degenerate embryos. In total four miRNAs - 25,302c, 192a2 and 181 were found to be more prevalent in culture medium of degenerating embryos. It is apparently also possible to detect sex determining mRNAs, such as Xist and Sry, in the conditioned medium of in vitro–produced embryos cultured individually, which could be used for sexing (Saadeldin et al. 2015).

Paracrine communication between embryos and somatic cells in vitro: role of maternally-derived extracellular vesicles
In mammals, maternal-embryo communication is considered the basis for the success of any reproductive event (Rizos et al. 2002). The oviduct, or Fallopian tube, which is connecting the ovary to the uterus, plays a vital role in these interactions. In fact, it holds the first maternal cross-talk with gametes and early embryos and provides an optimal environment for fertilization and early embryo development. The oviductal epithelium is composed of ciliary and secretory cells responsible for the secretion of proteins and other factors that together with constituents derived from plasma, contribute to the formation of the oviduct fluid (OF) (Buhi et al. 2000; Leese et al. 2008). Later, the embryo will migrate to the uterus, and this dialogue will continue with the endometrium to ensure proper implantation. The role of the oviduct has been underestimated based on the ability to produce competent embryos in vitro which after transfer to the uterus establish a pregnancy and live calves, lambs, kids, and babies are born. However, it has been evidenced that embryos cultured in the oviducts of different species are of superior quality to those produced in vitro, in terms of morphology, gene expression, cryotolerance and pregnancy rate after transfer (Lazzari et al. 2010; Rizos et al. 2010; Besenfelder et al. 2012), indicating that the oviduct is not merely an organ of transit.

Despite the fact that in vitro culture conditions are capable of supporting a “relatively high” percentage of blastocysts (30 to 40%), they provide a suboptimal environment reflected on the quality of the produced embryos with short and long term consequences (Rizos et al. 2008). Thus, the goal of in vitro embryo production is to simulate as closely as possible the conditions in vivo to obtain high quality embryos capable of continued development and implantation, and resulting in viable births (Menezo et al. 1998). Moreover, studying the oviductal environment is crucial to improve our understanding of the regulatory mechanisms controlling early
reproductive events (Avilés et al. 2015). While *in vitro* models provide a simple and defined context to study maternal interactions with gametes and embryos, their advantages are not limited to their simplicity. As Van Soom *et al.* (2010) pointed out, when choosing an *in vitro* model, the aim of the experiment is an important consideration. In studies of gamete and embryo interaction with the reproductive tract, the use of BOEC, OF and their EVs may be considered as the most appropriate *in vitro* models to mimic the physiological conditions pertaining *in vivo*.

The *in vitro* culture of BOEC has been considered a suitable model to produce embryos of better quality and also to study oviductal-embryo interaction (Ulbrich *et al.* 2010). These cells can be cultured as monolayers or cell suspension (Fig. 4). The drawback of monolayers is that they dedifferentiate losing important morphological characteristics (Rottmayer *et al.* 2006), including reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Thibodeaux *et al.* 1992), whereas short-term (24 h) epithelial cell suspension culture maintained morphological characteristics as well as gene markers present in the cells *in vivo* such as *OVGp1*, oestrogen and progesteron receptors (Rottmayer *et al.* 2006).

In the present review we will merely focus on the *in vitro* model consisting of coculture of primary BOEC with *in vitro* produced bovine embryos. Using this *in vitro* system, Schmaltz-Panneau and colleagues demonstrated that BOEC adapted their transcriptomic profile in response to the presence of embryos (Schmaltz-Panneau *et al.* 2014). Most of the genes regulated in BOEC by the presence of embryos are known to be interferon regulated, but other pathways may also be involved and triggered by other embryonic signals. Moreover, when the levels of expression of genes suspected to be involved in embryo development support were evaluated (GPX4, OVGP, C3) in different regions of the oviduct (ampulla and Isthmus), a regional
difference was found (Cordova, personal communication).

Also the other way around, from BOEC to embryo, communication could be detected: Cordova et al. (2014) showed that the use of BOEC in embryo culture *in vitro* at the early stages of embryo development, up to day 4, improves embryo development and embryo quality in terms of specific gene transcripts. This period of culture coincides with the *in vivo* conditions where the embryo is still in the oviduct. Furthermore, BOEC co-culture with embryos for the first 4 days accelerated the kinetics of blastocyst development, with a significant increase in the number of blastocysts at days 6 and 7 compared to control and coculture during 8 days. BOEC from the isthmus were more capable of supporting early embryo development than BOEC from the ampulla, demonstrating a regional specialization of the oviduct in supporting embryo development (Cordova, personal communication). In addition, embryo transcriptomic analysis revealed that the level of expression of several genes related to embryo quality were altered as a result of the presence of BOEC, reflecting reduced embryo apoptosis and increased capacity to adapt against oxidative stress after coculture.

Taken together, these *in vitro* studies have shown the existence of a real dialogue between the early embryo and the oviduct, as a result of which, the embryo regulates its own environment in the maternal tract but also during *in vitro* culture. Soluble factors are probably involved in this cross-talk, binding to receptors on both embryo and maternal sides. However, recent studies indicate that there is room for other players in this embryo-maternal dialogue. Extracellular vesicles have been proposed as intercellular vehicles in the embryo-maternal dialogue in the uterus (Ng *et al.* 2013; Burns *et al.* 2014, 2016; Ruiz-Gonzalez *et al.*, 2015) and might also mediate the maternal-gametes/embryo interactions in the oviduct. To date, little is
known on how EVs could be taken up by gametes and early embryos and whether they modulate the maternal interactions to promote successful pregnancy. Recently, we demonstrated that an extended culture BOEC monolayer can be used successfully for embryo co-culture and conditioned media (CM) production, improving embryo development and embryo quality, most likely due to the presence of EVs secreted by the cells (Lopera-Vasquez et al. 2016a). This hypothesis was confirmed by the presence of $3 \times 10^5$ EVs/ml of a relatively homogeneous population of 150-200 nm in diameter obtained by ultracentrifugation from BOEC CM and assessed by transmission electron microscopy and nanoparticle tracking analysis (Nanosight) (Fig. 5). Also, it was verified by Western blot and bead-assisted flow cytometry analysis that these EVs expressed the classical markers of exosomes like tetraspanins CD9 and CD63, TSG101 and ERM proteins (Fig. 5). Furthermore, embryos cultured with EVs, irrespective of concentration ($3 \times 10^5=100\% ; 1.5 \times 10^5=50\% ; 7.5 \times 10^4=25\%$ EVs/ml) or processing (fresh or frozen/thawed) had similar blastocyst yield on Day 7, 8 or 9 (range on Day 8: 37.8-43.4%) when compared with controls. Likewise, the survival rate after vitrification/warming was higher at all points in time compared to controls (range at 72h; 48.7-56.5% vs 22.3% respectively). Blastocysts cultured with EVs displayed a higher number of total cells and expressed several genes related with embryo quality. On the other hand, EVs derived from FCS exerted a deleterious effect on embryo quality. Based on this evidence it can be concluded that EVs from BOEC may have an important function in the communication between the oviduct and the embryo during early stages of development. (Lopera-Vasquez et al. 2016a).

An important component of the oviductal environment is the OF. The composition of OF is very complex, containing simple and complex carbohydrates, ions, lipids, phospholipids and
proteins (Avilés et al. 2010). Some of these components are metabolic substrates (lactate, pyruvate, amino acids and glucose) and their concentrations are different from those in the uterine fluid and serum (Leese 1988; Hugentobler et al. 2007; Leese et al. 2008). It has been shown that specific oviductal secretions have an effect on oocyte and sperm function (Killian 2011; Mondejar et al. 2013), since oviductins, osteopontin, glycodelins and lactoferrin may play a role in gamete interaction (Ghersevich et al. 2015). When porcine oocytes were treated with OF before fertilization, a significant increase in cleavage rate and blastocyst yield was evident, suggesting protection of the embryo by OF against apoptosis and against adverse effects on mitochondrial DNA transcription or replication (Lloyd et al. 2009). When bovine oocytes were exposed to OF before fertilization, no effect was visible on embryo development and morphology of the resulting blastocysts; but differences appeared in specific transcripts of the embryos produced from oocytes treated with OF (Cebrian-Serrano et al. 2013).

It is worth to mention that, until recently, the OF was only used before fertilization. In a recent study we investigated the developmental competence of bovine zygotes and the quality of blastocysts produced after culture in SOF without FCS, but supplemented with different concentrations of OF. It was clear that >5% OF supplementation was detrimental for embryo development, while low concentrations of OF (1.25%) had a positive effect on development and quality of the produced blastocysts in terms of cryotolerance, cell number and expression of qualitatively related genes (Lopera-Vasquez et al. 2015). Thus, enhancing the post fertilization environment in vitro with substances present in the oviduct may diminish the limitations of in vitro embryos and make them comparable to their in vivo counterparts. This enhanced development may also be brought about by extracellular vesicles present in the OF. Almiñana
and colleagues isolated exosomes from bovine OF and co-cultured them with *in vitro* produced embryos to demonstrate the existence of oviductal-embryo communication via exosomes (Almiñana *et al.* 2015; 2016 published communication IETS). Extracellular vesicles were isolated by serial ultracentrifugation and measured by dynamic light scattering analysis and transmission electron microscopy, detecting exosomes (63-97 nm) and microvesicles (>100nm), both in OF (Fig. 3a) and culture media from BOEC primary culture (Fig. 3b). To demonstrate the existence of the oviductal-embryo communication via exosomes, oviductal exosomes were labelled with green fluorescent dye (PKH67), filtered (0.22µm) to remove microvesicles and co-incubated with *in vitro* produced blastocysts for 20 h, under 5% CO$_2$ and 5% O$_2$ conditions. Confocal microscopy observations confirmed that exosomes were internalized by blastocyst cells, demonstrating the existence of an oviductal-embryo communication via exosomes (Fig. 6).

Lopera-Vasquez *et al.* (2016b) evaluated the developmental competence and the mRNA abundance of specific genes on bovine blastocysts produced *in vitro* with EVs obtained by ultracentrifugation from ampullary and isthmic OF. EVs from both oviduct regions had a similar size of a mean around 200 nm as quantified with NTA and transmission electron microscopy. Blastocyst rate was not affected by the supplementation of EVs compared to controls (SOF+BSA and SOF+FCS). However, bovine isthmic OF EVs supplementation had a positive effect on gene expression patterns of developmental related genes (*AQP3, LDLR, DNMT3A* and *SNRPN*) compared with serum supplementation suggesting an association between the oviductal environment and the developing embryo (Lopera-vasquez *et al.* 2016b).

In an attempt to decipher the role of oviductal derived EVs, the contents of EVs was analyzed at the proteomic level (Almiñana *et al.* 2015). Knowing that *in vitro* culture could alter
the gene expression profile of OEC (Rottmayer et al. 2006; Schmaltz-Panneau et al. 2015), EVs were analysed from both in vivo oviductal fluid and in vitro BOEC conditioned medium (Almiñana et al., 2015). For this purpose, the same primary BOEC culture system was used as explained previously. EVs secreted by OEC in vivo in the oviductal fluid and by OEC in vitro in the conditioned media after primary culture were collected by serial ultracentrifugation. Preliminary results by dynamic light scattering analysis revealed different size distribution profiles compatible with exosomes and microvesicle populations from in vivo preparations and mostly microvesicle populations from in vitro preparations. Protein profile analysis by SDS-PAGE showed quantitative and qualitative differences between both EV samples. In addition, exosomes of in vivo and in vitro origin exhibited distinct proteomic profiles. Indeed, western blot analysis demonstrated that (i) both types of exosomal protein samples were positive for HSP70, a known exosomal protein; and (ii) in vivo exosomes contained OVGP and heat shock protein A8 (HSPA8), oviductal proteins with known roles in fertilization and early development. However, OVGP was not detected in in vitro exosomes. This is not surprising since the OVGP gene is known to be downregulated during BOEC culture under these conditions. High throughput analysis of the proteomic content of the in vivo vesicles by LC1D-nanoESI-LTQ-Orbitrap revealed 480 proteins in the oviductal EVs. Gene ontology (GO) analysis revealed that a high number of these proteins were involved in metabolism (24.9%), cellular process (19.3%) and 0.8% reproductive processes. Further analysis revealed that more than 56% of EVs proteins involved in cellular processes were associated with cell-to-cell communication (Almiñana et al. 2016).

In addition to the identification of proteins that may be involved in embryo-embryo communication or embryo-maternal interaction, the analysis of the content of these EVs at
mRNA and miRNA levels will bring new insights into the dialogue of the embryos with its environment. Moreover, a better understanding of the molecular mechanisms by which these EVs are recognized and internalized by embryos may contribute to their therapeutic applications in ARTs. Mechanisms involving membrane fusion or endocytosis (Del Conde et al. 2005; Parolini et al. 2009) have been proposed, but it is still unclear whether these vesicles could use more than one route or whether the vesicular uptake is cell type specific (Feng et al. 2010). It becomes more and more apparent that EVs represent ideal natural nanoshuttles for carrying specific in vivo molecules that are not present in classical in vitro culture media. EVs supplementation could bring a “cocktail” of in vivo oviductal proteins, miRNA and lipids to overcome the absence of maternal environment or to complement a deficient coculture system involving partially dedifferentiated BOEC (Fig. 7). Increasing our understanding of the content and function of EVs will highlight the great potential for the use of these vesicles as non-invasive biomarkers in embryo culture or as therapeutic assets in infertility and early pregnancy loss.

Conclusion

In conclusion, beyond classical ways of cell communication involving ligands binding to membrane receptor to trigger intracellular cascades of phosphorylations, EVs, and especially exosomes, predominate as new players of a complex networking activity of cells and tissues. Indeed, EVs are able to deliver a complex cargo, including proteins, RNA and lipids, to target cells and bypass the classical receptor step to induce deep changes in various cell functions. Number of recent works highlighted the presence and possible functions of such EVs in the reproductive
organs and fluids, including oviduct and uterus, as well as in embryonic secretions. Deciphering this newly described communication paradigm will open the way to a better understanding of the regulation of early embryo development and implantation by maternal tissues and by embryos themselves. It will also provide new tools for evaluating the success of these different steps and to improve assisted reproduction biotechnologies.

Acknowledgements

The authors are members of the COST Action FA1201 EPICONCEPT and are collaborating in H2020 Marie Sklodowska Curie (MSCA) Innovative Training Network (ITN) project ‘Biology and Technology of Reproductive Health or REP-BIOTECH project. KP is financed by Rep-Biotech, and DR & MAR are financed by the Spanish Minister of Economy and Competitiveness AGL2015-70140-R. CA was financed by the EU in the framework of the Maria-Curie FP7 COFUND People Programme through the award of an AgreenSkills fellowship, under grant agreement n° 267196.
References


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<table>
<thead>
<tr>
<th>Reference</th>
<th>IVM and IVF conditions</th>
<th>Protein supplement</th>
<th>Individual culture</th>
<th>Group culture</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Embryo density</td>
<td>Blastocyst % D8</td>
</tr>
<tr>
<td>Carolan et al. 1996</td>
<td>Group</td>
<td>10 % FCS (D2)</td>
<td>1:1</td>
<td>0</td>
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<tr>
<td></td>
<td>Individual</td>
<td>10 % FCS (D2)</td>
<td>1:20</td>
<td>20-35*</td>
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<tr>
<td>Donnay et al. 1997</td>
<td>Group</td>
<td>10 % FCS (D2)</td>
<td>1:20</td>
<td>0</td>
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<tr>
<td>Hagemann et al. 1998</td>
<td>Individual</td>
<td>3.2 % BSA</td>
<td>1:10</td>
<td>23</td>
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<td></td>
<td>Idem + 1 µl FCS (D5)</td>
<td>1:10</td>
<td>39</td>
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<td></td>
<td></td>
<td>Idem + Glutamax</td>
<td>1:10</td>
<td>24</td>
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<tr>
<td></td>
<td></td>
<td>Idem + glucose</td>
<td>1:10</td>
<td>24</td>
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<tr>
<td>Fukui et al. 2000</td>
<td>Small group</td>
<td>0.8 % BSA</td>
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<td>5% FCS + cumulus</td>
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### Table 2. Broad classification of extracellular vesicles.

<table>
<thead>
<tr>
<th>Vesicle Types</th>
<th>Diameter(nm)</th>
<th>Density(g/ml)</th>
<th>Morphology (TEM)</th>
<th>Cellular Origin</th>
<th>Origin</th>
<th>Composition</th>
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</thead>
<tbody>
<tr>
<td>Exosomes</td>
<td>40–150 1-4</td>
<td>1.13–1.19 1,3</td>
<td>Rounded 1-3,5</td>
<td>Most cell types</td>
<td>Endolysosomal pathway, intraluminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane, Plasma membrane, Endosomes 6-9</td>
<td>mRNA, miRNA, non coding RNAs, most proteins and lipids not unique for exosomes 1,2,5,11-15,26</td>
</tr>
<tr>
<td>Microvesicles</td>
<td>100–1000 2,14-17</td>
<td>Unknown</td>
<td>Rounded</td>
<td>Most cell types</td>
<td>Cell surface, outward budding of cell membrane, Plasma membrane Cytoplasmic proteins and membrane proteins, including receptors 27</td>
<td>Histones, DNA, nuclear fractions, cell organelles 14,22-25</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>1000–5000 13,14,22,23</td>
<td>1.16–1.28 14</td>
<td>Heterogeneous 23</td>
<td>All cell types</td>
<td>Plasma membrane endoplasmic reticulum 24</td>
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</tr>
</tbody>
</table>

Fig. 1 Droplet of 50 µl medium containing 25 embryos (a) or a single embryo (b). It is obvious that embryonic secretions are diluted in case of single embryo culture.
Fig. 2. Schematic illustration of extracellular vesicles: Microvesicles that are considered to be budded off from the surface of secreting cell with surface receptors attached to it, which are attached to other cell finally obtained inside the recipient cell. Exosomes were considered to be secreted by multi vesicular endosomes in which each exosomes are filled with different types of cargo, which were engulfed by the recipient cells. Apoptotic bodies are released from the cells undergoing apoptosis.
Fig. 3a Oviductal exosomes from *in vivo* origin observed by TEM after ultracentrifugation.
Fig. 3b Oviductal exosomes derived from Bovine Oviduct Epithelial cells (BOEC) cultured *in vitro* as observed by TEM after ultracentrifugation.
Fig. 4 *In vitro* embryo culture systems using oviduct (A) components in cattle. (a) Bovine oviduct epithelial cell (BOEC) monolayer (●); (b) BOEC suspension; (c) BOEC conditioned media (●); (d) Extracellular Vesicles purified from BOEC conditioned media (●); (e) Oviduct Fluid (OF) supplementation; (f) Extracellular Vesicles purified from OF (●).

- Embryotrophic factors released from BOEC
- Proteins, ions, energy substances from OF
Fig. 5 Characterization of vesicles isolated from BOEC-CM.

Fig. 6 Oviductal exosomes labelled with green fluorescent dye (PKH67) and internalized by embryos at blastocyst stage after 20h of co-culture. Nuclei are stained by Hoechst 33342.
Fig. 7 Exosomes labelled with green fluorescent dye (PKH67) and internalized by partially dedifferentiated BOEC after 24 h of coculture. Nuclei are stained by Hoechst 33342.