Anim. Reprod., v.12, n.3, p.366-374, Jul./Sept. 2015

Snooping on a private conversation between the oviduct and gametes/embryos

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Abstract

After a long journey travelling up the maternal tract the spermatozoa will meet the oocyte. As a result, an early embryo will promptly commence its development while travelling down the oviduct. These short but vital journeys of gametes and embryos are accompanied by important changes in the maternal tract. In particular, from the oviduct, which provides an optimal environment for gamete maturation and transport, fertilization and early embryo development. In fact, to achieve a successful pregnancy the oviduct should keep a fruitful dialogue with the gametes followed by an appropriate communication with the embryo(s). In the present review, the transcriptomic and proteomic changes induced by gametes and embryos in the oviduct as a result of this early dialogue will be reported. A special mention of the differential conversation between the oviduct and X and Y-chromosome-bearing spermatozoa, which might be at the basis of gender selection, will be provided. Subsequently, the ability of the embryo to modulate its own oviductal environment thus avoiding its maternal rejection will be discussed. Ultimately, a third player will be introduced in this dialogue, exosomes/microvesicles, which have been proposed as early mediators of these maternal-gamete/embryo interactions. Snooping on the private conversation between the oviduct and gametes/embryo may provide some molecular clues about the mechanisms that mediate these interactions. Moreover, knowing the genes and proteins that pilot the success of the early reproductive events will offer great opportunities for the improvement of assisted reproductive technologies and animal breeding efficiency.

Keywords: embryos, exosomes, gametes, oviduct interactions.

Introduction

In mammals, maternal interactions with gametes and embryos are the basis for the success of any reproductive event. The oviduct, or Fallopian tube, which is the maternal tube connecting the ovary and the uterus, plays a vital role in these interactions. It holds the maternal dialogue with gametes and early embryos and provides an optimal environment for gamete maturation and transport, fertilization and early development of the embryo (Hunter, 2005).

The oviduct can be seen as a bidirectional route, where the spermatozoa travel up to meet the oocyte while the early embryo travels down towards the uterus. In most mammals it is divided anatomically into three parts: 1) the utero tubal junction, that connects the oviduct to the uterus; 2) the isthmus, the region associated with the storage of spermatozoa before ovulation and where spermatozoa bind to the oviduct epithelial cells (OEC) on their way to meet the oocyte and; 3) the ampulla, where fertilization takes place. Spermatozoa from most mammals can reside in the oviduct from a few hours up to a maximum of 5-7 days (Holt and Fazeli, 2010). Bats are exceptional among mammals having the ability to store spermatozoa for several months in the uterus or oviducts during hibernation (Bernard and Cumming, 1997). By contrast, the embryo spends only a few days (2-5) in the oviduct, which also varies depending on the species: in mouse 2-3 days (Rafferty, 1970); in pigs 1-3 days (Pomeroy, 1955; Oxenreider and Day, 1965); in cows 2-4 days (Hamilton and Laing, 1946; Crisman *et al*.,1980); in sheep 2-3 (Holst, 1974) and in mares 5-6 days (Freeman *et al*., 1991). To adapt to these different scenarios, the oviduct is spatially and temporally regulated by hormones and also by its interactions with gametes and embryos (Fig. 1).

However, modulation of the oviduct by gametes and embryos is poorly understood. Focusing on these interactions is also a matter of two sides. On one side, there is a modulatory effect of OEC on spermatozoa (Ellington *et al*., 1991) and the oviductal secretions on embryo development (Gandolfi, 1989). On the other side, spermatozoa and the embryo can also modulate the gene and protein expression of the oviduct (Ellington *et al*., 1993; Thomas *et al*., 1995; Fazeli *et al*., 2004; Georgiou *et al*., 2005, 2007; Almiñana *et al*., 2012; Schmaltz-Panneau *et al*., 2014; Yeste *et al*., 2014). Emerging studies are suggesting a third player in these interactions, exosomes/microvesicles, which could act as mediators in the two-way communication system that takes place in the maternal tract (Ng *et al*., 2013; Burns *et al*., 2014).

For simplicity, this review will focus on oviduct-gamete/embryo interactions in mammals. The role of gametes and embryos as modulators of the maternal tract will be addressed in the following pages. In recent years an increasing number of publications have examined this side of the maternal interactions,

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Received: May 25, 2015
Accepted: July 7, 2015

which reflects the importance of these interactions. Snooping on the private conversation between the oviduct, gametes and embryos may reveal the mechanisms that mediate these interactions. Understanding this complex dialogue will shed some light into infertility problems, reduce early pregnancy loss and may even identify the factors that influence the development of the offspring into adulthood.

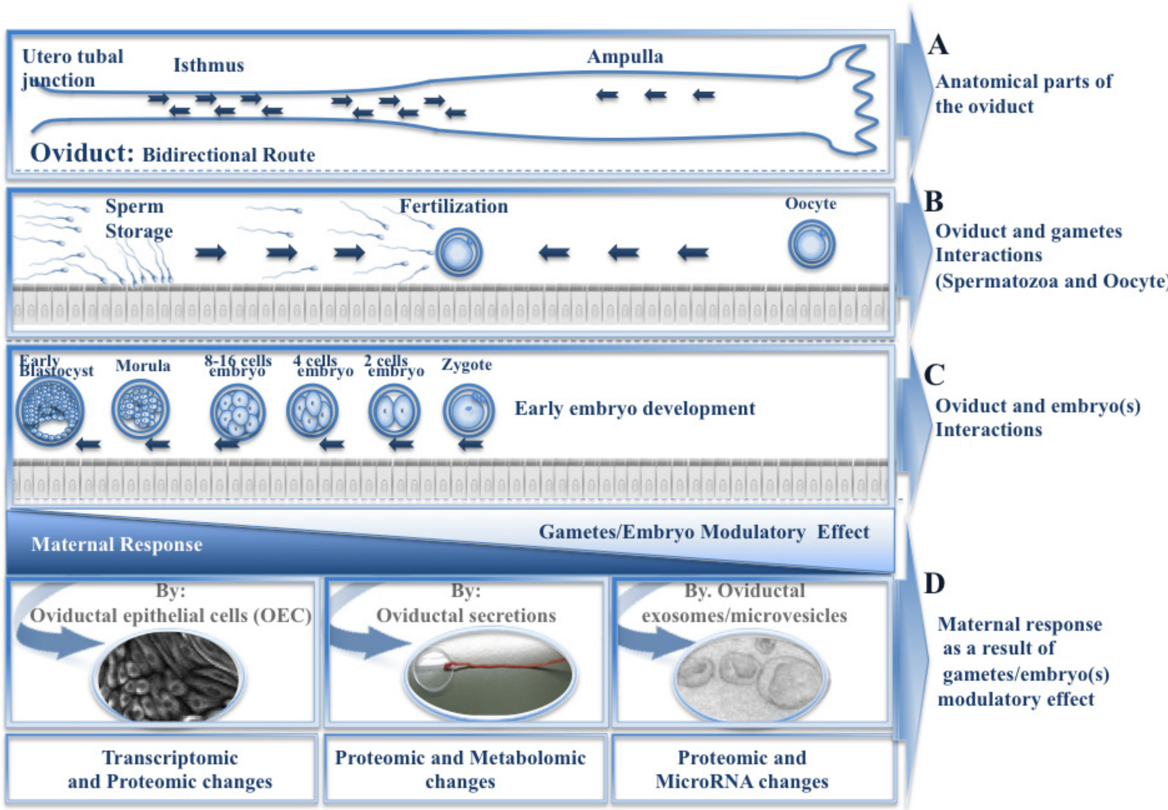

Figure 1. Diagram of anatomic parts of the oviduct and gametes/embryos interactions with the oviduct.

Oviduct and gametes interactions: conversations or negotiations?

The interactions between the oviduct and the gametes involve close and specific contact between them (Hunter and Nichol, 1983; Fazeli *et al.*, 1999, 2003). As a result of this contact, a confidential dialogue between the OEC and the gametes takes place. Solid evidence allows us to state that this dialogue is not univocal, and must be seen as a two-way communication system that ensures the success of early reproductive events. On one side, the oviduct and its secretions influence the physiology of the gametes (Avilés *et al.*, 2010). On the other side, gametes also modulate the oviductal environment (Fazeli *et al.*, 2004; Georgiou *et al.*, 2007).

There is no doubt of the vital role of the oviduct in the preparation of male and female gametes for their successful meeting (Coy *el al.*, 2012; Avilés *et al.*, 2015) but less extensive and detailed research exists on the ability of the gametes to modulate their own oviductal environment. Initial evidence about the way that spermatozoa control the oviductal environment revealed that the attachment of sperm cells to the bovine OEC during co-culture changed the types and quantities of proteins secreted into the conditioned medium (Ellington *et al.*, 1993). Several studies using mouse or pig models have further demonstrated a maternal response to spermatozoa (Fazeli *et al.*, 2004; Georgiou *et al.*, 2005, 2007). Fazeli *et al.* (2004) revealed that the arrival of spermatozoa into the oviduct after mating resulted in alterations of the oviductal transcriptome. Those same alterations were not found when infertile mice, which produce seminal plasma but no spermatozoa (T145H mutant mice), were used in the experiment (Fazeli *et al.*, 2004). Georgiou and co-workers showed that the presence of both gametes, spermatozoa and oocytes, altered the oviductal secretory profile (Georgiou *et al.*, 2005, 2007). The oviductal response to spermatozoa was different from that induced by oocytes. Spermatozoa induced a specific oviductal proteomic response, modulating the expression of 20 proteins while only one protein was regulated by oocytes. Recently, Artemenko and colleagues using a refined mass-spectrometry-based approach reported an immediate response of the surface proteome of oviductal cells to spermatozoa, which was modulated over time (Artemenko *et al.*, 2015). Thirtyone cell surface proteins were found pronouncedly altered (≥2 fold change) immediately, 1 and 2 h after insemination compared to control. Functional analysis showed that those proteins were associated to structural reorganization of the oviductal epithelium cell surface. Interestingly, oviduct specific glycoprotein (OVGP), a crucial protein in fertilization processes (Buhi, 2002), was strongly increased at the cell surface 1 h after insemination. OVGP was also found up-regulated in response to spermatozoa in sow oviducts (Georgiou *et al*., 2007) but at 24 h after artificial insemination. These findings support the view that the complex transcriptomic and proteomics changes that occur in the oviduct are finely tuned through the dialogue between the oviduct and gametes.

Moreover, the sperm-oviductal dialogue could be at the basis of the intriguing selection of X or Y-chromosome bearing spermatozoa by the oviduct prior to fertilization. Sex allocation of offspring in mammals is usually considered as a matter of chance, being dependent on whether an X- or a Y-chromosome-bearing spermatozoon reaches the oocyte first. Evidence from the field and laboratory suggests that female mammals can bias the sex ratio of their offspring (Clutton-Brock and Lason, 1986; James, 2009). However, no biological mechanism(s) explaining this selection has yet been discovered. A recent study in pigs (Almiñana *et al*., 2014) provided an important mechanistic insight into this phenomenon. By introducing X- or Y-sperm populations into the two separate oviducts of single female pigs using bilateral laparoscopic insemination, Almiñana and co-workers found that the spermatozoa did indeed elicit sex-specific transcriptomic responses. Microarray analysis revealed that 501 from 24123 probes were consistently altered ($P < 0.05$) in the oviduct in the presence of Y-chromosome-bearing spermatozoa compared to the presence of X-chromosome-bearing spermatozoa. From these 501 transcripts, 271 transcripts (54.1%) were down-regulated and 230 transcripts (45.9%) were up-regulated when the Y- chromosome-bearing spermatozoa were present in the oviduct. Two fascinating ideas derived from our study: 1) spermatozoa carrying the Y- or X-chromosome can modulate the oviductal response by activating specific signalling pathways in a gender specific manner and 2) the female reproductive tract can sense the presence of X- or Y-chromosome-bearing spermatozoa in the oviduct before fertilization occurs. The fact that mothers can recognize the difference between X- and Y- bearing spermatozoon is a first prerequisite to allow only one preferred type of spermatozoa to reach the oocyte. Therefore, these sperm-oviduct interactions could be seen more as fruitful "negotiations" if, as a result, one type of spermatozoon might be selected. Although the precise mechanism that might bias the gender selection is not yet elucidated, the study by Almiñana *et al*. (2014) provides candidate genes that might be responsible of this gender selection.

After digging in X and Y-sperm features that could be read by the oviduct and might be involved in the sperm sex-selection, different topographic characteristics on the head of X- and Y-spermatozoa were observed by atomic force microscopy (Carvalho *et al*., 2013). In a similar way, differentially expressed proteins found between bull X- and Y-spermatozoa (Chen *et al*., 2012), might be sensed by the oviduct and help in the sex-selection. Furthermore, emerging studies on the microRNA population of spermatozoa suggest that they could be important players in these sperm-oviductal interactions. MicroRNAs are powerful regulators of gene and protein expression (Bartel, 2004; He and Hannon, 2004) and thus, sperm microRNA could modulate oviductal gene expression. The emerging new ways of embryo-to-embryo communication proposed by microRNA release via exosomes during *in vitro* culture (Saadeldin *et al*., 2014) could be also used by sperm microRNA to interact with the oviduct. To date, only differences in sperm microRNA between fertile and infertile spermatozoa (Lian *et al*., 2009: Abu-Halima *et al*., 2013) and, a potential role of sperm microRNA as chemoattractant-activated transduction signalling and their association to vesicles have been demonstrated (Das *et al*., 2013). But together, such evidence supports the view of microRNAs as "hot" candidates in gender-selection.

Oviduct and embryo(s) dialogue: what does the embryo say to the mother?

The oviduct also plays a direct role in supporting early embryonic development (Gandolfi *et al*., 1989). It provides the best environment for the embryo, matching its requirements, within the short but very vital period before entering the uterus (Besenfelder *et al*., 2012).

Previously, we have mentioned that the arrival of spermatozoa in the oviduct and their binding to oviductal cells initiates a sperm-oviduct signalling dialogue. By contrast following fertilization, the resulting embryo spends the next few days in the oviduct while it is "free-floating" in the maternal tract, and has no direct contact with the mother while travelling down the oviduct to reach the uterus (Hunter, 1980). Because of this, the embryo has been considered relatively autonomous during this early time of its life. The fact that embryos can be routinely produced and developed up to the blastocyst stage *in vitro,* due to the great advancement of reproductive biotechnologies, has reinforced this idea. All together, these facts have encouraged into the view that the oviduct is merely a passive tube for the transport of the embryo on its way to the uterus (Marston *et al*., 1977), rather than an essential organ that offers protection and nutrition for the normal embryo development. However, evidence demonstrating the superior competence of the *in vivo* embryos compared to the *in vitro* embryos (Rizos *et al*., 2008, 2010; Van Soom *et al*., 2014) and the epigenetic effects of the *in vitro* culture on the embryo developmental potential (Hou *et al*., 2007; Reis e Silva *et al*., 2012; Beaujean, 2014; Bertoldo *et al*., 2014) has made researchers rethink the undoubted role of the oviduct hosting the early developing embryos.

The early developing embryo undergoes a highly orchestrated series of events, such as the first mitotic cells divisions and genome activation. To encompass these early developmental events and allow the delivery of a competent conceptus to the endometrium, the oviductal lining is subjected to dynamic changes (Besenfelder *et al*., 2012). In this regard, researchers have examined the possibility that the embryo could act as a mediator of its own environment (Almiñana *et al*., 2012). However, the complex signals exchanged between the oviduct and the embryos that lead to alterations of the environment in response to embryo(s) are not yet fully understood.

Given the ethical and scientific obstacles associated with *in vivo* embryo-maternal studies, primary OEC cultures have been thoroughly used to study these early embryo-oviductal interactions. Using this model researches have confirmed the existence of a real dialogue between the early embryo and the oviduct (Cordova *et al*., 2014; Schmaltz-Panneau *et al*., 2014). Co-incubation of bovine OEC (BOEC) with bovine embryos induced changes in embryonic gene expression (Cordova *et al*., 2014). Moreover, BOEC from isthmus and ampullar regions increased cleavage rate and blastocyst rate over the control, with BOEC from the isthmus being more capable of supporting early embryo development than BOEC from the ampulla. In response, the embryo was also capable of modifying BOEC gene expression and protein secretion (Schmaltz-Panneau *et al*., 2014). In this regard, thirty-three genes were over-expressed in BOEC in the presence of embryos compared to the control counterpart. Only one gene was down-regulated. Most of the up-regulated genes corresponded to genes regulated or involved in interferon type I signalling pathway. A large number of these interferon tau (IFNT)-induced genes were also found in transcriptional profiling experiments in the bovine uterus (Bauersachs, 2006; Klein *et al*., 2006; Mansouri-Attia *et al*., 2009; Forde *et al*., 2011, 2012). These uterine changes have been mainly associated to pregnancy recognition signals in response to the secretion of IFNT by the conceptus. However, IFNT secretion by bovine embryo starts around 15-16 days after fertilization when the embryo is in the uterus (Bazer *et al*., 1997). Therefore it has been hypothesized that embryonic IFNT could play a key role in maternal pregnancy recognition in the oviduct and in the uterus by activating a set of specific genes before and at the implantation period (Schmaltz-Panneau *et al*., 2014).

Even though BOEC-embryo *in vitro* model studies have proved the existence of certain embryo-oviductal interactions, the question that arises is how far are these *in vitro* interactions from those that occur *in vivo* during early pregnancy. To date, only a few studies have provided evidence of the *in vivo* maternal-embryo interactions in the oviduct at the very early stages of embryo development (Lee *et al*., 2002; Almiñana *et al*., 2012; Maillo *et al*., 2015). Lee *et al*. (2002) compared the gene expression pattern of mouse oviducts containing early embryos and oviduct containing oocytes. The presence of embryos altered the transcriptome profile of the oviduct compared to oocytes. Using a pig model Almiñana and co-workers showed that the changes observed in the oviductal gene expression were dependent on the embryo developmental stage (Almiñana *et al*., 2012), demonstrating a more specific response of the oviduct towards the embryo. Additionally, these authors observed that when the embryo migrated from the oviduct to the uterine horn, the mRNA levels of a selected transcript related to immunity (TICAM2) was down-regulated in both the oviduct and the uterine horn samples. The uterine down-regulation of the immune related genes while the embryo is still in the oviduct might function as in preparing the uterus to accept the embryo.

In a more holistic study of the oviductal changes, Maillo *et al*. (2015) have demonstrated that the early bovine embryo elicits an oviductal response during its transit through the oviduct that may contribute to its subsequent development. Although these authors have used a non-physiological model to prove this dialogue by transferring 50 embryos into the oviduct of a cow, the presence of multiple embryos in the oviduct induced differential transcriptional changes in OEC when compared to the gene expression responses to oocytes. Furthermore, Maillo *et al*. (2015) observed that the presence of multiple embryos in the cow oviduct down-regulated the maternal immune system, confirming previous results obtained by Almiñana *et al*. (2012). Taken together, these studies demonstrated that as a result of the early embryo maternal dialogue the embryo mediates its own environment in the maternal tract. Furthermore, the embryo seems to contribute to its maternal tolerance by modulating the maternal immune system.

On the other hand, the transcriptomic changes observed in the oviduct in response to the presence of the embryo (Lee *et al*., 2002; Almiñana *et al*., 2012; Maillo *et al*., 2015), may be possibly associated with changes in the oviductal secretions at the very early stages of pregnancy. Therefore, it seems imperative to investigate the temporal and spatial secretions triggered by the embryos while they are free floating in the oviduct. So far, much emphasis has been paid to the uterine fluid surrounding the blastocyst or early conceptus (Muñoz *et al*., 2012; Gomez *et al*., 2013; Forde *et al*., 2014), even though early embryonic mortality might occur before embryo reaches the uterus.

Exosomes/microvesicles: mediators of gamete/embryo interactions

Exosomes are small (30-100 nm) membrane vesicles of endocytotic origin that have been identified *in vivo* in all body fluids including follicular (da Silveira *et al*., 2012; Sohel *et al*., 2013), uterine (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) and oviductal fluids (Al-Dossary *et al*., 2013) and can be secreted by most cell types *in vitro*. They specifically carry proteins, lipids, and genetic materials such as DNA, RNA, and microRNA that could be transferred to recipient cells, and may induce epigenetic changes. Exosomes together with microvesicles (bigger vesicles around 50-1000 nm with similar content; Dragovic *et al*., 2011; Gyö̈rgy *et al*., 2011; Turiák *et al*., 2011; Braicu *et al*., 2015) play fundamental biological roles in the regulation of physiological as well as pathological processes, which make them interesting therapeutic vectors (Suntres *et al*., 2013).

Recent studies indicate that exosomes/microvesicles could act as intercellular vehicles in the embryo-maternal dialogue in the uterus (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) and might also mediate the maternal-gametes/embryo interactions in the oviduct. Oviductosomes (Al-Dossary *et al*., 2013) and uterosomes (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) have been identified recently, but it is still a mystery how they are taken up by gametes and embryos and whether they modulate the maternal interactions to promote successful pregnancy. On the embryo side, only one recent study has shown that *in vitro* produced embryos can secrete exosomes as a possible way of communication among them (Saadeldin *et al*., 2014).

As mentioned above, OEC from primary *in vitro* culture have been thoroughly used as *in vitro* models to study oviduct-embryo interactions in different species and therefore, could be the model of choice to study the role of the exosomes in this unique communication system. However, knowing the large differences between *in vivo* and *in vitro* embryos in terms of embryo quality and gene expression and the different morphologic characteristics and protein expression of OEC from *in vivo* and *in vitro* origin (Rottmayer *et al*., 2006), our laboratory began to characterize the bovine oviductal exosomes from both *in vivo* and *in vitro* origin (Almiñana *et al*., 2015). For this purpose, exosomes secreted by OEC *in vivo* in the oviductal fluid and by OEC *in vitro* in the conditioned media after OEC 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 among the exosomes samples, their cells of origin and the milieu (conditioned media or flushing). In addition, exosomes of *in vivo* and *in vitro* origin exhibited distinct proteomic profiles. Western blot analysis demonstrated that (i) both types of exosomal protein samples were positive for HSP70, a known exosomal protein (ii) *in vivo* exosomes expressed OVGP and heat shock protein A8 (HSPA8), oviductal proteins with known roles in fertilization and early pregnancy. However, only HSPA8 was detected in *in vitro* exosomes. These results have contributed to the first characterization of oviductal exosomes of *in vivo* and *in vitro* origin. In depth analysis of the content of these vesicles will bring new insights into the embryo-oviductal dialogue and will increase our knowledge of the oviductal environment that supports the early stages of embryo development.

In addition, further studies aimed at understanding the molecular mechanisms by which exosomes/microvesicles are internalized by cells may contribute to their therapeutic applications. 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). To date, it is known that oocytes can take up exosomes from the follicular fluid, showing a cell-to-cell communication system during oocyte growth (da Silveira *et al*., 2012; Sohel *et al*., 2013). In addition, it has been shown that sperm can take up a Ca2+ regulatory protein, PMCA4, from oviductosomes (Al-Dossary *et al*., 2013). PMCA4 is involved in the capacitation and acrosome reaction, suggesting than oviductosomes may have an important role in gamete-oviduct interactions and fertility. Moreover, embryos can take up exosomes released from other embryos during the *in vitro* culture as a way of embryo-embryo communication (Saadeldin *et al*., 2014). Ultimately, trophectoderm ovine cells, an established Tr1 cell line from day 15 conceptuses, internalized exosomes collected from uterine fluids (Ruiz-Gonzalez *et al*., 2015). However, the possibility that the early developing embryo takes up exosomes from the oviductal fluid or the OEC internalize embryo-derived exosomes, to the best of our knowledge, has not yet been shown.

Why should we snoop on these conversations? Why does it matter what they say?

By snooping on the private conversation between the oviduct and gametes/embryo a number of genes and proteins participating in these oviductal interactions have been revealed. While the biological nature of this oviductal cross-talk with gametes and embryos is interesting for its own sake, knowing the molecules and mechanisms that pilot these processes offers great opportunities for the improvement of assisted reproductive technologies (ARTs).

The use of ARTs such as intracytoplasmic sperm injection (ICSI), or *in vitro* fertilization (IVF), bypasses the early maternal interactions in the oviduct**.** Despite all our efforts in improving the procedures or culture media used in these techniques, evidence has shown genomic imprinting disorders (Cox *et al*., 2002; Le Bouc *et al*., 2010; Lazaraviciute *et al*., 2014). Since there is a lack of oviductal interactions in these scenarios, harnessing the molecular clues obtained from snooping on the conversation between the oviduct and gametes/embryos could improve the success of ARTs.

Here a few examples: (i) A solid molecular basis of the maternal mechanisms involved in sperm selection will help to develop advanced selection methods on sperm quality and improve ART outcomes and animal breeding efficiency; (ii) Identify oviductal proteins that enhance sperm survival, will offer great opportunities for the development of long-life semen diluents; (iii) Determining oviductal proteins that support the development of the early embryo will be used in designing new *in vitro* culture media or in reformulating the current ones.

Although the idea of using the identified oviductal proteins seems quite straightforward, in practice, it is not. Some hurdles need to be overcome: the difficulty in the isolation of oviductal proteins; the fact that once the proteins are isolated they may not exert the same effect as *in vivo*; and the fact that gametes and embryos are remarkably resistant towards the uptake of exogenous substances, including drugs, biomolecules, and intracellular markers.

In this regard, the exosomes represent ideal natural nanoshuttles for carrying specific *in vivo* molecules that are not expressed in the *in vitro* cultures. Exosome supplementation will bring a “cocktail” of *in vivo* oviductal proteins, miRNA and lipids to overcome the *in vitro* cultures deficiencies and promote successful pregnancy. Increasing our understanding of the exosome/microvesicle content and function will highlight the great potential for the use of these vesicles as non-invasive biomarkers or as therapeutic assets in infertility and early pregnancy loss.

Acknowledgments

The author thanks William V. Holt for their constructive reading and advice about this manuscript. This work was supported by the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship, AgreenSkills programme: under grant agreement n° 267196.

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