Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature

Natalia Barkalina1, Celine Jones1, Matthew J.A. Wood2, and Kevin Coward1,*

1Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3, Women’s Centre, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK 2Department of Physiology, Anatomy and Genetics, University of Oxford, Le Gros Clark Building, South Parks Road, Oxford OX1 3QX, UK

*Correspondence address. Nuffield Department of Obstetrics and Gynaecology, Level 3, Women’s Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK. Tel: +44-1865-728278; E-mail: kevin.coward@obs-gyn.ox.ac.uk

Submitted on March 24, 2015; resubmitted on May 15, 2015; accepted on May 21, 2015

TABLE OF CONTENTS
• Introduction
  - Background
  - Research into the mechanisms of gamete function: current challenges
• Methods
  - Nanoparticle-mediated delivery for reproductive biology: a potential strategy to improve uptake into gametes
    - Biomedical nanomaterials represent versatile small-scale platforms for targeted delivery
    - Nanomaterial-mediated delivery into gametes is an encouraging, yet controversial field
    - Cell-penetrating peptides act both as targeting tools for nanomaterials and independent delivery platforms
  - Exosomes and microvesicles in reproductive biology: natural delivery vectors and mediators of cell function
    - EVs are universal mediators of cell communication with specialized roles in pregnancy
    - EVs regulate sperm maturation via the direct transfer of essential proteins to sperm
    - Emerging evidence of the role of EVs in regulating female reproductive function
  - EV-mediated delivery: bridging the gap between nature and nanotechnology
• Conclusions

BACKGROUND: Currently, even the most sophisticated methods of assisted reproductive technology (ART) allow us to achieve live births in only approximately 30% of patients, indicating that our understanding of the fine mechanisms underlying reproduction is far from ideal. One of the main challenges associated with studies of gamete structure and function is that these cells are remarkably resistant towards the uptake of exogenous substances, including ‘molecular research tools’ such as drugs, biomolecules and intracellular markers. This phenomenon can affect not only the performance of reproductive biology research techniques, but also the outcomes of the in vitro handling of gametes, which forms the cornerstone of ART. Improvement of intra-gamete delivery in a non-aggressive fashion is vital for the investigation of gamete physiology, and the advancement of infertility treatment. In this review, we outline the current state of nanomaterial-mediated delivery into gametes and embryos in vitro, and discuss the potential of a novel exciting drug delivery technology, based upon the use of targeted ‘natural’ nanoparticles known as extracellular vesicles (EVs), for reproductive science and ART, given the promising emerging data from other fields.

METHODS: A comprehensive electronic search of PubMed and Web of Science databases was performed using the following keywords: ‘nanoparticles’, ‘nanomaterials’, ‘cell-penetrating peptides’, ‘sperm’, ‘oocyte’, ‘egg’, ‘embryo’, ‘exosomes’, ‘microvesicles’, ‘extracellular vesicles’, ‘delivery’, ‘reproduction’, to identify the relevant research and review articles, published in English up to January 2015. The reference lists of identified publication were then scanned to extract additional relevant publications.
RESULTS: Biocompatible engineered nanomaterials with high loading capacity, stability and selective affinity represent a potential versatile tool for the minimally invasive internalization of molecular cargo into gametes and embryos. However, it is becoming increasingly clear that the translation of these experimental tools into clinical applications is likely to be limited by their non-biodegradable nature. To allow the subsequent use of these methodologies for clinical ART, studies should utilize biodegradable delivery platforms, which mimic natural mechanisms of molecular cargo trafficking as closely as possible. Currently, EVs represent the most physiological intracellular delivery tools for reproductive science and medicine. These natural mediators of cell communication combine the benefits of engineered nanomaterials, such as the potential for in vitro production, targeting and loading, with the essential feature of biodegradability.

CONCLUSION: We anticipate that future investigations into the possibility of applying EVs for the intentional intracellular delivery of molecular compounds into gametes and embryos will open new horizons for reproductive science and clinical ART, ultimately leading to improvements in patient care.

Key words: extracellular vesicles / nanomaterials / delivery / gametes / assisted reproductive technology

Introduction

Background

Assisted reproductive technology (ART) has revolutionized the field of infertility treatment, resulting in the birth of >5 million children worldwide ever since its first successful use in humans in 1978 (Adamson et al., 2013). Over the last four decades, pregnancy rates following ART have increased by nearly 6-fold, from 6% (Wang and Sauer, 2006) to ~35% (ESHRE, 2014; HFEA, 2014). In these years, ART has expanded and improved, perhaps even more than anticipated in its early days, and transformed from a controversial experimental procedure to a routine medical treatment. However, its success rates, from the modern perspective, remain remarkably insufficient to consider this technique a reliable solution to the problem of infertility. According to recent estimations, the average live birth rate after ART globally still remains reasonably low, and does not exceed 30% per started cycle (ESHRE, 2014). At the same time, the demands for successful ART, especially in post-industrial economies, are continuously growing. The main driving forces for this trend are the increasing prevalence of age-related infertility due to the voluntary postponement of parenthood, and the expansion of assisted reproduction into non-infertility indications, such as the preimplantation genetic diagnosis of hereditary diseases and fertility preservation for medical or social reasons (reviewed in Barkalina et al., 2014a).

The sub-optimal success rates of ART are generally attributed to two key factors. Firstly, the conventional techniques for selection of embryos to be transferred back into the patient’s uterus have inherent limitations, since they are based exclusively upon the morphological assessment of embryos, and not the evaluation of their chromosomal status and, therefore, developmental potential in the long-term (Fragouli et al., 2014). Secondly, in vitro handling of gametes and embryos, which forms an integral part of ART, has been reported to induce microstructural and functional damage in these delicate structures, with consequential reduction in developmental competency. There is mounting evidence that gamete processing in vitro during ART also promotes the fragmentation of sperm DNA (Toro et al., 2009; Matsuura et al., 2010; Rougier et al., 2013), reduces the levels of sperm-borne oocyte-activating factor phospholipase C zeta (PLCζ; Kashir et al., 2011; Yelumalai et al., 2013), and facilitates oxidative stress in unfertilized oocytes (Martin-Romero et al., 2008; Otsuki et al., 2009); all of which, in the case of gametes with already compromised fertility, can have profound negative effects. Optimization of in vitro culture conditions, such as the supplementation of culture media with antioxidants, small molecules and growth factors (Kawamura et al., 2012; Yun et al., 2013; Tardif et al., 2014), or embryo incubation in time-lapse monitoring systems, which do not require repeated interruptions of culture for morphology assessment (Meseguer et al., 2012), has been reported to increase gamete/embryo survival and improve developmental potential. These observations elegantly indicate that the potential to improve ART success rates via the wider adoption of such approaches is both exciting and necessary. Nevertheless, substantial breakthroughs in the field of clinical ART can only be achieved via ongoing fundamental reproductive biology studies into the physiological mechanisms underlying reproduction, enabling the discovery of targeted molecular tools to investigate and manipulate these fine mechanisms at the cellular level.

Research into the mechanisms of gamete function: current challenges

The use of molecular research tools, including oligonucleotides, nucleic acids, peptides, antibodies, fluorescent markers and small molecules, forms the cornerstone of experimental studies in developmental and reproductive biology. These tools allow the precise mapping of specific cellular structures and molecular pathways, and tracking of their activity and fate at the different stages of gamete/embryo development. However, this seemingly straightforward approach, which is universally applied for experiments in biology, is associated with substantial challenges when used for studies of gamete structure and function in vitro. These highly specialized cells, especially in their mature form and after isolation from the natural microenvironment, acquire remarkable resistance towards the uptake of exogenous compounds. The specific molecular structure of the sperm membrane, characterized by an increased proportion of polyunsaturated fatty acids and the presence of rare ether-linked phospholipids, plasmalogens (Lenzi et al., 2000; Tapia et al., 2012), along with high structural and functional compartmentalization (James et al., 2004) and low activity of endocytotic processes (Jones et al., 2013), render sperm a particularly difficult target for the intracellular delivery of investigative compounds in vitro. Similarly, the oocyte, throughout its maturation in vivo, maintains an intimate relationship with the surrounding cumulus cells, which deliver essential nutrients into the oocyte via a system of gap junctions between the long cumulus cell processes penetrating the zona pellucida and the oocyte plasma membrane (Eppig et al., 2005). Studies of oocyte structure and function in vitro often require the mechanical removal of these surrounding nurturing cells to facilitate visualization of the female gamete, and, subsequently, compromise the physiological mechanisms of cargo internalization.
In its current form, the in vitro intracellular delivery of research compounds into gametes, and particularly into sperm, often requires the use of powerful membrane-disrupting agents, such as cholamidopropyldimethylammonio propane sulfonate hydrate (CHAPS), Tween 20 and Triton X-100 (Jakop et al., 2009) with subsequent fixation, which renders the gametes entirely unsuitable for further use (Garcia-Vazquez et al., 2009; Yamauchi et al., 2012). Consequently, this approach does not allow for the evaluation of how the differences in gamete structure relate to their functionality, especially the ability to initiate and sustain normal embryo development. Improvement of intracellular delivery into gametes in a non-aggressive fashion and without effects upon developmental potential is, therefore, pre-requisite for the improvement of our existing knowledge of reproductive biology, and, consequently, the advancement of ART.

From a rather more applied perspective, tools for efficient and non-damaging intra-gamete delivery could hold a therapeutic promise for patients with infertility caused by specific molecular deficiencies in gametes, for example deficiency of the sperm-borne oocyte-activating factor PLCζ, resulting in oocyte activation failure post-fertilization, even following the intracytoplasmic injection of sperm into oocytes (ICSI) (Amdani et al., 2013). Similarly, these tools could be used in applied ART to supplement gametes with fertility-enhancing compounds, either promoting sperm motility or protecting gametes from deterioration during long-term culture in vitro (Kawamura et al., 2012; Yun et al., 2013; Tardif et al., 2014), especially for such indications as the in vitro maturation of oocytes or the in vitro culture of oocytes from primordial follicles for experimental fertility preservation programmes (Teffer and McLaughlin, 2012).

In this review, we outline the current state of nanomaterial-mediated delivery into gametes and embryos in vitro, and discuss the potential of a novel exciting drug delivery technology, based upon the use of targeted ‘natural’ nanoparticles, known as extracellular vesicles (EVs), for reproductive science and ART, given the promising emerging data from other fields.

**Methods**

A comprehensive electronic search of PubMed (US National Library of Medicine, National Institute of Health; http://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (Thomson Reuters, http://webofknowledge.com/) databases was performed using the following keywords: ‘nanoparticles’, ‘nanomaterials’, ‘cell-penetrating peptides’, ‘sperm’, ‘oocyte’, ‘egg’, ‘embryo’, ‘exosomes’, ‘microvesicles’, ‘extracellular vesicles’, ‘delivery’, ‘re-production’ to identify the relevant research and review articles published in English up to January 2015. The reference lists of identified publications were then scanned to extract potential additional relevant publications.

**Nanoparticle-mediated delivery for reproductive biology: a potential strategy to improve uptake into gametes**

**Biomedical nanomaterials represent versatile small-scale platforms for targeted delivery**

Nanotechnology is a novel and rapidly developing field of science, positioned at the interface of physical, chemical, biological, materials and computer sciences, which investigates and manipulates physical matter at the nanoscale (1–100 nm). From the biomedical perspective, the revolutionary nature of nanotechnology lies in its ability to design a customisable small-scale biocompatible delivery platform with large loading capacity, stability and highly specific affinity towards selected cell populations (Riehemann et al., 2009; Lehner et al., 2013; Tsai et al., 2014). Biomedical nanomaterials offer enormous targeting options, which can be achieved either via the modification of the physicochemical properties of nanomaterials, such as size, shape, surface charge and chemistry (‘passive’ targeting), or intentional functionalisation of the surface of nanomaterials with specific affinity moieties, for example peptides, antibodies and aptamers (‘active’ targeting), which selectively bind with the complimentary ligands on the surface of target cells (Riehemann et al., 2009; Petros and DeSimone, 2010; Albanese et al., 2012). Nanomaterials are universally characterized by their small size, comparable to the size of biological molecules and/or intracellular organelles, and their vast surface area, allowing the nanocarrier to be loaded with large amounts of almost any type of biological cargo, including a combination of contrast and therapeutic agents for the simultaneous detection and targeted treatment of pathologic lesions (‘nanotheranostic’) (Lammers et al., 2011). The small size enables straightforward internalization of nanomaterials inside the cells using the innate physiological mechanisms of uptake, with subsequent intracellular transport and metabolism (Petros and DeSimone, 2010; Kunzmann et al., 2011). Furthermore, nanomaterials are robust and, therefore, capable of carrying payloads to distant target locations following systemic administration. Collectively, these features, summarized in Table I, render biomedical nanomaterials strong candidates for the targeted delivery of diagnostic and therapeutic agents, including those with poor bioavailability after systemic use or high non-specific cytotoxicity.

Over the last decade, the use of nanomaterials for diagnostic imaging and drug delivery into pathological lesions has consistently proven advantageous in such fields as oncology, and infectious and chronic internal diseases (Ulrich and Lamprecht, 2010; Brakman et al., 2012; Psarros et al., 2012; Holmes, 2013; Tsai et al., 2014). This success has triggered an expansion of nanobiotechnological ‘vision’ to other scientific disciplines, including reproductive biology (Barkalina et al., 2014a). Indeed, universal evidence that nanomaterials improve the selectivity and efficacy of cargo delivery across a variety of cell types (Ryan and Brayden, 2014; Tsai et al., 2014) and do not compromise cell function, render them particularly attractive candidates for intracellular delivery into gametes and embryos.

**Nanomaterial-mediated delivery into gametes is an encouraging, yet controversial field**

The number of studies utilizing nanomaterials for the transfer of molecular compounds into gametes has been steadily growing since the mid-2000s, however the total number of publications still remains relatively low (Table II). Currently, the spectrum of nanomaterials with favourable biocompatibility with gametes/embryos includes polyvinylalcohol-functionalised iron oxide (Ben-David Makhluf et al., 2006; Makhluf et al., 2008), magnetic (Kim et al., 2010) and polystyrene (Fyne et al., 2007) nanoparticles, mesoporous silica (Barkalina et al., 2014b), cerium dioxide (Falchi et al., 2014), perfluorocarbon (Jallouk et al., 2014), halloysite clay nanotubes and commercial polymeric nanotransfectants (Campos et al., 2011a, b), specialized CdSe/ZnS quantum dots (Feugang et al., 2012), and nanogold (Taylor et al., 2014b; Tiedemann et al., 2014). Most of
these studies have consistently demonstrated that the use of nanomater-
ials improves the efficacy of research techniques, based upon the internal-
ization of molecular compounds into gametes. These techniques primarily
involved loading sperm with exogenous genetic constructs for subsequent
sperm-mediated gene transfer into the oocyte at the time of fertilization
(Kim et al., 2010; Campos et al., 2011a, b), proof-of-principle transfer of
proteins into sperm (Makhluf et al., 2008), labelling of preimplantation
embryos during in vitro culture (Fynewever et al., 2007), sperm bioimaging
(Feugang et al., 2012), and sorting into subpopulations (Odhiambo et al.,
2014; Barchanski et al., 2015)—all with positive outcomes.

However, even in view of these encouraging findings, substantial con-
cerns associated with the use of nanomaterials for intra-gamete delivery
remain. Thus far, most studies evaluating the potential effects of nanoma-
terials in gametes have focused specifically on sperm since this cell
represents the main target for loading with exogenous compounds,
either for sorting purposes or for sperm-mediated gene/protein delivery
into the oocyte. Secondly, the nanomaterials, which have been tested for
safety in sperm almost exclusively belong to the non-biodegradable cat-
egory, which raises legitimate concerns about their potential long-term
effects in the case of stable integration into embryonic cells. Although
the studied nanomaterial-based intra-gamete delivery platforms have
been reported to exert their transport function primarily via anchoring
to the surface of the gamete plasma membrane or by intra-membrane
sequestration, rather than cytoplasmic internalization, a small propor-
tion of nanomaterial has been reported to reach the intracellular com-
partment in most cases (Kim et al., 2010; Feugang et al., 2012; Courbiere
et al., 2013; Barkalina et al., 2014b; Taylor et al., 2014b; Barchanski
et al., 2015). These observations, along with the contradictory nature

---

### Table I Key features of nanomaterials which favour their use in biomedicine [reproduced with permission from Barkalina et al. (2014a)].

| Feature          | Relevance for biomedical applications                                                                                                                                 |
|------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Small size       | Comparability with the size of biological molecules                                                                                                                                                                                |
|                  | Potential for a straightforward integration into cellular processes and physiological pathways                                                                                                                                 |
| Large surface    | Capacity to carry large amounts of biological cargo, including simultaneous transport of various types of cargo on one nanocarrier                                                                                           |
| Versatility      | Adjustable physicochemical properties (size, shape, surface charge and architecture) for increased efficacy of targeting                                                                                                     |
|                  | Adjustable surface chemistry (addition of specific functional groups and/or coatings) for the covalent or non-covalent absorption of a particular type of payload                                         |
|                  | Options for the ‘fine-tuning’ of surface chemistry through the addition of highly specific ligands for molecular recognition and further enhanced selectivity of targeting         |
| Targeted action  | High sensitivity and specificity                                                                                                                                                                                                   |
|                  | Decreased ‘off-target’ effects of cargo                                                                                                                                                                                            |
|                  | Improved accuracy of detection profiles for diagnostic agents                                                                                                                                                                    |
| Stability        | Distance of action                                                                                                                                                                                                                 |
|                  | Options for systemic administration                                                                                                                                                                                                |
|                  | Protection of ‘sensitive’ payloads and optimized biodistribution                                                                                                                                                                 |

---

### Table II Nanoparticle-mediated delivery into gametes and cell-labelling in vitro: experimental studies in animal models.

| Study            | Nanomaterial                                                                 | Application                                                                                          |
|------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| Fynewever et al. | Polystyrene and polyacrylonitrile NPs                                      | External and intracytoplasmic labelling of preimplantation embryos during in vitro culture             |
| (2007)           |                                                                             | Proof-of-principle transfer of anti-protein kinase C-antibody into sperm                             |
| Makhluf et al.   | Polynvinylalcohol-coated magnetic iron oxide (Fe₃O₄) NPs                   | Facility of SMGT                                                                                     |
| (2008)           |                                                                             | Facility of SMGT (‘NanoSMGT’)                                                                        |
| Kim et al. (2010)| Magnetic NPs (commercial agent)                                             | Facility of SMGT                                                                                     |
| Campos et al.    | Nanopolymer (commercial agent)                                              | Facility of SMGT (‘NanoSMGT’)                                                                        |
| (2011a)          |                                                                             |                                                                                                       |
| Campos et al.    | Nanopolymer (commercial agent) and halloysite clay nanotubes               |                                                                                                       |
| (2011b)          |                                                                             |                                                                                                       |
| Feugang et al.   | CdSe/ZnS quantum dots                                                       | ‘Live’ bioimaging of sperm                                                                          |
| (2012)           |                                                                             | Magnetic removal of defective sperm subpopulation from the ejaculate (‘nanopurification’)            |
| Odhiambo et al.  | Magnetic iron oxide (Fe₃O₄) NPs                                            |                                                                                                       |
| (2014)           |                                                                             |                                                                                                       |
| Barchanski et al.| Nanogold                                                                    | Proof-of-principle investigation of the potential to label the specific DNA sequences in viable sperm |
| (2015)           |                                                                             |                                                                                                       |

NPs, nanoparticles; SMGT, sperm-mediated gene transfer.
of data regarding the long-term effects of non-biodegradable nanomaterials upon the embryo/fetal development, which arise from the methodologically diverse studies utilizing different protocols and animal models (Cela´ et al., 2014), form the main reasons for general caution towards the application of non-biodegradable nanomaterials for intra-gamete delivery outside the experimental setting. Therefore, discovery of an alternative small-scale versatile delivery platform, which would interact with gametes and transport molecular compounds inside these cells in a fashion similar to previously studied inorganic nanomaterials but at the same time undergo biodegradation, would be highly advantageous for reproductive biology and ART.

Cell-penetrating peptides act both as targeting tools for nanomaterials and independent delivery platforms

In recent years, the search for a targeted biodegradable delivery tool, capable of transporting biological cargo into gametes and embryos, prompted an investigation into the potential benefits of cell-penetrating peptides (CPPs). CPPs are a specific class of short cationic/amphipathic peptides (<30 amino acids), capable of undergoing the energy- and receptor-independent translocation across the plasma membrane and transporting a considerably larger molecular cargo inside the cell, bypassing the traditional internalization pathways (Patel et al., 2007; Jones and Sayers, 2012). To date, a number of CPPs have been described as possessing affinity towards mammalian gametes and embryos, with several CPPs also demonstrating a promising delivery capacity (Table III). Apart from their innate delivery potential, these CPPs can be applied as functionalisation tools for active targeting of nanomaterials towards a particular cell population. For example, in a recent study, Dr Coward’s group have identified that functionalisation of mesoporous silica nanoparticles with the CPP C105Y results in an increase of their binding rate with mammalian sperm in vitro and changes in binding profiles, which start to mimic those previously described for free C105Y (Jones et al., 2013; Barkalina et al., 2015). Other authors have utilized the functionalisation with nona-arginine R9 for CdSe/ZnS quantum dots (Feugang et al., 2012) and deca-arginine (R10), transactivator of transcription and simian-virus 40 large T antigen nuclear localization signal peptide for gold nanoparticles (Barchanski et al, 2015), to target mammalian sperm, although the efficacy of these CPPs in improving the sperm-particle interaction has not been demonstrated consistently.

Although CPPs are biodegradable and demonstrate a cargo delivery capacity, the high cost of production, need for specialized peptide synthesis equipment and dependence upon a third-party manufacturer, along with a lower loading capacity and limited potential for additional functionalisation, restrict their use as delivery tools primarily to a large research laboratory setting. An ideal delivery vehicle for reproductive science and medicine would represent a nano-sized multifunctional biodegradable cargo carrier, which can be produced in most laboratories in a straightforward fashion, targeted towards gametes/embryos and loaded with different types of cargo. In fact, a prototype of this platform exists in nature already, and is known to cell biologists as EVs. There is increasing understanding that these natural nanoparticles, universally secreted by pro- and eukaryotic cells, are involved in the crucial processes underlying gamete development, maturation and acquisition of fertilization potential. Moreover, these processes appear to be highly conserved across a variety of biological species (Sohel et al., 2013; Sullivan and Saez, 2013). These observations form another justification for studies into the feasibility of manipulating these key processes using similar ‘recombinant’ nanoplatforms.

Exosomes and microvesicles in reproductive biology: natural delivery vectors and mediators of cell function

EVs are universal mediators of cell communication with specialized roles in pregnancy

Exosomes and microvesicles (MVs), collectively referred to as EVs, are nanoscale-sized phospholipid bilayer-enclosed particles, naturally released

| Table III | Cell-penetrating peptides with affinity towards reproductive tissues and gametes and their delivery potential. |
|-----------|--------------------------------------------------------------------------------------------------|
| Author    | Peptide                  | Target                                   | Intracellular translocation | Delivery potential |
| Jones et al. (2013) | Penetratin | Bovine sperm | Yes | No |
|           | Tat                        | Yes | N/A |
|           | C105Y                     | Yes | N/A |
|           | Mitoparan                   | Yes | N/A |
|           | Inverso mitoparan           | Yes | N/A |
|           | Inverso mastoparan          | Yes | N/A |
|           | Transportan 10              | Yes | N/A |
| Yang et al. (2014b) | Poly-arginine 11R | Fish (Takifugu rubripes) spermatic cells | Yes | (biologically active Oct4) |
| Kwon et al. (2013) | LDP12                   | Mouse oocytes and embryos | Yes | (EGFP) |
| Yang et al. (2014a) | 7X-arginine R7             | Mouse oocytes and embryos | Yes | (estrogen-related receptor β) |
| Campelo et al. (2014) | Crotamine                | Bovine embryos | Yes | N/A |

LDP12, human papillomavirus L1 capsid protein; EGFP, enhanced green fluorescent protein.
by a variety of cell types into their microenvironment. According to the most recent views, the main difference between exosomes and MVs lies not in their size, from 40 nm to ~100 nm for exosomes and up to 1 μm for MVs, as it was assumed previously, but in the mechanism of production (Raposo and Stoorvogel, 2013). Exosomes represent derivatives of multivesicular bodies (MVBs), and are first formed as intra-luminal vesicles (ILVs) inside these enclosed intracellular compartments via inward budding of the MVB membrane. These ILVs undergo release from cells upon the fusion of MVBs with the plasma membrane, and form exosomes. In contrast, MVs originate via direct budding from the plasma membrane (Akers et al., 2013). Ever since exosomes were first described in the 1980s as reticulocyte-secreted vesicles involved in the process of transferrin receptor recycling (Harding et al., 1983; Pan and Johnstone, 1983), our understanding of the fundamental role of these natural organic nanoparticles in cellular communication has evolved enormously. Exosomes have been demonstrated to have a complex tissue-specific organic content, including bioactive lipids (Record et al., 2014), proteins (Fontana et al., 2013), cytokines, growth factors, messenger RNAs (mRNAs) and non-coding transcripts, such as microRNAs (miRNAs) (Braicu et al., 2015). However, the composition of MVs has been studied to a far lesser extent (Raposo and Stoorvogel, 2013). Today, EVs are universally recognized as powerful mediators of maternal immunosuppression, prevention of the semi-allogenic fetus by the mother’s immune system, and also as vasoactive messengers involved in endothelial dysfunction during pre-eclampsia (Knight et al., 1998; Taylor et al., 2006). Such seemingly conflicting roles were later attributed to the contrasting functions of two distinct pregnancy-specific EV subpopulations: placental exosomes with multiple immunomodulatory properties, favouring successful pregnancy, and pro-inflammatory syncytiotrophoblast-derived microvesicles/microparticles (STBMs), closely involved in pre-eclampsia (Redman et al., 2012; Mincheva-Nilsson and Baranov, 2014). Today, pregnancy-specific EVs are recognized as paramount mediators of fetomaternal cross-talk, responsible for the orchestration of a series of events leading to the establishment and maintenance of mammalian pregnancy. In particular, placental exosomes have been reported to promote vascular smooth muscle cell and endothelial migration, which is essential for the remodelling of uterine spiral arteries and the establishment of physiological fetomaternal placental circulation (Salomon et al., 2013, 2014), and triggering apoptosis in activated immune cells (Stenqvist et al., 2013). STBMs, in contrast, are characterized by their proinflammatory, anti-endothelial, and procoagulant effects, and the total concentration and levels of expression of associated ‘endogenous danger molecules’, such as heat shock protein 70 (HSP70) and high mobility group box 1 (HMGB1), have been reported to directly correlate with the severity of pre-eclampsia (Redman et al., 2012).

**EVs regulate sperm maturation via the direct transfer of essential proteins to sperm**

Over recent years, and in addition to their role in mammalian pregnancy, the significant contribution of EVs to the fine processes of gamete maturation and the acquisition of fertilization potential are beginning to be elucidated (Table IV). Furthermore, the fact that these processes are highly conserved across many species is being increasingly recognized (Corrigan et al., 2014). Although prostate-derived EVs (‘prostasomes’) were first discovered in human semen in 1978, the highly important contribution of EVs (produced in various portions of the male reproductive tract) to post-testicular sperm maturation across the variety of mammalian species was not recognized until the 1990s (reviewed in Saez et al., 2003; Sullivan et al., 2005). Post-testicular sperm maturation involves structural and functional reorganization of the sperm membrane during its passage through the epididymis, and is essential for the acquisition of motility and fertilization ability. At this stage, the sperm will have already lost the capacity for active protein synthesis. Therefore, the modification of sperm surface structure with protein targets for the recognition of zona pellucida is largely dependent upon the direct transfer of essential compounds from the epididymal microenvironment. The limited endocytic activity of sperm at this stage necessitates the non-conventional molecular translocation mechanisms, which are currently considered to be mediated by the EVs of epididymal origin, also referred to as epidysomes. Epidysomes are involved in the enrichment of the sperm surface membrane with a wide range of proteins: glycosylphosphatidylinositol (GPI)-anchored proteins, including the proteins P26h, P25b, and P34H, essential for fertilization in hamster, bull, and human, respectively (Legare et al., 1999; Frenette et al., 2002); a disintegrin and metalloprotease (ADAM) 7 (Oh et al., 2009) and glialia pathogenesis-related I-like protein 1 (GIPrL1) (Caballero et al., 2012), both of which are involved in the interaction of sperm with zona pellucida; tyrosine kinase cSrc, playing a role in sperm capacitation (Krapf et al., 2012); epidydymal sperm binding protein 1 (ELSPBP1), serving as a ‘molecular tag’ for dead epididymal sperm in certain animals (D’Amour et al., 2012); plasma membrane Ca²⁺-ATPase (PMCA) with important functions for male fertility (Schwarz et al., 2013). In addition, recent observations show that epidysomes are also involved in the regulation of post-transcriptional gene expression within the epididymal epithelium via the intercellular transport of miRNAs between the portions of male reproductive tract (Belleannee et al., 2013). The mechanisms of protein transfer between epidysomes have not been yet elucidated in detail. However, it has been shown that epidysomes, similarly to sperm, demonstrate compartmentalization of proteins on the surface membrane and contain similar detergent-resistant domains, or lipid rafts, enriched in cholesterol and sphingomyelin, which can directly exchange proteins with the corresponding domains on the sperm surface membrane and thereby facilitate sperm interactions and protein transfers (Schwarz et al., 2013).

Prostasomes, secreted by the prostate gland epithelium and rich in sphingomyelins and cholesterol, have also been reported to ‘supplement’ sperm with complement-inhibitory molecules (CD59), shielding the sperm from immune recognition in the female genital tract (Rooney et al., 1993) via the pH-dependent fusion mechanism (Arienti et al., 1997), interact with polymorphonuclear and mononuclear leucocytes (Arienti et al., 1998) and reduce the overall reactive oxygen species production by polymorphonuclear neutrophils via the inhibition of NADPH oxidase activity by lipid transfer (Saez et al., 2000). Prostasomes enhance sperm motility (Fabiani et al., 1994a, b; Arienti et al., 1999; Wang et al., 2001) and influence sperm capacitation (Cross and Mahasreshti, 1997;
Secretory pathway Ca\(^{2+}\)-adenosine diphosphate (ADP) ribosyl cyclase (CD38), V-ATPase A1, are delivered to sperm by prostasomes, include the enzymes required for fertilization. The Ca\(^{2+}\)-signalling machinery, capacitation and acquisition of its specific patterns associated with fertilization. The Ca\(^{2+}\)-signalling tools are currently considered to be provided to sperm by prostasomes, include the enzymes adenosine diphosphate (ADP) ribosyl cyclase (CD38), V-ATPase A1, secretory pathway Ca\(^{2+}\)-ATPase, progesterone receptors, ryanodine receptors (Park et al., 2011) and free Ca\(^{2+}\) (Palmerini et al., 1999). This molecular machinery, acquired by sperm during fusion with prostatesomes, has been shown to play an essential role in the promotion of sperm hyper-activation, a crucial motion pattern required for penetration of the zona pellucida, and the acrosome reaction (Park et al., 2011). The mechanism of prostasomes-sperm transport appears to be similar to the epididymosomes-sperm exchange of molecular compounds. Similarly to epididymosomes, the prostasomes have been shown to fuse with sperm at acidic or low pH (Arienti et al., 1997; Palmerini et al., 1999), and deliver the molecular cargo. Apart from the proteins, prostasomes have been recently demonstrated to contain fragments of DNA randomly selected from the genome and rapidly, within 15 min of co-incubation, transfer these fragments into the sperm head, neck and tail under the physiological vaginal pH—an important observation, highlighting yet another potential biological role of these mediators of cell communication (Ronquist et al., 2011).

### Emerging evidence of the role of EVs in regulating female reproductive function

More recently, EVs have been identified in the follicular fluid of ovarian follicles where they have been shown to harbour a wide array of granulosa and cumulus cell-derived miRNAs and common exosomal and cell-type-specific proteins, suggesting a role in cell communication within mammalian ovarian follicles and in the regulation of follicular maturation (da Silveira et al., 2012, 2014; Sohel et al., 2013; Santonocito et al., 2014). Exosomes have also been isolated from mammalian oviducts (Al-Dossary et al., 2013; Alminana et al., 2014) and uterine cavity fluid (Ng et al., 2013; Ruiz-Gonzalez et al., 2015), and in all cases have been shown to transport molecular cargo (miRNAs/proteins) with important functions for fertilization, implantation and early pregnancy or the induction of specific sperm motility patterns, required for penetration of the zona pellucida (‘hyperactivation’). In the same way as pregnancy-specific exosomes and STBMs, EVs in the male and female reproductive tract are structurally heterogenous and form multiple subpopulations (Poliakov et al., 2009; Aalberts et al., 2012; Brouwers et al., 2013; Caballero et al., 2013). It is hypothesized that different subpopulations of these EVs have different functional roles, however the exact mechanisms involved remain to be characterized.

Interestingly, the powerful effects of natural EV-mediated paracrine regulation were intentionally exploited by Saadeldin et al. (2014), who reported that the co-culture of cloned porcine embryos, produced...
using the nuclear transfer technique, improved substantially during co-culture with parthenogenetic embryos releasing EVs containing multiple pluripotency gene mRNAs, which could be internalized into the cloned embryos. This promising observation further strengthens the hypothesis that EVs could represent an attractive candidate for intracellular delivery in reproductive biology and medicine, and improve the efficacy of existing investigative and therapeutic techniques.

**EV-mediated delivery: bridging the gap between nature and nanotechnology**

The concept of using EVs therapeutically is the focus of intense research in the fields of cancer treatment, regenerative medicine and infectious, inflammatory and neurodegenerative diseases (El-Andaloussi et al., 2013). The beneficial effects of EV-mediated paracrine signalling form the cornerstone of mesenchymal stromal cell-based therapies for the treatment of neural, cardiac or acute generalized tissue damage (Cashman et al., 2013; Monsel et al., 2014; Xin et al., 2014). These cells are characterized by strong paracrine activity rather than differentiation potential, and produce large amounts of EVs, potentiating cell proliferation, regenerative reprogramming, angiogenesis and immunomodulation in affected areas (El-Andaloussi et al., 2013). In addition to these approaches involving the indirect uses of EVs, an increasing number of studies describe the intentional production of EVs for subsequent use as drug delivery platforms.

There is mounting evidence that EVs are naturally produced by a variety of cell types (El-Andaloussi et al., 2012), and can be purified from culture media using relatively straightforward ultracentrifugation protocols, labelled with fluorescent probes (Nazarenko et al., 2013; Takahashi et al., 2013) and loaded with molecular cargo via co-incubation (Sun et al., 2010) or electroporation (Alvarez-Erviti et al., 2011; El-Andaloussi et al., 2012; Tian et al., 2014). Furthermore, cells can be engineered through transfection to secrete modified EVs, either loaded with specific cargo (Akao et al., 2011; Mizrahi et al., 2013) or expressing targeting moieties on their surface, which allows users to direct EVs towards a particular cell population in order to improve selectivity and range of action (Alvarez-Erviti et al., 2011; Rountree et al., 2011;
El-Andaloussi et al., 2012; Ohno et al., 2013; Tian et al., 2014). The expression of targeting moieties on the EV surface could be extensively explored in reproductive science, especially considering emerging evidence that certain CPPs promote the internalization of compounds into gametes and embryos and improve the outcome of in vitro culture (Jones et al., 2013; Kwon et al., 2013; Yang et al., 2014a; Barkalina et al., 2015). Interestingly, in response to growing interest in EV-mediated drug delivery, several research groups have proposed alternative approaches for EV production, involving the passage of source cells through a series of filters (Jang et al., 2013; Jo et al., 2014b) or microfluidic channels (Jo et al., 2014a) to create artificial exosome-mimetic nanovesicles. These nanovesicles have been demonstrated to have a similar composition and delivery capacity to secreted EVs, and contain mRNAs and intracellular and plasma membrane proteins.

Thus far, purified and loaded EVs have been successfully applied for the delivery of anti-inflammatory compounds into activated myeloid cells and microglial brain cells as prototype treatments for autoimmune/inflammatory diseases (Sun et al., 2010; Zhuang et al., 2011), targeting of chemotherapeutics, suicide gene mRNAs/proteins, miRNAs and investigative therapeutic cancer vaccines towards malignant cells (Rountree et al., 2011; Mizak et al., 2013; Ohno et al., 2013; Tian et al., 2014), and the targeted systemic delivery of small interfering RNAs into the brain as an experimental therapy for Alzheimer’s and Parkinson’s disease (Alvarez-Erviti et al., 2011; El-Andaloussi et al., 2012; Cooper et al., 2014). In these studies EVs have been consistently shown to have highly promising features for intracellular delivery, including high specificity and selectivity, and significant potential for the systemic delivery of experimental agents with otherwise unfavourable biodistribution profiles.

**Figure 2** Translational aspects of EV-mediated transfer of molecular compounds into gametes and embryos in vitro: potential applications. EVs loaded with molecular cargo could be applied for (a) targeted delivery of sperm motility-activating compounds to facilitate fertilization during IVF; (b) loading of sperm, characterized by molecular deficiencies of oocyte-activating factors (SOAFs), with novel and more physiological exogenous compounds acting as oocyte activators (recombinant versions of SOAFs, small molecules with similar activity, etc.) for subsequent delivery into the oocyte at the time of fertilization and assisted oocyte activation (AOA) as an alternative to currently applied physical, mechanical or chemical AOA; (c) facilitation of sperm-mediated gene transfer—a phenomenon based upon the property of mammalian sperm to bind and incorporate exogenous DNA upon co-incubation in vitro and delivery into the oocyte at the time of fertilization to produce genetically-edited embryos; (d) direct supplementation of oocytes with molecular compounds, enhancing their developmental capacity, and increasing the chances to sustain early embryo development; (e, f) delivery of gene editing tools into the oocytes or embryos, respectively, as a tool to treat hereditary diseases. The use of EVs as versatile delivery tools could allow us to bypass invasive micromanipulation procedures, which are currently considered the gold standard for gamete manipulation in assisted reproduction.
Collectively, the relative ease of production, biodegradability and the possibility for targeting and loading with a variety of compounds relevant for reproductive biology and science (including small molecules, nucleic acids and proteins) along with the enormous physiological role of EVs in cell communication processes underlying reproduction, as well as encouraging evidence from other fields, render these natural ‘nanoplatforms’ particularly attractive candidates for compound delivery into gametes and embryos (Figs 1 and 2).

Conclusions

Today, ART is viewed not only as a routine approach for infertility treatment but also, progressively, as a state-of-art guarantee of successful conception and childbirth at any chosen time in an individual’s life. However, despite the ever-growing success of ART in the field of infertility treatment, this technique still only results in the live birth of healthy children in approximately 30% of couples starting treatment. The increasing reliance of medical practitioners and the general public upon ART justifies further extensive investigation into the fundamental mechanisms of reproduction to improve our existing levels of knowledge and facilitate the continuous improvement of current medical technology. However, it is highly unlikely that a substantial breakthrough in the field of reproductive science and medicine can be achieved without the discovery of novel research tools that allow us to systematically study and manipulate gamete and embryo function in a real-time setting, while fully preserving their viability. In recent years, biomedical nanotechnology has offered potent solutions to the problem of delivering molecular compounds into gametes. Nevertheless, the predominant use of non-biodegradable nanoparticles to promote the uptake of DNA and proteins into gametes continues to raise concerns, which limits the use of these techniques to a purely investigative platform. To allow the subsequent translation of these methodologies to clinical ART, studies should utilize biodegradable delivery platforms, which mimic natural mechanisms of molecular cargo trafficking as closely as possible. In this view, the field of reproductive science could be substantially advanced by actively developing EV-mediated delivery technology.

Currently, EVs represent the most physiological intracellular delivery tools available for reproductive science and medicine. These natural universal mediators of cell communication combine the benefits of engineered nanomaterials, such as the potential for in vitro production, targeting and loading, with the essential feature of biodegradability. Furthermore, the high degree of involvement of EVs in the essential processes underlying gamete maturation, the acquisition of fertilization potential and the establishment/maintenance of pregnancy, renders the use of similar ‘modified’ nanoplatforms particularly exciting. We anticipate that future investigations into the possibility of applying EVs for the intentional intracellular delivery of molecular compounds into gametes and embryos will open new horizons for reproductive science and clinical ART, ultimately leading to significant improvements in patient care.

Acknowledgements

The authors would like to acknowledge Professor Christopher Barratt (Division of Cardiovascular & Diabetes Medicines, University of Dundee) for a critical discussion of the original draft of this manuscript.

Authors’ roles

N.B. designed the manuscript, performed the literature search and drafted the manuscript. C.J. participated in manuscript drafting, performed revisions and critically discussed the manuscript. M.J.A.W. performed revisions and critically discussed the manuscript. K.C. revised and critically discussed the manuscript. No writing assistance was utilized in the production of this manuscript.

Funding

N.B. is funded by the Clarendon, Scatcherd European and Cyril & Phillis Long Schemes, the Nuffield Department of Obstetrics and Gynaecology and an EPSRC Pathways to Impact Award (University of Oxford). No specific funding was sought for the preparation of this manuscript.

Conflict of interest

N.B., C.J. and K.C. have a patent pending related to the work discussed in this article entitled ‘Delivery Method’ (PCT Patent Application Number PCT/GB13/053394 filed on the 20th December 2013). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

References

Aalberts M, van Dessel-Emiliami PM, van Adrichem NP, van Wijnen M, Wauben MH, Stout TA, Stoovogel W. Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. Biol Reprod 2012; 86:82.

Adamson G, Tabangin M, Macaluso M, de Mouzon J. The number of babies born globally after treatment with the assisted reproductive technologies (ART). Fertil Steril 2013; 100:S42.

Alkao Y, Iio A, Itoh T, Naguchi S, Itoh Y, Ohtsuki Y, Naote T. Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages. Mol Ther 2011; 19:395–399.

Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol 2013; 113:1–11.

Ali-Dossier AA, Strehler EE, Martin-Deleon PA. Expression and secretion of plasma membrane Ca2+-ATPase 4α (PMCA4a) during murine estrus: association with oviductal exosomes and uptake in sperm. PLoS One 2013; 8:e80181.

Albanese A, Tang PS, Chan WC. The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng 2012; 14:1–16.

Almimana C, Corbin E, Tsikis G, Soleilhavoup C, Gallo L, Sandra O, Mermillod P. 108 characterization of bovine oviductal exosomes from in vivo and in vitro origin. Reprod Fertil Dev 2014; 26:147.

Alvarez-Erviti L, Seow Y, Yin H, Bettis C, Lalakal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 2011; 29:341–345.

Amdani SN, Jones C, Coward K. Phospholipase C zeta (PLCzeta): oocyte activation and clinical links to male factor infertility. Adv Biol Regul 2013; 53:292–308.

Arienti G, Carlino E, Palmerini CA. Fusion of human sperm to prostasomes at acidic pH. J Membr Biol 1997; 155:89–94.

Arienti G, Carlino E, Saccardi C, Palmerini CA. Interactions between prostasomes and leukocytes. Biochim Biophys Acta 1998; 1425:36–40.

Arienti G, Carlino E, Niculucci A, Cosmi EV, Santi F, Palmerini CA. The motility of human spermatozoa as influenced by prostasomes at various pH levels. Biol Cell 1999; 91:51–54.
Barchanski A, Taylor U, Saçi CL, Gamrad L, Kues WA, Rath D, Barcikowski S. Bioconjugated gold nanoparticles penetrate into spermatozoa depending on plasma membrane status. J Biomed Nanotechnology 2011;1:1597–1607.

Barkalina N, Charalambous C, Jones C, Coward K. Nanotechnology in reproductive medicine: emerging applications of nanomaterials. Nanomedicine 2014a;10:921–938.

Barkalina N, Jones C, Kashir J, Coote S, Huang X, Morrison R, Townley H, Coward K. Effects of mesoporous silica nanoparticles upon the function of mammalian sperm in vitro. Nanomedicine 2014b;10:859–870.

Barkalina N, Jones C, Townley H, Coward K. Functionalisation of mesoporous silica nanoparticles with a cell-penetrating peptide to target mammalian sperm in vitro. Nanomedicine (Lond.) 2015;10:1539–1553.

Belleeunera C, Calvo E, Caballero J, Sullivan R. Epipriomosomes convey different repertoires of microRNAs throughout the bovine epididymis. Biol Reprod 2013;89:30.

Ben-David Makhfuf S, Qasem R, Rubinstein S, Gedanken A, Breitbart H. Loading magnetic nanoparticles into sperm cells does not affect their functionality. Langmuir 2006;22:9480–9482.

Braico C, Tomuleasa C, Monroig P, Cucuianu A, Benindna-Neagoe I, Calin GA. Exosomes as divine messengers: are they the Hermes of modern molecular oncology? Cell Death Differ 2015;22:34–45.

Brakman G, Winslet M, Seifalian AM. Systematic review: the applications of nanotechnology in gastroenterology. Aliment Pharmacol Ther 2012;36:213–221.

Brouwers JF, Aalberts M, Jansen JW, van Niel G, Wauben MH, Stout TA, Helms JB, Stoovogel W. Distinct lipid compositions of two types of human protasomes. Proteomics 2013;13:1660–1666.

Caballero J, Fenrette G, D’Amours O, Belleeunera C, Lacroix-Pepin N, Robert C, Sullivan R. Bovine sperm raft membrane associated Glioma Pathogenesis-Related 1-like protein (GliPrL1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida. J Cell Physiol 2012;227:3876–3886.

Caballero JN, Fenrette G, Belleeuna C, Sullivan R. CDP-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. PLoS One 2013;8:e65364.

Campelo IS, Pereira AF, Alcantara-Neto AS, Canel NG, Souza-Fabjan JM, Teixeira DI, Camargo LS, Mele LM, Radis-Baptista G, Salamone DF. Extracellular vesicle for intra-gamete delivery in ART. Fertil Steril 2014;101:276–287.

Cooper JM, Wiklander PB, Nordin JZ, Al-Shawi R, Wood MJ, Vithlani M, Schapira AH, da Silveira JC, Camargo LS, Melo LM, Radis-Baptista G, Salamone DF. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. Biol Reprod 2012;86:71.

da Silveira JC, Carnevale EM, Winger QA, Bouma GJ. Regulation of ACVR1 and ID2 by cell-secreted exosomes during follicular maturation in the mare. Reprod Biol Endocrinol 2014;12:44.

El-Andalousi S, Lee Y, Lakhal-Littleton S, Li J, Seow Y, Gardner C, Alvarez-Erviti L, Sargent IL, Wood MJ. Exosome-mediated delivery of siRNA in vitro and in vivo. Nat Protoc 2012;7:112–126.

El-Andalousi S, Mager I, Breakfield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 2013;12:347–357.

Eppig JJ, Pendola R, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperation between granulosa cells and oocytes: amino acid transport. Biol Reprod 2005;73:351–357.

European Society of Human Reproduction and Embryology (ESHRE). ART fact sheet (June 2014). 2014. http://www.eshre.eu/Guidelines-and-Legal/ART-fact-sheet.aspx (accessed on 2 March 2015).

Fabiani R, Johansson L, Lundkvist O, Ronquist G. Enhanced recruitment of motile spermatozoa by protasome inclusion in swim-up medium. Hum Reprod 1994a;9:1485–1489.

Fabiani R, Johansson L, Lundkvist O, Ullmsten U, Ronquist G. Promotive effect by protasomes on normal human spermatozoa exhibiting no forward motility due to buffer washings. Eur J Obstet Gynecol Reprod Biol 1994b;57:181–188.

Falchi L, Bogliolo L, Galleri V, Vlachoupolou G, Murrone O, Epifani G, Pinna A, Innocenzi P. Ledda S. 266 biocompatibility of nanoceria in ram sperm during 24 hours of incubation. Reprod Fertil Dev 2014;27:222.

Feugang JM, Youngblood RC, Greene J, Fahad AS, Monroe WA, Willard ST, Ryan PL. Application of quantum dot nanoparticles for potential non-invasive bio-imaging of mammalian spermatozoa. J Nanobiotechnology 2012;10:45.

Fontana S, Saieva L, Taverna S, Alessandro A. Contribution of proteomics to understanding the role of tumor-derived exosomes in cancer progression: state of the art and new perspectives. Proteomics 2013;13:1581–1594.

Fragnoli E, Affarawati S, Spah K, Wells D. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. Mol Hum Reprod 2014;20:177–182.

Frenette G, Lessard C, Sullivan R. Selected proteins of ‘prostasome-like particles’ from human seminal plasma prevent sperm from becoming acrosomally responsive to the agonist progesterone. J Cell Physiol 2012;229:267–278.

Garcia-Vazquez FA, Garcia-Rosello E, Gutierrez-Adan A, Gadea J. Effect of sperm proteomics to extracellular vesicles in equine ovarian follicular fluid contain miRNAs and proteins. Reprod Biol Endocrinol 2012;10:51.

Garcia-Vazquez FA, Garcia-Rosello E, Gutierrez-Adan A, Gadea J. Effect of sperm proteomics to extracellular vesicles in equine ovarian follicular fluid contain miRNAs and proteins. Reprod Biol Endocrinol 2012;10:51.

Giroud J, Fenrette G, Sullivan R. Compartmentalization of proteins in epididymosomes coordinates the association of epididymal proteins with the different functional structures of bovine spermatozoa. Biol Reprod 2009;80:965–972.

Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 1983;97:329–339.

Holmes D. The next big things are tiny. Lancet Neurol 2013;12:31–32.

Human Fertilisation and Embryology Authority (HFEA). Fertility treatment in 2013: trends and figures. 2014. http://www.hfea.gov.uk/docs/HFEA_Fertility_Trends_and_Figures_2013.pdf (accessed on 2 March 2015).

Jakovup L, Fuchs B, SuSzz R, Wibbelt G, Braun B, Muller K, Schiller J. The solubilisation of boar sperm membranes by different detergents—a microscopic, MALDI-TOF MS, 31P NMR and PAGE study on membrane lysis, extraction efficiency, lipid and protein composition. Lipids Health Dis 2009:8:49.

Jalilak AP, Moley KH, Omurtaj K, Hu G, Lanza GM, Wicklaine SA, Hood JL. Nanoparticle incorporation of melittin reduces sperm and vaginal epithelium cytotoxicity. PLoS One 2014;9:e95411.

James PS, Hennessy C, Berge T, Jones R. Compartmentalisation of the sperm plasma membrane: a FRAP, FLIP and SPCI analysis of putative diffusion barriers on the sperm head. J Cell Sci 2004;117:6485–6495.

Jang SC, Kim OY, Yoon CM, Choi DS, Roh TY, Park J, Nilsson J, Lotvall J, Kim YK, Gho YS. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. ACS Nano 2013;7:6768–7710.

Jo W, Jeong D, Kim J, Cho S, Jang SC, Han C, Kang JY, Gho YS, Park J. Microfluidic fabrication of cell-derived nanovesicles as endogenous RNA carriers. Lab Chip 2014a;14:1261–1269.
Jo W, Kim J, Yoon J, Jeong D, Cho S, Jeong H, Yoon YJ, Kim SC, Gho YS, Park J. Large-scale generation of cell-derived nanovesicles. Nanoscale 2014b; 6:10256–10264.

Jones AT, Sayers EJ. Cell entry of cell penetrating peptides: tales of tail wagging dogs. J Control Release 2012;161:582–591.

Jones S, Lukanowska M, Suhorutenko J, Oxenham S, Barratt C, Publicover S, Copolovic DM, Langel U, Howl J. Intracellular translocation and differential accumulation of cell-penetrating peptides in bovine spermatozoa: evaluation of efficient delivery vectors that do not compromise human sperm motility. Hum Reprod 2013;28:1874–1889.

Kashir J, Heynen A, Jones C, Durrans C, Craig J, Gadea J, Turner K, Parrington J, Coward K. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. Reprod Biomed Online 2011;23:263–267.

Kawamura K, Chen Y, Shu Y, Cheng Y, Qiao J, Behr B, Pera RR, Hsieh A/JW. Promotion of human early embryonic development and blastocyst outgrowth in vitro using autocrine/paracrine growth factors. PLoS One 2012;7:e93328.

Kim TS, Lee SH, Gang GT, Lee YS, Kim SU, Koo DB, Shin MY, Park CK, Lee DS. Exogenous DNA uptake of boar spermatozoa by a magnetic nanoparticle vector system. Reprod Domest Anim 2010;45:e201–e206.

Knight M, Redman CWG, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. BJOG 1998; 105:632–640.

Krapf D, Ruan YC, Wertheimer EV, Battistone MA, Pawlak JB, Sanjay A, Pilder SH, Cuasnicu P, Breton S, Visconti PE. Src is necessary for epididymal development and is incorporated into sperm during epididymal transit. Dev Biol 2013;369:43–53.

Kunzmann A, Anderson ER, Thurner T, Krug H, Schweynus A, Fabel D. Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. Biochim Biophys Acta 2011;1810:361–373.

Kwon S, Kwak A, Shin H, Choi S, Kim S, Lim HJ. Application of a novel cell-permeable peptide-driven protein delivery in mouse blastocysts. Reproduction 2013; 146:15–153.

Lammers T, Aime S, Hennink WE, Storm G, Kiessling F. Theranostic nanomedicine. Acc Chem Res 2011;44:1029–1038.

Legare C, Berube B, Boue F, Lefevre V, Morales CR, El-Alfy M, Sullivan R. Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. Mol Reprod Dev 1999;52:225–233.

Lehner R, Wang X, Marsch S, Hunziker P. Intelligent nanomaterials for medicine: carrier platforms and targeting strategies in the context of clinical application. Nanomedicine 2013;9:742–757.

Lenai A, Gandini L, Picardo M, Tramer F, Sandri G, Panfili E. Accumulation of cell-penetrating peptides in bovine spermatozoa: evaluation of efficient delivery vectors that do not compromise human sperm motility. Hum Reprod 2013;28:1874–1889.

Martin-Romero FJ, Miguel-Lasobras EM, Dominguez-Arroyo JA, Gonzalez-Carrera E, Lenzi A, Gandini L, Picardo M, Tramer F, Sandri G, Panfili E. Lipoperoxidation damage of spermatozoa. Asian J Androl 2013;15:483–488.

Makhluf SB, Abu-Mukh R, Rubinstein S, Breitbart H, Gedanken A. Modified Cu+signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. Sci Signal 2011;4:ra31.

Patel LN, Zaro JL, Shen WC. Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. Pharm Res 2007;24:1977–1992.

Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov 2010;9:615–627.

Pfeihl LL, Fischman ML, Hellman U, Cisale H, Miranda PV. Boar seminal plasma exosomes: effect on sperm function and protein identification by sequencing. Theriogenology 2013;79:1071–1082.

Polakova A, Siplman M, Dokland T, Amling CL, Mobley JA. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. Prostate 2009;69:159–167.

Pons-Rejrahi H, Autonne C, Sion B, Brugnon F, Canil CL, Mobley JA. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. Prostate 2009;69:159–167.

Psarros C, Lee R, Margaritis M, Antoniades C. Nanomedicine for the prevention, treatment and imaging of atherosclerosis. Nanomedicine 2012;8:Suppl 1:S59–S68.

Ragoș G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200:373–383.

Record M, Carayon K, Poirot M, Silvante-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochim Biophys Acta 2014;1841:108–120.

Redman CW, Tannetta DS, Dragovic RA, Gardiner C, Southcombe JH, Collett GP, Sargent IL. Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia. Placenta 2012;33:Suppl:S48–S54.

Rehemann K, Schneider SW, Luger TA, Godin B, Ferrari M, Fuchs H. Nanomedicine—challenge and perspectives. Angew Chem Int Ed Engl 2009;48:872–897.

Ronquist GK, Larsson A, Ronquist G, Isaksson A, Hreinsdottir J, Carlsson L, Staveuers-Evers A. Prostasomal DNA Characterization and Transfer Into Human Sperm. Mol Reprod Dev 2011;87:467–476.

Rooney IA, Atkinson JP, Krul ES, Schonfeld G, Polakoski K, Saffitz JE, Morgan BP. Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes, and inhibits complement-mediated lysis. J Exp Med 1993;177:1409–1420.

Rouger N, Uhrondo H, Papier S, Checa MA, Sueldo C, Alvarez Sedo C. Changes in DNA fragmentation during sperm preparation for intracytoplasmic sperm injection over time. Fertil Steril 2013;100:65–74.

Rountree RB, Mandl SJ, Nachtewy JM, Dalpozzo K, Do L, Lombardo JR, Schoonmaker PL, Brinkmann K, Diermeier U, Lau S et al. Exosome targeting of tumor antigens expressed by cancer vaccines can improve antigen immunogenicity and therapeutic efficacy. Cancer Res 2011;71:5235–5244.

Ruiz-Gonzalez I, Xu J, Wang X, Burghardt RC, Dunlap KA, Bazer FW. Exosomes, endogenous retroviruses and toll-like receptors: pregnancy recognition in ewes. Reproduction 2015;149:281–291.
Ryan SM, Brayden DJ. Progress in the delivery of nanoparticle constructs: towards clinical translation. Curr Opin Pharmacol 2014;18:120–128.

Saadelldin IM, Kim SJ, Choi YB, Lee BC. Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. Cell Reprogram 2014;16:223–234.

Saez F, Motta C, Boucher D, Grizzard G. Prostasomes inhibit the NADPH oxidase activity of human neutrophils. Mol Hum Reprod 2000;6:883–891.

Saez F, Frenette G, Sullivan R. Epidydymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. J Androl 2003;24:149–154.

Salomon C, Ryan J, Sobrevilla L, Kobayashi M, Ashman K, Mitchell M, Rice GE. Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. PLoS One 2013;8:e68451.

Salomon C, Yee S, Scholz-Romer K, Kobayashi M, Vaswani K, Kwaskoff D, Illanes SE, Mitchell MD, Rice GE. Extravillous trophoblast cells-derived exosomes promote vascular smooth muscle cell migration. Front Pharmacol 2014;5:175.

Santonocito M, Vento M, Guglielmino MR, Battaglia R, Walsgreen J, Ragusa M, Barbagallo D, Borzi P, Rizzi S, Maugeri M et al. Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. Fertil Steril 2014;102:175–181.

Schwarz A, Wenemuth G, Post H, Brandenburger T, Aumuller G, Wilhelm B. Vesicular transfer of membrane components to bovine epididymal spermatozoa. Cell Tissue Res 2013;353:549–561.

Siciliano L, Marcano V, Carpino A. Prostasome-like vesicles stimulate acrosome reaction of pig spermatozoa. Reprod Biol Endocrinol 2008;6:5.

Soehl MM, Hoelker M, Noferesti SS, Salliew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K et al. Exosomal and non-exosomal transport of extra-cellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. PLoS One 2013;8:e78505.

Stenqvist AC, Nagaeva O, Baranov V, Mincheva-Nilsson L. Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules, in human sperm are inert envelope. J Biotechnol 2013;159:1551–1552.

Sullivan R, Saez F. Epidydymosomes, prostasomes, and liposome: their roles in mammalian male reproductive physiology. Reproduction 2013;146:R21–R35.

Sullivan R, Saez F, Giroud J, Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis 2005;35:1–10.

S. D. Zhang, X. Zhuang, X. Xiang, X. Wang, Y. Liu, C. Song, Y. Zhang, S. Barnes, S. Grizzle, W. Miller, D. Zhang. H. G. Xin H, L. Y. Chopp M. Exosomes/miRNAs as mediating cell-based therapy of stroke. Front Cell Neurosci 2014:377.

Takahashi Y, Nishikawa M, Shintotsuka H, Matsui Y, Ohara S, Imai T, Takakura Y. Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. J Biotechnol 2013;165:77–84.

Tapia J, Macias-Garcia B, Pihro-Moran A, Ortega-Ferrusola C, Salido G, Pena F, Aparicio I. The membrane of the mammalian spermatozoon: much more than an inert envelope. Reprod Domest Anim 2012;47 Suppl 3:65–75.

Tardif S, Madamidola OA, Brown SG, Frame L, Lekevire L, Wyatt PG. Exosomes: clinical relevance of human sperm motility modulation with compounds with reported phosphodiesterase inhibitor activity. Hum Reprod 2014;29:2123–2135.

Taylor DD, Akyol S, Gercel-Taylor C. Pregnancy-associated exosomes and their modulation of T cell signaling. J Immuno 2006;176:1534–1542.

Taylor U, Barchanski A, Petersen S, Kues WA, Baulain U, Gamrad L, Sajit L, Barcikowski S, Rath D. Gold nanoparticles interfere with sperm functionality by membrane adsorption without penetration. Nanotoxicology 2014;8 Suppl 1:118–127.

Taylor U, Garrells W, Barchanski A, Peterson S, Sajit L, Lucas-Hahn A, Gamrad L, Baulain U, Klein S, Kues WA et al. Injection of ligand-free gold and silver nanoparticles into murine embryos does not impact pre-implantation development. Beilstein J Nanotechnol 2014b;5:677–688.

Telfer EE, McLaughlin M. Strategies to support human oocyte development in vitro. Int J Dev Biol 2012;56:901–907.

Tian YH, Li SP, Song J, Ji T, Zhu MT, Anderson GJ, Wei JY, Nie GJ. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials 2014;35:2383–2390.

Tiedemann D, Taylor U, Rehbock C, Jakob J, Klein S, Kues WA, Barcikowski S, Rath D. Reprotoxicity of gold, silver, and gold-silver alloy nanoparticles on mammalian gametes. Analyst 2014;139:931–942.

Toro E, Fernandez S, Colomar A, Casanovas A, Alvarez JG, Lopez-Teijon M, Velilla E. Processing of semen can result in increased sperm DNA fragmentation. Fertil Steril 2009;92:2109–2112.

Tsai N, Lee B, Kim A, Yang R, Pan R, Lee D-K, Chow EK, Ho D. Nanomedicine for global health. JALA 2014:pii:2211068214538263.

Ulbrich W, Lamprecht A. Targeted drug-delivery approaches by nanoparticulate carriers in the therapy of inflammatory diseases. J R Soc Interface 2010;7 Suppl 1:555–566.

Wang J, Sauer MV. In vitro fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. Ther Clin Risk Manag 2006;2:353–364.

Wang J, Lundqvist M, Carlsson L, Nilsson O, Lundkvist O, Ronquist G. Prostasome-like granules from the PC-3 prostate cancer cell line increase the motility of washed human spermatozoa and adhere to the sperm. Eur J Obstet Gynecol Reprod Biol 2001;96:88–97.

Xin H, Li Y, Chopp M. Exosomes/miRNAs as mediating cell-based therapy of stroke. Front Cell Neurosci 2014a:877.

Yamauchi Y, Riel JM, Ward MA. Paternal DNA damage resulting from various sperm treatments persists after fertilization and is similar before and after DNA replication. J Androl 2013;34:229–238.

Yang NJ, Seol DW, Jo J, Jang HM, Yoon SY, Lee DR. Effect of cell-penetrating peptide-conjugated estrogen-related receptor beta on the development of mouse embryos cultured in vitro. Clin Exp Reprod Med 2014a;41:1–8.

Yang XX, Hou XN, Xu B, Hao X, Jiang GJ, Fan TJ. Cell-penetrating peptide delivery of biologically active ocyt6 protein into cultured Takifugu rubripes sperm cells. J Fish Biol 2014b;85:1369–1380.

Yelumalai S, Jones C, Mounce G, McVeigh E, Fatum M, Coward K. Levels of the oocyte activation factor, phospholipase c zeta, in human sperm are enhanced when encapsulated in exosomes. Mol Ther 2010;18:1606–1614.

Yin J, Gong SP, Song YH, Lee ST. Effects of combined antioxidant supplementation on human sperm motility and morphology during sperm manipulation in vitro. Fertil Steril 2013;99:373–378.

Zhuang X, Xiang X, Grizzle W, Sun D, Zhang S, Axtell RC, Ju S, Mu J, Zhang L, Steinman L et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Mol Ther 2011;19:1769–1779.