REVIEW

Cell-secreted vesicles containing microRNAs as regulators of gamete maturation

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Abstract

Mammalian gamete maturation requires extensive signaling between germ cells and their surrounding somatic cells. In the ovary, theca cells, mural granulosa cells, cumulus cells and the oocyte all secrete factors throughout follicle growth and maturation that are critical for ovulation of a high-quality oocyte with the competence to develop into an embryo. Similarly, maturation of sperm occurs as it transits the epididymis during which epididymal epithelium and sperm exchange secretory factors that are required for sperm to gain motility and fertility. Recent studies in a variety of species have uncovered the presence of cell-secreted vesicles in follicular fluid (microvesicles and exosomes) and epididymal fluid (epididymosomes). Moreover, these cell-secreted vesicles contain small non-coding regulatory RNAs called microRNAs, which can be shuttled between maturing gametes and surrounding somatic cells. Although little is known about the exact mechanism of how microRNAs are loaded into these cell-secreted vesicles or are transferred and modulate gene expression and function in gametes, recent studies clearly suggest that cell-secreted vesicle microRNAs play a role in oocyte and sperm maturation. Moreover, a role for cell-secreted vesicular microRNAs in gamete maturation provides for novel opportunities to modulate and discover new diagnostic markers associated with male or female fertility. This manuscript provides an overview of cell-secreted vesicles in ovarian follicular fluid and epididymal fluid and microRNAs and discusses recent discoveries on the potential function of cell-secreted vesicles as carriers of microRNAs in oocyte and sperm maturation.

Key Words

- ▶ microRNAs
- ▶ extracellular vesicles
- ▶ sperm
- ▶ oocyte

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Introduction

Proper cell function requires the coordinated regulation of a myriad of genes and proteins necessary for maintenance of tissue homeostasis and organism fitness. MicroRNAs are small non-coding RNA molecules that regulate gene expression and function by binding complementary sequences in target transcripts and either promoting degradation or inhibiting translation. It has been

estimated that microRNAs regulate 10–50% of genes in the human genome (Lu & Clark 2012). Moreover, a single microRNA can regulate hundreds of transcripts, and at the same time, a single transcript can be regulated by many microRNAs (reviewed in Lu & Clark 2012). MicroRNAs thus are responsible for regulating and fine-tuning many gene regulatory networks, with the overall result of controlling

cell differentiation, proliferation, apoptosis, metabolism, responsiveness and secretion. It is not surprising that microRNAs also play important roles in regulation of gene function in various aspects of reproductive biology, including development and function of the reproductive tracts, germ cell development and maturation, fertilization and early embryo development (reviewed in [Luense *et al.* 2011](#), [Hilz *et al.* 2016](#)). The recent findings that microRNAs also are present in blood and can be transferred from one cell to another (e.g., [György *et al.* 2011](#)), adds another layer of intricacy of microRNA function in reproductive physiology.

A new mechanism in context of cell communication is the release and uptake of extracellular vesicles. Cells secrete a wide variety of membranous vesicles, often characterized by size, shape or content. Their nomenclature still is obscure and cell-secreted vesicles have been referred to as microparticles, ectosomes, shedding vesicles, microvesicles or exosomes ([Cocucci & Meldolesi 2015](#)). The notion that cells release membrane-enclosed vesicles and these vesicles are present in extracellular space has existed for several decades ([Raposo *et al.* 1996](#)). Initially considered 'cellular garbage bags' and/or the result of cellular degeneration, it is now clear that cells release vesicles into extracellular fluid both constitutively and following induction. Moreover, these extracellular vesicles contain a variety of bioactive molecules, including mRNAs and microRNAs that can be transferred from one cell to another.

The presence of microRNAs in these vesicles and their transfer and uptake by target cells suggests these vesicles can have pronounced effects on the regulation of genetic networks and ultimately cell phenotype and function ([Fevrier *et al.* 2004](#), [Mathivanan *et al.* 2010](#)). This review will focus on recent discoveries on the presence and potential function of cell-secreted or extracellular vesicles as carriers of microRNAs in oocyte and sperm maturation.

Small non-coding microRNAs: an overview

MicroRNAs are small, 18–24 nt long non-coding RNA molecules that regulate target mRNA expression and function through base-pairing between a ~8 nt seed sequence and the 3' untranslated region (UTR) ([Bartel 2009](#), [Lee *et al.* 2009](#), [Lu & Clark 2012](#)). The first step in microRNA biosynthesis is transcription of a microRNA gene by RNA polymerase II generating a primary transcript (pri-microRNA). Next, cropping of pri-microRNA by the RNase III endonuclease Drosha yields a ~70 nucleotide

long precursor microRNA (pre-microRNA). The pre-microRNAs are imperfect hairpin structures that are exported from the nucleus into the cytoplasm. In the cytoplasm, pre-microRNAs are processed further by the RNase III endonuclease Dicer into imperfect double-stranded RNA (dsRNA) complexes. The dsRNA complexes are loaded into a ribonucleoprotein complex called RNA-induced silencing complex (RISC), which consists of Argonaute proteins, and only retains the mature microRNA strand. Although Dicer is a critical enzyme involved in mature microRNA synthesis, pre-microRNAs can also be directly loaded into the RISC complex and processed by Argonaute 2 ([Cheloufi *et al.* 2010](#)). RISC forms a complex with target RNA and causes gene (transcript) silencing. Downregulation of target gene expression occurs by base-pairing interaction with a mRNA target via one of the two mechanisms; mRNA cleavage if there is high sequence complementarity with the microRNA or translational repression if there is less sequence complementarity with the microRNA. MicroRNA binding sites reside in the 3' UTR (untranslated region) of mRNA transcripts; however, binding to the coding region of a target transcript and 5'UTR also has been observed ([Lee *et al.* 2009](#), [Hausser *et al.* 2013](#)). MicroRNAs have been cloned, sequenced and/or identified in numerous plants, viruses and animals, and to date, hundreds of mammalian microRNA sequences have been identified that are highly conserved between species (e.g., [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article).

MicroRNAs in gamete maturation

Taking advantage of conditional gene targeting approaches in mice, a variety of studies have illustrated a critical role for microRNAs within germ cells. Using different promoter-driven Cre recombinase transgenes, Dicer has been deleted specifically in primordial germ cells. Absence of Dicer early on in development only mildly affects PGC proliferation in ovaries, but significantly decreased germ cell number in fetal testes by the time gonadal differentiation has completed ([Hayashi *et al.* 2008](#)). Furthermore, it now is evident that Dicer, and as such microRNAs, in germ cells are necessary for meiosis ([Luense *et al.* 2011](#), [Wang & Xu 2015](#)) and absence of microRNAs in oocytes leads to meiotic I and II spindle defects, suggesting a potential role during chromosome segregation. Contrary to oocytes, effects of microRNA absence on male germ cell maturation appear more widespread, and include apoptosis, chromosomal

misalignment, acrosome defects, morphological defects and impaired motility. Clearly microRNAs in germ cells play a variety of critical roles in germ cell development and maturation (reviewed in [Hilz *et al.* 2016](#)).

The process of gamete maturation requires extensive communication with surrounding somatic cells. In oocytes, this involves coordinate signaling processes between the maturing oocyte, cumulus cells and mural granulosa cells. The presence of cell-specific microRNAs within the ovarian follicle indicates distinct functions in controlling follicle growth and oocyte maturation. Using the horse as a model, we detected 22 microRNAs exclusively in granulosa cells, and 35 microRNAs in cumulus cells collected from antral follicles that are also consistently present in cell-secreted vesicles isolated from follicular fluid ([Silveira *et al.* 2012](#)).

Sperm maturation occurs within the epididymis and is accompanied by transfer of material between sperm and epididymal epithelial cells. Similar to the ovarian follicle, regional specific microRNAs in epididymal epithelial cells suggests microRNA involvement in epididymal function and gamete maturation. For example, microRNAs were detected within distinct areas of the stallion epididymis with the caput containing 11 microRNAs, proximal corpus 6 microRNAs, the distal corpus 8 microRNAs and cauda 19 microRNAs unique to these regions ([Twenter *et al.* 2017](#)). Moreover, microRNA content in sperm changes as they travel through the epididymis, and these changes also are associated with cell-secreted vesicles present

in epididymal fluid (Twenter HM & Bruemmer JE, unpublished observations). The presence of these microRNAs within different cells types of the ovarian follicle or epididymal regions suggests important roles for microRNAs in gamete maturation.

Cell-secreted vesicles: microvesicles and exosomes

Microvesicles (MV) and exosomes (EXO) are two different classes of cell-secreted, membranous, extracellular vesicles and have been studied more extensively in the context of intercellular communication, whereas epididymosomes are vesicles unique to the epididymis and discussed later ([Table 1](#)). Microvesicles (also called microparticles, ectosomes, shedding vesicles) are a heterogeneous population of cell-secreted membrane vesicles roughly between 100 and 1000 nm in size. These vesicles are released through outward budding and fission of the plasma membrane; however, do not contain cellular organelles or DNA fragments as seen in apoptotic bodies. Microvesicles have been studied extensively as secreted products by red blood cells, platelets and tumors and appear to function in blood coagulation, inflammation and maternal–fetal communication ([Tannetta *et al.* 2014](#)). Exosomes on the other hand are smaller, ~40–160 nm in size, and are derived through formation of intraluminal vesicles inside late endosomes (i.e., multivesicular bodies (MVBs)). Following fusion of MVBs with the plasma

Table 1 Summary of key characteristics of microvesicles, exosomes and epididymosomes.

	Microvesicles	Exosomes	Epididymosomes
Intracellular origin	Plasma membrane	Multivesicular body (MVBs)	Intraluminal compartment
Size (nm)	~100–1000	~40–160	50–250
Contents	miRNAs, mRNAs and proteins	miRNAs, mRNA and proteins	miRNAs, mRNAs and proteins
Membrane characteristic		Enriched in cholesterol, sphingomyelin, hexosylceramides	Enriched in phospholipids, sphingomyelin, arachidonic acids
Isolation procedure	Differential ultracentrifugation	Differential ultracentrifugation, commercial precipitation reagent (e.g. Exoquick, System Biosciences), immunocapture	Differential ultracentrifugation, CD9 immunocapture
Buoyant density	Not determined	1.13–1.19 g/cm ³	Not determined
Transmission electron microscopy appearance	Irregular shaped	Cup-shaped	Irregular shaped
Selected protein markers	CD40, integrins	CD63, CD81, CD9, HSP70	CD9, CD26, CD224, P25b, GliPriL1, MIF, AKRB1
Mode of release	Plasma membrane blebbing	Fusion of MVBs with the plasma membrane	Apocrine secretion

References; [Thery *et al.* \(2006\)](#), [Mathivanan *et al.* \(2010\)](#), [György *et al.* \(2011\)](#), [Raposo and Stoorvogel \(2013\)](#), [Sullivan and Saez \(2013\)](#), [Sullivan \(2015\)](#).

membrane, intraluminal vesicles (now called exosomes) are exocytosed and released into extracellular space (Raposo & Stoorvogel 2013). Similar to microvesicles, functions related to immune system and tumor cells have been ascribed to exosomes.

Both microvesicles and exosomes are surrounded by a phospholipid bilayer containing transmembrane proteins and carry RNAs and proteins. Characteristic protein constituents of exosomes include the tetraspanins CD63, CD81 and CD9, ALIX, heat shock proteins HSP70 and HSP90, adhesion molecules integrin $\alpha 4 \beta 1$ and laminin $\alpha 4$, cytoskeletal proteins actin and tubulin and proteins related to gene expression such as elongation factor. For a more comprehensive overview of extracellular vesicle content, the reader is referred to excellent reviews by Fvrier & Raposo (2004), Gyrgy *et al.* (2011), Muller *et al.* (2016), as well as the recently established Exosome Protein, RNA and Lipid Database 'ExoCarta' (<http://www.exocarta.org>) and Vesiclepedia, a compendium of RNA, lipid and protein data cell-secreted vesicles including microvesicles and exosomes (<http://www.microvesicles.org>). Both exosomes and microvesicles contain material characteristic of the cell of origin, further supporting the idea that these vesicles can contribute to complex signaling pathways between cells.

Interest in exosomes and microvesicles has greatly expanded with recent discoveries that mRNAs and microRNAs are present and transferred to target cells (Valadi *et al.* 2007), illustrating these vesicles enable genetic exchange between cells at distant sites. Moreover, a number of cancer-related studies reveal the ability of microvesicles/exosomes to induce phenotypic changes in cell behavior, including invasion and migration. For example, Yang and coworkers reported that macrophage-secreted vesicles contain miR-223, which when transferred to breast cancer cells *in vitro*, lead to increased cell invasiveness (Yang *et al.* 2011). Although the potential role of cancer cell-secreted vesicles in tumorigenesis is becoming better understood, less is known about the role of microvesicles and exosomes in normal physiological processes.

MicroRNA packaging in cell-secreted vesicles

Since the identification of RNAs in cell-secreted vesicles and their transfer to target cells much interest and effort was placed on finding out how RNAs are packaged into these vesicles, as well as if and how selective this process is.

Although progress has been made many questions remain unanswered. The following highlights a number of potential mechanisms identified so far.

First, in general, it appears that microRNA cargo in cell-secreted vesicles reflect their type and level in donor cells. Squadrito and coworkers demonstrated that overexpression (physiologically activated or artificial) of microRNA target sequences leads to depletion of microRNAs from MVBs where exosomes are formed, and instead their enrichment in P-bodies (site of microRNA function) (Squadrito *et al.* 2014). Alternatively, overexpression of microRNAs leads to their increased presence in MVBs and secreted exosomes.

Secondly, inhibition of ceramide synthesis by targeted inhibition of neutral sphingomyelinase 2 leads to reduced secretion of exosomal microRNAs, whereas its overexpression results in increased secretion (Kosaka *et al.* 2010). Interestingly, the endosomal sorting complex (ESCRT) is not involved in this process, suggesting that at least in HEK293 and COS7 cells, exosomal microRNA secretion involves a ceramide-dependent pathway.

Thirdly, it is possible that microRNAs are selectively sorted into exosomes as evident from RNAseq analysis, and their relative abundance in vesicles does not resemble donor cells. Villarroya-Beltri and coworkers found that sumoylated hnRNPA2B1 (a heterogenous nuclear riboprotein) recognizes and binds a GGAG motif in microRNAs that is over-represented in T cell-secreted exosomes (Villarroya-Beltri *et al.* 2013). This suggests that microRNAs destined for loading and secretion in vesicles contain distinct sequence signatures that maybe cell or tissue specific. Alternatively, human B-cells secrete exosomal microRNAs containing 3' end uridylated isoforms whereas 3' end adenylated microRNAs are overrepresented in cells (Koppers-Lalic *et al.* 2014). This suggests that posttranscriptional modification involving uridylation of microRNAs regulates their sorting into MVBs and exosomes.

It is unclear if these different mechanisms are/can be complementary, or if these represent cell-specific mechanisms, or responses to different stimuli, of packing microRNAs in cell-secreted vesicles.

Cell-secreted vesicles containing microRNAs and regulation of oocyte maturation

The nomenclature for these cell-secreted vesicles is still confusing and controversial, and often experimental procedures are such that observed effects can not solely

be attributed to one vesicle type over the other. Therefore, we will use extracellular vesicles (EVs) in the following section (Lötvall *et al.* 2014).

Presence of EVs containing microRNAs in follicular fluid

The presence of EVs containing microRNAs has been described in equine, bovine and human ovarian follicular fluid (da Silveira *et al.* 2012, Sang *et al.* 2013, Soheli *et al.* 2013, Santonocito *et al.* 2014, Navakanitworakul *et al.* 2016). Using transmission electron microscopy and flow cytometry, da Silveira and coworkers reported the presence of a heterogeneous sized population of EVs in equine follicular fluid (da Silveira *et al.* 2012). Furthermore, real-time PCR analysis revealed the presence of at least 120 different microRNAs in EVs. Importantly, using both *in vitro* and *in vivo* approaches, uptake of these follicular fluid-derived EVs was observed by pre-ovulatory equine granulosa cells. These data suggest EV secretion and uptake between different follicular cells is part of the complex, intricate cell communication process that underlies follicle development and oocyte maturation (Silveira *et al.* 2012) (Fig. 1).

Two separate studies reported that EVs containing microRNAs were also present in follicular fluid from women undergoing ICSI treatment (Sang *et al.* 2013, Santonocito *et al.* 2014). Isolated EVs contained 120 microRNAs according to TaqMan microRNA Array (Sang *et al.* 2013). Although many of the EV-associated microRNAs were also detected in EV-free follicular fluid, 38 microRNAs were only detected in the isolated EV fraction. Furthermore, Santonocito and coworkers isolated EVs that were on average 40 nm in size from follicular fluid, and using a similar TaqMan PCR array, identified

22 microRNAs that were higher in EVs compared to plasma (Santonocito *et al.* 2014).

Soheli and coworkers isolated EVs from follicular fluid of growing and fully grown bovine follicles (Soheli *et al.* 2013). PCR array analysis of 748 known microRNAs uncovered 509 microRNAs present in follicular fluid EVs, with 40 microRNAs that are present at different levels between growing and fully grown follicles, suggesting a role for these EV microRNAs in follicular growth. Furthermore, recent data from RNA sequencing experiments yielded a comprehensive list of microRNAs present in EVs isolated from small, medium and large bovine follicles (Navakanitworakul *et al.* 2016). Presence of 249 previously known and 455 novel microRNAs was detected in this study, and target pathway analysis tools identify cell proliferation as a major pathway predicted to be targeted by microRNAs present in EVs isolated from follicular fluid in small follicles. Interestingly, this study also revealed that not only did microRNA content change, vesicle number in follicular fluid decreased during bovine follicle development.

Although the abovementioned studies used different experimental approaches and paradigms to isolate EVs and identify microRNAs, it is clear that EVs are present in ovarian follicular fluid, and these vesicles likely play a role in cell-to-cell communication by transferring microRNAs. Pathway analysis indicates that microRNA targets include members of the WNT and TGF β signaling pathways, well-known regulatory pathway of folliculogenesis (Van Den Hurk & Zhao 2005, Knight & Glister 2006, da Silveira *et al.* 2012). EVs mediated delivery of molecular cargo between the different follicular cells represents a novel mechanism of regulating follicle growth and development and oocyte maturation. In addition, the fact that these EVs can be isolated relatively non-invasively allows for novel opportunities to use these naturally occurring nanovesicles

Figure 1

General overview of follicle growth and development. This process is characterized by a switch from high FSH to high LH levels and is accompanied by changes in relative amounts of cell-secreted vesicular microRNAs. Highlighted as black circles are extracellular vesicles.

as diagnostic markers of infertility or as molecular tool in ART clinics (Barkalina *et al.* 2015, Machtinger *et al.* 2016).

Hormonal regulation of EV microRNAs and their potential function in folliculogenesis

Ovarian follicular development is a coordinated process, beginning with recruitment then selection and growth of follicles followed by atresia or dominance, ovulation and formation of the corpus luteum and finally luteolysis. These events are tightly regulated by various endocrine and paracrine signals coming from the pituitary (FSH and LH) as well as the ovary itself (e.g., androgens, estradiol, activin and inhibin). In order to examine the potential role of EV microRNAs during follicle development and oocyte maturation, we isolated EV from follicular fluid of ovaries at early maturation (deviation), mid-estrus and pre-ovulatory stages in mares. MicroRNA real-time PCR profiling analysis revealed that a number of EV microRNAs are present at different levels at early maturation and preovulation stages compared to mid-estrus (da Silveira *et al.* 2015).

Transition from mid-estrus to pre-ovulatory period coincides with increased LH levels (Fig. 1); therefore, it is possible the rise in LH controls EV microRNA content in follicular fluid. For example, EV miR-125 and miR-199 are increased in follicular fluid (EV) from pre-ovulatory follicles and are present in equine follicular somatic cells such as granulosa cells (Silveira *et al.* 2012). Both microRNAs also were identified in ovine follicles using a microRNA sequencing platform and detected more frequently in pre-ovulatory follicles (McBride *et al.* 2012). miR-125b and miR-199-3p target cell cycle regulators (*LIF*) and eicosanoids biosynthesis enzyme, (*PTGS2*), respectively, genes known to be involved in oocyte maturation and cumulus expansion (Assou *et al.* 2006, McBride *et al.* 2012). Similarly, miR-21, miR-132 and miR-212 are examples of microRNAs regulated by hCG/LH in periovulatory mouse granulosa cells and in follicular fluid and granulosa cells of mares (Fiedler *et al.* 2008, Schauer *et al.* 2013). Also, these miRNAs are present in granulosa/cumulus cells and EVs collected from follicular fluid of pre-ovulatory follicles in the mare (Silveira *et al.* 2012). Based on results by Schauer and coworkers, these miRNAs are altered by LH induction in follicular fluid as well as in granulosa cells and are involved in cell survival and final differentiation. Predicted targets include *PTEN* and *BMP2* (miR-21; Qin *et al.* 2009, Schauer *et al.* 2013), involved in regulating granulosa cell proliferation (Fan *et al.* 2008) and

maturation of the cumulus–oocyte-complex (Assou *et al.* 2006) and *TJP1* (miR-212; Tang *et al.* 2008) involved in cell–cell interactions and regulation of tight junctions in ovarian surface epithelial cells (Zhu *et al.* 2004).

In an effort to identify a physiological function for follicular fluid EVs, Hung and coworkers isolated EVs from small (3–5 mm) and large (>9 mm) bovine antral follicles (Hung *et al.* 2015). Incubating either mouse or bovine cumulus–oocyte complexes with EVs from small or large antral follicles resulted in cumulus–oocyte complex expansion. However, even though expansion of cumulus–oocyte complexes was associated with changes in *PTGS2*, *TNFAIP6* and *PTX3*, at present, it is unclear if EV microRNAs are involved in this.

These studies clearly highlight the possibility that EV microRNAs present in follicular fluid contribute to various aspects of follicular growth and maturation (Fig. 2).

EV microRNAs and oocyte maturation

Although the exact role of these EV microRNAs and the importance of EV-mediated transfer through follicular fluid during follicle growth are unclear, they likely play a role in the process of oocyte maturation and acquisition of developmental competence (Fig. 1). In an effort to study the role of EV microRNAs in oocyte maturation, EVs were isolated from follicular fluid of follicles collected from young (3–10 years) and old (>20 years) mares and profiled for microRNAs. The aged mare is a well-established model of poor/decreased oocyte quality (Carnevale 2008). Real-time PCR analysis of EV microRNA content revealed 26 microRNAs that were differentially expressed at deviation, mid-estrus and pre-ovulatory in follicular fluid of young mares (da Silveira *et al.* 2015). Most of the differences were observed when comparing EV miRNAs between mid-oestrous and preovulation stages. Alternatively, a total of 53 EV miRNAs were differentially expressed at deviation, mid-oestrous and pre-ovulatory in follicular fluid of old mares (da Silveira *et al.* 2015). In old mares, most differences were observed when comparing EV miRNAs between deviation and the mid-oestrous and pre-ovulatory stages of follicle maturation. Among these EV microRNAs, miR-23a, a validated regulator of *XIAP* in human was increased in EVs isolated from follicular fluid at mid-estrus in old mares compared to young mares. miR-23a was also increased in ovaries of patients with premature ovarian failure and increased circulating FSH levels (Yang *et al.* 2012). In addition, miR-222 a predicted regulator of *ESR1* and *CDK1B* was

Figure 2

An overview of selected EV microRNAs and their possible role in follicle growth and oocyte maturation. For simplicity, EVs are represented as containing specific, individual microRNAs.

decreased in EVs isolated from FF of old mares compared to young mares. miR-222 was found in FF of human follicles and demonstrated to stimulate secretion of estradiol in KGN cells (a steroidogenic human ovarian granulosa-like tumor cell line) *in vitro* (Sang *et al.* 2013). Moreover, twelve microRNAs were validated *in vitro* as modulators of steroidogenesis (e.g., miR-24 decreased estradiol production by KGN cells), while miR-132, miR-320, miR-520c-3p and miR-222 promoted estradiol secretion (Sang *et al.* 2013). miR-24, miR-19b, miR-222 and miR-132 were present in EVs isolated from follicular fluid of mares, and miR-132 is also higher in EVs isolated from old compared to young mares (da Silveira *et al.* 2012), and increased in follicular fluid of luteinizing follicles compared to dominant follicles before induction of follicle maturation (Sang *et al.* 2013, Schauer *et al.* 2013). This suggest that the well-known changes in oocyte maturation between young and old mares (as seen in women) may be due to altered secretion and/or delivery of EV microRNA between follicular cells. Of interest again is the observation that many EV microRNAs identified are predicted to target the TGF β signaling pathway (da Silveira *et al.* 2012). Furthermore, we demonstrated that EV miR-181a was 15-fold higher in follicular fluid from pre-ovulatory (mature) compared with mid-oestrous follicles (immature) from young mares (da Silveira *et al.* 2014). In mice miR-181 leads to suppression of activin A receptor type IIA, which can induce SMAD phosphorylation and block in cell proliferation (Zhang *et al.* 2013).

Finally, we identified changes in EV microRNAs and potential targets belonging to the TGF β signaling member family (*IL6*, *COL1A2*, *ID2*, *STAT1* and *CDC25A*) in follicle growth and development in young and old mares (da Silveira *et al.* 2015).

EV microRNAs also were assessed in follicular fluid from young (<31 years old) and older (>38 years old) women (Diez-Fraile *et al.* 2014). Real-time PCR profiling identified at least 91 EV microRNAs, with miR-21-5p only detected in EVs isolated from follicular fluid of young women, whereas miR-134 significantly higher and miR-190b and miR-99b-3p only in EVs from follicular fluid of old women. These microRNAs are different than the ones observed in young vs old mares, which possible is due to species differences or differences in analysis platforms. However, miR-21-5p also targets the TGF β pathway.

Overall, these results suggest that EV microRNAs play an important role in follicular development and cellular communication within the mammalian ovarian follicle by regulating critical signaling pathways, including TGF β and WNT signaling (Table 2). In addition, EVs could serve as vehicles to carry microRNAs to the maturing oocyte and play roles in early embryo activation and/or development.

Epididymosomal microRNAs and sperm maturation and function

EVs found in epididymal fluid are called epididymosomes (Sullivan 2015) and have been detected in bovine, ovine,

Table 2 Examples of ovarian follicle compartment and extracellular vesicular microRNAs and their predicted targets and role in oocyte maturation.

microRNAs	Target genes	Signaling pathways and processes	Effect	Species	Reference
miR-24	<i>TGFβ1</i> <i>EGFR</i> <i>RAB5B</i>	Reproductive aging, cell proliferation, and metabolic diseases Reproductive aging, cell proliferation, and metabolic diseases	Decreased estradiol secretion Decreased estradiol secretion	Human Equine	da Silveira et al. (2012) Sang et al. (2013)
miR-132	<i>HMGA2</i> <i>PTEN</i> <i>RASA1</i>	Etiology of PCOS Cell signaling Reproductive system diseases Endocrine system disorders	Increased estradiol secretion Regulated by hCG/LH in periovulatory granulosa cell Differed between small and large follicles	Human Equine Mice Bovine	Fiedler et al. (2008) da Silveira et al. (2012) Sang et al. (2013) Schauer et al. (2013) Navakanitworakul et al. (2016) Machtinger et al. (2017)
miR-320	<i>IL-1A</i> <i>IL-10</i> <i>IL-12B</i> <i>IL-37</i> <i>IL-8</i> <i>RAB5B</i>	Immune system Etiology of PCOS WNT	Increased estradiol secretion Embryonic development improvement	Human	Sang et al. (2013)
miR-520c-3p	<i>ESR1</i>	Steroidogenesis	Increased estradiol secretion	Human	Sang et al. (2013)
miR-222	<i>ESR1</i> <i>PTEN</i>	Steroidogenesis Tumor suppressor, insulin signaling and glucose metabolism in adipose tissue	Increased estradiol secretion Differed between small and large follicles	Human Equine Bovine	da Silveira et al. (2012) Sang et al. (2013) Navakanitworakul et al. (2016)
miR-193b	<i>ESR1</i>	Steroidogenesis	Decreased progesterone secretion	Human	Sang et al. (2013)
miR-483-5p	<i>IL-1A</i> <i>IL-10</i> <i>IL-12B</i> <i>IL-37</i> <i>IL-8</i>	Immune system	Decreased progesterone secretion	Human	Sang et al. (2013)
miR-19b	ND	WNT TGFβ MAPK	Folliculogenesis regulation Differed between small and large follicles	Equine Bovine	da Silveira et al. (2012) Navakanitworakul et al. (2016)
miR-125	<i>LIF</i> <i>CDKN1A</i>	Cell cycle	Differed between small and large follicles Follicular–luteal transition	Equine Bovine Ovine	da Silveira et al. (2012) McBride et al. (2012) Navakanitworakul et al. (2016)
miR-199	<i>PTGS2</i>	Oocyte maturation and cumulus expansion	Differed between small and large follicles Follicular–luteal transition	Equine Bovine Ovine	da Silveira et al. (2012) (McBride et al. 2012) Navakanitworakul et al. (2016)
miR-21	<i>PTEN</i> <i>BTG2</i> <i>RASA1</i> <i>MECP2</i>	Cell signaling, survival and proliferation/differentiation	Regulated by hCG/LH in periovulatory granulosa cell Differed between small and large follicles Follicular–luteal transition	Mice Equine Bovine Ovine	Fiedler et al. (2008) McBride et al. (2012) da Silveira et al. (2012) Schauer et al. (2013) Navakanitworakul et al. (2016)
miR-212	<i>PTEN</i> <i>RASA1</i>	Cell signaling, survival and proliferation/differentiation	Regulated by hCG/LH in periovulatory granulosa cells	Mice Equine	Fiedler et al. (2008) da Silveira et al. (2012) Schauer et al. (2013)
miR-23a	<i>XIAP</i>	Apoptosis in granulosa cells	Higher in exosomes from follicular fluid of old compared with young mares Increase FSH levels	Equine Human	da Silveira et al. (2012) Yang et al. (2012) da Silveira et al. (2015)
miR-181a	<i>BMPR2</i> <i>ACVR2A</i>	Cell proliferation, ovarian follicle development	Inhibition of the cellular proliferation	Equine Mice	da Silveira et al. (2012) Zhang et al. (2013) da Silveira et al. (2014) da Silveira et al. (2015)

ND, not determined.

human, mouse and rat (reviewed in Sullivan & Saez 2013, Belleannée 2015). Epididymosomes range between 50 and 250 nm in size and contain a variety of proteins that can be transferred to sperm during epididymal transit (Table 1) (Sullivan *et al.* 2007, Sullivan 2015). There are at least two populations of epididymosomes with distinct functions within the epididymis; one containing tetraspanin proteins such as CD9, which can interact with live sperm and a second population containing proteins such as ELSPBP1 (epididymal sperm binding protein 1), that interact with dead sperm (Sullivan *et al.* 2007, Sullivan 2015). Secretion of these epididymosomes appears to occur by the Principal cells through an apocrine mechanism; release of apical cytoplasm (blebs) that contain free ribosomes and small membrane vesicles. When these blebs disintegrate in the epididymal lumen, vesicles (epididymosomes) are released (reviewed in Sullivan & Saez 2013).

In addition to proteins, epididymosomes carry microRNAs within epididymal fluid. Belleannée *et al.* (2013) demonstrated that bovine epididymosomes collected from the epididymal caput (head) and cauda (tail) regions contain different microRNA profiles and do not always reflect the repertoire present in epididymal cells. Epididymosomal microRNAs from let-7 and miR-200 families as well as miR-26a, miR-103 and miR-191 were present in high abundance in both epididymosomal populations (Belleannée *et al.* 2013). Additionally, miR-145, miR-143, miR-214 and miR-199 were present in higher amounts in epididymosomes from caput, while miR-654, miR-1224 and miR-395 are higher in epididymosomes collected from the cauda region (Belleannée *et al.* 2013). Moreover, epididymosomes secreted within the epididymis intraluminal environment can be taken up by surrounding epithelial cells possibly regulating transcription/translation within these cells (Belleannée *et al.* 2013, Belleannée 2015). Of interest here is that miR-145 is one of several known microRNAs that target and regulate expression of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Gillen *et al.* 2011), an anion-selective channel expressed in the epididymis and sperm (Diao *et al.* 2013). Moreover, impaired CFTR function is associated with poor sperm quality and infertility (Diao *et al.* 2013). Similarly, miR-200a and Let-7b were highly abundant in epididymosomes (Belleannée *et al.* 2013). These microRNAs are of interest considering their tumor suppressor function, and the observation that epididymal tumors are exceedingly rare (0.03% of all male cancers; Yeung *et al.* 2012). In a recent set of experiments, spermatozoa and epididymosomes were also isolated from equine epididymal

luminal fluid along 4 different regions of the epididymis (Twenter HM & Bruemmer JE, unpublished observations). 324 microRNAs were identified in spermatozoa from the epididymal caput or cauda or both regions. Importantly, 33 microRNAs were discovered in caudal epididymal sperm but not within sperm from the caput. This suggests epididymosomal transport and delivery of microRNAs. The 324 miRNAs within epididymal spermatozoa were compared to the microRNAs within epididymal tissue from the caput or cauda; 92 microRNAs were found within both epididymal spermatozoa and tissue. These 92 microRNAs were compared to epididymosomes from the lumen of the caput, proximal corpus, distal corpus and cauda epididymis. Although 69 microRNAs were noted in epididymal spermatozoa from one or more regions, in epididymosomes, or in epididymal tissue, there were many microRNAs that were only identified in a specific region. Most notably, 11 microRNAs were found within epididymal tissue of either the caput or cauda that were also detected in epididymosomes of the corresponding region. These 11 microRNAs were also observed in spermatozoa collected from the caudal region suggesting these could have been transferred to sperm during their transit from the caput to the caudal region of the epididymis. Notable pathways predicted to be targeted by epididymosomal microRNAs included those with potential roles in oocyte and embryos, specifically embryogenesis, resumption of oocyte meiosis, protein processing in endoplasmic reticulum, and mTOR signaling pathway (Twenter HM & Bruemmer JE, unpublished observations). These data further suggest possible transfer of microRNAs between epididymal cells and sperm through epididymosomes.

Jerczynski *et al.* (2016) used both *in vivo* and *in vitro* approaches to demonstrate the role of DICER1 in epididymal and epididymosome microRNA expression and function. Conditional knockout of *Dicer1* in Principal cells decreased expression of 114 mature microRNAs, including miR-210, miR672, miR-191 and miR-204. Of interest here is that these microRNAs also are present in EVs secreted by cultured Principal cells, and whose predicted target genes include genes associated with lipid metabolism, calcium transport and cytoskeletal formation.

A recent study demonstrated the presence of >350 microRNAs in mouse epididymosomes, with the majority (~60%) also present in sperm (Reilly *et al.* 2016). In addition, 5 microRNAs (miR-191, miR-375, miR-467a, miR-467d and miR-467e) increased in caput epididymosomes were increased in sperm following exposure to these epididymosomes, demonstrating that

Figure 3

Overview of cell-secreted vesicles and their microRNA cargo in epididymal fluid. Highlighted and color coded are examples of selected epididymosomal microRNAs present in somatic cells, reproductive fluids and germ cells.

epididymosomes can deliver microRNAs to spermatozoa in transit through the epididymis (Reilly *et al.* 2016). Moreover, it appears that epididymosomes carrying microRNAs are transferred between epididymal epithelial cells and spermatozoa by epididymosomes thereby regulating sperm maturation (Fig. 3). Interestingly, miR-191 and miR-210 in epididymosomes secreted by Principal cells (Jerczynski *et al.* 2016) and transferred to sperm (Reilly *et al.* 2016) are microRNAs that are also present in EVs isolated from follicular fluid (da Silveira *et al.* 2012). Possibly these microRNAs are transferred to both sperm and oocyte and play a role in early embryogenesis. miR-210 is a well-known regulator of stem cell differentiation and cell cycle progression (Huang *et al.* 2010).

Finally, recent studies demonstrated that paternal metabolic phenotype can modulate epididymosomes content and thus affect offspring. Protein restriction in mice alters small RNA levels in mature sperm, with a decrease in microRNA let-7 and increase in 5' fragments of glycine tRNAs levels. These are scarce in testicular sperm but acquired as sperm mature in the epididymis after fusion with epididymosomes (Sharma *et al.* 2015). Furthermore, mice fed with western-like diet (high-fat or high-sugar) presented with altered sperm microRNA profiles, and the subsequent transfer of altered microRNAs through ICSI led to the establishment of western-like-diet-induced metabolic phenotype in offspring (Grandjean *et al.* 2015).

In summary, it appears that similar to EVs in follicular fluid, epididymosomes in epididymal fluid are important

contributors to gamete maturation and function. These endogenous small cell-secreted vesicles appear to aid in cell communication between epithelial cells and sperm by delivering microRNAs. Since sperm are transcriptionally inactive, it is likely that microRNAs transferred to sperm as they transit the epididymis play a role in embryo activation and/or early development.

Future directions

Although the presence of EVs has been known for many years, their content analyzed and catalogued extensively, many basic questions remain. For example, how are different microRNAs selectively loaded into these vesicles? Aside from their biogenesis, are there differences in microvesicle- and exosome-mediated delivery of microRNAs and/or function? How specific is this communication process? How is molecular cargo actually transferred? How useful are these EV microRNAs as diagnostic markers? The packing of distinct microRNAs within vesicles allows for characterization of microRNA signatures that likely better reflect certain cell phenotypes instead of a single microRNA.

Continuing efforts in vesicle isolation procedures, especially sensitivity as it relates to volume requirements to isolate these vesicles, will further advance our understanding of vesicular microRNAs and proteins in cell communication processes. For example, vesicles are released into extracellular fluid (space). Sampling

extracellular space to characterize vesicular morphology and content can provide novel insight into critical processes such as theca-granulosa cell communication and Sertoli cell-germ cell interactions. Finally, the identification of small RNA regulatory molecules within cell-secreted vesicles provides for exciting new opportunities by generating 'delivery tools' to transfer molecular cargo and modulate cell function. For example, elegant experiments by Woods and colleagues reveal that exosomes can be manipulated to provide an immunologically inert vesicle carrier of small interfering RNAs to specific target sites (Alvarez-Erviti *et al.* 2011). Therefore, in addition to its potentially important, under-studied role in reproductive biology and physiology, and utility as a novel class of biomarkers in reproductive disease, these vesicular microRNAs also can be used as novel tools in reproductive biotechnology and medicine.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-17-0200>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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