DISSERTATION

THE ROLE OF CELL-SECRETED VESICLES IN EQUINE OVARIAN FOLLICLE DEVELOPMENT

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ABSTRACT

THE ROLE OF CELL-SECRETED VESICLES IN EQUINE OVARIAN FOLLICLE DEVELOPMENT

Ovarian follicular development is a process responsible for generating a gamete and steroid hormones, which are important for reproduction and general health. Failure of intrafollicular cell communication is one of the causes behind infertility. Recently, cell-secreted vesicles (microvesicles and exosomes) were described as mediators of cell communication through the transfer of bioactive material such as protein, mRNA and miRNA. Cell-secreted vesicles are present in different body fluids. The overall hypothesis is that cell-secreted vesicles are present in ovarian follicular fluid and are involved in regulating TGF-β signaling members during follicular development. In order to test this hypothesis we utilized the mare as an animal model due to the well described follicular dynamics and the easy access to sufficient experimental material. Firstly, we described the presence of microvesicles and exosomes in ovarian follicular fluid from pre-ovulatory follicles. Further we demonstrated the presence of cell-secreted vesicles markers such as miRNA and proteins. We also demonstrated that microvesicles are taken up by granulosa cell in vitro and in vivo. Secondly, we demonstrated the role of exosomes mediating regulation of TGF-β signaling members during follicular development at mid-estrous and pre-ovulatory stages. Thirdly, we demonstrated association between relative levels of TGF-β signaling members and exosomal miRNAs during follicular development in young and old mares. The data indicates that cell-secreted vesicles play an important role mediating cell proliferation and differentiation through the regulation of TGF-β signaling members during follicular development.

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INTRODUCTION

Ovarian follicle development is a dynamic and coordinated process and involves follicle selection and dominance, follicular growth, ovulation and formation of a corpus luteum. Problems during any of these steps are associated with infertility in humans and domestic animals. Ovarian follicles are recruited in follicle cohorts or follicular waves. Each follicle is composed of different cell types, which are enclosed by a basement membrane; follicular cells have to communicate with each other in order to achieve oocyte maturation and ovulation. Following selection, the dominant follicle is mainly stimulated by two gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH), released by the anterior pituitary. These two gonadotropins stimulate theca cells to produce androstenedione (precursor of testosterone) and granulosa cells to produce estradiol. The acquisition of FSHR is important to select the most FSH responsive follicle, which will become the dominant follicle. Production of estradiol by the dominant follicle will act on the hypothalamus and pituitary causing a negative feedback. This negative feedback becomes a positive feedback and causes an LH surge responsible for ovulation. All these events are necessary for follicular selection, growth and ovulation.

This PhD dissertation will present new data on the role of cell-secreted vesicles and the regulation of TGF-β signaling members during follicular development in the mare. The mare is a good model to study follicular development due to the many similarities between the reproductive physiology in the mare and woman, as well as the size of the dominant follicle, which can achieve up to 45 mm in the pre-ovulatory stage providing enough material to study

ovarian physiology. Some of the questions addressed are related to cell communication and to the role of cell-secreted vesicles in the follicular fluid, their contents (miRNAs, mRNAs, and proteins), and their role regulating members of the TGF-β signaling family. Cell-secreted vesicles are divided into different subtypes; and in this dissertation I will investigate the presence of microvesicles (~100-1000nm) and exosomes (~50-150nm) within ovarian follicular fluid. Also, I will demonstrate the relation between cell-secreted vesicle content and oocyte competence associated with aging and also present their role in regulating members of the TGF-β signaling family during the pre-ovulatory stage.

TGF-β signaling members play an important role during ovarian follicle selection, growth, and oocyte maturation. Their function is important to coordinate granulosa cell proliferation in the beginning of follicular selection as well as coordinate granulosa cell differentiation during the final stages of follicular maturation. Members of this family present a coordinated and dynamic expression pattern, according with follicular stage, where lack or over expression of some of these members are associated with infertility problems. Therefore, it is clear that ovarian follicular development is a complex process coordinated by endocrine, paracrine and autocrine signals. Since cell-secreted vesicles can mediate cell communication through the transfer of bioactive material, it also is possible that cell-secreted vesicles mediate transfer of molecular signals as part of this complicated process.

Recently, we discovered that ovarian follicular fluid contain cell-secreted vesicles and these vesicles can be take up by granulosa cells. Thus we believe that this is a new type of intercellular communication within the ovarian follicle. This discovery provides the basis for future questions that need to be answered. For example, what intracellular signals are responsible for loading these vesicles with mRNAs, miRNAs and proteins? Are the contents of vesicles

secreted by different follicular cell types different? What makes this type of communication system specific? Do different cells take up different vesicles or is this a random event? In this dissertation, I began to address some of the questions about vesicle types within follicular fluid, vesicular contents and their effects on granulosa cells during different follicular stages.

CHAPTER I: LITERATURE REVIEW

The importance of cell communication and TGF-β signaling in follicle growth and development

Ovarian follicle selection

Ovarian follicular growth and development is a coordinated process, beginning with cyclic recruitment, selection and growth of follicles followed by atresia or dominance, ovulation, formation of the corpus luteum and finally luteolysis (Edson et al., 2009). Recruitment is a term utilized to define the growth of follicles beyond the stage at which most follicles undergo atresia (Fortune, 1994). This event is not random or isolated, but occurs in groups or a cohort of follicles. In different species the beginning of this event is associated with increases in circulating FSH levels and low levels of inhibin (Fortune, 1994). Inhibin is an inhibitor of FSH, and peripheral administration of follicular fluid containing inhibin prevents recruitment of the next cohort of follicles in rats (Fortune, 1994). Also dramatic elevations of plasma FSH achieved during hormonal regimes for superovulation in woman and domestic animals clearly increased the numbers of follicles recruited (Fortune, 1994; Ginther, 2004b). The development of primary follicles into late pre-antral/early antral stage follicles involves oocyte enlargement, zona pellucida formation, granulosa cell proliferation to form a multilayered tissue, formation of a basal lamina, condensation of stromal cells around the basal lamina to form enclosing theca cell layers and the development of fluid-filled spaces that gradually coalesce to form a single antral cavity (Knight and Glister, 2006; Oktem and Urman, 2010). Different endocrine, paracrine and

autocrine signals are important to achieve the completion of all of these events. One example of paracrine/autocrine signaling involves the TGF-β signaling family. The canonical signaling pathway for the TGF-β family is through the intracellular SMAD transcription factors which are divided into 1) receptor-associated SMADs (SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8) that are activated upon specific serine-threonine kinase receptor complexes; 2) a common mediator SMAD4; and 3) the inhibitory SMADs (SMAD6 and SMAD7), which regulate SMAD signaling competing for SMAD4 binding or blocking receptor phosphorylation (Pangas, 2012). Within the TGF-β signaling family, ligands such as BMPs have been implicated in pre-antral follicle development mediating signaling between granulosa cells and theca cells including cell proliferation. BMP-4 and BMP-7 produced by theca cells stimulate pre-antral follicle development (Buratini and Price, 2011; Oktem and Urman, 2010). Activins produced by granulosa cells, and GDF-9 and BMP-15 produced by oocytes play crucial roles in the growth of primary follicles into pre-antral and antral stages by stimulating granulosa cell proliferation (Oktem and Urman, 2010). Another important paracrine signal is mediated by AMH produced by granulosa cells of growing follicles, which act by suppressing primordial follicle recruitment (Knight and Glister, 2006; Edson et al., 2009; Buratini and Price, 2011). AMH inhibits KITL, FGF2 and FGF7 thereby preventing follicle recruitment (Buratini and Price, 2011).

Exposure of rodent and human ovarian tissue to GDF-9 promotes primary follicle progression (Knight and Glister, 2006; Edson et al., 2009). Another member of the TGF-β signaling family BMP-15 stimulates proliferation of granulosa cells and lack of this oocyte derived signal causes infertility in sheep due to the follicle developmental arrested at the primordial stage (Knight and Glister, 2006). Other important players during follicular development are activin, inhibin, and follistatin. For example, in rodents granulosa cells from

pre-antral follicles secrete activin A and enhance pre-antral follicle growth and granulosa cell proliferation in a follistatin-reversible manner. Also inhibin KO mice over produce activin protein and cause uncontrolled proliferation of granulosa cells leading to ovarian tumor development (Knight and Glister, 2006; Edson et al., 2009; Buratini and Price, 2011). Finally, AMH has been shown to inhibit FSH-dependent growth of late pre-antral follicles in mouse and a marked increase in recruitment of primordial follicle into the growing pool (Knight and Glister, 2006). These studies clearly reveal the importance of TGF-β signaling in follicle growth and development.

TGF-β signaling and ovarian follicle growth and maturation

Follicle progression through the late pre-antral/antral stage of development is associated with continued proliferation of granulosa and theca cells, increased thecal vascularization, further oocyte enlargement and relatively rapid increase in diameter and volume (Knight and Glister, 2006). The selection and growth of follicles is tightly controlled by FSH and LH secreted by the anterior pituitary under the influence of gonadotropin releasing hormone (GnRH) (Fortune, 1994; Clement and Monniaux, 2012). The follicular phase of the ovarian cycle involves growth of follicles towards ovulation or atresia. During the beginning of the follicular phase, an increase in FSH levels and low levels of LH causes production of estradiol, which together with inhibin from granulosa cells will feedback to the pituitary and diminish FSH secretion. This decrease in FSH levels is important to allow only the follicle with the largest number of FSH receptors (FSHR) to continue to grow and ovulate (Buratini and Price, 2011). Follicles with fewer FSHR will be repressed, also due to the effects of AMH secreted by the dominant follicle, and undergo atresia. Recent evidence suggests these events are accomplished

based on the autocrine/paracrine actions, between granulosa cells and oocyte. For example, BMP-6, BMP-15 and GDF-9 are secreted by oocytes, BMP-2, BMP-5 and BMP-6 by granulosa cells and BMP-2, BMP3b, BMP-4 and BMP-7 by theca cells (Knight and Glister, 2006). The influence of granulosa cell derived activin, BMP-6, oocyte derived GDF-9, BMP15 and theca derived BMP-2, -3b, -4 and -7 are crucial for follicle selection and growth (Knight and Glister, 2006; Oktem and Urman, 2010; Buratini and Price, 2011). Activin is a key regulator of granulosa cell responsiviness to FSH in antral follicles, and in vivo experiments have demonstrated that activin increases expression of FSHr in granulosa cells, stimulating granulosa cells proliferation and differentiation (Knight and Glister, 2001; Buratini and Price, 2011). Transgenic animals lacking ActRIIB or overexpressing follistatin demonstrate arrested follicular development and recruitment (Matzuk et al., 1995; Edson et al., 2009; Knight and Glister, 2006; Oktem and Urman, 2010). Also an increase in activin A:inhibin A and activin A:follistatin ratios occur in bovine antral follicles and is associated with FSH-dependent follicle selection mechanism (Oktem and Urman, 2010). In terms of follicle maturation, Activin A accelerates, while inhibin A has a negative effect on both oocyte maturation and development competence (Knight and Glister, 2006). Regarding FSH responsiveness, BMP-6 and BMP-15 have been shown to attenuate FSH action on granulosa cells, BMP-15 acts by suppressing FSH receptor expression, while BMP-6 acts by down-regulating adenylate cyclase activity, and BMP-2 and BMP-9 act on granulosa cells to promote follicle survival by maintaining cell proliferation and preventing premature luteinization and/or atresia (Knight and Glister, 2006). GDF-9 and BMP-15 have a suppressive effect inhibiting the stimulatory action of FSH on aromatase expression and estradiol production (Oktem and Urman, 2010). In conclusion, all these events modulate granulosa cell responsiveness to FSH causing follicle dominance, growth and prevention of granulosa cell

luteinization. Figure I.1 illustrates a summary of the events necessary for follicular recruitment and growth.

TGFβ signaling and final follicle maturation and ovulation

The ovarian follicle selected to continue to grow also differentiates functionally in order to prepare for ovulation (Fortune, 1994). Ovulation, luteinization and corpus luteum formation are three final events leading to the end of a follicular cycle. Secretion of increasing amounts of estradiol by the dominant follicle seems to be the difference between dominant and subordinate follicles (Fortune, 1994). In order for the dominant follicle to achieve this state, it needs cooperative action of follicular endocrine cells (theca and granulosa), as well as the presence of the gonadotropins (LH and FSH) secreted by the pituitary gland (Fortune, 1994; Clement and Monniaux, 2012). Theca cells are responsible for producing androgens in response to LH binding the LH receptor on the cell membrane. In response to FSH, granulosa cells express CYP19A1 (aromatase) (Fortune, 1994; Clement and Monniaux, 2012). This enzyme is responsible for converting androstenedione from theca cells into estradiol in the granulosa cells (Fortune, 1994; Clement and Monniaux, 2012). LH stimulates further differentiation of the theca cell layer, increasing the capacity of androgen secretion by these cells. Aromatization of androgens in granulosa cells increases the production of estradiol causing further differentiation of granulosa cells (Fortune, 1994). Increased vascularization occurs in the thecal layer around the dominant follicle, and plays an important role during the final steps towards ovulation by increasing delivery of gonadotropins to the largest follicle (Clement and Monniaux, 2012). High amounts of estradiol secreted by the pre-ovulatory follicle impact the secretion of GnRH from the hypothalamus and finally trigger the GnRH ovulatory surge, resulting in the pituitary LH

surge and causing ovulation (Clement and Monniaux, 2012). Pre-ovulatory follicles express LH receptors at high concentration in granulosa cells, allowing them to respond to the LH surge, causing a cascade of events responsible for oocyte meiotic resumption, cumulus expansion, follicle rupture, and terminal differentiation of the remaining granulosa and theca cells to generate the corpus luteum (Edson et al., 2009). Interestingly, cumulus cells (subpopulation of granulosa cells) do not express LH receptors like mural granulosa cells. Therefore, LH binding to the LH receptor (LHR) in the mural granulosa cells stimulates the expression and release of EGF factors (Ereg and Areg), which are cleaved off the plasma membrane and travel through the follicular fluid to EGF receptors on the membrane of cumulus cells (Edson, 2009). Once at the cumulus cells, EGF ligands bound to EGF receptors stimulate expression of cumulus expansion genes (*Ptgs2, Has2, Tnfaip6*) (Edson et al., 2009). The ability of the follicle to secrete androgen and estradiol decreases after ovulation and instead progesterone is produced by the corpus luteum (former granulosa cells) (Fortune, 1994; Edson et al., 2009). In summary a switch from proliferation to differentiation of granulosa cells characterizes an ovulatory trajectory, while massive cell death through apoptosis characterizes an atretic trajectory (Clement and Monniaux, 2012).

TGF-β signaling members also play an important role preventing premature granulosa cells luteinization during final pre-ovulatory follicle maturation (Knight and Glister, 2006; Oktem and Urman, 2010). Oocyte-derived factors GDF-9, BMP-15 and -6 are responsible for stimulating granulosa cells proliferation and inhibiting luteinization acting in concert with granulosa cell-derived factors BMP-2, -5 and -6 (Knight and Glister, 2006; Oktem and Urman, 2010). Cumulus cells secrete Inhibin A, Activin A and follistatin, while the oocyte expresses Activin receptor I and II, which suggests a role for these factors in oocyte maturation (Knight et

al., 2012). Addition of Activin A to oocytes in vitro accelerated the maturation process and improved development competence (Pangas, 2012). Follistatin (an inhibitor of Activin) secreted by granulosa cells in the ovarian follicle is associated with oocyte competence and embryonic cleavage after fertilization (Xia and Scheneyer, 2009; Patel et al, 2007). Follistatin may favor luteinization by bio-neutralizing intrafollicular activin and BMPs (Knight and Glister, 2003). Equally important is the role of oocyte-secreted factors on cumulus expansion. Oocyte-derived factor GDF-9 is responsible for up-regulation of HAS2, PTGS2, PTGER2, TNFAIP6 and PTX3, important for mediating cumulus expansion (Edson et al., 2009). GDF-9 acts through TGF-β receptors causing activation of SMAD2/3 to oppose FSH induction of LHR and Cyp11a1, suggesting that GDF-9 may be an important inhibitor of luteinization (Pangas, 2012; Edson et al., 2009). Although, there is sufficient evidence that GDF-9 is necessary for cumulus expansion, BMP-15 also is implicated in the regulation of this event. Oocyte derived BMP-15 is responsible for up-regulation of EGF-like growth factors in cumulus cells in vitro, and BMP-15 levels in human follicular fluid are associated with increased fertilization and embryo development following IVF (Edson et al., 2009).

Equine ovarian follicle development and similarities with woman reproductive cycle

The equine ovarian follicle develops an antrum when it reaches approximately 0.3 mm in diameter, and as in farm species and humans, the development of the antral follicle is characterized by periodic growth of cohorts of follicles or follicular waves (Donadeu and Pedersen, 2008). Follicular wave emergence is defined for experimental purposes between 6 or 13 mm and a daily growth rate between 2 and 3 mm/day (Donadeu and Pedersen, 2008; Aurich, 2011). Follicle deviation from the follicle cohort is manifested when the largest follicle reaches

approximately 22 mm (Ginther, 2003; Beg and Ginther, 2006; Donadeu and Pedersen, 2008). Deviation begins approximately 7 days before ovulation, once the largest follicle reaches 35-45 mm, and the dominant follicle normally ovulates or ceases to grow, depending on the presence of an LH surge (Donadeu and Pedersen, 2008). Follicular waves in the mare as well as in other species are stimulated by a FSH surge, and in the mare this surge reaches the peak when the largest follicle is approximately 13 mm (Donadeu and Pedersen, 2008). The decline in FSH results from an increase in circulating Inhibin-A and estradiol secreted by the largest follicle (Donadeu and Pedersen, 2008; Aurich, 2011). The further down-regulation of FSH production by the pituitary will consequently lead to the presence of a large follicle with the highest number of FSHr, which becomes the dominant follicle (Beg and Ginther, 2006; Donadeu and Pedersen, 2008; Aurich, 2011). After reaching the dominant stage, the antral follicle becomes gonadotropin dependent (Donadeu and Pedersen, 2008; Aurich, 2011). In the mare, the ovulatory follicle is larger than in other species and ruptures at a specific region of the ovary called ovulation fossa (Aurich, 2011).

The mare is a good model to study ovarian physiology due to different characteristics described bellow. Compared to other domestic animal species, the ovary of the mare has a unique structure characterized by an extremely large size and weight (35-120 cm^3 in volume; 40-48 g in weight), and an inverted location of its cortex and medulla (Aurich, 2011). The mare is seasonally polyestrus, with regular reproductive cycles occurring during periods of long daylight and transitional intervals into and out of the ovulatory season (Carnevale, 2008). Younger mares and women have inter-ovulatory intervals of 21 days for mares between 3 and 13 years, and 27 days for women between 19 and 43 years (Carnevale, 2008). Similar changes in inter-ovulatory interval are associated with advanced age in women and mares and characterize the mares as

good models to study reproductive aging. Folliculogenesis begins with deviation at the end of a common growth phase and is characterized by continued growth of the developing dominant follicle and regression of subordinate follicles (Ginther et al., 2004a). During the major ovulatory follicular wave, mares and women present a major and minor anovulatory wave (Ginther et al., 2004a; Carnevale, 2008). Follicular emergence and deviation is temporally associated with changes in FSH concentrations in mares and women (Ginther et al., 2004a; Carnevale, 2008). These characteristics suggests the mare is a good animal model to understand ovarian follicular development in mammals, as well as the advantage of the size of the ovarian follicle, which allows for collection of sufficient material to study follicle development.

Small non-coding RNA in follicle development

During a normal reproductive cycle, different development processes are in place to support generation of a healthy oocyte and pregnancy. The ovarian follicle and endometrium rapidly differentiate in order to support these processes. Rapid cell proliferation and differentiation in these tissues requires intricate intracellular signaling and gene regulation pathways to obtain a healthy oocyte and generate a pregnancy. The mammalian ovarian follicle is a tissue unit formatted by different cell types such as theca, granulosa, cumulus cells and oocyte (Fortune, 1994). Once the dominant follicle is selected out of the cohort of follicles it needs to be responsive to external stimuli (LH and FSH) as well as have intracellular responses in order to achieve its function (cell proliferation and differentiation) (Beg and Ginther, 2006). The results of these events are an endocrine organ producing androgen and estradiol, and the maturation of the ovarian follicle culminating with ovulation and formation of the corpus luteum. Hormones produced by the ovary have a pronounced effect on endometrium tissue causing it to

change and prepare to support a pregnancy (Lessey, 2010). In order to establishing and sustain a pregnancy, a receptive endometrium is a prerequisite (Altmae et al., 2012). Endometrial receptivity is a complex process, as it is a spatially and temporally restricted phenomenon occurring at different times in different species, referred as the "window of implantation" (Altmae et al., 2012). Ovarian folliculogenesis as well as endometrium tissue receptivity are under a tightly regulated network of genes, and miRNAs could be involved as mediators of these processes due to their extensive involvement in post-translational mRNA regulation.

In the last decade, small non-coding RNAs called miRNAs have been implicated in gene regulation of differentiating and developing tissues. In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes and participate in the regulation of almost every cellular process investigated to date (Fabian et al., 2010). MiRNAs are ~21 nt long, single-strand RNA molecules that can bind to the 3'-untranslated region (3'UTR) of specific target mRNA and, due to imperfect matching or perfect matching of the "seed" region, cause mRNA repression or degradation affecting protein levels, respectively (Fabian et al., 2010). In animals, most studied miRNAs form imperfect hybrids between sequences in the mRNA 3'UTR of the transcript, and the 5'-proximal "seed" regions (positions 2-8) of the miRNA, providing most of the pairing specificity (Fabian et al., 2010). MiRNAs are generated from endogenously transcribed, hairpin structures, transcribed by RNA polymerase II (pol II) and the transcript is named primary miRNAs (pri-miRNA), from which nucleotides are cleaved by Drosha (RNase enzyme) and its partner DiGeorge syndrome critical region gene 8 (DGCR8), inside the nucleus (Racz et al., 2011; Fazi and Nervi 2008) (Figure I.2). This second product is called pre-miRNAs and is recognized by nuclear export factor Exportin-5 (Exp5/Xpo5), which traffics pre-miRNAs into cytoplasm, once in the cytoplasm Dicer (RNase III enzyme) and its

partner TAR (HIV) RNA binding protein (TRBP), cleaves the hairpin structure and forms the miRNA-specific ends, generating a double-stranded, 18-25 nucleotide long, dsRNA mature miRNA (Racz et al., 2011). The antisense strand keeps its contact with the RNA-induced silencing complex (RISC), while the other strand is degraded (Racz et al., 2011). In terms of genomic distribution of miRNAs, they are individually encoded by their own set of genes, and many of them also are evolutionary conserved (Fazi and Nervi, 2008). Data on miRNA genes distribution revealed their presence in clusters transcribed as polycistonic primary transcripts, or within regions transcribed as independent units, including intergenic regions, exon sequences of non-coding transcription units or intronic sequences of either protein coding or non-coding transcription units (Fazi and Nervi, 2008). Intronic miRNAs oriented in the same direction as the surrounding genes are generally transcribed concomitantly with their host genes and excised by the splicing machinery from the larger transcript in which they are embedded (Fazi and Nervi, 2008).

The importance of miRNAs in the ovary was evident by the observation that knockout of Dicer in the ovary resulted in folliculogenesis dysfunction and infertility (Baley and Li, 2012, Hossain et al., 2012). Also, target deletion of DGCR8 in maturing oocytes indicated that the role of miRNAs synthesized via this pathway in the oocyte is limited, indicating that miRNAs play a role in somatic cells during follicle development (Baley and Li, 2012). The focus of this review is to demonstrate the role of miRNAs on follicular cells and their involvement in FSH and LH regulation of mRNA upon stimulation by gonadotropins.

Gonadotropin responsive miRNAs in the ovarian follicle

The ovaries are endocrine organs responsible for general health as well as follicle development and ovulation. In order to fulfill these functions, theca cells and granulosa cells have to produce androgens, estrogens and progesterone. Briefly, binding of LH to the surface receptors of theca cells stimulates enzymes (STAR, CYP11A1, CYP17A1) responsible for androgen production, and once androgen is produced, it is transferred to granulosa cells where it is converted to estradiol. This requires FSH-mediated up-regulation of CYP19A1. During the final stages of follicle maturation, the acquisition of LH receptors by granulosa cells is an important role modulating ovulation.

Recently, the role miRNAs in the ovary was revealed by the fact that knockout of Dicer in the ovary resulted in follicular dysfunction and infertility (Baley and Li, 2012). Granulosa cells are responsible for producing estradiol early during the follicular phase and therefore are responsive to FSH. Later before ovulation, granulosa cells acquire LH receptors and become responsive to LH. FSH treatment of rat granulosa cells revealed 31 miRNAs that were altered following FSH exposure (Yao et al., 2010b). Analysis of miRNA levels 12 h after FSH treatment demonstrated that miR-29a and 30d were down-regulated, whereas 48 h after treatment, levels of miR-29a and miR-30d were increased in granulosa cells (Yao et al., 2010b). Furthermore protein levels of two mRNA targets of miR-29a (Col4A1 and BMF) and of miR-30d (RNF2 and EED) were affected due to an increase in these miRNAs between 12 and 48 h after FSH (Yao et al., 2010b). An important function of granulosa cells is the production of estradiol, which is a result of FSH stimulation of enzymes including aromatase (CYP19A1). Recently, miR-378 was identified as a regulator of aromatase in pig granulosa cells due to the spatiotemporally inverse expression profile between miRNA and mRNA target (Xu et al., 2011). In vitro overexpression

and inhibition experiments revealed that aromatase expression, and therefore estradiol production by granulosa cells, was down-regulated by miR-378 (Xu et al., 2011). Furthermore two binding sites in the 3'UTR of the aromatase mRNA were identified and tested to confirm the action of miR-378. Overall these data indicates that FSH and miRNAs are involved in the regulation of granulosa cell function during folliculogenesis.

Later in follicle development granulosa cells are responsive to LH. In order to understand the effects of LH treatment on miRNA expression in granulosa cells, mice were injected with an ovulatory dose of hCG and the ovaries collected before and 4 h after the injection (Fiedler et al., 2008). MiRNA array analysis identified 212 mature miRNAs expressed and 13 miRNAs as differentially expressed in granulosa cells before and after treatment (Fiedler, 2008). MiRNA-132 and miR-212, which have the same seed sequence, were found to be highly up-regulated following LH/hCG induction (Fiedler et al., 2008). Further analysis of mRNA target and protein levels, revealed that knockdown of these two miRNAs decreased CTBP1 protein with no change in mRNA levels (Fiedler et al., 2008). Using the same model, miR-21 was identified as an important miRNA involved in transition from proliferative granulosa cells to a terminally differentiated luteal cells state (Carletti et al., 2010). Furthermore absence of miR-21 in granulosa cells increased apoptosis, while an increase in miR-21 was protective against granulosa cell apoptosis, leading to the conclusion that miR-21 has an anti-apopitotic role in granulosa cells (Carletti et al., 2010). Granulosa cells apoptosis is a normal event in folliculogenesis, and regulation of this process plays an important role in female fertility. Therefore, FSH and LH stimulation modulate miRNA regulation of folliculogenesis, suggesting that miRNAs are involved in regulation of proliferation, differentiation and apoptosis of granulosa cells.

TGF-β is one of the most studied signaling pathways in granulosa cells proliferation and differentiation (Edson et al., 2009, Knight and Glister, 2006, Richards and Pangas, 2010). Recently, researchers have focused on the role of this pathway controlling miRNAs expression as well as miRNAs controlling TGF-β members during folliculogenesis (Yao et al., 2010a). Perturbation of TGF-β signaling leads to reproductive abnormalities (reviewed by Richards and Pangas, 2010). Recent reports demonstrated an association between TGF-β signaling pathway and miRNA (Yao et al., 2010a). TGF-β1 is a ligand molecule involved in granulosa cell proliferation and differentiation (Yao et al., 2010a). Recently, treatment of mouse granulosa cells with TGF-β1 lead to up-regulation of 3 miRNAs and down-regulation of 13 miRNAs (Yao et al., 2010a). Among the up-regulated miRNAs, miR-224 was the second most significantly elevated miRNA (Yao et al., 2010a). Forced expression of miR-224 and knockdown of miR-224 in mouse granulosa cells decreased and increased Smad4 protein levels without altering mRNA levels, respectively (Yao et al., 2010a). In addition, both miR-224 and TGF-β1 can promote estradiol release from granulosa cells, by increasing *CYP19A1* levels (Yao et al., 2010a).

Another important member of the TGF-β signaling family is the Activin receptor IB (ACVRIB), an important receptor mediating Activin signaling and regulating oocyte maturation and granulosa cell proliferation (Yan et al., 2012). Recently miR-145 was associated with inhibition of cell proliferation in different cell types, which also is true in granulosa cells (Yan et al., 2012). One of the targets of miR-145 is ACVRIB and ectopic expression of miR-145 reduced the levels of both ACVRIB mRNA and protein and interfered with activin-induced Smad2 phosphorylation (Yan et al., 2012). These studies reveal that miRNAs are modulating granulosa cell proliferation and differentiation through TGF-β1 stimulation and/or repression of ACVRIB.

Therefore miRNAs play an important role in follicular somatic cells; however, it is important to mention that miRNAs are present in oocytes and cumulus cells as well (Miles et al., 2012, da Silveira et al., 2012). The role of miRNAs in the oocyte still is unclear. Knockout of Dicer in mouse oocytes demonstrated up-regulation of 3'UTR transcripts that lacked miRNA binding sites, implicating a weak impact of miRNAs on the maternal transcriptome (Ma et al., 2010). These data indicate that miRNA function is down-regulated during oocyte development, which is supported by normal meiotic maturation of oocytes lacking Dgcr8, which is required for the miRNA but not the RNAi pathway (Ma et al., 2010). On the other hand, analysis of bovine cumulus-oocyte complexes (COCs) demonstrated the presence of 64 miRNA clusters (Miles et al., 2012). Among these miRNAs, let-7b and let-7i were not statistically significant between different cell populations analyzed from various sized follicles (Miles et al., 2012). However, miR-106 expression was significantly higher in oocytes compared to COC and granulosa cells (Miles et al., 2012). Furthermore, all miRNA processing genes (XPO5, DICER1, DGCR8, TARBP2, RNASEN, EIF2C2) have greater expression in oocytes compared to COCs and granulosa cells, and the expression of potential target mRNAs for let-7b and let-7i (MYC) and miR-106a (WEE1A) were decreased in oocytes compared to COCs and granulosa (Miles et al., 2012). Together these results indicate that miRNAs play different roles in oocyte and cumulus cells of different species such as mice and bovine.

Follicular to luteal transition of granulosa cells is a dynamic and coordinated process involving different signaling pathways and little is known about post-transcriptional mRNA regulation (Donadeu et al., 2012). In order to address some of the underlying pathways involved in this process, miRNA libraries were produced from healthy growing and pre-ovulatory follicles, as well as from early and late corpora lutea of ovine (McBride et al., 2012). A total of

211 miRNAs were identified by sequencing of these libraries, among them miR-21, miR-125b, let-7a and let-7b were the most abundant miRNAs accounting for 40% of all miRNAs sequenced (McBride et al., 2012). Analysis of the cloning frequencies identified nine miRNAs with decreased expression associated with follicular-luteal transition, and eight miRNAs with increased expression during the same transition (McBride et al., 2012). In a different study, deletion of Dicer was responsible for corpus luteum insufficiency in part the result of impaired growth of new capillary vessels in the ovary (Otsuka et al., 2008). This impaired angiogenesis was associated with lack of miR-17-5p and let-7b, two miRNAs that participate in angiogenesis by regulating expression of TIMP1 (Otsuka et al., 2008). Furthermore, injection of miR-17-5p and let-7b into the ovaries of *Dicer*$^{d/d}$ mice partially normalized *TIMP1* expression and CL angiogenesis (Otsuka et al., 2008).

miRNAs in the Endometrium

The endometrium is a dynamic tissue that undergoes cyclic changes regulated by ovarian secreted steroid hormones estradiol (E2) and progesterone (P4) (Kuokkanen et al., 2010). E2 elicits a wave of epithelial cell proliferation, while P4 inhibits E2 induced changes and promotes epithelial cell differentiation (Kuokkanen et al., 2010). Coordinated and synchronized action of E2 and P4 are essential for controlled proliferation of endometrium and for uterine receptivity at the time of implantation (Kuokkanen et al., 2010). Similar to other tissues, miRNAs likely are important mediators of endometrium function in response to E2 and P4 stimuli (Lessey, 2010). In order to identify the effects of estradiol on miRNAs expression in uterine tissue, ovariectomized mouse were treated with E2 or ER antagonist (ICI 182,780) and tissues were collected (Nothnick and Healy, 2010). Real time PCR analysis revealed that miR-155, miR-429

and miR451 were significantly increased by estrogen administration. In the same study, miR-181b, miR-204 were down-regulated following treatment. No validation of possible mRNA targets was conducted in this study, however it suggests that estrogen regulates miRNAs through ER (Nothnick and Healy, 2010). In a different study endometrium samples obtained from late proliferative (E2 stimulation) and mid-secretory (P4 stimulation) endometrium phases were used to analyze miRNA expression (Kuokkanen et al., 2010). A total of 12 miRNAs (miR-29b, miR-29c, miR-30b, miR-30d, miR-31, miR-193-3p, miR-203, miR-204, miR-200c, miR-210, miR-582-5p and miR-345) were significantly up-regulated in the mid-secretory phase (Kuokkanen et al., 2010). These up-regulated miRNAs were predicted to target many cell cycle genes, among them E2F3 (target of miR-204) an important transcription factor that induces expression of cell cycle-regulated genes and promotes cell cycle progression, which decrease in mid-secretory phase compared to late proliferative phase (Kuokkanen et al., 2010). MiRNAs also seems to be involved in the implantation site on the endometrium, two miRNAs (miR-101 and miR-199a) were pointed as regulators of *Cox-2* at the implantation site in mouse (Chakrabarty et al., 2007). MiRNA-101 and miR-199a are spatiotemporally expressed in the mouse uterus during implantation in coincidence with expression of *Cox-2* (Chakrabarty et al., 2007). Furthermore using in vitro gain and loss-of-function experiments reveal that *Cox-2* expression is post-transcriptionally regulated by these two miRNAs (Chakrabarty et al., 2007). These data suggest that miRNAs in the endometrium are regulated by estradiol and progesterone and potentially play an important role in embryo implantation. Recently, miRNAs packaged in cell-secreted vesicles were suggested as biomarkers for different tumors (Raposo and Stoorvogel, 2013). Thus secretion of cell-secreted vesicles packaged with miRNAs can play important roles on

reproductive physiology upon hormone action on reproductive tissues such as ovary and endometrium, and also serve as biomarkers for reproductive events.

Cell-secreted vesicles and cell communication

Cell-secreted vesicles are membrane-enclosed vesicles secreted by different cell types in body fluids. Release of membrane vesicles is a process conserved in both prokaryotes and eukaryotes, and represents an evolutionary link, which suggests essential functions of a dynamic extracellular vesicular compartment (including exosomes, microvesicles and apoptotic bodies) (Gyorgy et al., 2011). For the purpose of this review, I will focus on two types of cell-secreted vesicles called microvesicles (MVs) and exosomes (EXO) (Figure I.3). Initially they were considered the same population with a size between 40-1000 nm (Raposo and Stoorvogel, 2013). Later the nomenclature changed, and MVs are considered vesicles with a size around ~1000 nm and EXO considered vesicles between 40-100 nm with variations depending on the cell of origin (Pan et al., 1985; Therry et al., 2006; Raposo and Stoorvogel, 2013). They also are distinguished based on biogenesis pathway. Whereas MVs are secreted by shedding of the plasma membrane, EXO are vesicles originating within the multivesicular endosome (MVE) and are secreted by the fusion of MVE with the plasma membrane (Raposo and Stoorvogel, 2013). These two populations of cell-secreted vesicles also differ in content. It is important to highlight that cell-secreted vesicles contain material that mirror the genetic and proteomic content of the secreting cell (Akers et al., 2012). In general, MVs contain cell membrane proteins (Anexin V) and some intracellular proteins depending on the cell of origin, mRNA, and miRNA (Gyorgy et al., 2011). EXO in general will be enriched with tetraspanin proteins (CD63, CD9, CD81), and also contain mRNA and miRNAs (Gyorgy et al., 2011). MVs and EXO share similar functions. MVs main

functions are intercellular communication, pro-coagulant activity, pro-invasive character of tumors, induction of oncogenic cellular transformation, and feto-maternal communication (Gyorgy et al., 2011, Raposo and Stoorvogel, 2013). EXO main functions are intercellular communication, antigen-presenting vesicles, stimulating anti-tumoral immune responses, sperm-egg interaction and feto-maternal communication (Gyorgy et al., 2011, Raposo and Stoorvogel, 2013). Compelling evidence supports the significance of these vesicles in a broad range of physiological and pathological processes (Gyorgy et al., 2011). These cell-secreted vesicles represent an important mode of intercellular transport and communication by serving as vehicles for membrane and cytosolic proteins, lipids and RNA, between cells (Raposo and Stoorvogel, 2013).

Biogenesis and contents sorting

Cell-secreted vesicles content from different cells are similar to the cell of origin; however cell-secreted vesicles can be different from the donor cell. MVs are formed as the result of dynamic interplay between phospholipid redistribution and cytoskeletal protein contraction in cell membranes (Akers et al., 2013). The protein distribution within the plasma membrane is not uniform and forms micro-domains (Akers et al., 2013). The distribution of phospholipids within the plasma membrane is regulated by aminophospholipid translocases, which are enzymes responsible for translocating phospholipids from one leaflet to the other (e.g.: flipases that transfer phospholipids from the outer leaflet to the inner leaflet) (Akers et al., 2013). This event is mediated by the translocation of phosphotidylserine to the outer-membrane leaflet (Akers, 2013; Hugel, 2005). The budding process is completed through the contraction of cytoskeletal

structures by actin-myosin interactions that form the secretory vesicles (Akers et al., 2013; Huge et al., 2005).

EXOs are formed within the endosomal network, a membraneous compartment that sorts various intraluminal vesicles and directs them to their appropriate destinations (Akers et al., 2013; Raposo and Stoorvogel, 2013). Some of the destinations include lysosomes, protein/lipids for degradation, or protein/lipids targeted to recycling or exocytosis (Akers et al., 2013; Raposo and Stoorvogel, 2013). Endosomes are divided in three compartments, early endosomes, late endosomes, and recycling endosomes (Huotari and Helenius, 2011; Akers et al., 2013; Raposo and Stoorvogel, 2013). Early endosomes fuse with endocytotic vesicles and incorporate their content into those destined for recycling, degradation, or exocytosis (Akers et al., 2013; Raposo and Stoorvogel, 2013). The contents destined for recycling are sorted into recycling endosomes, while others undergo a series of modifications to become late endosomes (Akers et al., 2013; Raposo and Stoorvogel, 2013). During modifications some content destined for degradation are directed to lysosomes, whereas content fated for secretion are sorted into intraluminal vesicles (ILVs) in late endosomes, generating what is known as multivesicular bodies (MVBs) or multivesicular endosomes (MVEs) (Akers et al., 2013; Raposo and Stoorvogel, 2013).

Rab GTPases serves as master regulators of vesicular membrane transport of both the exo- and endocytic pathways (Stein, 2003). Rab GTPases, specifically Rab5 and Rab7, provide the most important identity markers of exosomes (Huotari and Helenius, 2011). Endosome maturation involves a conversion from Rab5 to Rab7 (Stein et al., 2003; Huotari and Helenius, 2011). In regards to exosomes biogenesis, it is not clear what the role is of Rab5 and Rab7, however data indicates their importance in endosome maturation and intracellular movement of proteins (Stein et al., 2003; Huotari and Helenius, 2011). Recent data suggest that Rab GTPases

play an important role during exosome secretion. Deletion or repression of Rab11, Rab35, Rab27a and Rab27b causes decreased secretion of exosomes in different cell types (Raposo and Stoovorgel, 2013).

The cargo within cell-secreted vesicles varies between vesicular populations. For MVs there is not a system described responsible for sorting and loading cargo during shedding of the vesicles. However, in EXO more is known about the mechanisms involved in sorting and loading of vesicles in the different cell types. Briefly this process requires two distinct steps (Akers et al., 2013). The first step involves the enrichment of the endosome membrane with proteins called tetraspanins (CD9, CD63, CD81) (Akers, 2013). The second step in ILV/exosome formation involves a series of complexes called endosomal-sorting complex required for transport, or ESCRTs (Akers et al., 2013). The ESCRT protein complex recognizes membrane proteins modified with ubiquitin molecules and marked for sorting (Marsh and van Meer, 2008). ESCRT complexes sort these cargoes to specific domains of endosomes and regulate both the inward invagination of these membrane regions, and the scission of invaginated membrane buds to form ILVs (Marsh and van Meer, 2008). There are four multi-protein complexes required for ILV formation termed ESCRT 0, I, II and III (Akers et al., 2013). In vitro experiments indicate that recruitment of ESCRT-I and II drive membrane budding and ESCRT-III is required for completion of budding (Akers et al., 2013). ESCRT-III is recruited to the site of ESCRT-I and II via Alix, a protein that simultaneously binds to the TSG101 component of the ESCRT-I complex (Akers et al., 2013). Therefore, ESCRT machinery is involved in exosomes formation, through the association of ESCRT-Alix, which interacts with transferrin receptors during exosome formation in reticulocytes (Marsh and van Meer, 2008).

Cell-secreted vesicles and intercellular communication

Cell-secreted vesicles were described almost 3 decades ago initially as microvesicles with 5'-nucleotidase activity released from neoplastic cell lines (Trams et al., 1981; Thery et al., 2002). In the same decade, others reported the involvement of these vesicles in the endocytic pathway and recycling of cell membrane proteins in sheep reticulocytes (Pan et al., 1985; Thery et al., 2002). Since their discovery, cell-secreted vesicles are thought the be involved in recycling of membrane proteins as a mechanism for release of unwanted cell contents, mediators of immune response, mediators of tumor progression and more recently mediators of cell communication (Simpson et al., 2009; Kosaka and Ochiya, 2011; Gyorgy et al., 2011; Raposo and Stoorvogel, 2013; Thery et al., 2002). Cell-secreted vesicles also have been identified in different body fluids such as, saliva, serum, plasma, tears, semen, and breast milk (Raposo and Stoorvogel, 2013). Since the discovery of circulating vesicles in body fluids, and the discovery of RNA and protein in body fluids, it was proposed that cell-secreted vesicles protected RNA and proteins against RNAses and proteases, respectively (Valadi et al., 2007; Wang et al., 2010; Kosaka et al., 2010a).

A breakthrough about circulating RNA was the discovery of mRNA and miRNA in exosomes (Valadi et al., 2007). Since this discovery researchers have been trying to address the role of cell-secreted vesicles mediating cell communication. In order to confirm that exosomes contain protein, mRNA and miRNAs and could mediate cell communication between cells, an interesting experiment was conducted by Valadi et al., (2007). Following the transfer of mouse exosomes to human mast cells, new mouse proteins were found in the recipient human cells, indicating that transferred exosomal RNA can be translated after transfer and uptake by other target cells (Valadi et al., 2007, Kosaka and Ochiya, 2011). It also is important to mention that

microvesicles contain miRNAs and are able to transfer them. Zhang et al., (2010) reported that miR-150 is contained inside microvesicles and is secreted from a cultured monocyte/macrophage cell line (THP-1). These microvesicles delivered miR-150 into human microvascular endothelial cell line (HMEC-1) and effectively caused reduction of c-Myb expression, enhancing cell migration (Zhang et al., 2010; Kosaka and Ochiya, 2011). Another example was the treatment of prostate cancer cells (PC-3M) with microvesicles containing miR-146 obtained from monkey fibroblast cell line (COS-7) (Kosaka et al., 2010b). MiRNA-146a is considered a tumor-suppressor miRNA, and is down-regulated in the PC-3M cell line (Kosaka et al., 2010b). Following treatment microvesicles containing miR-146a were able to knockdown ROCK1 protein levels and caused growth inhibition after 4 days of treatment (Kosaka et al., 2010b).

Cell-secreted vesicles also contain mRNAs that can be transferred to recipient cells. The first example was demonstrated using exosomes from human and mouse mast cell lines (Valadi et al., 2007). Microarray assessment revealed the presence of mRNA from approximately 1300 genes, many of which are not present in the cytoplasm of the donor cell (Valadi et al., 2007). In vitro translation demonstrated that mRNAs contained in exosomes were functional after transfer to human recipient cells (Valadi et al., 2007). Finally after transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in recipient cells, indicating that transferred exosomal RNA are translated after entering another cell (Valadi et al., 2007). Interrestingly, human bone-marrow mesenchymal stem cells (hBM-MSC), which are known to repair tubular damage in acute kidney injury by locally releasing growth factors including insulin-like growth factor-1 (IGF-1), released exosomes containing IGF-1 receptor (IGF-1R) mRNA, but not IGF-1 mRNA, while hBM-MSC contained both mRNAs. Furthermore, treatment of proximal tubular epithelial cells with exosomes containing IGF-1R followed by incubation with exogenous IGF-1,

increase cell proliferation, suggesting the transfer of functional IGF-1R via exosomes (Tomasoni et al., 2013).

Cell-secreted vesicles also contain protein that can be transfered to recipient cells. Proteins are amongst the most studied functional elements of cell-secreted vesicles (Lee et al., 2011). In this regard, chemokine receptors (CCR5 or CXCR4), which are known as portals for viral (HIV) infection, have recently been shown to undergo vesicular transfer to heterotypic cells (Lee et al., 2011; Rak and Guha, 2012). Microvesicles also contain and transfer regulatory polypeptides such as IL-1β and CCL5/RANTES to cells that do not express these proteins themselves resulting in changes in cellular responses (Lee et al., 2011). In processes such as angiogenesis, regulated dissolution of the vascular basement membrane and surrounding extracellular matrix is thought to be facilitated by microvesicle-mediated delivery of proteases (e.g. MMP9, MMP2, and MT1-MMP) and their activators (Lee et al., 2011). Microvesicles also carry oncogenic proteins (EGFR) capable of modulating and reprogramming endothelial cell responses in vitro (Lee et al., 2011; Al-Nedawi et al., 2008). Another example is the tumor-derived tetraspanin (Tspan-8)-rich exosomes present in general circulation of tumor-bearing mice can interact with endothelial cells and provoke their systemic pro-angiogenic conditioning (Rak and Guha, 2012). Many more oncogenic and proto-oncogenic proteins have been identified in cell-secreted vesicles cargo; among these are PDGFR, Met, β-catenin, Ras and LMP1 (Rak and Guha, 2012). Also many more protein receptors have been identified in cell-secreted vesicles and have been associated with intercellular transfer, including CCR5, CXCR4, PAR-1, Tspan28, TF and ACL receptor kinase (Rak and Guha, 2012).

Use of cell-secreted vesicles for diagnostic and therapeutics

Due to their presence in different body fluids and their bioactive contents, cell-secreted vesicles not only are proposed to provide a new form of cell communication but also serve as diagnostic biomarkers (Thery et al., 2011, Raposo and Stoorvogel, 2013). Interestingly, the comparison of exosomes present in plasma from patients with different types of cancers to plasma from normal patients, revealed higher levels of exosomes in plasma of cancer patients (Simpson et al., 2009). Increased levels of tumor-derived exosomes in plasma of patients with cancer suggest that exosomes may play an important role in tumor progression, and indicates that circulatory exosomes can be used in cancer diagnosis and discovery of blood-biomarkers (Simpson et al., 2009). In order to link cell-secreted miRNA profile with the cell of origin, different groups analyzed miRNA profiles of cell-secreted vesicles obtained from known cancer cell cultures. For example, analysis of exosomes obtained from colorectal cancer cells (SW480) identified 241 miRNAs enriched in microvesicles compared to the donor cells, among those 27 were involved in cell cycle-related processes (Hong et al., 2009). In a different study, miRNA profiles of ovarian tumors were compared to those of tumor exosomes isolated from plasma of the same patients and demonstrated similar miRNAs between tumor and exosomes (Taylor and Gercel-Taylor, 2008). Ovarian cancer has a low incidence, however it is the most lethal gynecologic malignancy, typically due to the poor prognosis (Giusti et al., 2013). Therefore constant efforts are underway to uncover a diagnostic marker to identify ovarian cancers in early stages. Recently, it was suggested that miRNA profiles can be used to distinguish between various histological ovarian cancer subtypes, and some profiles also appear to be closely related to early relapse in patients with advanced-stage tumors (Giusti et al., 2013). MicroRNAs from ovarian cancer cells and exosomes from the same patients were positive for 218 of 467 mature

miRNAs analyzed (Taylor and Gercel-Taylor, 2008). The levels of miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214 were similar between cellular and exosomal miRNAs (Taylor and Gercel-Taylor, 2008). Using EpCam antibody as a marker for ovarian cancer exosomes, they verified that EpCam-positive exosomes were detectable in serum of both patients with benign ovarian disease and ovarian cancer. Exosomal miRNAs from ovarian cancer patients exhibited similar profiles, which were significantly different form profiles observed in benign disease (Taylor and Gercel-Taylor, 2008). Exosomal miRNAs associated with ovarian cancer could not be detected in normal controls patients (Taylor and Gercel-Taylor, 2008). Furthermore, some miRNAs are consistently and significantly up-regulated in ovarian cancer, including miRNAs belonging to to the miR-200 family (i.e., miR-200a, miR-200c and miR-200b), whereas miRNAs of the let-7 family, miR-140, miR-145, and miR-125b are consistently down-regulated in ovarian cancer (Giusti et al., 2013). Altered expression levels also have been reported for other miRNAs, such as miR-21, miR-99a, miR-125b, and miR-199a (Giusti et al., 2013). Therefore the association between cell-secreted vesicle miRNA profiles and cancer type and stage, as well as the stability of miRNAs in blood and other biological fluids, makes them useful markers for early cancer diagnosis.

Another advantage of cell-secreted vesicles is that they often are not recognized by the immune system, circulating freely, entering and exiting different body tissues, which makes them ideal as a new type of drug delivery system. Importantly, mRNA and miRNAs can be loaded inside the vesicles, and membrane protein can be added that can help target specific cells and increases sensitivity. The principal advantage of using this type of delivery system is that exosomes provide a protective barrier against premature transformation and elimination of RNAi or siRNA molecules (Lai et al., 2012; van Dommelen et al., 2011; El-Andaloussi, 2012).

According to Lai et al., (2012) exosomes have many features of an ideal drug delivery vehicle. Firstly, the presence of protein and genetic material in exosomes imply that such biological materials can be loaded into exosomes. Secondly, exosomes are well tolerated in the body as evidenced by their wide distribution in biological fluids. Thirdly, exosomes have been shown to cross the plasma membrane and deliver their cargo into target cells. Fourthly, exosomes have an intrinsic ability to home to target tissues without being destroyed leading to a longer circulating half-life and improved efficacy. Finally, exosomes are amenable to membrane modification that enhances cell type-specific targeting (Lai et al., 2012). As proof of principle, researchers overexpressed a fusion gene consisting of a neuron-specific protein in immature dendritic cells, dendritic-exosomes were able to cross the brain-blood barrier and deliver a siRNA to neurons and oligodendrocytes, causing a 60% knockdown of the siRNA-target gene (BACE-1) (Lai et al., 2012; El-Andaloussi, 2012; El-Andaloussi, 2013). This system is based on overexpression of a hybrid protein composed by a 29 peptide sequence originated from the rabies virus glycoprotein (RVG), which is known for targeting acetylcholine receptors expressed in the brain, and Lamp2b a lysosomal associated membrane protein-2, which is enriched in exosomes cell membrane (El-Andaloussi et al., 2013). The result was exosomes containing RVG protein in their membranes (Lai et al., 2012). The next step was to load the exosomes with the siRNA of interest using electroporation (El-Andaloussi et al., 2013). Following in vitro validation they performed an in vivo study, injecting mice (El-Andaloussi et al., 2013). Injection via the tail-vein caused no immune response and displayed significant gene silencing of BACE-1 in the mouse brain cerebral cortex at 48 h after i.v. delivery (El-Andaloussi et al., 2013). These data indicate that exosomes and microvesicles could be use as biomarkers for disease diagnostics as well as therapeutic delivery of siRNA.

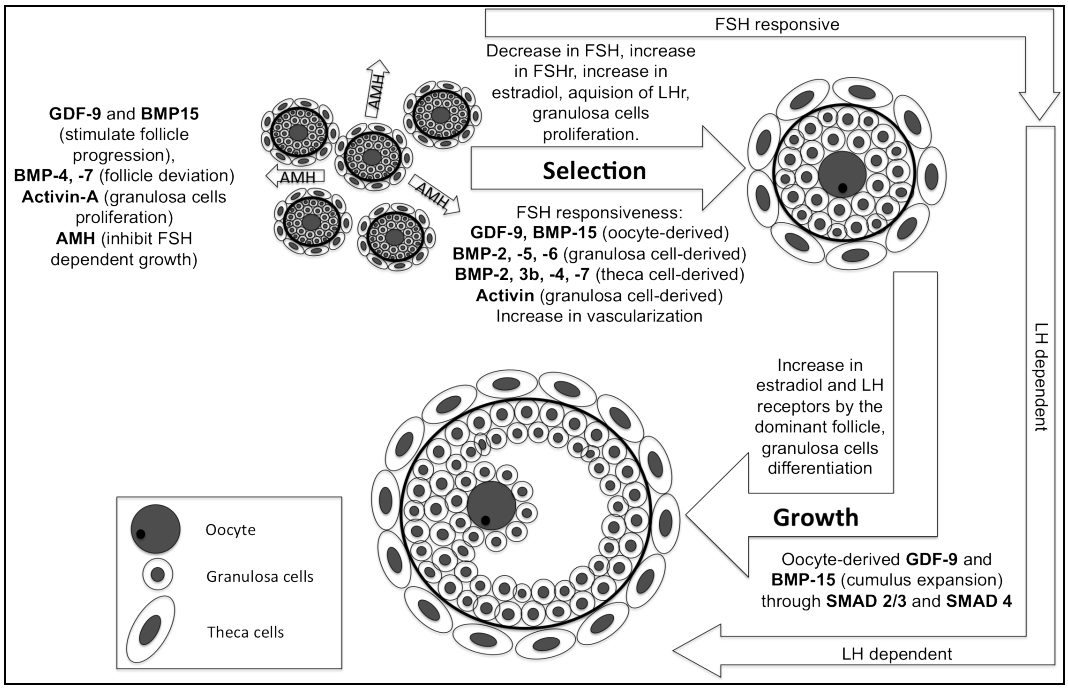

Figure I.1: Summary of the main events responsible for selection and growth of the dominant follicle.

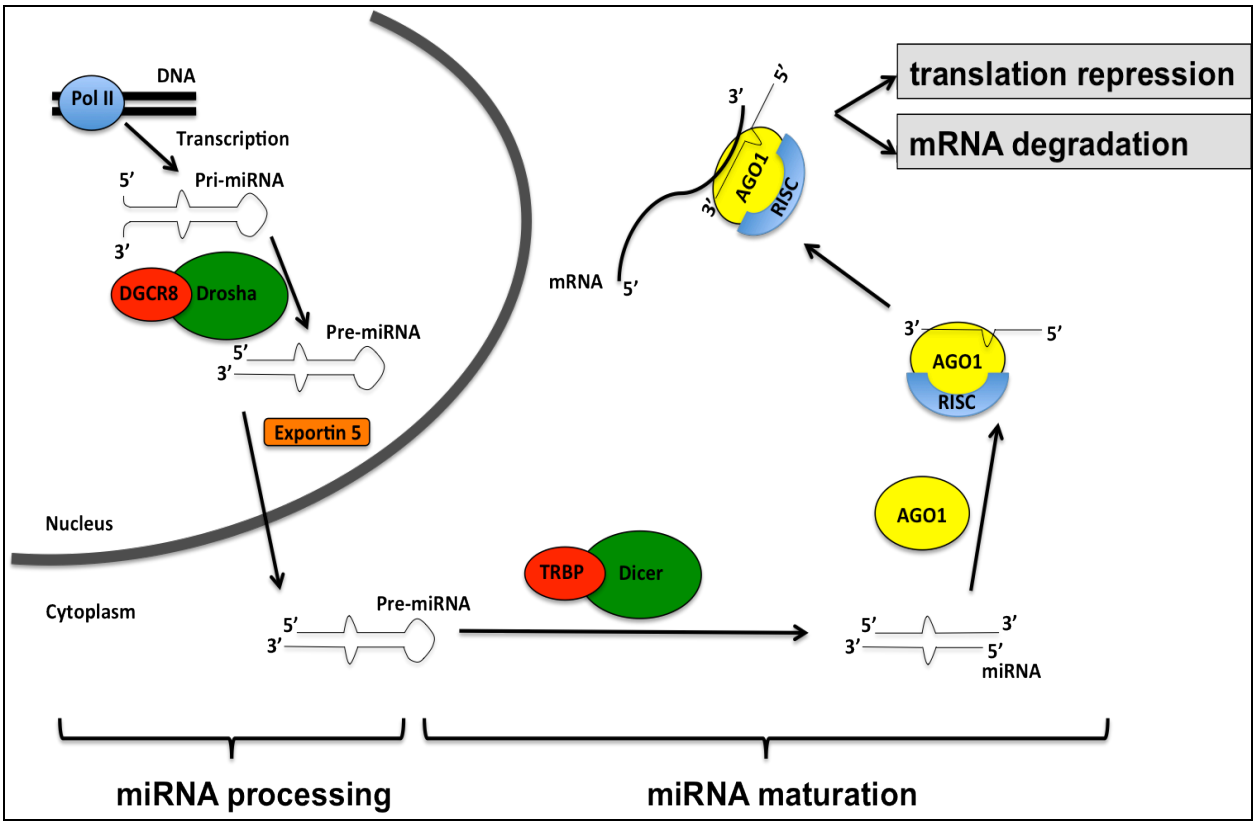

Figure I.2: MiRNA biogenesis and function (Adapted from Racz et al., 2011).

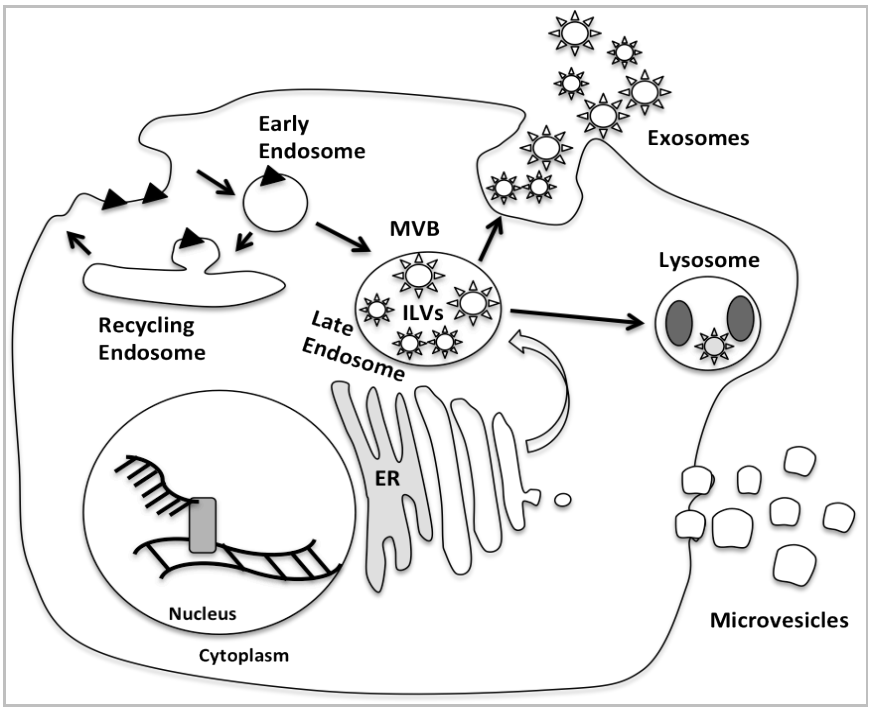

Figure I.3: Schematic picture of different pathways for formation and secretion of cell-secreted vesicles in mammalian cells. ILVs (Intraluminal vesicles).

Justification

Problems associated with ovarian follicle growth and oocyte maturation are associated with infertility and diseases. Ovarian follicles are selected and grow, ultimately leading to the production of a mature oocyte competent for fertilization. Follicle growth and development involves an intricate network of intercellular communication necessary to coordinate this process. Extragonadal hormones, FSH and LH, regulate this communication; however, intracellular and intercellular signals are necessary to achieve maturation and ovulation. It is important to highlight that a follicle is composed of different cells and that respond differently to external stimuli. Recently, miRNAs have been referred to as "fine tuners" of cell responses, based on their role in regulating mRNA translation. MiRNAs are capable of terminating a signal

or temporally repressing mRNA translation. Interestingly, in 2007 cell-secreted vesicles were identified as carriers of miRNAs, mRNAs and proteins, suggesting they can function as mediators of intercellular signaling. Therefore, we started to investigate the role of cell-secreted vesicles in follicular fluid in follicular development. First we identified cell-secreted vesicles in mammalian ovarian follicle utilizing the mare as an animal model due to the advantage of its known reproductive cycle, similarities with woman and the size of the ovarian follicle, which allowed sampling for in vitro and in vivo experiments. Second we determined if cell-secreted vesicles were taken up by granulosa cells. Third we demonstrated the ability of exosomes to regulate TGF-β signaling member. Fourth we demonstrated the association of exosomal miRNAs and TGF-β during ovarian follicular development in young and old mares.

Hypothesis

Cell-secreted vesicles containing mRNA, miRNA and protein are present in ovarian follicular fluid and are involved in follicle development by modulating TGF-β signaling members.

Aim 1: Determine the presence of cell-secreted vesicles (microvesicles and exosomes) in ovarian follicular fluid and its uptake by granulosa cells in vitro and in vivo.

Aim 2: Determine the role of exosomes during ovarian follicle development and its role regulating TGF-β signaling members.

Aim 3: Determine if altered exosomes content underlie changes in TGF-β signaling during follicle development in aged mares.

CHAPTER 2: CELL-SECRETED VESICLES IN EQUINE OVARIAN FOLLICULAR FLUID CONTAIN MIRNAS AND PROTEINS: A POSSIBLE NEW FORM OF CELL COMMUNICATION WITHIN THE OVARIAN FOLLICLE[1]

Summary:

Proper cell communication within the ovarian follicle is critical for the growth and maturation of a healthy oocyte that can be fertilized and develop into an embryo. Cell communication within the follicle involves many signalling molecules and is affected by maternal age. Recent studies indicate that cell communication can be mediated through secretion and uptake of small membrane-enclosed vesicles. The goals of this study were to (1) identify cell-secreted vesicles (microvesicles and exosomes) containing miRNAs and proteins within ovarian follicular fluid, and (2) determine if miRNA level differs in exosomes isolated from follicular fluid in young compared to old mares. We demonstrate the presence of vesicles resembling microvesicles and exosomes in ovarian follicular fluid using transmission electron microscopy, CD63-positive and RNA containing vesicles using flow cytometry. Moreover, proteomics analysis reveals that follicular fluid-isolated exosomes contain both known exosomal

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proteins, and proteins not previously reported in isolated exosomes. MiRNAs were detected in microvesicle and exosomes preparations isolated from follicular fluid by real-time PCR analysis. Uptake of fluorescent-labelled microvesicles by granulosa cells was examined using in vitro and in vivo approaches. MiRNA expression profiling reveals that miRNAs in microvesicle and exosomes preparations isolated from follicular fluid also are present within surrounding granulosa and cumulus cells. These studies revealed that cell communication within the mammalian ovarian follicle may involve transfer of bioactive material by microvesicles and exosomes. Finally, miRNAs present in exosomes from ovarian follicular fluid varied with the age of the mare and a number of different miRNAs were detected in young versus old mare follicular fluid.

***Keywords**:* Follicle, non-coding RNA, microvesicles, exosomes, mare, cell communication

***Short title**:* Cell-secreted vesicles in ovarian follicular fluid

***Summary sentence**:* Microvesicles and exosomes are present in equine ovarian follicular fluid and contain miRNAs and proteins that are taken up by surrounding granulosa cells.

Introduction

The process of ovarian follicular growth and maturation (folliculogenesis) involves extensive cell-to-cell communication between somatic cells (theca, granulosa, and cumulus cells) and somatic cells and oocytes (e.g., Eppig, 2001; Matzuk, 2002). Members of the TGFβ and WNT signalling family are involved in regulating follicular growth and maturation and comprise many of the signalling molecules that are part of the cell communication process (reviewed in Knight, 2006; Boyer, 2010). Oocyte competence is acquired during folliculogenesis, and ultimately the coordinated communication and signalling between the different follicular cell

types is critical for the growth, maturation and release of an oocyte that can be fertilized and develop into an embryo.

Follicular fluid has been recognized as a source of biochemical factors that can be predictive of oocyte quality. Follicular fluid provides an important microenvironment for oocyte maturation, and contains hormones (e.g., FSH, LH, GH, inhibin, activin, estrogens and androgens), anti-apoptotic factors (e.g., TNF and Fas-ligand), proteins, peptides, amino acids, and nucleotides (reviewed in Revelli, 2009). Thus, it is logical to think that products contained within follicular fluid play a role in follicle growth and oocyte maturation. Studies revealed that follicular fluid is a product of both blood plasma and the secretory activity of granulosa cells (Fortune, 1994; Rodgers, 2010). However, it is unclear if or how surrounding follicular cells respond to these factors during follicle development.

Recent studies have demonstrated that membrane-enclosed vesicles called microvesicles are released by cells and contain bioactive molecules (i.e., proteins and RNAs (mRNAs and miRNAs)) (Muralidharan_chari, 2010; Camussi, 2010; Simpson, 2009; Valadi, 2007). Microvesicles have been identified in body fluids such as blood plasma, urine, ascites fluid, and saliva. Microvesicles are secreted by cells through unconventional exocytosis. These vesicles are heterogeneous in size (~100-1000nm) and are released from cells by outward budding and fission of the plasma membrane (Cocucci, 2009). Microvesicles are distinct from apoptotic blebs, which contain cytosolic organelles and/or nuclear fragments (Taylor, 2008). Similar to microvesicles, exosomes also are membrane-enclosed vesicles, but are more homogeneous and smaller in size (50-100nm) than microvesicles, and are derived from late endosomal compartments (multivesicular bodies; MVBs) in cells (Keller, 2006; Lakkaraju, 2008). Endocytotic invagination leads to MVBs containing several exosomes that in turn are released

into the extracellular space when MVBs fuse with the plasma membrane. Similar to microvesicles, exosomes contain bioactive material (including mRNAs and miRNAs), can be transported over large distances through blood and are taken up by cells. It has been speculated that microvesicles and exosomes play an important role in cell communication by transferring proteins, RNA and/or miRNA molecules to target cells (Valadi, 2007; Yuan, 2009). Currently, a role for these vesicles and/or miRNAs in follicle growth and oocyte maturation is unknown.

MiRNAs (~22 nucleotides) serve as important regulators of gene function in a variety of different tissues, are highly conserved between species, and in many cases exhibit tissue-specific expression patterns. MiRNA regulation of target gene expression occurs by base-pairing interaction with the 3' untranslated region of mRNA targets leading to mRNA cleavage or translational repression (Bartel, 2009; Huntzinger, 2011). The presence of miRNAs (and possible endogenous siRNAs) is necessary for female fertility in mice (reviewed in Luense, 2009). Moreover, miRNAs are present in blood plasma and serum and can be used as diagnostic markers in prostate and ovarian cancers (Zen, 2010; Chen, 2008).

In this study, we explored the possibility that cell-secreted vesicles (microvesicles and exosomes) are present in equine ovarian follicular fluid and contain miRNAs and proteins. The mare is an excellent model to study human follicular growth and oocyte maturation (Carnevale, 2008); follicular waves and endocrine profiles are similar between women and mares, both are monovular, and there are similar age-related changes in the reproductive cycles. An additional benefit is the large size of the equine follicle, allowing follicular contents (i.e., follicular cells and fluid) to be sampled and collected with relative ease. Transmission electron microscopy (TEM), flow cytometry, and proteomics analysis revealed that cell-secreted vesicles (microvesicles, exosomes) are present in ovarian follicular fluid, and fluorescent microscopy

demonstrated that microvesicles are taken up by surrounding granulosa cells. Expression-profiling experiments identified miRNAs present in microvesicles and exosomes within ovarian follicular fluid likely originate from within the follicle, and a number of different miRNAs were present in exosomes isolated from ovarian follicular fluid from old compared to young mares.

Material and Methods

A series of experiments were conducted to investigate the presence and potential role of cell-secreted vesicles (microvesicles and exosomes) in ovarian follicular fluid. To this end follicular fluid and cells were aspirated from dominant follicles (≥ 35 mm) from young (3-13 yr) estrous mares and processed for TEM, flow cytometry, microvesicle/exosomes isolation, cell culture, and/or RNA and protein isolation. In addition, follicular fluid was aspirated from dominant follicles (≥ 35 mm) from old (≥ 20 yr) estrous mares and processed for exosomes and miRNA isolation.

Collection of ovarian follicular cells and fluid

Ovarian follicular fluid (10 ml) and cells (granulosa and cumulus cells) were obtained from light-horse, young and old mares. All procedures were done in accordance with the Institutional Animal Care and Use Committee. Mares were housed on dry lots with water and hay *ad libitum*. Follicular maturation was induced by administration of hCG and/or deslorelin (2500 IU and 1.5 mg, respectively, iv), and follicular contents (follicle ≥ 35 mm) were collected 32 h later by transvaginal, ultrasound-guided follicular aspirations (Carnevale, 2000). Follicular fluid was collected using a 12 GA needle, placed in an incubator at 37°C for 10-20 min, centrifuged at 1500 x g for 15 min and later stored at -80°C until processed for microvesicles/exosomes, RNA,

or protein isolation. Follicular cells (granulosa and cumulus cells) were rinsed (3x) to remove red blood cells in PBS containing 0.02% polyvinyl alcohol (PVA); approximately half of the sample was snap frozen and used for RNA isolation, whereas the rest was placed in medium (DMEM/F-12, (Invitrogen™ #11320-033) supplemented with 10% fetal bovine serum (FBS)) according to previously published methods (Roy, 1998; Carnevale, 2006). Cells were pipetted repeatedly to separate cells and placed in 2X freezing medium (50% FBS, 20% DMSO and 30% DMEM/F-12) in a freezing container overnight at -80°C, and stored until further use in microvesicle labelling and uptake experiments (see below).

Isolation of microvesicles and exosomes from follicular fluid

Cell-secreted vesicles (microvesicles and exosomes) were isolated from blood serum (used as a positive control) and ovarian follicular fluid through a series of differential ultracentrifugation steps as described in Thery et al. 2006. Samples were spun at 300 x g for 10 min and 2000 x g for 10 min to remove residual cells and debris, at 10,000 x g for 30 min to remove microparticles, and two times at 100,000 x g for 70 min (33,000 rpm, rotor SW55) to pellet the microvesicles. The pellets were resuspended in 500 µl of PBS (pH7.4) and used for flow cytometry (n = 2), real time PCR analysis (n = 3), or fluorescent labelling (n = 3).

Exosomes were isolated using Exoquick™ (SBI System Biosciences, Inc). Exoquick is a polymer-based reagent created to enrich preparations for exosomes. Blood serum (used as a positive control) and follicular fluid were centrifuged at 3000 rpm for 15 min after collection, and 400 µl of the supernatant added to 100 µl of Exoquick. This preparation was incubated 12 h at 4°C, and centrifuged 1500 rpm for 30 min to obtain an exosome pellet. Exosome pellets were

resuspended in 500 µl of PBS (pH7.4) and used for flow cytometry (n = 2), real time PCR analysis (n = 3), or proteomics analysis (n = 3).

Flow cytometry

Microvesicle and exosomes preparations isolated from follicular fluid of young mares were incubated with 1 µg/µl CD63-FITC conjugated antibody (AHN16.1/46-4-5 Santa Cruz Biotechnology, Inc.) and 5 µl/ml Acridine Orange (AO) (BD-Diagnostic System). CD63 is a tretraspanin superfamily protein member present in exosomes, and AO is a cell-permeable stain that binds RNA. CD63-FITC antibody and AO were added for 30 min prior to flow cytometry.

Samples were filtered through a sterile mesh with pore size 122 µm to remove any clumps. Flow cytometry was conducted on a MoFlo Legacy Flow Cytometer, by Dako-Cytomation (Beckman-Coulter), using a laser at 488 nm and 100 mW power. Dyes used were AO (high voltage = 608) and FITC (high voltage = 527), and fluorescence was analyzed for forward scatter and side scatter measured using log histograms and log scale. Isolated microvesicle and exosome preparations from follicular fluid samples were compared to microvesicle and exosome preparations from blood samples that were used as controls.

Transmission electron microscopy

Isolated cell-secreted vesicle preparations (n = 2 mares) were fixed in 2.5% glutaraldehyde, 5% sucrose, 0.1 M sodium cacodylate at pH 7.4 for 30 min at room temperature. The pellet was post-fixed in a solution of 1% osmium tetroxide in 0.1 M cacodylate buffer and centrifuged. Osmicated pellets were dehydrated through a graded series of ethanol, rinsed in propylene oxide, and embedded in Poly/Bed 812 (Polysciences Inc. Warrington, PA). From these tissue blocks,

~80 nm-thin sections were cut, mounted on 300-mesh nickel grids, stained with uranyl acetate and lead citrate, and examined using a JEOL-JEM-1200EX transmission electron microscope.

Protein isolation and mass spectrometry

Exosomes were isolated from follicular fluid collected from dominant follicles (32 h following hCG/deslorelin administration) of three young mares. Following an overnight precipitation, exosomal proteins were isolated using TRI reagent BD (Molecular Research, Inc.) and resuspended in 8 M urea. Samples were depleted of albumin and IgG using the ProteoExtract^{TM} Removal Kit (Calbiochem) and concentrated using Amicon Ultra centrifugal filters (Millipore Corp.). Protein samples (50 μg) underwent in-solution digestion using Protease Max (Promega, Madison, WI) and urea. Briefly, samples were solubilised in 8 M urea, 0.2% protease max, then reduced, alkylated, and digested with 1% protease max and trypsin at 37°C for 3 h. Samples were dried in a Speed Vac® vacuum centrifuge, desalted using Pierce PepClean C18 spin columns (Pierce, Rockford, IL), dried, and resuspended in 10 μl acetonitrile (ACN; 3%), formic acid (0.1%). Online 2-dimensional LC-MS/MS with SCX (strong cation exchange) and subsequent reverse phase chromatography was performed as follows. Digested peptides (10 μg) from each sample were loaded onto a Zorbax BIO-SCX II 3.5 μm, 50 x 0.8 mm column (Agilent Technologies, Santa Clara, CA). Peptides were eluted off from the SCX column step-wise using increasing concentrations of NaCl in 0.3% ACN, 0.1% FA (NaCl salt injections: 15, 30, 45, 60, 75, 90, 120, 150, 300, 500 mM). Peptides from each individual salt injection are then purified and concentrated using an on-line enrichment column (Agilent Zorbax C18, 5 μm, 5 x 0.3 mm). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Agilent 1100 nanoHPLC, Zorbax C18, 5 μm, 75 μm ID x 150 mm column) using a 60

min linear gradient from 25 - 55% buffer B (90% ACN, 0.1% formic acid) at a flow rate of 300 nl/min. Peptides were eluted directly into the mass spectrometer (Thermo Scientific LTQ linear ion trap), and spectra were collected over a m/z range of 200-2000 Da using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 sec (exclusion duration of 90 sec). Compound lists of the resulting spectra were generated using Bioworks 3.0 software (Thermo Scientific) with an intensity threshold set at 5,000 and 1 scan/group. This workflow generates 10 raw data files per sample. MS/MS spectra were searched against the appropriate protein database (NCBI 2008, equine taxonomy filter, 68020 entries) using the Mascot database search engine (version 2.3). The 10 data files per sample were then combined using the "mudpit" option in Scaffold 3 (Proteome Software, Portland, OR), generating a composite listing of all proteins identified from the 10 runs for each sample. Data files from the biological replicates were combined in Scaffold 3 generating one list of identified proteins. Thresholds were set to 99.9% protein probability, two peptide minimum, and a custom peptide threshold was used that combines Scaffold 3 algorithms and the Mascot score. Peptide false discovery rate (FDR) was less than 0.5%. All solvents, water, and acid used were LC-MS/MS grade from Sigma (St. Louis, MO).

Western blot analysis

Exosomal proteins (with and without albumin and IgG depletion) were isolated using TRI reagent®BD (Molecular Research, Inc., Cincinnati, OH, USA) and resuspended in 8M Urea. Protein concentration was determined using the Bradford assay, and 50 µg was loaded and resolved in 10% SDS-PAGE polyacrylamide gels (Bio-Rad,Corp., Hercules, CA, USA). Protein samples were run at 30 mA for 30 min. and transferred to nitrocellulose membranes (Biotrace

NT, Pall life Sciences, Pensacolla, FL, USA) for 1 h at 100 V. Membranes were incubated in blocking buffer (5% non-fat dried milk in TBST) for 1 h at room temperature, and presence of INHA was assessed by exposing the membranes to a monoclonal antibody (2 μg/ml, sc-22048, Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4˚C. In addition, membranes were incubated with antibodies raised against HSP70 (1:5000, ab79852, Abcam, San Francisco, CA) and CYT C (0.4 μg/ml, sc13156, Santa Cruz Biotechnology Inc). Membranes were washed three times in 1X TBST for 5 min, and incubated for 1 h at room temperature with a horseradish peroxidase conjugated anti-goat secondary antibody (0.4 μg/ml, sc-2020, Santa Cruz Biotechnology Inc.), horseradish peroxidase conjugated anti-rabbit secondary antibody (0.2 μg/ml, sc-2004, Santa Cruz Biotechnology Inc.) or horseradish peroxidase conjugated anti-mouse secondary antibody (0.4 μg/ml, sc-13156, Santa Cruz Biotechnology Inc.), respectively. Membranes were washed three times in 1X TBST for 5 min and incubated for 5 min in ECL Plus Western Blotting Detection System solution (Amersham™, Buckinghamshire, UK) for color development.

Vesicle labelling

Cell-secreted vesicles obtained by differential ultracentrifugation (microvesicles, exosomes) were collected from follicular fluid of the dominant follicles of three young mares and were labelled with PKH67 (Molecular Probes, Invitrogen), a green fluorescent dye that labels the lipid membranes. This preparation containing primarily microvesicles (see results below) was incubated in 2 μl PKH67 (2 μM) for 5 min, followed by incubation in 1% BSA for 1 min at room temperature, washed four times with FBS free medium (DMEM F-12) to remove excess dye, and resuspended in DMEM F-12 medium according manufacturer's instructions. For

a negative control, sterile PBS was incubated with PKH67 and treated the same as described above. The labelled microvesicles or PBS (negative control) was used to determine their uptake by granulosa cell in vitro and in vivo (described below).

Microvesicle uptake by granulosa cell culture

Granulosa cells from the dominant follicles of three young mares were thawed at 37°C for 5 min and placed in 1:1 freezing medium to DMEM/F-12 overnight in 60 mm dishes. Medium was replaced after the first day to remove any dead cells, and then replaced every 2 days. To study microvesicle uptake, granulosa cells were collected using 500 μl trypsin, transferred to chamber slides (Bab-Tek® Chamber Slide™ System 154534 NUNC™; ~45,000 cells per chamber), and cultured overnight in 200 μl DMEM/F-12 medium. Microvesicle uptake was determined after granulosa cells had attached to the slides, and replacing medium with DMEM/F-12 without FBS, supplemented with labelled microvesicles (3.6 μg/μl protein; diluted in 50 μl DMEM/F-12 media). After 22 h cells were fixed with 70% ethanol and presence of fluorescent-labelled microvesicles in granulosa cells was ascertained using a Nikon® Eclipse E800 fluorescence microscope. These experiments were performed three times, each time using labelled microvesicles isolated from follicular fluid of a different young mare, and labelled PBS as negative control.

Microvesicle uptake in vivo

Four hours after collection of follicular fluid of estrous mares, fluorescent-labelled microvesicles (obtained by differential ultracentrifugation) were injected back into the antrum of the same follicle from which it originated. Deslorelin (1.1 mg, im) was administered to mares,

and follicular cells were aspirated 22-24 h later. Granulosa cells were fixed in 70% ethanol for 10 min, and stained with 4',6-diamidino-2-phenylindole (DAPI). Presence of fluorescent-labelled microvesicles within granulosa cells was evaluated using LSM 510 Meta 405 Confocal Microscope Sytem Zeiss® or Olympus® FSX100. A series of images were collected at 0.05 μm intervals and used to generate a z-stack. This experiment was performed each time using labelled microvesicles isolated from follicular fluid from three independent young mares, and labelled PBS as negative control in an additional three mares.

MiRNA isolation and reverse transcription

Total RNA and miRNA were isolated from follicular fluid, microvesicle and exosome preparations isolated from follicular fluid, and granulosa and cumulus cells using TRI REAGENT®BD (Molecular Research Center Inc), according to the manufacturer's instructions. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -80°C.

Quantifiable cDNA was generated using the QuantiMir™ RT Kit (Systems Biosciences (SBI), Mountain View, CA) according to the manufacturer's instructions. Briefly, approximately 500 ng of total RNA, including the small RNA fraction, was anchor-tailed with polyA by incubating RNA, 5X PolyA Buffer, 25 mM $MnCl_2$, 5 mM ATP, and polyA polymerase at 37°C for 30 min. Oligo (dT) adaptors were annealed at 60°C for 5 min, and reverse transcription was carried out by incubating the samples at 42°C for 60 min followed by 95°C for 10 min.

Real-time PCR expression analysis of miRNAs

The expression of mature miRNAs was examined in microvesicle and exosome preparations isolated from three different animals in each group (i.e., young and old mares) using Human miRNome Profiler plates (SBI). In addition, expression of mature miRNAs also was examined in granulosa and cumulus cells from young mares (n = 3). The human miRNome Profiler plate contains 381 mature miRNA sequences (used as forward primers), and three endogenous controls; U6 snRNA, RNU43 snoRNA, and U1 snRNA. Comparing these mature miRNA sequences to recently available equine mature miRNA sequences (http://www.mirbase.org/), 221 are identical in sequence, 11 miRNAs were different in size (5' or 3' nucleotides added or removed), and 27 miRNA sequences contained nucleotide differences (18 miRNAs with only a single nucleotide difference). For 122 miRNAs, no equine sequence was available (Supplemental Table II.1). Each analysis was performed in 6 µl reactions containing 2X SYBR Green I master mix (Roche Applied Sciences), 10 µM Universal reverse primer and miRNA specific forward primer (SBI), and 0.1 µl cDNA. Real-time PCR was conducted using the LightCycler480 PCR system (Roche Applied Sciences) with 384-well plates. The PCR cycle conditions were as follows: 95°C for 5 min, 45 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec followed by a melt curve analysis to confirm amplification of single cDNA products. To identify differences in the presence of exosomal miRNAs isolated from follicular fluid of young and old mares, raw Cp values were normalized to U1 snRNA, and statistical differences were assessed at $P < 0.05$ using a Student's t-test.

Results

Presence of microvesicles and exosomes in equine follicular fluid.

Differential ultracentrifugation and Exoquick precipitation of equine follicular fluid followed by TEM revealed that different sized vesicles resembling microvesicles and exosomes were present in ovarian follicular fluid (Figure II.1A and B). Differential ultracentrifugation yielded a preparation containing primarily microvesicles and few exosomes (heron referred to as microvesicle preparation), whereas Exoquick precipitation yielded a highly enriched-preparation of exosomes. Furthermore, flow cytometric analysis demonstrated that these isolated preparations both contained vesicles positive for CD63 and RNA (Figure II.1C and D).

Proteomics analysis revealed 73 proteins in exosomes isolated from follicular fluid, with 44 proteins previously identified in exosomes isolated from a variety of fluids, including serum, plasma, urine, and saliva (ExoCarta, Mathivanan, 2009). In addition, peptide sequences were detected corresponding to 29 proteins not previously reported in exosomes, including Inhibin alpha (INHA) chain precursor (Table II.1). Western blot analysis using an antibody designed against the C-terminal region of INHA, revealed a ~18 kDa band (mature INHA) in addition to the predicted ~37 kDa for INHA precursor (Figure II.2). Furthermore, the exosomes protein preparations were positive for HSP70 (positive control) and negative for CYT C (negative control).

Presence of miRNAs in microvesicles and exosomes isolated from follicular fluid and surrounding granulosa and cumulus cells.

The presence of miRNAs in cell-secreted vesicles was examined by real time RT-PCR. MiRNAs were considered to be present when the Cp value (number of cycles it takes to detect a

fluorescence signal above background) was ≤ 37. Real-time PCR analysis revealed that microvesicles and exosomes isolated from follicular fluid contain miRNAs (n=3; Supplemental Tables II.2 and II.3); specifically, 79 miRNAs and 41 miRNAs were detected in microvesicles and exosomes, respectively (Figure II. 3; Table II.2). Twenty five miRNAs were present in microvesicles and exosomes isolated from follicular fluid. These 25 miRNAs are predicted to target several signalling pathways (DIANA-mirPath; http://diana.cslab.ece.ntua.gr/), with the Wnt signalling pathway the most significantly targeted pathway (Supplemental Table II.4).

In addition to the miRNAs present in both microvesicles and exosomes, 54 miRNAs were enriched in the microvesicle preparation and 16 miRNAs in the exosome preparation (Figure II.3; Table II.2). In silico target prediction (DIANA-mirPath; http://diana.cslab.ece.ntua.gr/) revealed that the 54 microvesicular miRNAs target pathways including TGFβ and MAPK signaling, whereas the 16 exosomal miRNAs target pathways including Focal adhesion and Regulation of actin cytoskeleton (Supplemental Table II.5 and II.6).

Presence of miRNAs also was examined in granulosa and cumulus cells. Table II.3 illustrates that of the 381 miRNAs examined, 22 were detected (n=3; Cp value ≤ 37; standard deviation ≤ 1.0) only in granulosa cells and 35 only in cumulus cells. In addition, 17 miRNAs were detected in both granulosa and cumulus cells. Comparing the miRNAs that were detected in the microvesicle and exosomes preparation in follicular fluid to the ones present in follicular cells, 21 miRNAs in granulosa cells and 12 miRNAs in cumulus cells also were present in microvesicles and/or exosomes (Table II.3). In general, miRNAs that were consistently detected in microvesicles or exosomes also were consistently present in granulosa cells or cumulus cells (see Table II.2 and Table II.3).

Microvesicle uptake by granulosa cells in vitro and in vivo

Granulosa cells were cultured with green fluorescent-labelled (PKH67) microvesicles added to the culture media. Following a 24 h incubation, PKH67 labelled-microvesicles were evident in cultured granulosa cells (Figure II.4). Importantly, granulosa cells cultured in media with PKH67-treated sterile PBS were negative for green fluorescence.

Because granulosa cells took up fluorescent-labelled microvesicles in vitro, we explored the possibility this also occurred in vivo. Microvesicles were isolated, labelled with PKH67, and injected back into follicular fluid of the same dominant follicle from which it originated. Green fluorescent-labelled microvesicles were evident within the granulosa cells when follicles were injected with PKH67-labelled microvesicles (Figure II.5), whereas no staining was observed when follicles were injected with PBS-incubated PKH67. Importantly, z-stack images obtained by confocal microscopy clearly revealed that the fluorescent-labelled vesicles were inside the granulosa cells (Figure II.5C).

Exosomal miRNAs in follicular fluid collected from young and old mares

Real-time PCR analysis revealed a total of 22 and 13 miRNAs were present (Cp value $\leq$ 37; standard deviation $\leq$ 1.0) in exosomes isolated from young and old mares, respectively (Table II.4). Moreover, 14 miRNAs were detected only in exosomes isolated from follicular fluid of young mares, and five miRNAs were detected only in exosomes from old mares. Finally, using normalized expression values, three miRNAs (miR-181a, miR-375, and miR-513a-3p) were expressed in significantly ($P \leq 0.05$) higher amounts in exosomes isolated from follicular fluid of old compared to young mares (Table II.4).

The most significantly targeted pathways by the exosomal miRNAs present only in follicular fluid from either young or old mares are MAPK signalling and focal adhesion (Supplemental Tables II.7 and II.8). The three miRNAs present at significantly higher amounts in exosomes collected from old compared to young mares were predicted to target TGFβ signalling as the most significant pathway (Supplemental Table II.9).

Discussion

Cells secrete microvesicles and exosomes that can travel over long distances when released into the bloodstream, and potentially modulate cell function at remote target sites. Recent interest in microvesicles and exosomes comes from the observation that tumor cells secrete microvesicles/exosomes that have potential roles in cancer progression (Valenti, 2007; Iero, 2008; Al-Nedawi, 2009). Microvesicles and exosomes are present in a variety of body fluids in health and disease (urine, blood, ascites fluid) suggesting that they have a role in normal homeostasis and also are released under disease conditions. To our knowledge, this is the first report demonstrating that microvesicles and exosomes are present in ovarian follicular fluid.

Differential ultracentrifugation and Exoquick^{TM} precipitation were used to isolate cell-secreted vesicles from ovarian follicular fluid. TEM, considered the 'gold standard' in the identification/visualization of cell-secreted membrane vesicles (reviewed in Gyorgy, 2011), revealed that differential ultracentrifugation of ovarian follicular fluid collected from young mares contained a variety of different-sized vesicles (Figure II.1A) that corresponded both to the size and shape reported for exosomes (50-100 nm) and microvesicles (100-1000 nm; irregular shaped) isolated from serum (Raimondo, 2011; Mathivanan, 2010), with most vesicles present in this preparation larger than 100 nm. Furthermore, TEM indicated that Exoquick^{TM} precipitation

of ovarian follicular fluid yielded a highly enriched fraction containing exosomes (Figure II.1B). Using flow cytometry, CD63-positive vesicles were identified in follicular fluids that also were positive for AO, a dye that binds RNA. CD63 is enriched in exosomes (Valadi, 2007; Mathivanan, 2007) indicating that microvesicles and exosomes carrying RNA are present within ovarian follicular fluid, similar to what has been reported for blood.

Proteomic profiling studies (Simpson, 2009; Simpson, 2008) revealed that exosomes contain a common set of membrane and cytosolic proteins, in addition to cell-type specific proteins. Using online 2-dimensional LC-MS/MS, peptide sequences resembling 73 proteins were identified in exosomes isolated from follicular fluid from young mares. Of these, 44 proteins are known to be exosomal proteins and have been identified previously in exosomes isolated from urine, plasma, serum, saliva, or culture media from different cell types (ExoCarta, Mathivanan, 2009). Moreover, these exosomal protein preparations were positive for HSP70, and negative for CYT C (Figure II.2), supporting our findings that exosomes are present within ovarian follicular fluid. Twenty-nine exosomal proteins are identified that appear specific to exosomes within ovarian follicular fluid (i.e., they have not been identified previously according to ExoCarta, a large compendium of exosomal proteins). INHA and Inhibin beta A (INHBA) chain precursors are two proteins present in the exosomes preparation obtained from ovarian follicular fluid, according to the proteomic screen. Western blot analysis using a specific antibody for INHA, revealed a ~18 kDa band in addition to the predicted ~37 kDa band. The antibody does not distinguish between the mature or precursor form of INHA, and the ~18 kDa band corresponds to the size predicted for the mature form of INHA. The fainter ~18 kDa bands in the exosomal protein sample preparations following albumin and IgG depletion, indicates that the mature form of INHA maybe attached (directly or indirectly through albumin) to the exosomes. Inhibins have

been detected in follicular fluid of cattle and horses (Donadeu, 2002; reviewed in Beg, 2006), and although its role in the process of dominant follicle selection still is unclear, inhibin A secretion by granulosa cells has been used as a indicator for the presence of a dominant follicle and corpus luteum function (reviewed in Knight, 2006; Knight, 2003). Moreover, inhibin levels reflect granulosa cell function and are used as diagnostic markers in female reproductive disease (Bowies, 2008). The identification of INHA (precursor) protein in exosome preparations within follicular fluid supports the idea that intrafollicular (paracrine) signaling by inhibins involves exosomal transport of INHA, something worth investigating.

Previous studies revealed that RNA, including miRNAs, present in blood also are present in microvesicles and exosomes. In this study we used a human miRNome PCR profiling assay to demonstrate that miRNAs also are detected in follicular fluid (without isolating vesicles), and microvesicles and exosomes present in follicular fluid. When these experiments were initiated, no equine miRNA sequences were available. However, many mature miRNA sequences are conserved across species, and we previously used the miRNome PCR profiling approach to identify the presence of miRNAs in a domestic animal species (Torley, 2011). Recently equine miRNA sequences were deposited in miRBase database (http://www.mirbase.org), which revealed that, of the mature miRNAs that are common between human and horse, >80% were identical in sequence, and only 3% were different by more than one nucleotide in sequence. Using this PCR profiling assay, 25 miRNAs were identified that were present in both microvesicular and exosomal preparations. Interestingly, the most significant pathway targeted by these 25 miRNAs is the Wnt signaling pathway (88 genes in this pathway are targeted). Wnt molecules are glycoproteins involved in fetal ovarian development and adult ovarian function including follicle growth, luteogenesis, and steroidogenesis (Boyer, 2010). Whereas 54 miRNAs

were enriched in microvesicles and 16 miRNAs enriched in exosomes. Microvesicles and exosomes are proposed to play a role in cell communication by delivering RNAs (miRNAs) and proteins from one cell to another and modulating cell function (Camussi, 2010). Although cell-cell communication is implicated in the development of the follicle and ovum, the precise mechanisms are yet to be delineated. Of particular interest is the observation that the predicted pathways targeted by miRNAs in microvesicles and exosomes include TGFβ, Wnt, and MAPK signaling pathways, pathways well known to be involved in regulating folliculogenesis.

To further investigate the possibility that microvesicular and exosomal miRNAs within follicular fluid play a role in ovarian follicle growth and development, we determined if granulosa cells take up these cell-secreted vesicles. In the first experiment, microvesicles were isolated from follicular fluid, fluorescently labelled and added to cultured equine primary granulosa cells in vitro. Importantly, primary granulosa cells were cultured in a medium without FBS because endogenous microvesicles possibly are present in FBS. Fluorescent microscopy revealed the presence of green fluorescent vesicles in cultured primary granulosa cells. Moreover, in a second experiment follicular fluid was collected, microvesicles were isolated, fluorescently labelled, and injected back into the antrum of the same dominant follicle in vivo. The following day, follicular (granulosa) cells were aspirated, and the presence of green fluorescent vesicles again was evident within granulosa cells. Examining z-stack images taken at 0.05 μm intervals, it is evident that these labelled-vesicles are present within the cells, and not adhered to the outside extracellular matrix. These data indicate that microvesicles (and likely exosomes) present within follicular fluid can be taken up by surrounding follicular cells. However, it is unclear where the microvesicles are originating from, although it is likely that

surrounding follicular cells (granulosa and cumulus cells) are cellular sources of microvesicles. Currently, these possibilities are being investigated in our laboratory.

To support the idea that microvesicles and exosomes in follicular fluid originate from within the ovarian follicle, we compared the miRNAs that are present within these vesicles to miRNAs detected in granulosa and cumulus cells. The miRNA PCR profile analysis indicates that both granulosa and cumulus cells contain distinct and overlapping sets of miRNAs. Importantly, miRNAs detected in microvesicles or exosomes isolated from follicular fluid also were detected in either granulosa cells or cumulus cells. A small number of miRNAs (11 in microvesicles and 7 in exosomes) did not appear to be present in granulosa or cumulus cells (compare Tables II.1 and II.3). However, this was due to our stringent criteria of defining miRNAs that are present in both vesicles and cells ($Cp \leq 37$ and a standard deviation of ≤ 1.0). These miRNAs were present ($Cp \leq 37$) in granulosa and/or cumulus cells; however, their level was more variable (standard deviation ≥ 1.0) between the samples (n=3). Therefore, the observation that cell-secreted vesicles in follicular fluid and surrounding granulosa and cumulus cells contain a common set of miRNA leads us to conclude that microvesicles and exosomes likely originate from within the ovarian follicle. Future studies will validate these observations, and focus on a functional role of these vesicular miRNAs.

The old mare has been used and is an excellent model to study age-related decline in ovarian function, specifically as relates to oocyte quality and competence (reviewed in Carnevale, 2002). In general, old mares (20 yr and older) have reduced fertility and compromised oocyte quality or developmental competence. To explore the possibility that exosomal miRNAs play a role in follicle growth and development, we examined the relative miRNA level in exosomes isolated from follicular fluid collected of young and old mares. Real

time PCR analysis revealed that 14 miRNAs in exosomes were consistently present in follicular fluid from young mares and five miRNAs in exosomes in follicular fluid from old mares. Bioinformatic analysis (DIANA-mirPath; http://diana.cslab.ece.ntua.gr/) revealed that both sets of miRNAs present only in exosomes of follicular fluid from young and old mares target MAPK signaling and focal adhesion as the most significant pathways; however, more genes in each pathway (104 and 85 compared to 47 and 37, respectively) were targeted by miRNAs in exosomes isolated from follicular fluid of young mares. It is unclear whether exosomes containing these miRNAs are released in follicular fluid by follicular cells to initiate these signaling pathways and/or as a way to signal to other follicular cells to suppress or fine tune MAPK signaling and focal adhesion signaling pathways.

Relative levels of miR-181a, miR-375, and miR-513a-3p in exosomes were significantly higher ($p \leq 0.05$) in follicular fluid from old compared to young mares. The top pathway predicted to be targeted by these three miRNAs is TGFβ signaling; 24 genes are potential targets in this pathway. The fact that the TGFβ signaling pathways is critical for proper follicle development and growth (Knight, 2006) suggests that in old mares exosomes containing these miRNAs could suppress this pathway leading to perturbed oocyte maturation. The identification of exosomal miRNAs expressed only or at relative higher expression in follicular fluid from old mares may lead to the development of novel biomarkers predictive of the age-related decline in oocyte quality and competence.

In summary, TEM and flow cytometry reveal the presence of microvesicles and exosomes within ovarian follicular fluid. Proteomics and real time PCR expression analysis indicate that proteins and miRNAs are present within microvesicles and exosomes isolated from follicular fluid. Moreover, the same miRNAs also are present in surrounding follicular cells. Importantly,

using both in vitro and in vivo approaches, we demonstrate that microvesicles isolated from ovarian follicular fluid could be taken up by surrounding granulosa cells. Based on these data, we propose that microvesicles and exosomes play a not previously recognized role in mediating cell communication within the mammalian ovarian follicle. Finally, using the mare as a model of age-related decline in oocyte quality, several exosomal miRNAs were identified that possibly correlate with age-related decline in oocyte quality.

Table II.1. Proteins identified in exosomes preparations isolated from ovarian follicular fluid collected from young mares by online 2-dimension tandem mass spectrometry. The exosomes database ExoCarta (http://exocarta.ludwig.edu.au/) was used to determine whether exosomal proteins from ovarian follicular fluid have been identified in exosomes from other fluids.

proteins previously identified in exosomes
serum albumin precursor [Equus caballus]
similar to Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) [Equus caballus]
similar to Complement C3 precursor [Equus caballus]
similar to alpha-1-antitrypsin; serine protease inhibitor [Equus caballus]
similar to carboxylesterase D1 isoform 1 [Equus caballus]
fibronectin [Equus caballus]
similar to Plasminogen precursor isoform 1 [Equus caballus]
similar to Ceruloplasmin precursor (Ferroxidase) [Equus caballus]
gelsolin [Equus caballus]
similar to Alpha-2-macroglobulin precursor (Alpha-2-M) (C3 and PZP-like alpha-2-macroglobulin domain-containing protein 5) [Equus caballus]
serum albumin precursor [Equus asinus]
similar to Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Alpha-2-Z-globulin) (Ba-alpha-2-glycoprotein) [Equus caballus]
clusterin precursor [Equus caballus]
similar to complement component C4A [Equus caballus]
similar to Fibrinogen beta chain precursor [Equus caballus]
alpha-2-macroglobulin [Equus caballus]
similar to apolipoprotein A-II [Equus caballus]
similar to Haptoglobin precursor [Equus caballus]
alpha-1-antitrypsin [Equus caballus]
hemopexin [Equus caballus]
inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Equus caballus]
similar to Alpha-2-antiplasmin precursor (Alpha-2-plasmin inhibitor) (Alpha-2-PI) (Alpha-2-AP) [Equus caballus]
similar to nexin-1 isoform 1 [Equus caballus]
complement component C9 precursor [Equus caballus]
similar to complement component C5 [Equus caballus]
similar to Alpha-2-macroglobulin precursor (Pregnancy zone protein) (Alpha-2-M) [Equus caballus]
similar to histidine-rich glycoprotein [Equus caballus]
apolipoprotein A-IV [Equus caballus]
fibrinogen A-alpha chain [Equus caballus]
similar to Leucine rich repeat containing 15 [Equus caballus]
similar to AMBP protein precursor [Equus caballus]
alpha-1-antitrypsin [Equus caballus]
similar to Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein) [Equus caballus]
similar to Fibrinogen alpha chain precursor [Equus caballus]
similar to Fibrinogen gamma chain [Equus caballus]
similar to fibulin 1 [Equus caballus]
similar to Fetuin-B precursor (Gugu) (IRL685) (16G2) [Equus caballus]
similar to transthyretin [Equus caballus]
similar to inter-alpha globulin inhibitor H2 polypeptide [Equus caballus]
similar to complement factor B [Equus caballus]
similar to porcine inhibitor of carbonic anhydrase [Equus caballus]
similar to angiotensinogen [Equus caballus]
immunoglobulin J chain [Equus caballus]
similar to antithrombin protein [Equus caballus]
proteins not previously identified in exosomes
lambda-immunoglobulin [Equus caballus]
similar to Group-specific component (vitamin D binding protein) [Equus caballus]
immunoglobulin gamma 1 heavy chain constant region [Equus caballus]
similar to prothrombin [Equus caballus]
similar to inter-alpha (globulin) inhibitor H1 [Equus caballus]
lambda-immunoglobulin [Equus caballus]
immunoglobulin alpha constant heavy chain [Equus caballus]
immunoglobulin gamma 2 heavy chain constant region [Equus caballus]
immunoglobulin mu heavy chain constant chain secreted form [Equus caballus]
immunoglobulin gamma 3 heavy chain constant region [Equus caballus]
immunoglobulin gamma 4 heavy chain [Equus caballus]
inhibin beta A chain precursor [Equus caballus]
inhibin alpha chain precursor [Equus caballus]
immunoglobulin G heavy chain [Equus caballus]
similar to Membrane copper amine oxidase (Semicarbazide-sensitive amine oxidase) (SSAO) (Vascular adhesion protein 1) (VAP-1) (HPAO) [Equus caballus]
similar to Plasma protease C1 inhibitor precursor (C1 Inh) (C1Inh) (C1 esterase inhibitor) (C1-inhibiting factor) [Equus caballus]
similar to Afamin precursor (Alpha-albumin) (Alpha-Alb) [Equus caballus]
similar to pre-procarboxypeptidase R [Equus caballus]
similar to Complement component C8 beta chain precursor (Complement component 8 subunit beta) [Equus caballus]
similar to Exosome complex exonuclease RRP42 (Ribosomal RNA-processing protein 42) (Exosome component 7) (p8) [Equus caballus]
Ig lambda chain V region - horse (fragment)
similar to Complement component 8, alpha polypeptide [Equus caballus]
similar to C-reactive protein [Equus caballus]
complement component 7 [Equus caballus]
serotransferrin precursor [Equus caballus]
immunoglobulin gamma 7 heavy chain [Equus caballus]
immunoglobulin gamma 5 heavy chain constant region [Equus caballus]
immunoglobulin kappa light chain [Equus caballus]
immunoglobulin gamma 6 heavy chain constant region [Equus caballus]

Table II.2. MiRNAs enriched in microvesicle (MV) and exosome (EXO) preparations obtained from follicular fluid. MiRNAs listed are present (Cp ≤ 37) in three samples collected from young mares.

miRNAs enriched in microvesicles			
miR-19b	*miR-139-5p*	*miR-326*	*miR-411*
miR-27a	*miR-149*	*miR-328*	*miR-455-5p*
miR-28-3p	*miR-181b*	*miR-331-5p*	*miR-488*
miR-30e	*miR-187*	*miR-342-3p*	*miR-493*
miR-31	*miR-193b*	*miR-345*	*miR-494*
miR-34c-3p	*miR-202*	*miR-346*	*miR-503*
miR-106a	*miR-210*	*miR-370*	*miR-512-3p*
miR-106b	*miR-212*	*miR-372*	*miR-513c*
miR-124	*miR-214*	*miR-373*	*miR-515-3p*
miR-125a-5p	*miR-222*	*miR-374a*	*miR-520d-3p*
miR-127-3p	*miR-296-3p*	*miR-377*	*miR-532-3p*
miR-129-3p	*miR-320b*	*miR-409-3p*	*miR-539*
miR-129-5p	*miR-324-3p*	*miR-409-5p*	*miR-548b-5p*
miR-134	*miR-324-5p*		
miRNAs enriched in exosomes			
miR-20a	*miR-23b*	*miR-152*	*miR-433*
miR-20b	*miR-30b*	*miR-215*	*miR-483-3p*
miR-22	*miR-93*	*miR-223*	*miR-499-3p*
miR-23a	*miR-101*	*miR-378*	*miR-513a-3p*
miRNAs present in microvesicles and exosomes			
miR-17	*miR-92b*	*miR-191*	*miR-362-5p*
miR-19b	*miR-132*	*miR-192*	*miR-375*
miR-21	*miR-135a*	*miR-195*	*miR-421*
miR-24	*miR-135b*	*miR-197*	*miR-485-3p*
miR-25	*miR-181a*	*miR-320a*	*miR-486-5p*
miR-30a	*miR-181c*	*miR-323-5p*	*miR-523*
miR-92a			

Table II.3. MiRNAs detected (Cp ≤ 37; Standard deviation ≤ 1.0) in granulosa cells (GC) and cumulus cells (CC) from dominant follicles of young mares (n = 3). The presence of miRNAs in GC and CC was compared to their presence in microvesicle (MV) and exosome (EXO) preparations isolated from follicular fluid.

+ = Cp ≥ 30; ++ = 25 ≤ Cp < 30; +++ = Cp < 25.

miRNAs detected only in GCs		GC	CC	MV	EXO
miR-20a	++	**miRNAs detected only in GC and CC**			
miR-28-5p	+	*miR-15b*	++	++	
miR-29a	+	*miR-25*	++	++	
miR-30c	++	*miR-26a*	++	++	
miR-30d	++	*miR-27b*	+	++	
miR-95	+	*miR-29c*	++	++	
miR-98	+	*miR-93*	++	++	
miR-99b	+	*miR-103*	+	++	
miR-128	+	*miR-140-5p*	+	++	
miR-153	+	*miR-362-3p*	+	++	
miR-154	+	*miR-365*	+	++	
miR-181d	+	*miR-374b*	+	++	
miR-193b	+	*miR-376a*	+	++	
miR-199b-5p	+	*miR-376c*	+	++	
miR-204	+	*miR-423-3p*	+	++	
miR-211	+	*miR-450b-5p*	+	+	
miR-221	+	*miR-484*	+	++	
miR-338-3p	+	*miR-545*	+	++	
miR-454	+	**miRNAs detected only in GC and MV**			
miR-487b	+	*miR-27a*	+		+
miR-543	+	*miR-125a-5p*	++		+
miR-544	++	*miR-129-3p*	+		+
miRNAs detected only in CC		*miR-192*	+		+
miR-7	+	*miR-374a*	+		+
miR-10a	++	**miRNAs detected only in CC and MV**			
miR-10b	++	*miR-21*		+++	+
miR-16	+++	*miR-30e*		++	+
miR-18a	++	*miR-106b*		++	+
miR-26b	++	*miR-342-3p*		++	+
miR-28-3p	++	**miRNAs detected only in GC and EXO**			
miR-31	++	*miR-20b*	++		+
miR-92a	++	*miR-23a*	+		+
miR-99a	++	*miR-135a*	+		+
miR-101	++	*miR-135b*	+		+
miR-107	+	*miR-181a*	+		+
miR-126	+	*miR-215*	+		+
miR-134	++	*miR-223*	++		+
miR-136	+	*miR-320a*	+		+
miR-148a	++	*miR-378*	++		+
miR-148b	++	**miRNAs detected only in CC and EXO**			
miR-151-5p	++	*miR-22*		++	+
miR-188-3p	++	*miR-523*		+	+
miR-194	+	**miRNAs detected only in GC, CC and MV**			
miR-218	+	*miR-30a*	++	++	+
miR-299-5p	++	*miR-106a*	++	+++	+
miR-331-3p	+	*miR-195*	++	+++	+
miR-363	+	**miRNAs detected only in GC, CC and EXO**			
miR-369-3p	+	*miR-152*	++	++	+
miR-376b	+	**miRNAs detected only in GC, MV and EXO**			
miR-379	++	*miR-375*	+	+	+
miR-424	++	**miRNAs detected only in GC, CC, MV and EXO**			
miR-425	++	*miR-24*	+	++	+	+
miR-502-5p	++	*miR-132*	++	++	++	+
miR-512-3p	++					
miR-532-3p	++					
miR-532-5p	++					
miR-539	+					
miR-542-3p	+					

Table II.4. MiRNAs detected (Cp ≤ 37; Standard deviation ≤ 1.0) in exosomes preparations isolated from follicular fluid (FF) of young compared to old mares. MiRNAs in bold are present in significantly different relative amounts.

MiRNAs in exosomes present in FF of only young or old mares	
Young	Old
miR-20b	*miR-25*
miR-22	*miR-188-5p*
miR-23b	*miR-192*
miR-24	*miR-320b*
miR-135a	*miR-320c*
miR-135b	
miR-152	
miR-181c	
miR-191	
miR-197	
miR-223	
miR-320a	
miR-323-5p	
miR-378	
miR-433	

MiRNAs in exosomes present in FF of old compared to young mares		
MiRNA	Fold Change	T-Test (P value)
miR-23a	2.7	0.24
miR-132	1.5	0.58
miR-181a	**3.4**	**0.03**
miR-215	1.2	0.83
miR-362-5p	2.0	0.23
miR-375	**4.0**	**0.05**
miR-513a-3p	**4.3**	**0.02**
miR-523	1.8	0.29

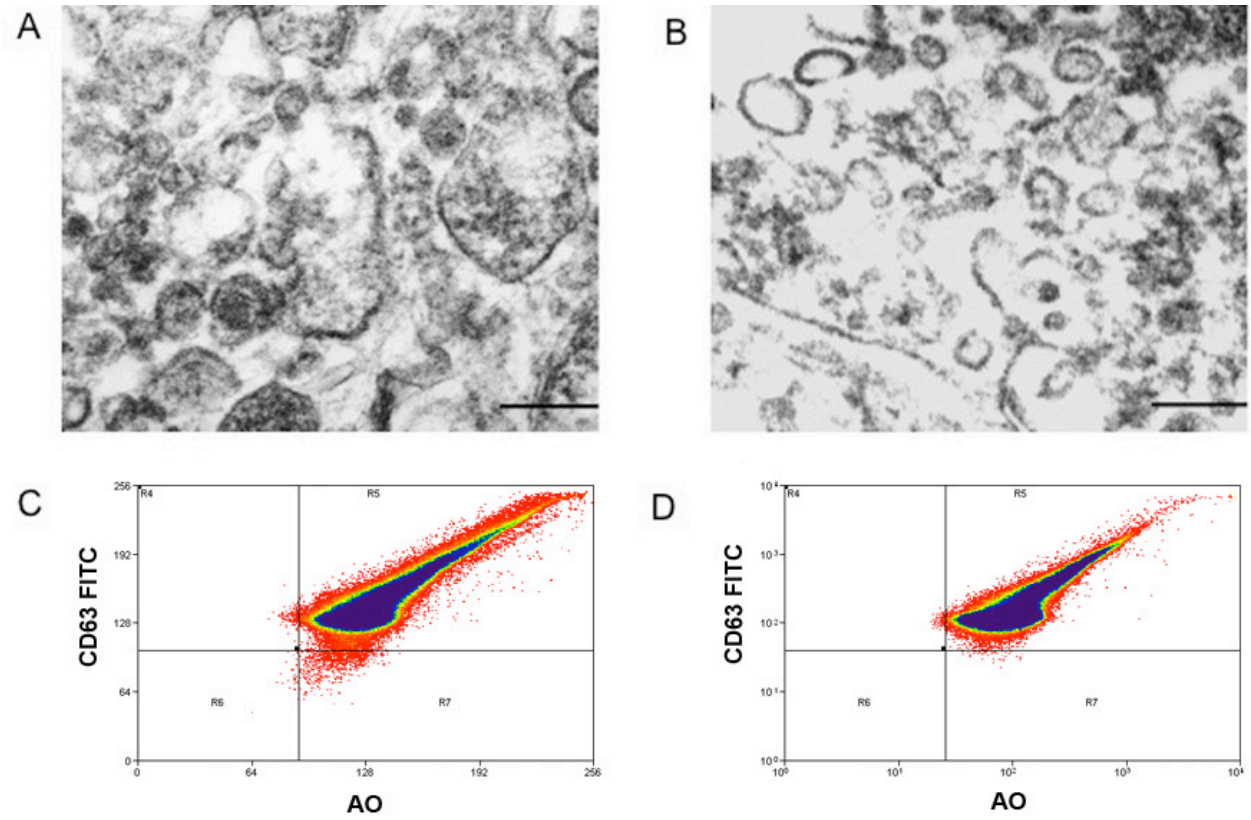

Figure II.1. Demonstration of presence of vesicular profiles resembling microvesicles and exosomes in ovarian follicular fluid from young mares by transmission electron microscopy (A and B) and flow cytometry (C and D). Whereas differential ultracentrifugation of follicular fluid yields vesicles of varying sizes including exosomes as well as microvesicles (A), precipitation using Exoquick™ yields more homogenous exosomes (50-100 nm; B). Flow cytometric analysis revealed that both preparations consist of vesicles positive for CD63 (y-axis) and RNA (acridine orange (AO); x-axis), similar to that reported for blood serum (Supplemental Figure II.1). Scale bar = 200 nm (original magnification 40,000x).

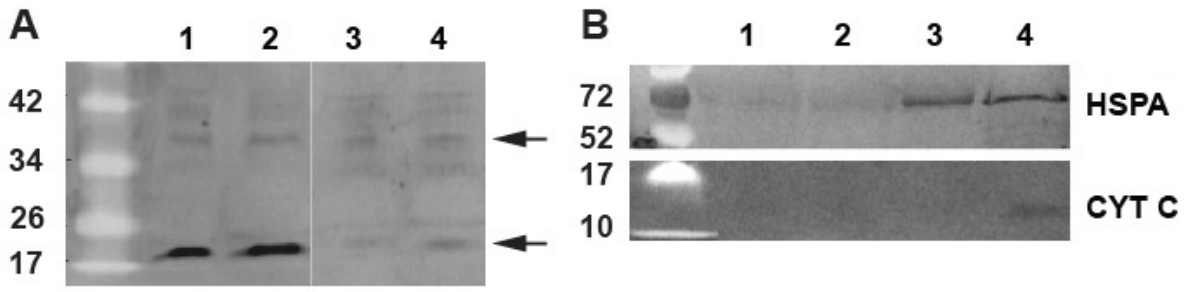

Figure II.2. Presence of INHA chain precursor in exosomes isolated from ovarian follicular fluid as demonstrated by Western blot analysis. A) The presence of a ~18 and ~ 37 kDa protein band is detected under reducing conditions in exosomal protein samples without (lanes 1 and 2) and with (lanes 3 and 4) albumin and IgG depletion, prepared from ovarian follicular fluid of different mares. B) These exosomal protein samples also were positive for HSP70 (a known exosomes protein), and negative for CYT C (cytosolic/mitochondrial protein). Lanes 1 through 3 are exosomal protein samples, whereas lane 4 is a granulosa cell protein sample.

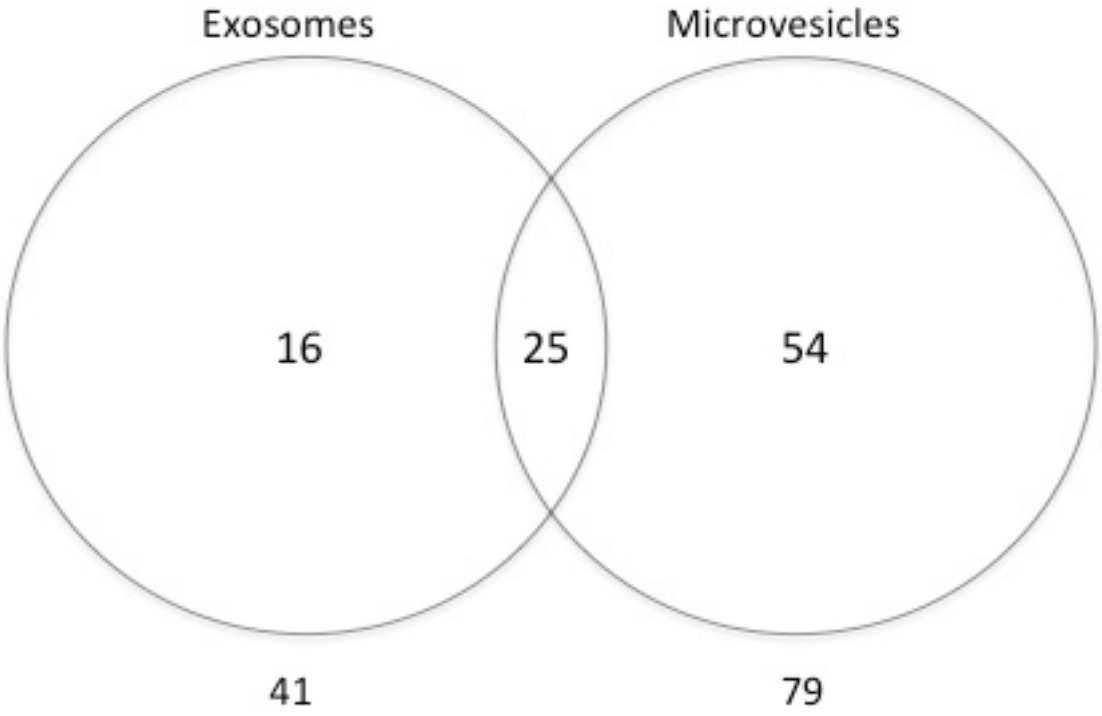

Figure II.3. Venn diagram indicating the number of miRNAs in microvesicles and exosomes isolated from follicular fluid.

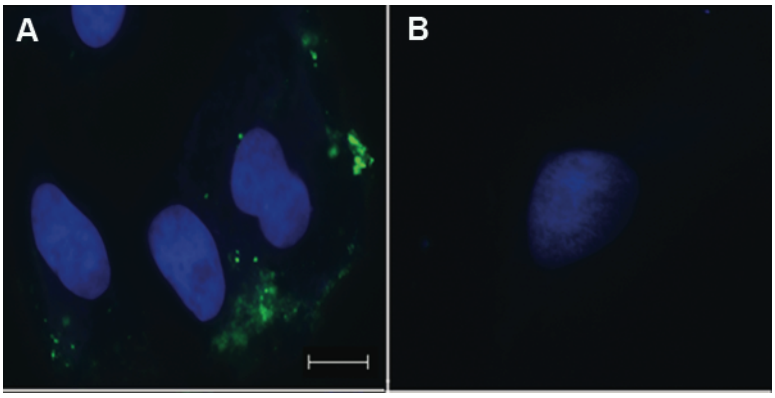

Figure II.4. Uptake of PKH67-labelled microvesicles by granulosa cells in vitro. A) Granulosa cells were cultured in FBS-free medium overnight containing PKH67-labelled microvesicles isolated from ovarian follicular fluid. Nuclei are stained purple (DAPI) and PKH67-labelled microvesicles are green. B) Granulosa cells cultured in FBS-free medium containing PKH67-labelled sterile PBS (negative control). Images were captured using 60X planapochromatic objective lenses. Scale bar = 10 μm.

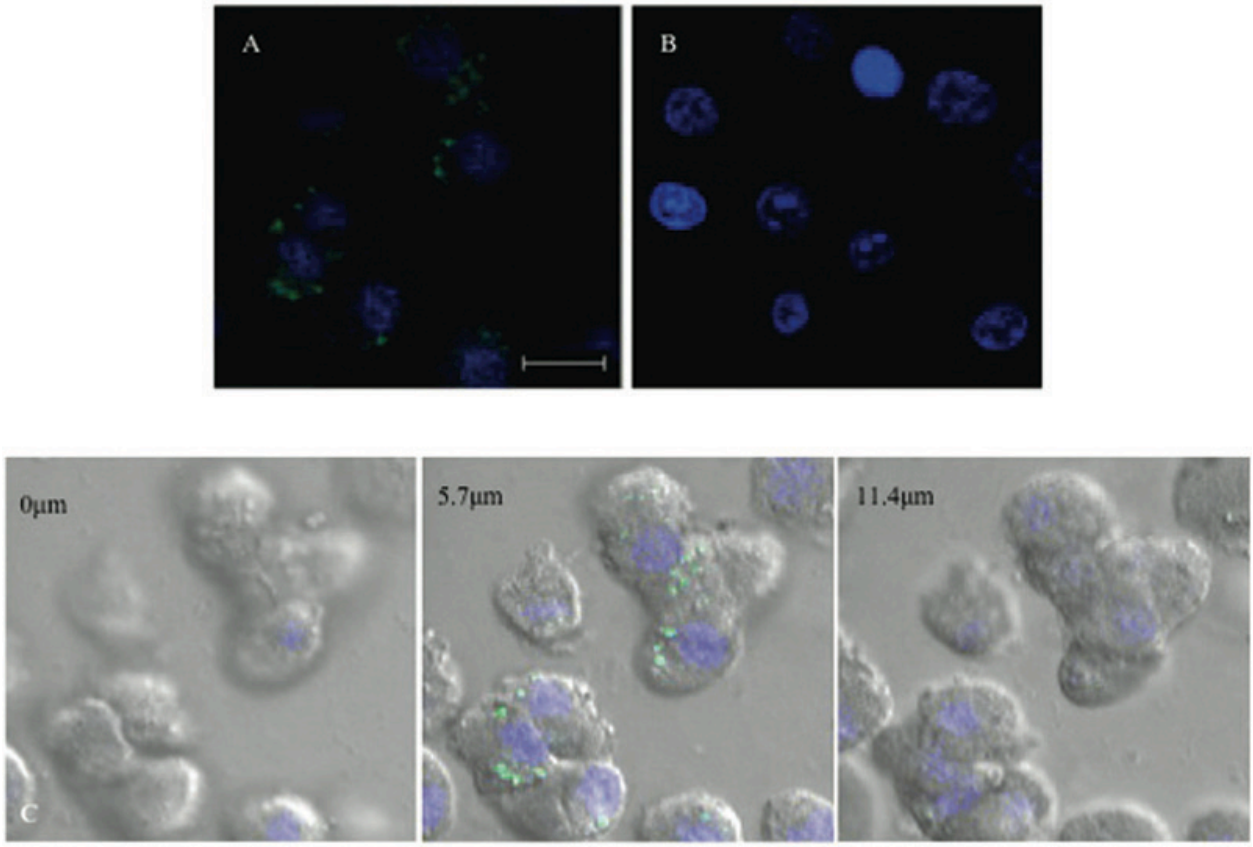

Figure II.5. Uptake of PKH67-labelled microvesicles by granulosa cells in vivo. A) Green (PKH67)-labelled microvesicles endocytosed by granulosa cells. Microvesicles were isolated from follicular fluid of a dominant follicle, labelled with PKH67, injected back into the antrum of the same follicle, and granulosa cells were collected the next day. B) Negative control. Granulosa cells collected from a dominant follicle injected with PKH67-labelled sterile PBS. C) Demonstration of localization of PKH67-labelled microvesicles in the interior of granulosa cells. That the labelled microvesicles indeed were present inside the cell (middle panel) and not simply coating the cell surface is evident in this confocal Z-stack generated from the cells shown in panel A. A series of 228 images were captured at 0.05 μm thickness interval spanning the entire depth of the cell. Numbers in each panel indicate the depth of the slice. Nuclei are stained purple

(DAPI) and microvesicles are green. Images were captured by confocal microscopy using a 63X objective. Scale bar = 10 μm.

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CHAPTER 3: A ROLE FOR EXOSOMES DURING OVARIAN FOLLICLE DEVELOPMENT: REGULATION OF TGFB SIGNALING MEMBERS[2]

Summary:

Ovarian follicle growth and maturation requires extensive communication between follicular somatic cells and oocytes. Recently, a new mechanism of intercellular cell communication was proposed involving cell-secreted vesicles called exosomes and microvesicles. Exosomes are cell-secreted vesicles between 50-150 nm in size, contain bioactive material such as miRNA, mRNAs and protein, and have been identified in a variety of body fluids, including ovarian follicular fluid. The goal of the current study was to investigate the role of exosomes during ovarian follicular development. More specifically, we tested the hypothesis that exosomes isolated from follicular fluid modulates TGFβ signaling in granulosa cells. Follicle contents (granulosa cells, follicular fluid) were collected from mid-estrous follicles (~35 mm, before induction of follicular maturation) and pre-ovulatory follicles (30-34 h after induction of follicular maturation). Following exosome isolation, a real time PCR miRNA profile screen revealed significant differences in the presence of 36 miRNAs in exosomes isolated from mid-estrous and pre-

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ovulatory follicles. Bioinformatics analysis of these exosomal miRNAs predicted their involvement in regulating different TGFβ signaling members. Analysis of selected TGFβ family members demonstrated significant lower levels of *ID2* an ACVR1 signaling induced target gene in granulosa cells from pre-ovulatory follicles. ACVR1 mRNA and protein levels in granulosa cells were not significantly different at mid-estrous or pre-ovulatory stages, however exposure to exosomes from follicular fluid of mid-estrous follicles affected ACVR1 and decreased *ID2* levels in granulosa cells. Moreover, exosomes isolated from mid-estrous and pre-ovulatory follicles, contain ACVR1 and miR-27b a predicted regulator of *ACVR1* and *ID2*, and were capable of altering ACVR1 and *ID2* levels in pre-ovulatory granulosa cells. These data indicate that exosomes isolated from follicular fluid can affect TGFβ signaling in granulosa cells, and play a role in regulating follicle development.

Keywords: Follicular fluid, ovarian follicle, exosomes, miRNAs, equine

Introduction

Mammalian antral follicular development is the last step of folliculogenesis and culminates in ovulation or atresia (Fortune, 1994). This dynamic process requires extensive cross talk between follicular cells (theca, granulosa, cumulus and oocyte) (Fortune, 1994; Knight and Glister, 2006; Matzuk et al., 2002), which is regulated by endocrine, paracrine and autocrine signaling. Recently, a new mechanism of intercellular communication mediated by cell-secreted vesicles was revealed (Raposo and Stoorvogel, 2013). Cell-secreted vesicles, called exosomes (~50-150 nm) and microvesicles (~100-1000 nm), carry bioactive material such as mRNAs, microRNAs (miRNAs), and proteins in different body fluids and deliver their contents to recipient cells (Raposo and Stoorvogel, 2013). Exosomes and microvesicles have been identified

in ovarian follicular fluid (FF) of mares and cows, and these vesicles contain microRNAs miRNAs and proteins (da Silveira et al., 2012; Seifi Noferesti et al., 2012; Sohel et al., 2012). MiRNAs, small (~22 nucleotide) non-coding RNAs, regulate gene expression by complementary base-pair interactions in the 3' untranslated region of mRNA targets, leading to mRNA cleavage or translational repression (Bartel, 2009; Huntzinger and Izaurralde, 2011). Studies involving specific deletion of Dicer (necessary for mature miRNA synthesis) in ovaries demonstrate miRNAs are necessary for adult ovarian function and fertility (Nagaraja et al., 2008). In addition, miRNA-21 is involved in regulation of granulosa cell apoptosis and corpus luteum formation in mice (Carletti et al., 2010), and gonadotropins regulate miRNA expression and consequently control estradiol production in sheep (Donadeu et al., 2012). Finally, studies in mice reveal that transforming growth factor β (TGFβ) induces miR-224 and miR-383 expression, which target *Smad4* and *Rbms1* thereby regulating estradiol production in response to gonadotropin stimulation (Yao et al., 2010; Yin et al., 2012). These studies clearly indicate that miRNAs are important regulators of ovarian function by controlling various aspects of follicular growth and development.

The TGFβ signaling family is necessary for follicle development and oocyte competence in mammals. Different studies have demonstrated the role of specific family members in theca cells, granulosa cells, cumulus cells and oocytes (Edson et al., 2009; Knight and Glister, 2006). Activins/Inhibins, BMPs, and GDFs are responsible for modulating the effects of both FSH and LH during antral follicle development. Therefore, understanding the mechanisms involved in regulating TGFβ signaling family members is important to provide insight into the process of follicle growth and development and oocyte maturation.

The overall goal of this study is to obtain a better understanding of the role of exosomes in follicle development and growth. Specifically, we will test the hypothesis that exosomes isolated from follicular fluid modulate TGFβ signaling in granulosa cells. To test this hypothesis we first identified exosomal miRNAs present in follicular fluid of mid-estrous and pre-ovulatory ovarian follicles, and used bioinformatics analysis to identify predicted targets of the TGFβ signaling pathway in granulosa cells. Second we determined if exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles are capable of altering expression of selected TGFβ signaling members in pre-ovulatory granulosa cells.

Material and Methods

Collection of ovarian follicular cells and fluid

Follicular fluid (10 ml) and granulosa cells were aspirated from dominant follicles (~35 mm before induction of follicular maturation- mid-estrus), and (30-34 h after induction of follicular maturation - pre-ovulatory) from young (3-12 yr) estrous mares. All procedures were done in accordance with the Colorado State University Institutional Animal Care and Use Committee. Mares were housed on dry lots with water and hay *ad libitum*. Reproductive tracts of the mares were examined using transrectal ultrasound. A synthetic Prostaglandin analog (Cloprostenol Sodium) was administered during two consecutive days beginning on day 5 or 7 (250 mg of Estrumate® Merck) following ovulation or aspiration, and dominant follicles were determined based on follicle growth and diameter, relaxed cervical tone and endometrial edema. For pre-ovulatory follicle collections follicular maturation was induced by administration of hCG and/or deslorelin (2500 IU and 1.5 mg, respectively, iv), and follicular contents were collected 32 h later by transvaginal, ultrasound-guided follicular aspirations (Carnevale et al., 2000). Upon

collection follicular fluid was collected using a 12-GA needle, placed in an incubator at 37°C for 10-20 min, centrifuged at 300 x g for 10 min, 2000 x g for 10 min, 10000 x g for 30 min and later stored at -80°C until processed for exosome isolation. To remove red blood cells, granulosa cells from young mares were rinsed (3x) in PBS containing 0.02% polyvinyl alcohol (PVA). Approximately half of the sample was snap frozen and used for total RNA and protein isolation, whereas the rest of the cells were pipetted repeatedly to separate cells and placed in DMEM/F-12, (Invitrogen™ #11320-033) with no addition of fetal bovine serum (FBS). Cells were cultured for 24 h and used in granulosa cell culture and exosome treatment experiments (see below).

Isolation of exosomes from follicular fluid

Exosomes were isolated from ovarian follicular fluid starting with a series of differential centrifugation steps as described (Thery et al., 2006), followed by Exoquick™ (SBI System Biosciences, Inc) precipitation, a polymer-based reagent that enriches preparations with exosomes. Briefly, following centrifugation, 400 µl of follicular fluid supernatant was added to 100 µl of Exoquick. This preparation was incubated overnight at 4°C and centrifuged at 1500 rpm for 30 min to obtain an exosome pellet. Exosome pellets were resuspended in 250 µl of PBS (pH 7.4) and used for miRNAs real time PCR analysis (n = 6 mid-estrus, and n = 6 pre-ovulatory).

Granulosa cell culture and exosome treatment

Granulosa cells from pre-ovulatory follicles were placed in 24-well dishes (Nunc, Inc. #142475) at 37°C and density of $5x10^4$ cells per well in 2 ml of DMEM/F-12 medium. Medium was replaced after the first day to remove any dead cells, and treatment started on the second day.

Treatment was composed of 1.75 ml of medium and 250 µl of exosomes isolated from 800 µl follicular fluid. Granulosa cells from pre-ovulatory follicles (n = 4) were treated with exosomes isolated from follicular fluid from pre-ovulatory (n = 4) or mid-estrus (n = 4) follicles. Cells were lysed using TRI Reagent®BD (Molecular Research, Inc., Cincinnati, OH, USA) and used for total RNA and protein isolation. Exosomal proteins were resuspended in 8M Urea.

Western blot analysis

Granulosa cell and exosome proteins were isolated, and protein concentration was determined using the Bradford assay. A total of 30 µg of protein was loaded and resolved in 12% SDS-PAGE polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Protein samples were run at 30 mA for 45 min and transferred to nitrocellulose membranes (Biotrace NT, Pall life Sciences, Pensacolla, FL, USA) for 1 h at 100 V. Membranes were incubated in blocking buffer (5% non-fat dried milk in TBST) for 2 h at room temperature, and the presence of ACVR1 was assessed by exposing membranes to a goat polyclonal antibody raised against a peptide mapping to the N-terminus of ACVR1 of human origin (0.4 µg/ml, sc-5671, Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4˚C. In addition, membranes were exposed to ACTB mouse monoclonal antibody raised against ACTB of avian origin (0.1 µg/ml, sc-47778, Santa Cruz Biotechnology Inc., Santa Cruz, CA), as a reference protein. Membranes were washed three times in 1X TBST for 5 min, and incubated for 1 h at room temperature with a horseradish peroxidase conjugated anti-goat secondary antibody (0.2 µg/ml, sc-2020, Santa Cruz Biotechnology Inc.) or horseradish peroxidase conjugated anti-mouse secondary antibody (1 µg/ml, ab-6789, Santa Cruz Biotechnology Inc.), respectively. Membranes were washed three times in 1X TBST for 5 min and incubated for 5 min in ECL Plus Prime Western Blotting Detection System solution

(Amersham™, Buckinghamshire, UK) for color development, and image and band analyses were performed using ChemiDoc MP Image System (Bio-Rad Laboratories, Inc).

mRNA and miRNA isolation and reverse transcription

Total RNA including miRNA was isolated from exosome preparations using TRI REAGENT®BD (Molecular Research Center Inc), according to the manufacturer's instructions. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -80°C until further use.

Quantifiable, reverse transcribed mRNA was generated using qScript™ cDNA Synthesis Kit (Quanta Biosciences Cat#95047) according to the manufacture's instructions. Briefly, the reverse transcription reaction was carried out using approximately 30 ng of total RNA for each selected gene. RNA was incubated with 5x qScript Reaction Mix, qScript Reverse Transcriptase, and DNase-free water at 22°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min.

Quantifiable, reverse transcribed miRNAs were generated using the miScript PCR System (Qiagen® #218193) according to the manufacturer's instructions. Briefly, the reverse transcription reaction was carried out with approximately 100 ng of total RNA for 384 miRNAs. Total RNA, including the small RNA fraction, was incubated with 5X miScript HiFlex Buffer, 10X miScript Nucleic Mix, RNase-free water, and miScript Reverse Transcriptase at 37°C for 60 min followed by 5 min at 95°C.

Real-time PCR expression analysis of mRNAs and miRNAs

Relative levels of mRNAs were examined in granulosa cells recovered from ovarian follicles of young mares at mid-estrus (n = 6) and pre-ovulation (n = 6) (Table III.1). In addition,

mRNAs levels were examined in granulosa cells from pre-ovulatory follicles exposed in culture for 24 h to exosomes isolated from follicular fluid collected at mid-estrus or pre-ovulation. Each analysis was performed in 10 µl reactions containing 2X SYBR Green I master mix (Roche Applied Sciences), 0.5 µM of forward and reverse primers (primers were designed against equine gene sequences, and are provided in Table III.1), and 1 µl cDNA. The PCR cycle conditions were as followed: 95°C for 5 min, 45 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec, followed by melt curve analysis to confirm amplification of single cDNA products. To identify differences in mRNA levels in granulosa cells before treatment or after treatment with exosomes from mid-estrus (n = 4) or pre-ovulatory (n = 4) follicles, raw Ct values were normalized to two internal controls (*ACTB*, *GAPDH*), and statistical differences were assessed at $P < 0.05$ using a Student's t-test.

The relative levels of mature miRNAs were examined in exosome preparations isolated from follicular fluid of different mares at mid-estrus and pre-ovulatory, using Human miRNome Profiler plates (SBI) with miRNA sequences conserved between horses and humans. In addition, levels of mature miRNAs predicted to target members of the TGF-β signaling family were examined in granulosa cells and exosomes isolated from follicular fluid, as well as cells in culture after treatment with exosomes from mid-estrus follicles (n = 4) and pre-ovulatory follicles (n = 4). Each analysis was performed in 6 µl reactions containing 2X SYBR Green I master mix (Roche Applied Sciences), 10 µM Universal reverse primer (Qiagen) and miRNA specific forward primer, and 0.03 µl cDNA. Real-time PCR was conducted using the LightCycler480 PCR system (Roche Applied Sciences) in 384-well plates. The PCR cycle conditions were as followed: 95°C for 5 min, 45 cycles of 95°C for 10 sec, 55°C for 15 sec, and 72°C for 15 sec followed by a melt curve analysis to confirm amplification of single cDNA

products. To identify differences in the presence of exosomal miRNAs isolated from follicular fluid or miRNAs in granulosa cells, raw Ct values were normalized to miR-99b (invariably and present in all samples), and statistical differences were assessed at $P < 0.05$ using a Student's t-test.

Results

Presence of exosomal miRNAs during equine follicle development

To investigate the role of exosomal miRNAs in equine ovarian follicle development, exosomes were isolated from follicular fluid of antral follicles at mid-estrus ~35 mm before induction of follicular maturation, and pre-ovulation ~30-34 h after follicular maturation based on previous findings that miRNAs are regulated by LH (Carletti et al., 2010; Fiedler et al., 2008; Lei et al., 2010; McBride et al., 2012; Xu et al., 2011). The presence of different miRNAs was examined using real-time PCR, and miRNAs were considered present if detected in at least three out of the six different biological samples, ($Ct < 37$) with single annealing peaks. Of the 380 miRNAs examined, 137 different miRNAs were detected in exosomes isolated from mid-estrous and pre-ovulatory follicles (data no shown). Of these, 36 miRNAs were present at different levels (fold change > 2; P value < 0.05) in exosomes collected from mid-estrous and pre-ovulatory follicles (Table III.2).

Predicted miRNA-regulated pathways

Since a single miRNA can target several mRNAs, and the identified miRNAs are packaged in exosomes, we choose to analyze exosomal miRNAs as a unit in their ability to target signaling pathways. Bioinformatics analysis of the 36 up-regulated exosomal miRNAs from

pre-ovulatory follicles (Table III.2) predicts regulation of 182 different signaling pathways (DIANA Lab-DNA Intelligent Analysis, http://diana.cslab.ece.ntua.gr/pathways/) (Supplemental Table III.1). According to this analysis, 44 genes of the TGFβ signaling pathway are predicted to be regulated by exosomal miRNAs, including receptors (*ACVR2B*, *ACVRI*), and SMAD target genes (*CDKN2B*, *ID1*, *ID2*, *COL1A2*, *COL3A1*, and *TGFβ7*). Therefore, we chose TGFβ signaling family members as potential targets of exosomal miRNAs in follicular fluid.

Expression of TGFβ family members during antral follicle development

Eighteen TGFβ family members, including receptors (*ACVR1*, *ACVR2B*, *TGFR2*, *BMPR2*), TGFβ/Activin responsive genes (*COL1A2*, *COL3A1*, *CDKN2B*, *cMYC*, *FOS*, *CDC25A*, *IL6*, *IGFBP3*, *ITGB7*, *TGFBI*, *TGFB1*), and BMP-responsive genes (*ID1*, *ID2*, *STAT1*), were selected for real time PCR analysis to investigate their relative level in equine granulosa cells. All 18 selected transcripts were present (Ct < 37) in granulosa cells. *IL6*, *TGFB1, ID2* levels were present at different levels in granulosa cells from mid-estrous compared to pre-ovulatory follicles (Figure III.1). *IL6* ($P < 0.02$) was lower in granulosa cells after LH induction of follicular maturation (pre-ovulatory), while *TGFB1* ($P < 0.01$) was higher in granulosa cells from pre-ovulatory follicles (Figure III.1). Also, *ID2* levels were ~3.6 fold lower in granulosa cells from pre-ovulatory compared to mid-estrous follicles ($P < 0.02$) (Figure III.1). ACVRI, a BMP receptor involved in regulating *ID2* expression, mRNA and protein were present at similar level in granulosa cells collected at mid-estrous compared to pre-ovulatory samples (Figure III.2 A and B).

Relative levels of miRNAs predicted to regulate ACVR1 and ID2 in granulosa cells and exosomes

MiRNAs predicted to regulate *ACVR1* (miR-27b, miR-372, miR-382) and *ID2* (miR-27b) were present in granulosa cells collected from mid-estrous and pre-ovulatory follicles (Supplemental Figure III.1). Interestingly, miR-382 only was detected in granulosa cells from mid-estrus follicles and not in granulosa cells from pre-ovulatory follicles, and miRNA-27b was not detected in granulosa cells.

Three miRNAs predicted to target *ACVR1* and *ID2* also were detected by real time PCR in exosomes isolated from follicular fluid collected from mid-estrous and pre-ovulatory follicles (Figure III.2C). Relative levels of miR-27b and miR-382 were higher ($P < 0.05$) in exosomes collected from follicular fluid of mid-estrous compared to pre-ovulatory follicles.

Primary granulosa cell culture and exposure to exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles

To determine if exosomes from different follicular stages are capable of regulating TGFβ family members in granulosa cells, exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles were added to pre-ovulatory granulosa cells in culture. Following a 24 h incubation period, mRNA levels of TGFβ members were examined by real time PCR analysis. Analysis of receptors belonging to the TGFβ family revealed differences in *ACVR1* and *ACVR2B* relative levels ($P < 0.05$) in granulosa cells following treatment with exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles compared to untreated granulosa cells (Figure III.3). In regards to TGFβ/ Activin responsive genes, *CDKN2B* levels in granulosa cells also were decreased ($P < 0.05$) following treatment with exosomes isolated from follicular fluid

of mid-estrous and pre-ovulatory follicles. Differences between untreated and exosomes-treated granulosa cells also were observed for *ITGB7*, *COL3A1*, and *COL1A2* (Figure III.3). *ITGB7* and *COL3A1* levels were lower ($P < 0.05$) in granulosa cells following treatment with exosomes from follicular fluid of mid-estrous follicles compared to untreated granulosa cells, whereas *COL1A2* levels were lower ($P < 0.05$) in granulosa cells treated with exosomes from follicular fluid of pre-ovulatory follicles compared to untreated granulosa cells. Relative levels of BMP-responsive genes *ID1* and *ID2* also were altered following exosomes treatment. *ID1* levels in cultured granulosa cells were increased ($P < 0.05$) following exposure to exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles, whereas *ID2* levels were decreased ($P < 0.05$) following exosome treatment from mid-estrous follicles compared to untreated granulosa cells (Figure III.3).

To complement changes observed in mRNA levels, relative protein levels were determined using Western blot analysis. Initial efforts focused on receptor (ACVR1), Western blot analysis using sc-5671 antibody detected a band of ~ 50 kDa band (equine ACVR1 protein contain 509 amino acids in length and an expected size of 57 kDa). Treatment of granulosa cells with exosomes collected from follicular fluid of mid-estrous and pre-ovulatory follicles did appear to increase ACVR1 levels compared to untreated granulosa cells, however Western blot results were variable (Figure III.4).

Relative levels of miRNAs were examined in granulosa cells predicted to regulate *ACVR1* and *ID2* following exposure to exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles (Supplemental Figure III.2). MiR-372 was increased ($P < 0.05$) in cultured granulosa cells following treatment with exosomes isolated from mid-estrous and pre-ovulatory follicles compared to untreated granulosa cells (Figure III.5). MiRNA-27b was

lowered in cultured granulosa cells after treatment with exosomes isolated from mid-estrus follicles ($P < 0.05$) compared to cultured granulosa cells not exposed to exosomes (Figure III.5).

Exosomal ACVR1

Possible presence of ACVR1 mRNA and protein in exosomes was assessed using real time PCR and Western blot analysis. *ACVR1* levels were ~ 3.7 fold decreased ($P = 0.09$) in exosomes collected from follicular fluid of pre-ovulatory follicles (Figure III.6A). Similarly, ACVR1 protein was present in exosomes isolated from mid-estrus and pre-ovulatory follicles, and although protein levels appeared higher in exosomes isolated from FF at mid-estrus, this was not significant (Figure III.6B).

Discussion

The main goal of this study was to demonstrate that exosomes isolated from follicular fluid of equine ovarian follicles at mid-estrous and pre-ovulatory stages are modulators of TGFβ signaling in granulosa cells during follicular development. Initially this was based on bioinformatics analysis, which revealed that exosomal miRNAs are predicted to target and regulate 44 genes of the TGFβ signaling family. Of the initial 18 genes profiled in equine granulosa cells, we focused on *ACVR1* and *ID2*. ACVR1 is a receptor involved in regulating *ID2* expression and the BMP response pathway, which in turn is known for its importance during follicular development (Edson et al., 2009; Knight and Glister, 2006).

Previously the presence of exosomes containing miRNAs in ovarian follicular fluid was described during pre-ovulatory stages in the mare (Silveira et al., 2012). In the current study, we extended these observations by conducting a miRNA profiling screen in exosomes isolated from

follicular fluid of mid-estrous (~35 mm, before induction of follicle maturation) and pre-ovulatory (30-34 h after induction of follicle maturation) follicles. Of 380 miRNAs screened, 137 miRNAs were detected in exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles. Of these, 36 miRNAs were higher ($P < 0.05$) in exosomes isolated from pre-ovulatory compared to mid-estrous follicles. This number of miRNAs was expected based on similar work done in rat (~200 miRNAs) and sheep (189 miRNAs) (Fiedler et al., 2008; McBride et al., 2012). It is important to note that both these studies involved granulosa cells or whole follicles to obtain RNA for miRNA profiling, instead of follicular fluid. Follicular fluid is a reservoir of factors secreted by follicular cells (Revelli et al., 2009; Rodgers and Irving-Rodgers, 2010). Using follicular fluid isolated from bovine and human ovarian follicles, (Sohel et al., 2012) and (Sang et al., 2013), also confirmed the presence of cell-secreted vesicles in ovarian follicular fluid. Analysis of miRNA profiling of exosomal miRNAs isolated from follicular fluid was conducted to uncover miRNAs associated with oocyte cytoplasmic maturation, suggesting that miRNA profile of exosomal miRNAs can be used as a biomarker for oocyte maturation (Sohel et al., 2012). It is evident that cell-secreted vesicles (microvesicles, exosomes) are present in follicular fluid and contain miRNAs. Furthermore, our previous studies indicate these cell-secreted vesicles likely have an intrafollicular origin, and importantly, can be taken up by surrounding granulosa cells (da Silveira et al., 2012). Still it is unclear if cumulus cells, granulosa cells, oocyte or all follicular cell types secrete these vesicles during intrafollicular cell communication, or their role during follicle growth and development.

In the current study, exosomes were collected from follicular fluid of mid-estrous and pre-ovulatory follicles, and analysis of miRNA content revealed 36 exosomal miRNAs that were significantly different ($P < 0.05$). These 36 miRNAs were predicted to target 44 genes of the

TGFβ signaling family. Most of these targets are involved in regulation of cell differentiation and proliferation, indicating exosomal miRNAs could play a role in follicular cell proliferation or differentiation during follicular development. Among these exosomal miRNAs, miR-181a was 15 fold higher in exosomes isolated from pre-ovulatory follicles (mature), compared to mid-estrous follicles (immature). Previously, miR-181a was detected at 3.4 fold higher levels in exosomes isolated from follicular fluid of pre-ovulatory follicles in old mares compared to young mares (da Silveira et al., 2012). An increase in miR-181a in mouse granulosa cells leads to suppression of ACVR2A-induced SMAD2 phosphorylation and a block in granulosa cell proliferation (Zhang et al., 2012). Transfection of miR-181a in human granulosa cells leads to a decrease in PCNA, a known regulator of cell proliferation (Sirotkin et al., 2010). During the final stage of follicular development (high LH levels), granulosa cells are not proliferating (Clement and Monniaux, 2012), suggesting that miR-181a in exosomes are part of a complex network regulating granulosa cell proliferation during final follicle maturation. These data also indicate an important role for exosomal miRNAs as regulators of TGFβ signaling in follicular cells during follicle development.

Initial analysis on the presence of selected members of the TGFβ family demonstrated significant differences ($P < 0.05$) in relative levels of *IL6*, *ID2* and *TGFβ1/1* in granulosa cells collected from mid-estrous compared to pre-ovulatory follicles. For example, *IL6* and *ID2* levels were decreased and *TGFβ1/1* was increased in pre-ovulatory granulosa cells. IL6 is known to regulate LH receptor (LHR) in granulosa cells during cell differentiation and if increased in follicular fluid, attenuates LH function on the granulosa cell maturation process (Tamura et al., 2001). During follicle selection high FSH levels stimulate *ID2* increase in granulosa cells which facilitates *FSHR* expression and, importantly, initiate *LHR* expression plus LH-induced

progesterone production (Johnson et al., 2008). The role of *TGFβ1/1* is unclear however, it possibly amplifies FSH-induced aromatase activity, inhibin production, progesterone production and LH receptor induction (Knight and Glister, 2006). These data illustrates the multifaceted roles of TGFβ signaling during follicle development.

BMP-signaling during antral follicle final maturation is an important step towards granulosa cell differentiation (Knight and Glister, 2006; Song et al., 2012). ACVR1 (ALK2) is a BMPs/GDFs ligand receptor that forms dimers with ACVR2, ACVR2B, and BMPR2, after binding of ligands (Hinck, 2012). The inhibitor of DNA binding ID2 is a well-characterized BMP responsive gene and is down-regulated following miR-148a mediated inhibition of ACVR1 (Song et al., 2012). Although *ACVR1* levels were not different between mid-estrous and pre-ovulatory granulosa cells, ACVR1 protein levels were increased ~ 3.3 fold in pre-ovulatory granulosa cells. ACVR1 increases with follicular size however, no significant difference was observed between immature and mature follicles in cattle (Glister et al., 2010).

Based on previous findings that miRNA-148a regulates ACVR1, and consequently down-regulate SMADs and *ID2* (Song et al., 2012), we determined the relative level of miRNAs predicted to target *ACVR1* and *ID2* in both granulosa cells and follicular fluid exosomes. We were unable to detect miR-148a in the isolated exosomes but did identify other exosomal miRNAs predicted to target *ACVR1* and *ID2*. MiRNA-27b (predicted regulator of *ACVR1* and *ID2*) was not detected in granulosa cells consistently, however was detected at higher levels ($P < 0.05$) in exosomes isolated from mid-estrous follicles. The source of exosomal miR-27b in follicular fluid is unclear; however, it is possible that exosomal miR-27b originates from cumulus cells. Using sheep ovarian follicles to create a miRNA cDNA library, McBride et al. (2012) also revealed that medium size follicles have increased abundance of miR-27b compared

to pre-ovulatory follicles; however, again it is not clear which cell type expresses this miRNA (Donadeu et al., 2012). Interestingly, miR-27b was present in higher levels in cumulus cells from pre-ovulatory follicles of young compared to old mares (da Silveira et al., 2012).

MiR-372 and miR-382 (predicted regulators of *ACVR1*) levels were elevated in granulosa cells from mid-estrous follicles compared to pre-ovulatory follicles (miR-382 was not detected in granulosa cells from pre-ovulatory follicles). This expression pattern was recapitulated in exosomes collected from follicular fluid. In addition to *ACVR1*, miR-372 is involved in regulation of cell cycle inhibitors p21, p27 and p53 (Sirotkin et al., 2010), both affecting cell proliferation and differentiation. Interestingly, miR-382 has been identified in the ovarian cortex of bovine, and not in the cumulus cells or corpus luteum, suggesting this miRNA originates from theca or stromal cells (Hossain et al., 2009).

To investigate the role of exosomes in mediating TGFβ signaling in granulosa cells, we performed an in vitro experiment using pre-ovulatory granulosa cells and treatment with or without exosomes isolated from follicular fluid of mid-estrous or pre-ovulatory follicles. Initially, we evaluated the level of *ACVR1* and verified that levels were significantly decreased in pre-ovulatory granulosa cells following exposure to exosomes from either mid-estrous or pre-ovulatory follicles. Combined with the observation that follicular fluid exosomes contain miR-372 and miR-382, and levels increase in granulosa cells following exosome treatment, it is possible these exosomal miRNAs regulate *ACVR1* levels in pre-ovulatory granulosa cells. Moreover, miR-27b also is predicted to target *ID2*, which was down-regulated in pre-ovulatory granulosa cells following exosomes treatment. However, analysis of ACVR1 protein level in pre-ovulatory granulosa cells indicates ACVR1 levels are ~ 3.3 fold higher compared to mid-estrous granulosa cells. This could be explained if exosomes contain ACVR1 themselves that is

transferred to granulosa cells. Previously *ACVR1* was identified in exosomes originating from MC/9 murine cells and SW480 human colorectal carcinoma cells (Hong et al., 2009; Valadi et al., 2007). We observed exosomes from mid-estrous follicles had higher, levels of ACVR1 mRNA (~ 3.7 fold) and protein (~ 1.8 fold) compared to exosomes from pre-ovulatory follicles. However, especially the relative level of ACVR1 protein was quite variable in exosomes, which could be due to heterogeneous population of follicular fluid derived exosomes, and it is unclear if granulosa cells, cumulus cells, or both release exosomes containing ACVR1. Overall these data indicate that exosomes isolated from follicular fluid of mid-estrous follicles possibly transfer ACVR1 mRNA and/or protein. Interestingly, *ACVR1* was decreased after 24 h following exosome treatment, and therefore it is more likely that the increase in ACVR1 is caused by delivery of exosomal ACVR1 protein into granulosa.

In conclusion, in the current study we identified dynamic changes in the presence of exosomal miRNAs during follicle growth and maturation in the mare. In addition, these miRNAs appear to target members of the TGFβ family members. To uncover the role of exosomes in regulating TGFβ signaling in follicular cells, exosomes isolated from follicular fluid were added to granulosa cells in vitro. These exosome treatment experiments resulted in altered levels of a number of TGFβ family members, and indicate a role for exosomes in regulating ACVR1 signaling and ID2 function in granulosa cells during follicle maturation possibly through direct delivery of ACVR1 present in exosomes. Future experiments will focus on identifying exosomes as delivery vehicles of TGFβ family members.

Table III.1: Primers utilized for real-time PCR analysis.

Gene	Primer Forward: 5'-3'	Primer Reverse: 5'-3	Annealing Temperature °C	Fragment Size (bp)
ACVR1	CCTCTCCTGTGGGAATGAGG	CTGGAAGCAGCCTTTCTGGT	60	100
ACVR2B	ATGTACCGGCATCGAAAACC	CGAGCCTTGATCTCCAACAG	60	119
TGFBR2	GACCCCAAGCTCACCTACCA	TGCACTCATCAGAGCTACAGGA	60	124
BMPR2	TTGCTTTGGCATCAGTCTCTG	CTGCTGCCTCCATCATGTTC	60	120
CDC25A	CTGGGCCCTTGGACAGTAAA	GTCGGAATGGCTCCTCTTCA	60	110
CDKN2B	CCGAGCTGCTACTGCTCCAC	CACCAGCGTGTCCAGGAAG	60	107
COL1A2	AGGTTTCCAAGGACCTGCTG	GGTTTTCCAGGGTGACCATC	60	117
COL3A1	GTCCCAACCCAGAGATTCCA	CGCTACTTTCATTTCCTTTCAGG	60	102
USF2	GGATACCACGGCTGTGTCAG	ATCGTCCTCTGCGTTCCTGT	60	114
IGFBP3	CAAGCGCGAGACAGAATACG	GAAGCCCTTCTTGTCGCAGT	60	124
IL6	GGCAGAAAAAGACGGATGCT	CACCCTTGAACTCGTTCTGGA	60	122
ITGB7	TGCCGAAGGATACCCTGTAGA	CTGCAGCTTCTCCAGCAAGG	60	112
MYCBP	GCCTTATATGAAGAACCAGAGAAACC	GCCAATTCTAGGCGAAGCAG	60	113
TGFB1	CTCAGTGCCCACTGCTCCT	CATCAATGGTGGCCAGATCA	60	100
TGFBI	ATGGTGAACAGACGGGTCCT	CCGTTGGGATAGTGGTGGAT	60	101
ID1	ACATGAACGGCTGCTACTCG	TCCAACTCCAGGTCCCAGAT	60	124
ID2	CATCCCCCAGAACAAGAAGG	TGGTGATGCAGGCTGACAAT	60	180
STAT1	GTTTCCCCATGGAAATCAGG	GAAGGTCGTGGAAACGGATG	60	108
ACTB	CGACATCCGTAAGGACCTGT	CAGGGCTGTGATCTCCTTCT	60	99
GAPDH	AGAAGGAGAAAGGCCCTCAG	GGAAACTGTGGAGGTCAGGA	60	87

Table III.2: Exosomal miRNAs present in follicular fluid isolated from mid-estrus and pre-ovulatory follicles. Values presented are normalized against miR-99b (mid-estrous 19.53 ± 0.63) and (pre-ovulatory 19.73 ± 0.54).

miRNAs	Mid-estrous (ΔCt)	STDEV	Pre-ovulatory (ΔCt)	STDEV	Fold change	P-value
let-7b	16.80	0.66	14.19	2.01	6.08	0.049
miR-24	14.69	2.36	10.21	2.45	22.32	0.039
miR-25	15.93	1.19	11.81	1.76	17.44	0.004
miR-27a	15.01	1.54	11.48	2.13	11.59	0.036
miR-30c	15.76	0.71	13.10	2.00	6.32	0.046
miR-93	16.59	0.94	12.47	1.68	17.35	0.013
miR-99a	16.99	0.54	12.93	1.33	16.72	0.002
miR-124	14.50	1.73	12.43	1.27	4.19	0.055
miR-125a-3p	15.04	0.87	10.68	1.76	20.47	0.002
miR-139-3p	16.17	0.44	12.52	2.23	12.52	0.042
miR-151-3p	16.54	1.15	13.63	1.28	7.52	0.015
miR-181a	15.99	0.86	12.01	3.91	15.82	0.037
miR-188-3p	15.96	1.04	12.75	1.84	9.30	0.013
miR-197	15.36	2.28	10.73	1.74	24.72	0.018
miR-205	16.16	1.52	11.85	2.55	19.80	0.027
miR-206	15.13	0.57	12.98	1.04	4.44	0.011
miR-210	14.42	1.97	9.71	1.08	26.17	0.004
miR-222	15.95	0.61	13.27	1.37	6.41	0.027
miR-320b	15.75	0.91	11.18	2.17	23.75	0.003
miR-320c	15.11	1.65	10.89	1.45	18.61	0.003
miR-324-3p	15.14	2.34	10.11	1.63	32.71	0.006
miR-324-5p	13.92	1.65	9.11	3.18	28.10	0.036
miR-328	14.88	0.74	12.29	1.62	6.05	0.022
miR-339-5p	16.09	0.92	13.01	1.55	8.45	0.004
miR-345	14.56	1.03	11.20	1.10	10.29	0.004
miR-346	14.57	1.44	10.56	1.11	16.14	0.002
miR-422a	16.03	1.31	13.00	2.30	8.18	0.053
miR-423-5p	15.44	0.98	11.62	1.75	14.20	0.009
miR-431	14.88	0.58	11.07	2.53	14.07	0.013
miR-432	15.31	1.75	12.11	1.40	9.24	0.029
miR-483-3p	16.15	0.51	12.62	1.34	11.55	0.003
miR-483-5p	15.58	1.53	10.58	0.94	31.86	0.001
miR-485-3p	12.78	2.62	7.22	1.40	47.34	0.014
miR-493	13.58	0.60	10.87	0.93	6.52	0.000
miR-505	16.66	0.38	12.31	2.29	20.44	0.024
miR-519b-5p	16.92	0.13	14.93	1.12	3.99	0.031

*ΔCt = normalized Ct value

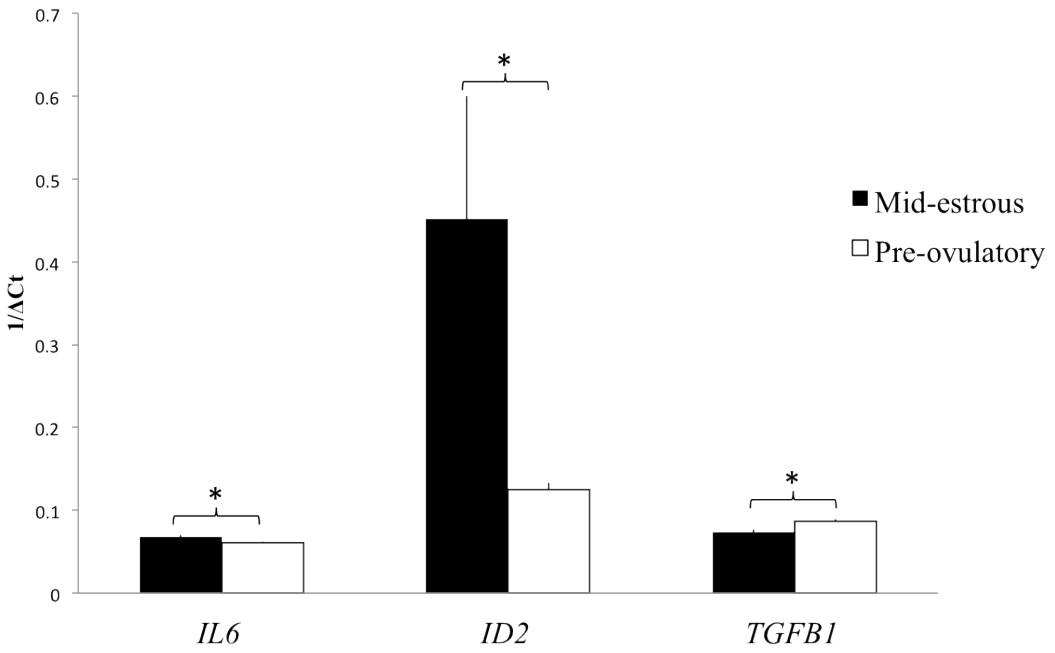


Figure III.1: TGFβ members during follicular development in equine granulosa cell samples. Values on y-axis indicate normalized (Δ) Ct values relative to geometric mean of *ACTB* and *GAPDH*. * = $P < 0.05$

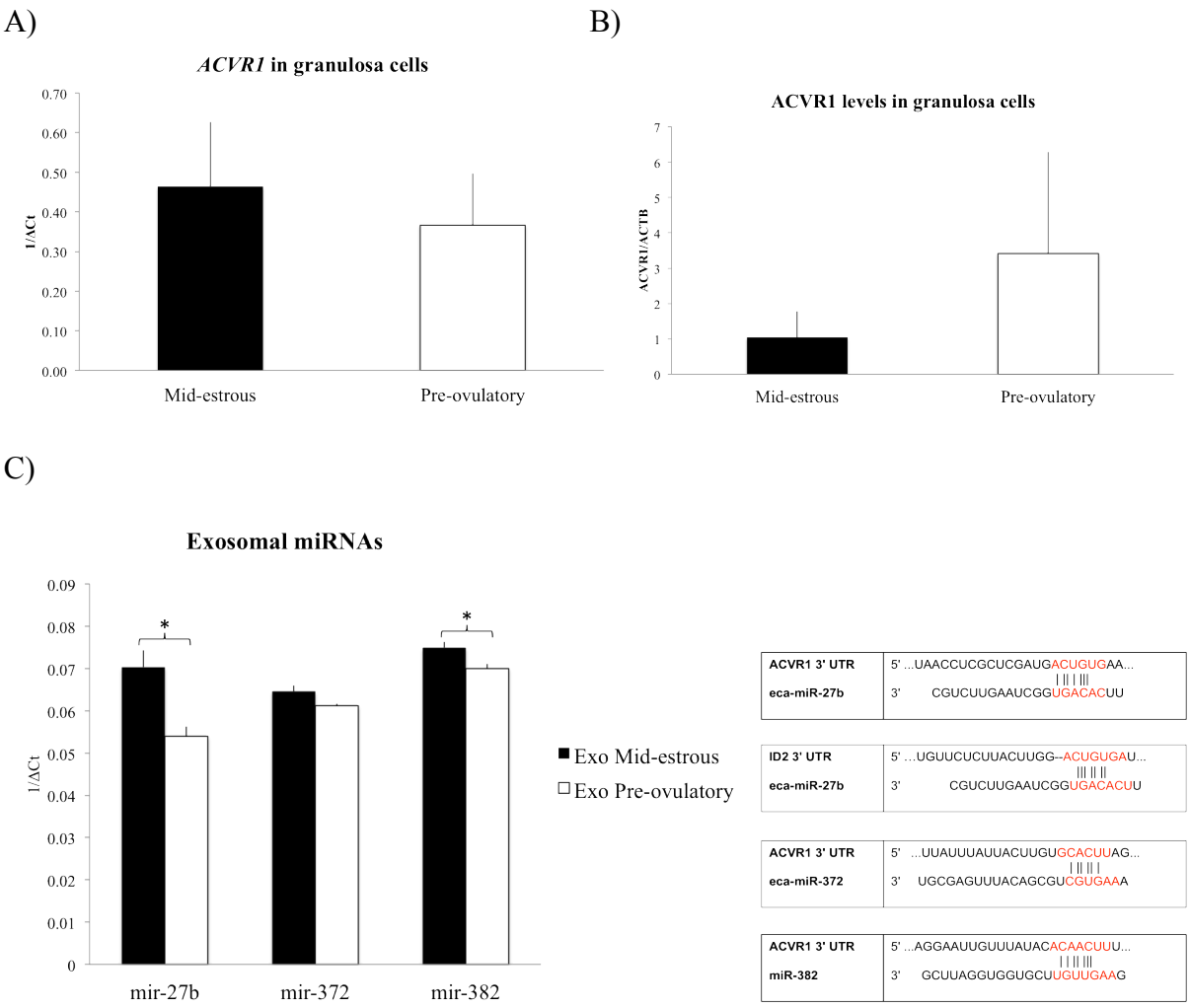


Figure III.2: ACVR1 mRNA (A) and protein (B) levels in granulosa cells isolated from mid-estrous and pre-ovulatory follicles. C) Relative levels of exosomal miRNAs predicted to regulate *ACVR1* and *ID2* isolated from follicular fluid. Values on y-axis indicate normalized (Δ) Ct values relative to geometric mean of *ACTB* and *GAPDH* or miR-99b and protein ratio. * = P < 0.05

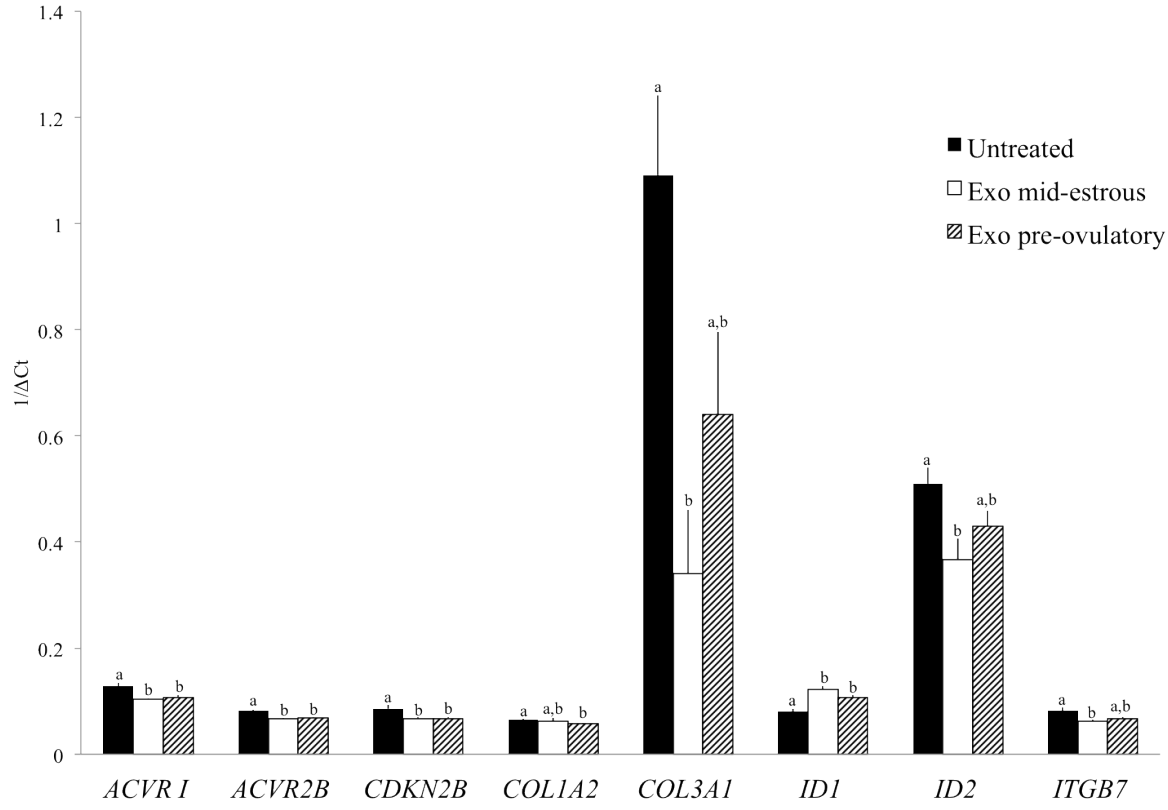


Figure III.3: Relative levels of TGFβ family members in granulosa cells following treatment with exosomes isolated from follicular fluid of mid-estrous and pre-ovulatory follicles. Values on y-axis indicate normalized (Δ) Ct values relative to geometric mean of *ACTB* and *GAPDH*.

Exo mid-estrous (exosomes isolated from follicular fluid of mid-estrous follicles)

Exo pre-ovulatory (exosomes isolated from follicular fluid of pre-ovulatory follicles)

Different letters $P<0.05$, similar letters $P>0.05$

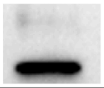
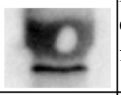
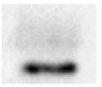
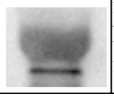
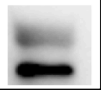
Replicate 1		Replicate 2		Replicate 3		Replicate 4	
Treatment	Normalized intensity	Treatment	Normalized intensity	Treatment	Normalized intensity	Treatment	Normalized intensity
Untreated	0.093 	Untreated	0.875 	Untreated	0.586 	Untreated	0.087 
Exosomes from FF of Pre-ovulatory follicles	1.570 	Exosomes from FF of Pre-ovulatory follicles	0.336 	Exosomes from FF of Pre-ovulatory follicles	0.677 	Exosomes from FF of Pre-ovulatory follicles	6.048 
Exosomes from FF of Mid-estrous follicles	3.870 	Exosomes from FF of Mid-estrous follicles	0.800 	Exosomes from FF of Mid-estrous follicles	0.884 	Exosomes from FF of Mid-estrous follicles	3.026 

Figure III.4: ACVR1 protein level in pre-ovulatory granulosa cells following follicular fluid exosome treatments.

- Granulosa cells isolated form pre-ovulatory follicles and treated with exosomes isolated from ovarian follicular fluid form pre-ovulatory or mid-estrous follicles

- Replicates are composed by granulosa cells from a unique pre-ovulatory follicle (n = 4) and exosomes isolated from follicular fluid of pre-ovulatory (n = 4) and mid-estrous follicles (n = 4)

- Upper band ACVR1 and lower band ACTB

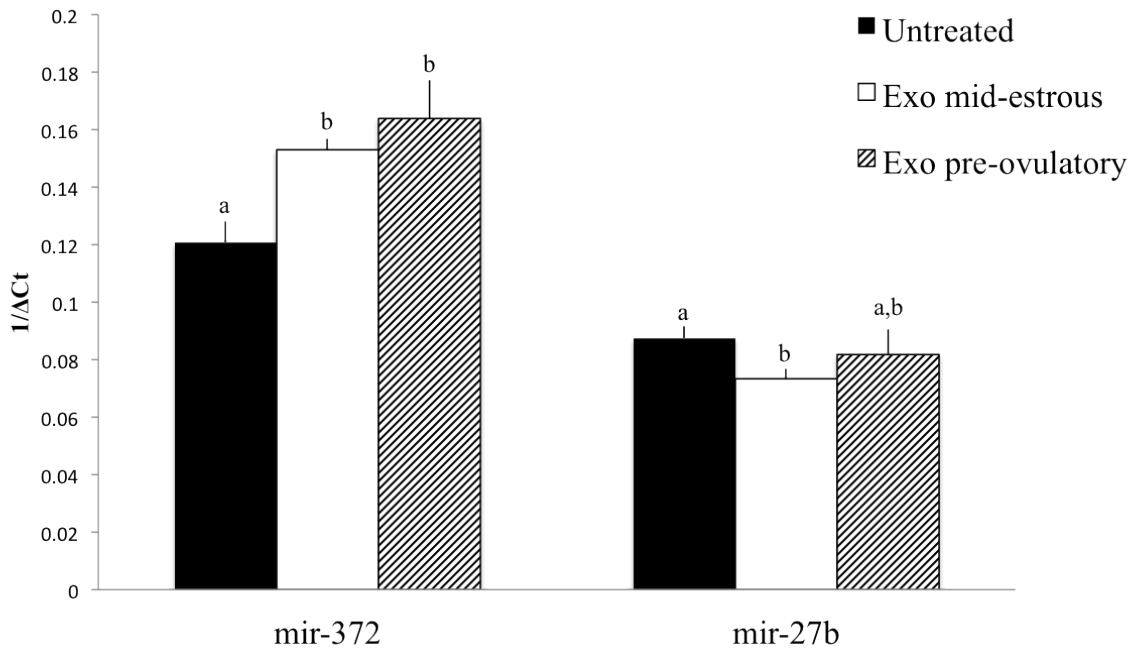


Figure III.5: MiRNA-27b and miR-372 levels in pre-ovulatory granulosa cells following exosome treatment. Values on y-axis indicate normalized (Δ) Ct values relative to miR-99b.

Exo mid-estrous = exosomes isolated from follicular fluid of mid-estrous follicles

Exo pre-ovulatory = exosomes isolated from follicular fluid of pre-ovulatory follicles

Different letters $P < 0.05$, similar letters $P > 0.05$.

A) B)

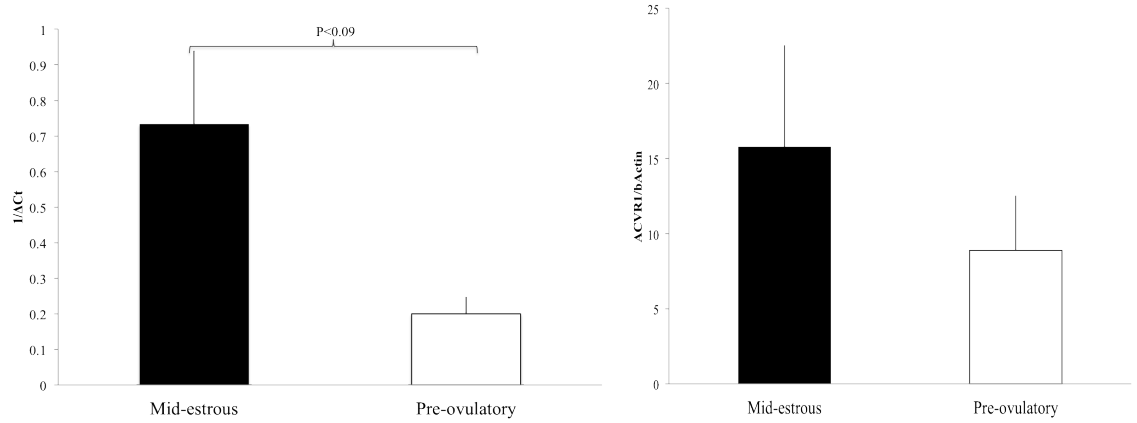


Figure III.6: ACVR1 mRNA and protein levels in exosomes. A) Relative levels of ACVR1 in exosomes isolated from mid-estrous and pre-ovulatory follicles, B) ACVR1 levels in exosomes isolated from mid-estrous and pre-ovulatory follicles. Values on y-axis indicate normalized (Δ) Ct values relative to geometric mean of *ACTB* and *GAPDH* or protein ratio.

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CHAPTER 4: AGE EFFECTS ON OVARIAN INTRAFOLLICULAR SIGNALING: EXOSOMAL MIRNAS AND FOLLICLE DEVELOPMENT[3]

Summary

Advanced maternal age in women and mares is associated with decline in fertility. This age-related decline in fertility is a consequence of low oocyte number and/or low oocyte competence resulting in difficulties to generate a pregnancy. This problem is exacerbated by postponement of pregnancy due to socioeconomic and cultural reasons in women. Another problem is the difficulty to determine when fertility begins to decline since it varies among individuals. Characteristics associated with the beginning of the decrease in fertility are high levels of FSH and irregular ovulations. High levels of pituitary hormones and irregular ovulation suggest that cell response to signaling within ovarian follicles must be affected by age. TGFβ signaling is a well-studied pathway involved in follicular development and ovulation. Recently, small non-coding RNAs (miRNAs) have been demonstrated to regulate several members of this pathway through repression or degradation. MiRNAs are produced by the cell, but also can be secreted inside vesicles such as exosomes. Exosomes are small vesicles (50-150 nm) that contain

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bioactive material, and deliver its contents to recipient cells. The overall goal of this study is to develop a better understanding on the effects of aging on mammalian ovarian follicular development. Specifically, we hypothesize that altered exosome content in old mares underlies changes in TGFβ signaling in granulosa cells during follicle development. To this end we identified exosomal miRNA content in follicular fluid from deviation, mid-estrous and pre-ovulatory follicles of young and old mares. Next, we performed real time PCR analysis of selected TGFβ family members in granulosa cells predicted to be targeted by exosomal miRNAs. Finally, we compared levels exosomal miRNAs and the predicted targeted TGFβ signaling member in granulosa cells collected from young and old mares. We observed 48 and 37 exosomal miRNAs that were altered in follicular fluid during follicle development from young and old mares, respectively. Analysis of selected TGFβ signaling members demonstrated significantly increased levels of *TGFb1* ($P < 0.01$) and *STAT1* ($P < 0.05$) in granulosa cells of pre-ovulatory follicles from young and old mares compared to mid-estrous. *ID2* was down-regulated ($P < 0.05$) at pre-ovulatory stages in granulosa cells from young and old mares compared to mid-estrous stages. Granulosa cells from old mares had significant ($P < 0.05$) altered levels of *ACVR1, BMPR2*, *MYCBP*, and *CDC25A*. Exosomal miRNA analysis identified miRNAs predicted to regulate selected TGFβ members, suggesting a role for exosomes in mediating cell-signaling within the ovarian follicle..

Keywords: equine ovarian follicle, aging, exosomes, miRNAs, and follicle development

Introduction

Over the past decades, demographic and socioeconomic factors have resulted in dramatic changes in human society, postponement of childbearing and a subsequent rise in mean female

age at first childbirth (Tatone *et al.* 2008). Reproductive problems (irregular ovulation and increase in FSH) predate ovarian oocyte depletion, and even the oocytes that survive and mature are of lower quality than those found in young women (Ellis and Wei 2010) (Tatone *et al.* 2008). One problem is to identify the beginning of the reproductive decline, which varies among women and includes low fertility and chromosomal disorders. Defects in oocytes from older women can lead to a decreased chance of fertilization and increased risk of miscarriage (Ellis and Wei 2010). The beginning of reproductive decline is associated with age, genetic factors, environmental changes and lifestyle, and therefore is considered to be a complex trait (Voorhuis *et al.* 2011). However, research with human subjects is limited, and consequently animal models have considerable utility (Carnevale 2008).

The mare is a domestic animal and presents similar reproductive characteristics as women. Irregular length of the cycle and increase in gonadotropins are present in the old mare as well as women approaching menopause (Carnevale 2008) (Butler and Santoro 2011). The initial sign of reproductive aging in mares and women is a shortening of the reproductive cycle with elevated concentrations of FSH (Carnevale 2008) (Butler and Santoro 2011). As reproductive aging advances, cycles become longer with irregular ovulations and elevated concentrations of FSH and LH (Carnevale 2008). In both species, reproductive life ceases with failure of follicular growth and elevated gonadotropins (Carnevale 2008) (Butler and Santoro 2011). The similarities in reproductive anatomy as well as the presence of a long follicular phase and single dominant follicle provide a foundation for studies using the mare as a model for oocyte and follicular development in women (Carnevale 2008).

Follicular development involves an intricate network of cellular signals as well as endocrine and paracrine signals (Buratini and Price 2011). Intrafollicular pathways involving the

TGFβ signaling family regulate recruitment, dominance, growth and ovulation of mammalian ovarian follicles (Edson *et al.* 2009). Knockout of specific TGFβ members such as SMAD2 increases reproductive lifespan in *Caenorhabditis elegans* (Ellis and Wei 2010; Luo *et al.* 2010). Knockout of TGFβ members in mice causes impaired fertility and embryonic mortality (Edson *et al.* 2009). The roles of TGFβ signaling members in female reproductive events are evolutionarily conserved, and thus it is important to understand the mechanisms involved in regulation of this signaling pathway. Recently, cell-secreted vesicles have been identified as mediators of intercellular communication (Raposo and Stoorvogel 2013). Cell-secreted vesicles called exosomes (50-150 nm) are within ovarian follicular fluid and are taken up by granulosa cells in vitro and in vivo (da Silveira *et al.* 2012). Exosomes contain miRNAs, which are small non-coding RNAs (Stoorvogel 2012) (Raposo and Stoorvogel 2013). Exosomal contents can be transferred from a donor cell to a recipient cell causing intracellular changes (Valadi *et al.* 2007). MiRNAs are involved in regulation of gene expression and function by complementary base-pairing interactions with the 3' untranslated region of mRNA targets, leading to mRNA cleavage or translational repression (Bartel 2009; Huntzinger and Izaurralde 2011).

The overall goal of this study is to develop a better understanding of the effects of aging in mammalian ovarian follicular development. Specifically, our hypothesis is that changes in exosomal content are associated with misregulation of TGFβ signaling during follicle development in aged mares. To test this hypothesis we identified exosomal miRNA contents during antral follicle development in young and old mares and performed real time PCR analysis of selected TGFβ family members predicted to be regulated by exosomal miRNAs in granulosa cells.

Material and Methods

Collection of ovarian follicular cells and fluid

Follicular fluid (~5 ml) and granulosa cells were aspirated from dominant follicles ~22 mm at deviation, ~35 mm at mid-estrus, and 30-34h after induction of follicular maturation for pre-ovulatory, from young (3-12 yr) and old (20-26 yr) estrous mares. All procedures were done in accordance with the Colorado State University Institutional Animal Care and Use Committee. Mares were housed on dry lots with water and hay *ad libitum*. Reproductive tracts of the mares were examined using transrectal ultrasound. Prostaglandin was administered on two consecutive days beginning on day 5 or 7 (1000 mg of Estrumate® MSD) following ovulation or aspiration, and dominant follicles were determined based on follicle growth and diameter. For pre-ovulatory follicle collections, follicular maturation was induced by administration of hCG and/or deslorelin (2500 IU and 1.5 mg, respectively, iv), and follicular contents (follicle $\geq$ 35 mm) were collected 30-34 h later by transvaginal, ultrasound-guided follicular aspirations. Follicular fluid was collected using a 12-GA needle, placed in an incubator at 37°C for 10-20 min, centrifuged at 300 x g for 10 min, 2000 x g for 10 min, 10000 x g for 30 min and later stored at -80°C until processed for exosome isolation. To remove red blood cells, granulosa cells from young mares were rinsed (3x) in PBS containing 0.02% polyvinyl alcohol (PVA), followed by snap freezing and stored at -80^{o}C until further use for total RNA isolation.

Isolation of exosomes from follicular fluid

Exosomes were isolated from ovarian follicular fluid starting with a series of differential centrifugation steps as described in (Thery *et al.* 2006). Exosomes were isolated using Exoquick™ (SBI System Biosciences, Inc), a polymer-based reagent that enriches preparations

with exosomes. Following centrifugation, 400 µl of follicular fluid supernatant was added to 100 µl of Exoquick. This preparation was incubated overnight at 4°C, and centrifuged at 1500 rpm for 30 min to obtain an exosome pellet. Exosome pellets were resuspended in 250 µl of PBS (pH 7.4) and used for miRNAs real time PCR analysis (n= 6 deviation, n = 6 mid-estrus, and n = 6 pre-ovulatory) young mares, and (n= 6 deviation, n = 6 mid-estrus, and n = 6 pre-ovulatory) old mares.

mRNA and miRNA isolation and reverse transcription

Total RNA including miRNA was isolated from exosome preparations using TRI Reagent®BD (Molecular Research Center, Inc.), according to the manufacturer's instructions. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Samples were stored at -80°C until further use.

Quantifiable reverse transcribed mRNA was generated using qScript™ cDNA Synthesis Kit (Quanta Biosciences #95047) according to the manufacture's instructions. Briefly, the reverse transcription reaction was done using approximately 30 ng of total RNA for each gene. RNA was incubated with 5x qScript Reaction Mix, qScript Reverse Transcriptase, and DNase-free water at 22°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min.

Quantifiable reverse transcribed miRNAs were generated using the miScript PCR System (Qiagen® #218193) according to the manufacturer's instructions. Briefly, the reverse transcription reaction was done using approximately 100 ng of total RNA for 384 miRNAs. Total RNA, including the small RNA fraction, was incubated with 5X miScript HiFlex Buffer, 10X miScript Nucleic Mix, RNase-free water, and miScript Reverse Transcriptase at 37°C for 60 min followed by 5 min at 95°C.

Real-time PCR expression analysis of mRNAs and miRNAs

Relative level of mRNAs (Table IV.1) was examined in granulosa cells recovered from ovarian follicles of young and old mares at deviation (n = 6) mid-estrus (n = 6) and pre-ovulation (n = 6). Each analysis was performed in 10 µl reactions containing 2X SYBR Green I master mix (Roche Applied Sciences), 0.5 µM of forward and reverse primers (sequences provided in Table IV.1), and 1 µl cDNA. The PCR cycle conditions were as followed: 95°C for 5 min, 45 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec, followed by melt curve analysis to confirm amplification of single cDNA products. To identify differences in mRNA levels in granulosa, raw Cp values were normalized to the geometric mean of two internal controls (*ACTB, GAPDH*), and statistical differences were assessed at $P < 0.05$ using a Student's t-test, comparing age groups or follicular stages.

The relative level of mature miRNAs was examined in exosome preparations isolated from the follicular fluid of different mares at deviation (young n = 6, old n = 6), mid-estrus (young n = 6, old n = 6) and pre-ovulatory (young n = 6, old n = 6), using Human miRNome Profiler plates (SBI). Each analysis was performed in 6 µl reactions containing 2X SYBR Green I master mix (Roche Applied Sciences), 10 µM Universal reverse primer (Qiagen) and miRNA specific forward primer, and 0.03 µl cDNA. Real-time PCR was conducted using the LightCycler480 PCR system (Roche Applied Sciences) in 384-well plates. The PCR cycle conditions were as followed: 95°C for 5 min, 45 cycles of 95°C for 10 sec, 55°C for 15 sec, and 72°C for 15 sec followed by a melt curve analysis to confirm amplification of single cDNA products. To identify differences in the presence of exosomal miRNAs isolated from follicular fluid, raw Cp values were normalized to miR-99b, and statistical differences were assessed at $P < 0.05$ using a Student's t-test, comparing age groups or follicular stages.

Results

Age effects on exosomal miRNAs during ovarian follicle development

In order to investigate the effects of aging on exosome contents during different stages of follicular development, we isolated exosomes from follicular fluid of antral follicles at deviation, mid-estrus and pre-ovulation of young and old mares. Based on previous findings suggesting that oocyte competence is altered due to age, we hypothesized that some of the age effects are mediated by changes in miRNA and mRNA levels in exosomes and granulosa cells, respectively (Carnevale 2008). The presence of different miRNAs and mRNA was examined using real-time PCR, and miRNAs and mRNAs were considered present if detected in at least three out of the six different biological samples, ($Cp < 37$) and single melt peaks. Of the 380 initial miRNAs, a total of 48 miRNAs were different ($P < 0.05$) among different follicular stages in exosomes isolated from follicular fluid of young mares (Table IV.2). Most of the differences were observed comparing exosomal miRNAs between pre-ovulatory and mid-estrous stages. A total of 37 miRNAs were different ($P < 0.05$) among different follicular stages in exosomes isolated from follicular fluid of old mares (Table IV.3). In old mares, most of the differences were observed comparing exosomal miRNAs between mid-estrous and deviation stages.

Predicted miRNA-regulated pathways

Since a single miRNA can target several mRNAs and the miRNAs we identified are packaged in exosomes, we choose to analyze the significantly different miRNAs as a unit in their ability to target signaling pathways. Bioinformatics analysis of 48 and 37 significantly up-regulated exosomal miRNAs from follicular fluid of the follicular stages of young and old mares, respectively, predicts regulation of different signaling pathways (DIANA Lab-DNA Intelligent

Analysis, http://diana.cslab.ece.ntua.gr/pathways/). Of the 48 exosomal miRNAs that changed during follicular development in young mares, a total of 8 miRNAs were significantly different in exosomes isolated from follicular fluid of deviation compared to mid-estrous follicles (Table IV.2). Together these 8 miRNAs are predicted to regulate 163 different signaling pathways (Supplemental Table IV.1). One exosomal miRNA, miR-485-3p, was differentially expressed between deviation and pre-ovulatory follicles from young mares (Table IV.2), and this miRNA was predicted to be involved in the regulation of 13 different signaling pathways (Supplemental Table IV.2). We also observed 39 miRNAs differentially expressed between exosomes isolated from follicular fluid at mid-estrous and pre-ovulatory follicles of young mares (Table IV.2). These miRNAs are predicted to regulate 182 different signaling pathways (Supplemental Table IV.3).

Of the 37 miRNAs differently expressed in exosomes from follicular fluid of old mares, 21 miRNAs were significantly different between deviation and mid-estrous follicles (Table IV.3). These 21 miRNAs together are predicted to be involved in the regulation of 181 different signaling pathways (Supplemental Table IV.4). Twelve exosomal miRNAs were differentially expressed between follicular fluid obtained from deviation and pre-ovulatory follicles (Table IV.3), together these miRNAs are predicted to regulate 163 different signaling pathways (Supplemental Table IV.5). Also four exosomal miRNAs were significantly different between mid-estrous and pre-ovulatory follicles (Table IV.3) and together they are predicted to regulate 114 different signaling pathways (Supplemental Table IV.6).

TGFβ signaling pathway was predicted by exosomal miRNAs from different follicular stages to be regulated based on target prediction analysis. We selected receptors (*ACVR, BMPR, TGFR*), SMAD target genes (*CDKN2B*, *CDC25A, ID1*, *ID2*, *IL6, TGFb1*) and SMADs

(SMAD3, 4, 7) as target genes to evaluate the affects of age and the stage of follicular development in young and old mares. Therefore, based on this information we selected members of the TGFβ signaling family as target candidates for analysis.

mRNA level of TGFβ signaling members during ovarian follicle development in young and old mares

Initially, 17 TGFβ signaling members including receptors (*ACVR1*, *ACVR2B*, *TGFR2*, *BMPR2*), TGFβ/Activin responsive genes (*COL1A2*, *CDKN2B*, *MYCBP*, *FOS*, *CDC25A*, *IL6*, *IGFBP3*, *ITGB7*, *TGFBI*, *TGFB1/1*), BMP-responsive genes (*ID1*, *ID2*), and transcription factor (*STAT1*) were selected, and real time PCR was used to investigate their relative level in equine granulosa cells. All selected genes were present in at least two of the follicular development groups (deviation, mid-estrous and/or pre-ovulatory stage) (Data not shown).

Analysis of granulosa cells from the ovarian follicles of young mares demonstrated significant different levels of *TGFb1, IL6, ID2* and *STAT1* during follicle development (Figure IV.1). *TGFb1* was not different between deviation and mid-estrous follicles, however was increased in pre-ovulatory compared to mid-estrous follicles ($P < 0.01$). *IL6* was not detected in granulosa cell samples from deviation follicles, but was present in mid-estrous and pre-ovulatory follicles, with lower levels in pre-ovulatory than mid-estrous follicles ($P < 0.03$). *ID2* presented dynamic changes in relative levels during follicular development. *ID2* level was increase at deviation ($P < 0.05$) and mid-estrus ($P < 0.04$) compared to granulosa cells from pre-ovulatory follicles. *STAT1* was not different at mid-estrus, but present at increased level in granulosa cells from pre-ovulatory stage compared to deviation ($P < 0.05$).

Analysis of granulosa cells obtained from ovarian follicles of old mares demonstrated significant changes in the levels of *ACVR1, BMPR2, TGFb1, ID2, MYCBP, STAT1,* and *CDC25A* during follicle development (Figure IV.2). *ACVR1* was present at higher level at deviation follicles compared to pre-ovulatory stage ($P < 0.01$). *BMPR2* level was increased in pre-ovulatory compared to mid-estrous granulosa cells ($P < 0.02$). *TGFb1* level increased in granulosa cells from pre-ovulatory compared to mid-estrous follicles ($P < 0.01$). *ID2* was increased in granulosa cells from deviation ($P < 0.01$) and mid-estrous ($P < 0.01$) follicles, compared to pre-ovulatory stage. *MYCBP* increased in granulosa cells from pre-ovulatory follicles compared to mid-estrous ($P < 0.01$). *STAT1* was increased in granulosa cells from pre-ovulatory stage compared to deviation ($P < 0.03$) and mid-estrous ($P < 0.01$). Finally, *CDC25A* levels were not different at deviation, but higher in granulosa cells from pre-ovulatory follicles compared to mid-estrous ($P < 0.01$). These results suggest that TGFβ signaling members including receptors and stimulated genes are affected differently by age during equine ovarian follicle development.

Age effects on expression of TGFβ signaling members at different stages of follicle development

We also compared expression levels of TGFβ signaling members at follicular stages between young and old mares. Initially, no differences were observed between TGFβ signaling members in granulosa cells from deviation follicles (Figure IV.3). On the other hand, significant differences were observed in relative levels of *TGFR2, ID1*, and *COL1A2* between age groups in granulosa cells from mid-estrous and pre-ovulatory stages (Figure IV.3). At mid-estrus, *COL1A2* was lower in granulosa cells from old mares compared to granulosa cells from young mares ($P <$

0.03). At the pre-ovulatory stage, *TGFR2* ($P < 0.05$) and *ID1* ($P < 0.01$) were lower in granulosa cells from old compared to young mares.

Expression of miRNAs predicted to regulated TGFβ signaling members and age effects

We used two different miRNAs databases Targetscan (http://www.targetscan.org/vert_61/) and miRTarbase (http://mirtarbase.mbc.nctu.edu.tw/index.html) to identify predicted and validated miRNAs involved in the regulation of *ACVR1, BMPR2, TGFB1* and *CDC25A*. BMP receptor *ACVR1* was significantly decreased in granulosa cells from pre-ovulatory stages compared to follicles at deviation in old mares ($P < 0.01$) whereas no significant difference was observed in granulosa cells from young mares (Figure IV.4A). Two predicted miRNAs targeting *ACVR1* are miR-27a and miR-346, and both were significantly different in exosomes isolated from follicular fluid of young and old mares ($P < 0.05$). A validated miRNA targeting *ACVR1* is miR-197, which was significantly different in exosomes isolated from follicular fluid of young mares ($P < 0.05$). Exosomal miR-27a was significantly up-regulated in exosomes from pre-ovulatory ($P < 0.04$) follicles in young mares and in exosomes collected at deviation ($P < 0.004$) in old mares compared to mid-estrous follicles, respectively (Figure IV.4B). Exosomal miR-346 was up-regulated in exosomes from pre-ovulatory ($P < 0.01$) follicles in young mares. In exosomes isolated from follicles of old mares miR-346 was down-regulated at mid-estrous ($P < 0.02$) and pre-ovulatory ($P < 0.05$) follicles compared to deviation (Figure IV.4B). Exosomal miR-197 present a similar pattern of expression, with increased levels at pre-ovulatory compared to mid-estrous follicles in young mares ($P < 0.02$) no significant difference was observed in exosomes from old mares (Figure IV.4B).

Another BMP receptor, *BMPR2* was up-regulated in granulosa cells from pre-ovulatory compared to mid-estrous follicles in old mares ($P < 0.01$) (Figure IV.5A). Bioinformatics analysis demonstrated miR-93, miR-99a, miR-181a, miR-181b, miR-181d and miR-494 as predicted miRNAs targeting *BMPR2*. Exosomal miR-93 ($P < 0.03$) and miR-99a ($P< 0.01$) were up-regulated at pre-ovulatory compared to mid-estrous stage in follicular fluid of young mares (Figure IV.6B), but no significant differences were observed in exosomes from follicular fluid of old mares. Exosomal miR-181a, miR-181b and miR-181d were significant different ($P < 0.05$) in exosomes isolated from follicular fluid of young and old mares, respectively. Exosomal miR-181a was significantly down regulated at mid-estrous compared to deviation ($P < 0.04$) and up-regulated at pre-ovulatory compared to mid-estrous ($P < 0.04$) follicular stages of young mares (Figure IV.5B). Exosomal miR-181b was significantly up-regulated at deviation compared to mid-estrous ($P < 0.05$) and pre-ovulatory ($P < 0.03$) follicular stages of old mares (Figure IV.5B). Exosomal miR-181d was significantly up-regulated at deviation compared to mid-estrous follicular stage of old mares ($P < 0.01$) (Figure IV.5B). Exosomal miR-494 a validated regulator of *BMPR2* was down-regulated at pre-ovulatory compared to exosomes collected at deviation ($P < 0.05$), however no significant differences were observed in exosomes from follicular fluid of young mares (Figure IV.6B).

TGFB1 a signaling molecule of the TGFβ signaling family was up-regulated in granulosa cells at pre-ovulatory compared to mid-estrous follicular stage in young ($P < 0.01$) and old mares ($P< 0.01$) (Figure IV.7A). Exosomal miR-24 is a validated regulator of TGFb1 and was up-regulated in pre-ovulatory compared to mid-estrous follicular fluid exosomes from young mares ($P < 0.05$) but no significant difference was observed in old mares (Figure IV.7B).

TGFβ regulated gene *CDC25A*, was up-regulated in granulosa cells at the pre-ovulatory stages compared to mid-estrous stages in old mares ($P < 0.01$) (Figure IV.8A). Bioinformatics analysis indicated let-7b, miR-196b and miR-339-5p as predicted miRNAs involved in the regulation of *CDC25A*. Exosomal let7b, miR-196b and miR-339-5p were significantly different in follicular fluid of young mares. Exosomal let-7b a validated miRNA regulating CDC25A was up-regulated in pre-ovulatory compared to mid-estrous follicular stage ($P < 0.05$) no significant differences were observed in follicular fluid exosomal let-7b content of old mares (Figure IV.8B). Exosomal miR-196b was up-regulated at deviation compared to mid-estrous in follicular fluid of young mares ($P < 0.02$) and no significant difference was observed in old mares (Figure IV.8B). Exosomal miR-339-5p was up-regulated at deviation ($P < 0.03$) and pre-ovulatory ($P < 0.01$) compared to mid-estrous follicular stages in young mares (Figure IV.8B) and no significant differences were observed in old mares.

Discussion

This study evaluated changes in exosomal miRNAs and TGFβ signaling members during deviation, mid-estrous and pre-ovulatory stages of ovarian follicular development in young and old mares. Our goal was to demonstrate changes in exosomal miRNAs that correlated with observed changes in TGFβ members from granulosa cells of young and old mares. Initially we identified 48 exosomal miRNAs significantly different during the different stages of follicular development in young mares. We also identified 37 exosomal miRNAs significantly different during the different stages of follicular development in old mares. Bioinformatics analysis suggested their involvement in regulation of several signaling pathways. Among the different pathways, TGFβ signaling pathway was a predicted target for exosomal miRNAs originated

from different follicular stages. We selected 17 members of the TGFβ family and analyzed their relative levels during ovarian follicular development. We identified four TGFβ members that had significant altered expression in granulosa cells during follicular development in young mares (*TGFb1*, *IL6*, *ID2* and *STAT1*). Similarly we identified seven TGFβ members that exhibited significant different changes in granulosa cells during follicular development in old mares (*ACVR1*, *BMPR2*, *TGFb1*, *ID2*, *cMYC*, *STAT1* and *CDC25A*). When comparing relative levels in granulosa cells between young and old mares, three genes *TGFR2*, *ID1* and *COL1A2* exhibit significant changes, with *TGFR2* and *ID1* levels higher in granulosa cells from young mares at pre-ovulation, and *COL1A2* levels higher at mid-estrus.

Gonadotropin hormones are altered in older mares, therefore we used real time PCR analysis to evaluate the relative levels of exosomal miRNAs possibly regulated by gonadotropins (FSH and LH) in young and old mares. Analysis of exosomal miRNAs originated from follicular fluid revealed 8 significant different miRNAs in young mares and 21 significant different miRNAs in old mares at deviation (FSH dominant phase) compared to mid-estrus (Table IV.2 and 3). Among exosomal miRNAs from young mares, miR-23a was increased at deviation compared to mid-estrous follicles but not significantly different ($P < 0.06$). Exosomal miR-23a was significantly higher at deviation compared to mid-estrous ($P < 0.02$) and pre-ovulatory ($P < 0.01$) follicles in old mares. MiRNA-23a also is high in plasma of women with premature ovarian failure, who contain high circulatory levels of FSH. MiRNA-23a was up-regulated in rat granulosa cells 12 h after FSH treatment, further demonstrating miR-23a regulation by FSH (Yao *et al.* 2010). Transfection of miR-23a in granulosa cells leads to down-regulation of XIAP (mRNA and protein) and an increase in cleaved caspase-3 protein level, suggesting its involvement in regulating apoptosis in human ovarian granulosa cells (Yang *et al.* 2012).

Exosomal miR-23a was 2.7 fold higher in exosomes from follicular fluid of old mares compared to young mares at the pre-ovulatory stage (da Silveira *et al.* 2012), and thus suggests impaired regulation of apoptosis in ovarian follicles of old mares.

Analysis of exosomal miRNAs at the pre-ovulatory stage (30-34 h after induction of follicular maturation) revealed 39 and 4 miRNAs significantly different in young and old, respectively compared to mid-estrous (Table IV.2 and IV.3). Among exosomal miRNAs significantly different in follicular fluid of young mares, miR-181a was up-regulated at pre-ovulatory compared to mid-estrous and deviation stages. Exosomal miR-181a was up-regulated 15 fold in follicular fluid from pre-ovulatory follicles (mature), compared to mid-estrous follicles (immature) (da Silveira, submitted for publication). In a different study, miR-181a was 3.4 fold higher in exosomes isolated from follicular fluid of pre-ovulatory follicles from old mares compared to young mares (da Silveira *et al.* 2012). In the present study miR-181a was not different among follicular stages in old mares (data not shown). An increase in miR-181a in mouse granulosa cells leads to suppression of ACVR2A induced Smad2 phosphorylation and a block in granulosa cell proliferation (Zhang *et al.* 2012). Transfection of miR-181a in human granulosa cells leads to a decrease in PCNA, a known regulator of cell proliferation (Sirotkin *et al.* 2010). During the final stage of follicular development (high LH), granulosa cells are not proliferating (Clement and Monniaux 2012), suggesting that miR-181a loaded exosomes are part of a complex network regulating granulosa cell proliferation during final follicle maturation. MiR-181b and miR-181d were significantly different in exosomes isolated from old mares at deviation. A common target for the miR-181 family is BMPR2, which is differently expressed in granulosa cells from old mares and the relative levels are constant in granulosa cells of young mares. The different levels of the miR-181 family members in exosomal miRNAs from follicular

fluid of young and old mares, suggests a important role for this miRNA during follicle development. The different levels of expression observed in exosomes isolated from follicular fluid of old mares in our previous (da Silveira *et al.* 2012) and present work, suggests the involvement of miR-181 in follicular health and consequently oocyte quality. These data indicate an important role for exosomes containing miRNAs regulators of important signaling pathways such as TGFβ during follicle development.

Ovarian aging and ultimately the end of reproductive function are the consequence of multifactorial events related to physiology, genetics and environment (Miao *et al.* 2009) (Fair 2010) (Tatone *et al.* 2008). Genetic analysis suggests that TGFβ signaling plays an important role mediating ovarian aging and oocyte quality, demonstrated by mutations within TGFβ members (Fair 2010) (Voorhuis *et al.* 2011) (Luo *et al.* 2010) (Ellis and Wei 2010). For example, mutations in AMHR2 are associated with an earlier onset of menopause in women (Voorhuis *et al.* 2011). Mutations in Sma-2 (SMAD2) are associated with increased reproductive lifespan in *C. elagans*. In equine granulosa cells, initial analysis demonstrated significant differences of four members in young mares (*TGFb1*, *IL6*, *ID2*, *STAT1*) and seven members in old mares (*ACVR1*, *BMPR2*, *TGFb1*, *ID2*, *MYCBP*, *STAT1*, *CDC25A*). Interestingly, *TGFb1*, *STAT1* and *ID2* levels were altered after induction of follicular maturation (Figures IV.2 and 3). Comparison of the relative levels of TGFβ signaling members between young and old mares across the different follicle stages revealed three genes that were significantly altered (*TGFR2*, *ID1*, *COL1A2*) (Figure IV.3). TGFR2, ID1 and COL1A2 are involved in cell proliferation and regulation of the cell cycle, thus the decreased levels of these genes in old mares indicates potential de-regulation of TGFβ signaling as an underlying factor in age-related decline in oocyte quality Since we know that some old mares are capable of produce a competent oocyte similar to old women,

post-transcriptional regulation events could be involved in restoring protein levels to normal. It is important to mention that in this study oocyte quality or competence itself was not evaluated, and that some of the findings can be related to rescue of follicular function.

Finally, we also investigated the relative levels of exosomal miRNAs in follicular fluid and the levels of their predicted mRNA targets in granulosa cells. This is the first study to address the relationship of exosomal miRNA in follicular fluid and gene expression of miRNA-targets in granulosa cells as a mechanism involved in regulation of follicular development. Analysis of relative levels of exosomal miRNAs revealed that miR-27a, miR-346 and miR-197 (target *ACVR1*); miR-93, miR-99a, miR-181a, miR-181b, miR-181d and miR-494 (target *BMPR2*), miR-24 (target *TGFβ1*) and let-7b, miR-196d and miR-339a-5p (target *CDC25A*) are present in exosomes isolated from follicular fluid of young and old mares. Further validation of miRNA and mRNA target interaction is necessary in order to confirm involvement of exosomes communication between follicular cells. Our data suggest that exosomal miRNAs could be involved in the regulation of selected TGFβ signaling targets during follicle development.

Two questions not answered in this study are: 1) Are exosomal miRNAs present in follicular fluid the result of uncontrolled exocytosis and involved in abnormal follicular maturation in old mares? 2) Are exosomal miRNAs part of intercellular communication process responsible for impaired follicular maturation? Studies related to oocyte quality and competence needs to be conducted in order to answer these questions and possibly create therapeutic treatments or biomarkers for oocyte quality. Recently exosomes have been used as drug delivery agents utilized to deliver siRNA to specific neuronal populations as a new mechanism of treatment for Parkinson's disease able to cross the blood-brain-barrier (El-Andaloussi *et al.* 2012) (Lai *et al.* 2012). Another feature of exosomes is their utility as biomarker for cell function

(Simpson *et al.* 2009) (Raposo and Stoorvogel 2013). Therefore, since the discovery of cell-secreted vesicles in follicular fluid, it is important to understand the role of these vesicles during follicular development and disease in order to create new therapeutic approaches and diagnostic markers for treatment and diagnostic of infertility.

In conclusion, in the current study we use the mare as a model to study age-related changes in ovarian function. Initially we identified differences in relative levels of exosomal miRNA at deviation, mid-estrous and pre-ovulatory follicles in the mare. Bioinformatics analysis identified TGFβ signaling pathway as a regulated pathway by exosomal miRNAs originated from different follicular stages in young and old mares. Analysis of TGFβ members verified similar changes in *TGFb1*, *ID2* and *STAT1* during follicular development in young and old mares. However, other members of this pathway were altered in granulosa cells from old mares (*ACVR1*, *BMPR2*, *cMyc*, *CDC25A*). We verified the presence of miRNAs predicted to regulate altered TGFβ signaling members.

Table IV.1: Primers utilized for real-time PCR analysis.

Gene	Primer Forward: 5'-3'	Primer Reverse: 5'-3	Annealing Temperature °C	Fragment Size (bp)
ACVR1	CCTCTCCTGTGGGAATGAGG	CTGGAAGCAGCCTTTCTGGT	60	100
ACVR2B	ATGTACCGGCATCGAAAACC	CGAGCCTTGATCTCCAACAG	60	119
TGFBR2	GACCCCAAGCTCACCTACCA	TGCACTCATCAGAGCTACAGGA	60	124
BMPR2	TTGCTTTGGCATCAGTCTCTG	CTGCTGCCTCCATCATGTTC	60	120
CDC25A	CTGGGCCCTTGGACAGTAAA	GTCGGAATGGCTCCTCTTCA	60	110
CDKN2B	CCGAGCTGCTACTGCTCCAC	CACCAGCGTGTCCAGGAAG	60	107
COL1A2	AGGTTTCCAAGGACCTGCTG	GGTTTTCCAGGGTGACCATC	60	117
USF2	GGATACCACGGCTGTGTCAG	ATCGTCCTCTGCGTTCCTGT	60	114
IGFBP3	CAAGCGCGAGACAGAATACG	GAAGCCCTTCTTGTCGCAGT	60	124
IL6	GGCAGAAAAAGACGGATGCT	CACCCTTGAACTCGTTCTGGA	60	122
ITGB7	TGCCGAAGGATACCCTGTAGA	CTGCAGCTTCTCCAGCAAGG	60	112
MYCBP	GCCTTATATGAAGAACCAGAGAAACC	GCCAATTCTAGGCGAAGCAG	60	113
TGFB1	CTCAGTGCCCACTGCTCCT	CATCAATGGTGGCCAGATCA	60	100
TGFBI	ATGGTGAACAGACGGGTCCT	CCGTTGGGATAGTGGTGGAT	60	101
ID1	ACATGAACGGCTGCTACTCG	TCCAACTCCAGGTCCCAGAT	60	124
ID2	CATCCCCCAGAACAAGAAGG	TGGTGATGCAGGCTGACAAT	60	180
STAT1	GTTTCCCCATGGAAATCAGG	GAAGGTCGTGGAAACGGATG	60	108
ACTB	CGACATCCGTAAGGACCTGT	CAGGGCTGTGATCTCCTTCT	60	99
GAPDH	AGAAGGAGAAAGGCCCTCAG	GGAAACTGTGGAGGTCAGGA	60	87

Table IV.2: Exosomal miRNAs significantly different during follicle development in young mares.

	Deviation (1/ΔCt)	STDEV	Mid-Estrus (1/ΔCt)	STDEV	Pre-ovulatory (1/ΔCt)	STDEV	T-test Dev/Mid (P-value)	T-test Dev/Pre (P-value)	T-test Mid/Pre (P-value)
let-7b	0.0797	0.0260	0.0596	0.0024	0.0714	0.0092	0.175	0.572	**0.047**
miR-24	0.0920	0.0369	0.0694	0.0112	0.1021	0.0230	0.276	0.645	**0.043**
miR-25	0.0889	0.0324	0.0630	0.0048	0.0860	0.0116	0.114	0.869	**0.005**
miR-27a	0.0773	0.0200	0.0671	0.0068	0.0891	0.0144	0.361	0.342	**0.033**
miR-30c	0.0806	0.0295	0.0636	0.0029	0.0776	0.0106	0.293	0.852	**0.043**
miR-93	0.0771	0.0190	0.0604	0.0035	0.0813	0.0106	0.188	0.702	**0.024**
miR-99a	0.0810	0.0354	0.0589	0.0019	0.0779	0.0076	0.254	0.889	**0.004**
miR-103-as	0.0779	0.0054	0.0583	0.0042	0.0678	0.0056	**0.024**	0.088	0.141
miR-124	0.0838	0.0098	0.0699	0.0096	0.0811	0.0080	**0.033**	0.631	0.069
miR-125a-3p	0.0931	0.0264	0.0667	0.0039	0.0954	0.0145	0.055	0.882	**0.004**
miR-139-3p	0.0803	0.0242	0.0619	0.0017	0.0815	0.0125	0.244	0.926	**0.046**
miR-151-3p	0.0746	0.0186	0.0607	0.0041	0.0738	0.0065	0.193	0.938	**0.014**
miR-181a	0.0832	0.0202	0.0627	0.0034	0.0906	0.0285	**0.034**	0.627	**0.040**
miR-185	0.0709	0.0117	0.0618	0.0018	0.0720	0.0023	0.355	0.889	**0.014**
miR-188-3p	0.0935	0.0330	0.0629	0.0041	0.0796	0.0103	0.073	0.448	**0.012**
miR-188-5p	0.1160	0.0386	0.0847	0.0260	0.1537	0.0566	0.129	0.223	**0.025**
miR-196b	0.0796	0.0059	0.0597	0.0070	0.0688	0.0070	**0.020**	0.086	0.148
miR-197	0.0843	0.0184	0.0662	0.0103	0.0948	0.0133	0.115	0.357	**0.014**
miR-202	0.0963	0.0302	0.0726	0.0123	0.0948	0.0145	0.136	0.928	**0.042**
miR-205	0.0783	0.0181	0.0623	0.0056	0.0873	0.0183	0.141	0.510	**0.039**
miR-206	0.0670	0.0112	0.0662	0.0025	0.0774	0.0062	0.886	0.133	**0.015**
miR-210	0.0879	0.0202	0.0703	0.0090	0.1039	0.0108	0.106	0.190	**0.001**
miR-211	0.0685	0.0084	0.0617	0.0065	0.0736	0.0089	0.177	0.354	**0.043**
miR-222	0.0716	0.0127	0.0627	0.0024	0.0760	0.0081	0.286	0.561	**0.043**
miR-320b	0.0876	0.0305	0.0637	0.0036	0.0917	0.0157	0.118	0.812	**0.006**
miR-320c	0.0864	0.0241	0.0668	0.0071	0.0929	0.0111	0.085	0.629	**0.002**
miR-323-5p	0.1793	0.0933	0.0871	0.0179	0.1368	0.0634	**0.039**	0.377	0.094
miR-324-5p	0.0867	0.0389	0.0726	0.0092	0.1178	0.0308	0.506	0.218	**0.031**
miR-328	0.0774	0.0193	0.0673	0.0032	0.0825	0.0103	0.339	0.611	**0.027**
miR-339-5p	0.0807	0.0165	0.0623	0.0035	0.0776	0.0086	**0.026**	0.751	**0.004**
miR-345	0.0818	0.0189	0.0689	0.0048	0.0900	0.0091	0.228	0.446	**0.006**
miR-346	0.0897	0.0242	0.0692	0.0070	0.0955	0.0102	0.074	0.667	**0.001**
miR-377	0.0294	0.0841	0.1198	0.0553	0.0212	0.0953	**0.046**	0.909	0.091
miR-422a	0.0750	0.0145	0.0627	0.0049	0.0786	0.0117	0.146	0.670	**0.040**
miR-423-5p	0.0825	0.0226	0.0650	0.0043	0.0875	0.0121	0.172	0.701	**0.013**
miR-431	0.0972	0.0337	0.0673	0.0026	0.0940	0.0215	0.081	0.871	**0.027**
miR-432	0.0742	0.0166	0.0660	0.0083	0.0834	0.0094	0.394	0.347	**0.032**
miR-483-3p	0.0801	0.0208	0.0620	0.0020	0.0799	0.0077	0.130	0.982	**0.004**
miR-483-5p	0.0839	0.0204	0.0647	0.0062	0.0950	0.0081	0.080	0.342	**0.000**
miR-485-3p	0.0930	0.0306	0.0803	0.0150	0.1425	0.0268	0.528	**0.030**	**0.016**
miR-490-3p	0.0683	0.0116	0.0584	0.0045	0.0700	0.0083	0.147	0.786	**0.042**
miR-493	0.1007	0.0342	0.0738	0.0032	0.0925	0.0075	0.084	0.653	**0.001**
miR-508-3p	0.0667	0.0025	0.0600	0.0033	0.0633	0.0018	**0.033**	0.205	0.270
miR-519b-5p	0.0666	0.0100	0.0591	0.0005	0.0673	0.0050	0.263	0.900	**0.041**
miR-541	0.0637	0.0043	0.0620	0.0029	0.0671	0.0013	0.593	0.249	**0.050**
Normalizer									
miR-99b	19.40	0.73	19.53	0.63	19.73	0.5382			

Table IV.3: Exosomal miRNAs significantly different during follicle development in old mares.

	Deviation (1/ΔCt)	STDEV	Mid-Estrus (1/ΔCt)	STDEV	Pre-ovulatory (1/ΔCt)	STDEV	T-test Dev/Mid (P-value)	T-test Dev/Pre (P-value)	T-test Mid/Pre (P-value)
miR-23a	0.091	0.003	0.077	0.007	0.077	0.004	**0.020**	**0.007**	0.959
miR-23b	0.078	0.003	0.064	0.008	0.066	0.004	**0.030**	**0.012**	0.734
miR-27a	0.096	0.003	0.076	0.012	0.074	0.016	**0.040**	0.081	0.854
miR-139-3p	0.071	0.004	0.073	0.007	0.061	0.005	0.683	**0.042**	**0.047**
miR-146b-3p	0.071	0.004	0.085	0.013	0.070	0.009	**0.034**	0.738	0.099
miR-149	0.261	0.011	0.135	0.071	0.160	0.083	**0.025**	0.105	0.673
miR-181b	0.080	0.004	0.071	0.005	0.069	0.005	**0.045**	**0.027**	0.620
miR-181d	0.074	0.003	0.066	0.009	0.062	0.006	0.199	**0.020**	0.380
miR-192	0.121	0.006	0.085	0.012	0.086	0.028	**0.003**	0.102	0.919
miR-211	0.088	0.001	0.070	0.010	0.070	0.015	**0.023**	0.112	0.956
miR-220c	0.059	0.001	0.067	0.004	ND	-	**0.017**	-	-
miR-221	0.075	0.005	0.063	0.003	ND	-	**0.025**	-	-
miR-222	0.069	0.004	0.065	0.005	0.059	0.003	0.228	**0.022**	0.123
miR-302d	0.063	0.004	0.071	0.008	0.063	0.003	**0.049**	0.722	**0.049**
miR-323-5p	0.172	0.012	0.124	0.041	0.105	0.040	0.091	**0.041**	0.487
miR-345	0.125	0.003	0.089	0.020	0.085	0.032	**0.023**	0.089	0.821
miR-346	0.117	0.002	0.085	0.017	0.079	0.024	**0.017**	**0.042**	0.670
miR-383	0.070	0.003	0.063	0.001	0.061	0.006	**0.018**	0.080	0.685
miR-409-3p	0.197	0.015	0.119	0.047	0.131	0.060	**0.034**	0.137	0.760
miR-429	0.057	0.002	0.063	0.001	ND	-	**0.002**	-	-
miR-431	0.079	0.021	0.075	0.005	0.065	0.002	0.807	0.321	**0.022**
miR-448	0.068	0.003	0.066	0.007	0.062	0.004	0.685	0.083	0.265
miR-449b	0.091	0.002	0.074	0.012	0.069	0.012	**0.047**	**0.035**	0.576
miR-455-5p	0.075	0.004	0.079	0.009	0.069	0.005	0.442	**0.039**	**0.045**
miR-494	0.216	0.051	0.199	0.054	0.154	0.040	0.584	**0.041**	0.135
miR-496	0.096	0.005	0.071	0.012	0.075	0.017	**0.015**	0.116	0.703
miR-501-5p	0.060	0.004	0.068	0.000	ND	**-**	**0.013**	-	-
miR-505	0.093	0.002	0.068	0.011	0.068	0.014	**0.008**	**0.032**	0.977
miR-510	0.083	0.006	0.064	0.008	ND	-	**0.013**	-	-
Normalizer									
miR-99b	19.85	1.40	19.62	0.86	19.72	1.11	0.739	0.861	0.867

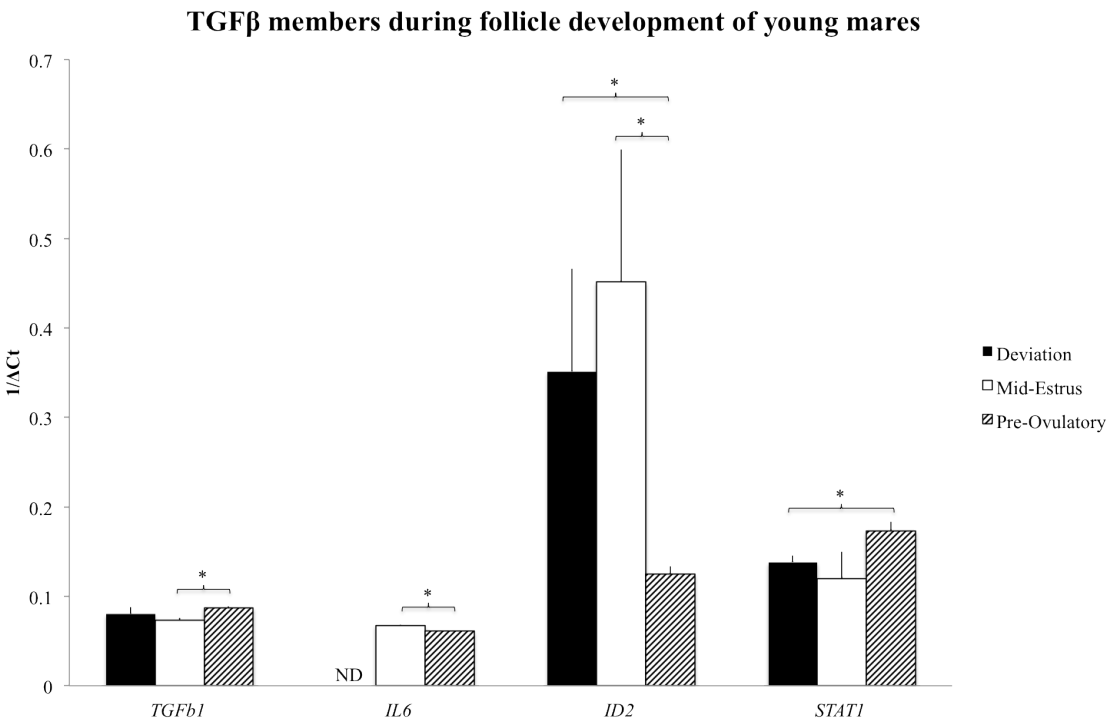


Figure IV.1: Expression levels of TGFβ signaling members in the granulosa cells of young mares. Significant differences are represented by *P<0.05.

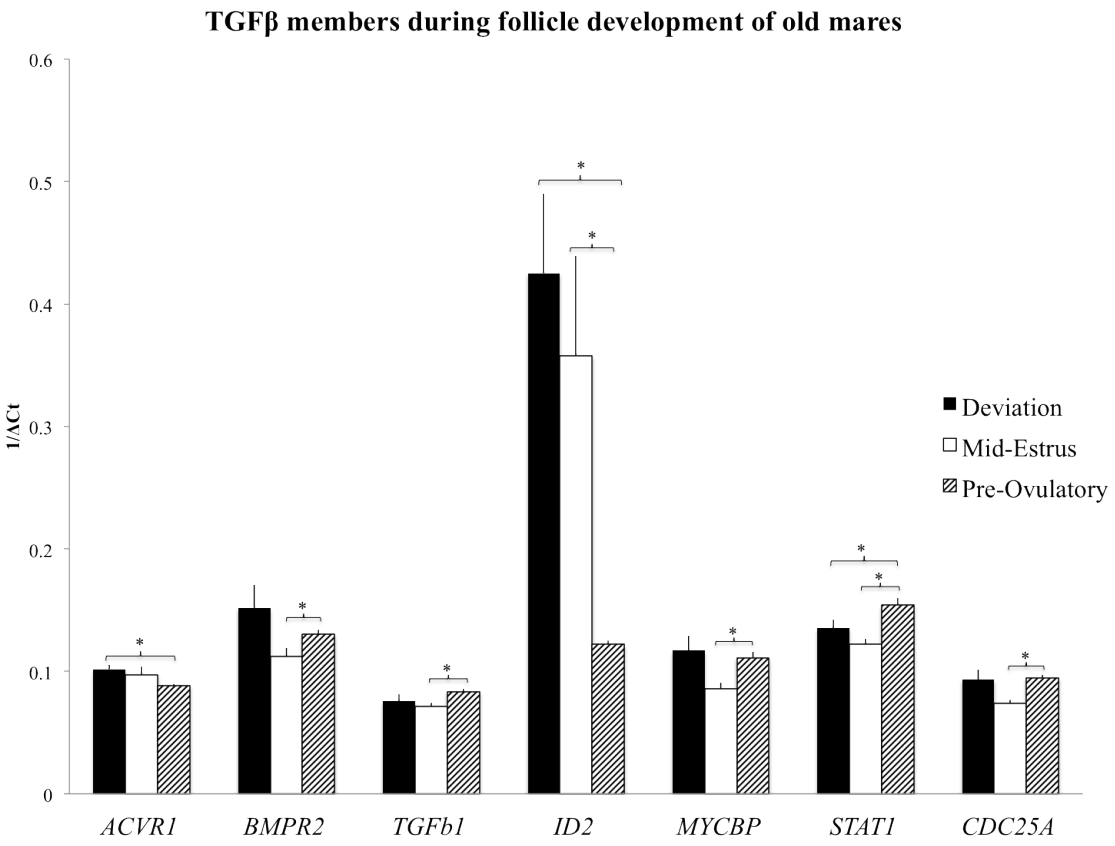


Figure IV.2: Expression levels of TGFβ signaling members in granulosa cells of old mares. Significant differences are represented by *P<0.05.

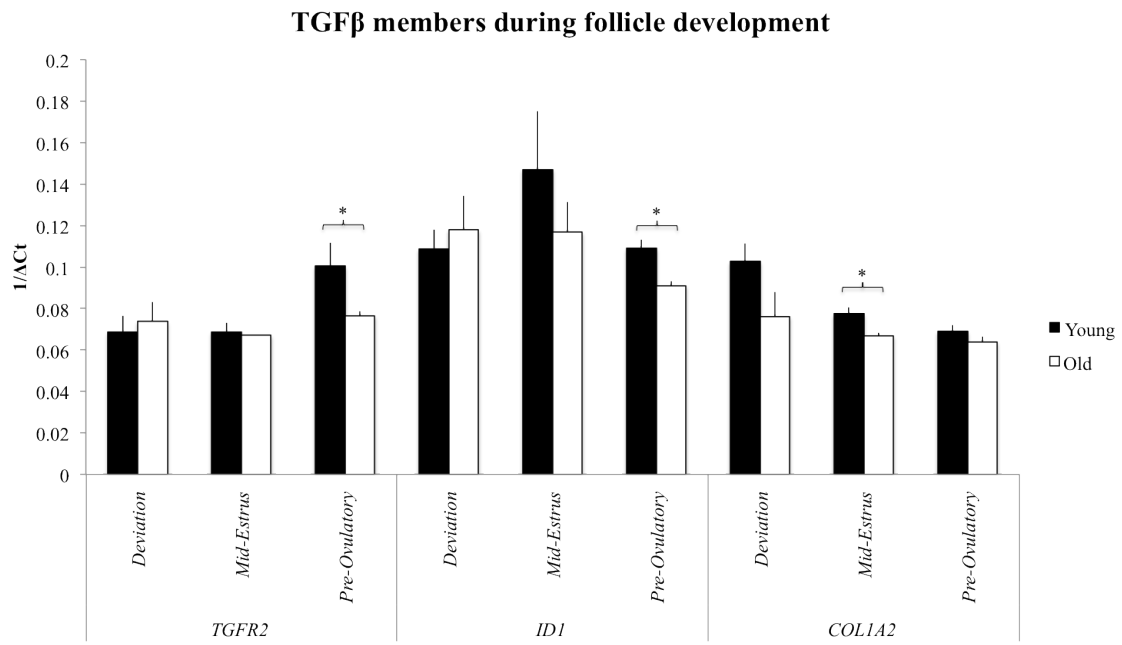
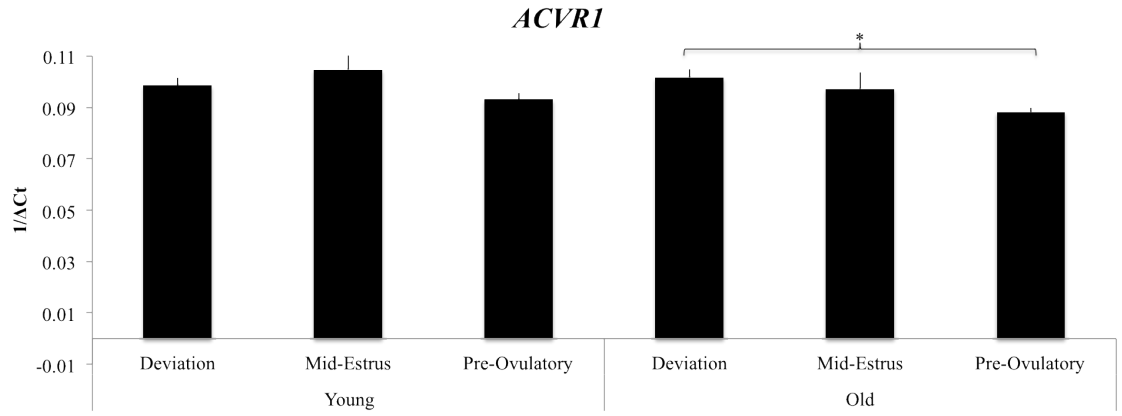


Figure IV.3: Comparison of the expression levels of TGFβ signaling members in granulosa cells between young and old mares. Significant differences are represented by *P<0.05.

A)



B)

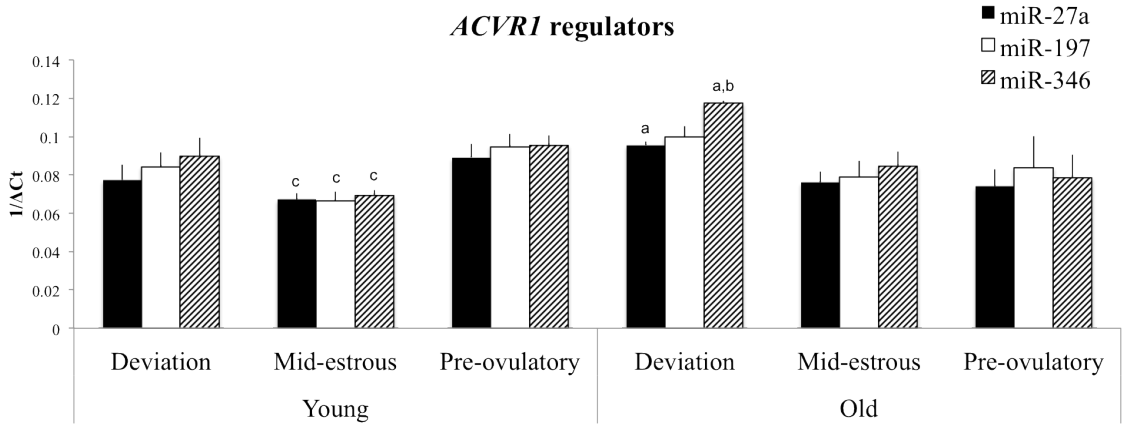


Figure IV.4: TGFβ signaling member *ACVR1* and its miRNA regulator. A) Relative levels of *ACVR1*. B) Relative levels of predicted miRNAs regulators of *ACVR1*.

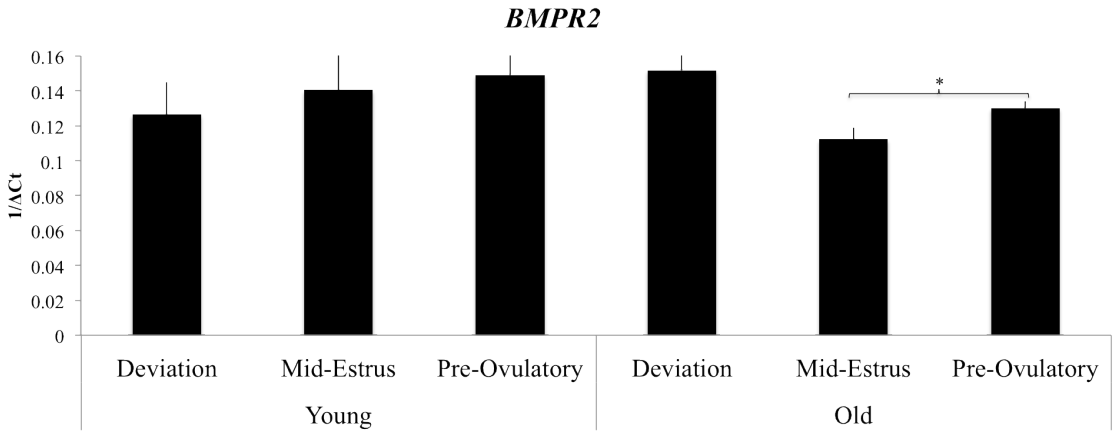
Significant differences are represented by *P<0.05

a= significant difference between deviation and mid-estrous (P < 0.05)

b= significant difference between deviation and pre-ovulatory (P < 0.05)

c= significant difference between mid-estrous and pre-ovulatory (P < 0.05)

A)



B)

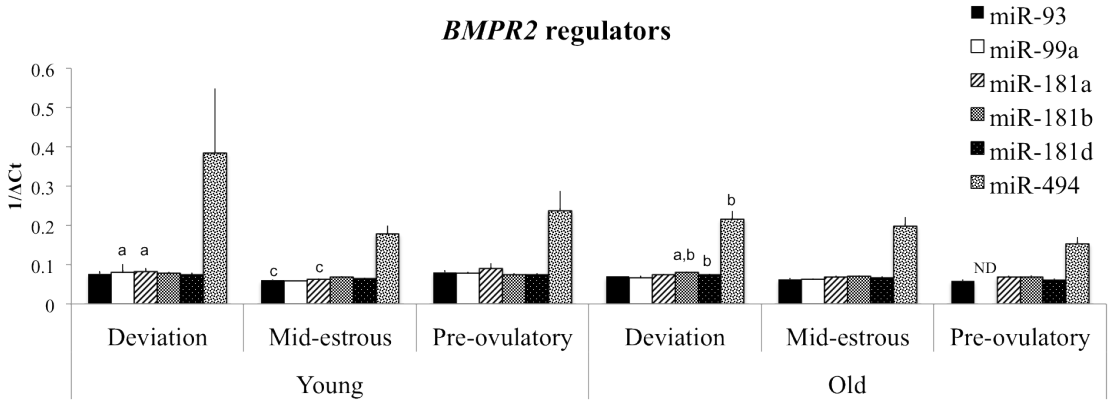


Figure IV.5: TGFβ signaling member *BMPR2* and its miRNA regulator. A) Relative levels of *BMPR2*. B) Relative levels of predicted miRNAs regulators of *BMPR2*.

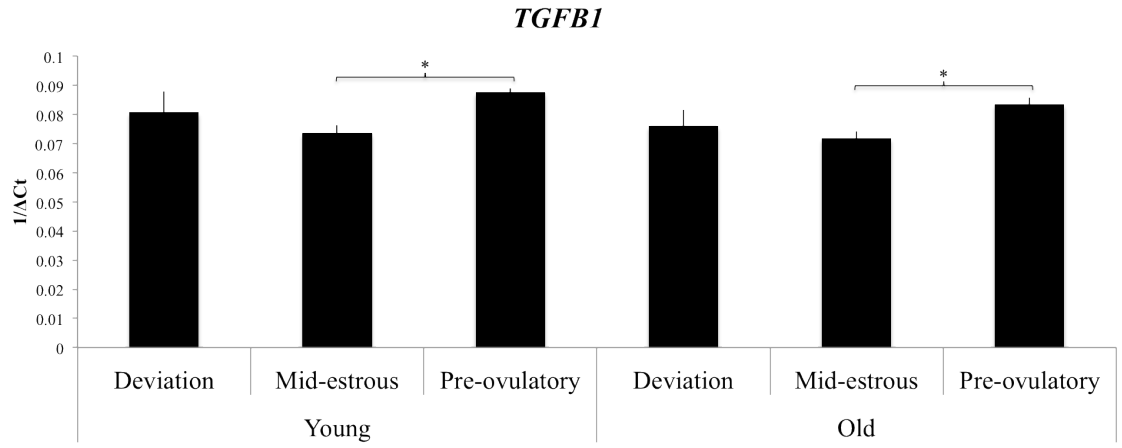
Significant differences are represented by *P<0.05

a= significant difference between deviation and mid-estrous ($P < 0.05$)

b= significant difference between deviation and pre-ovulatory ($P < 0.05$)

c= significant difference between mid-estrous and pre-ovulatory ($P < 0.05$)

A)



B)

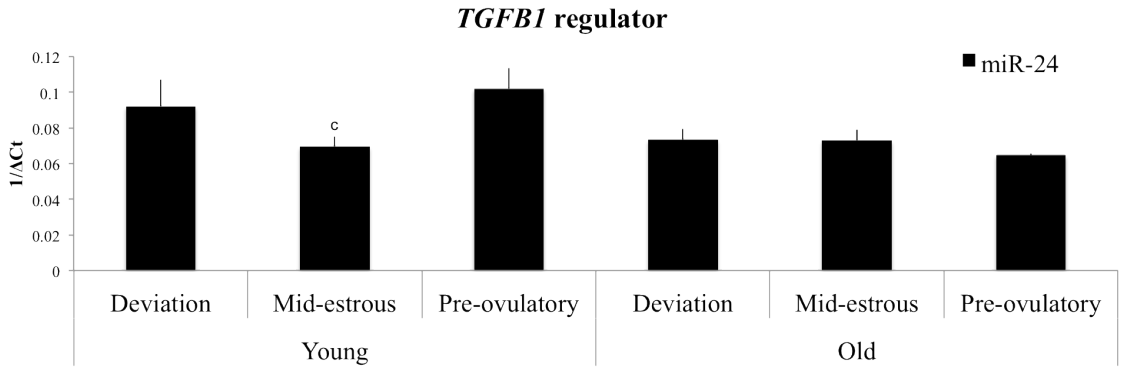


Figure IV.6: TGFβ signaling member *TGFβ1* and its miRNA regulator. A) Relative levels of *TGFβ1*. B) Relative levels of predicted miRNAs regulators of *TGFβ1*.

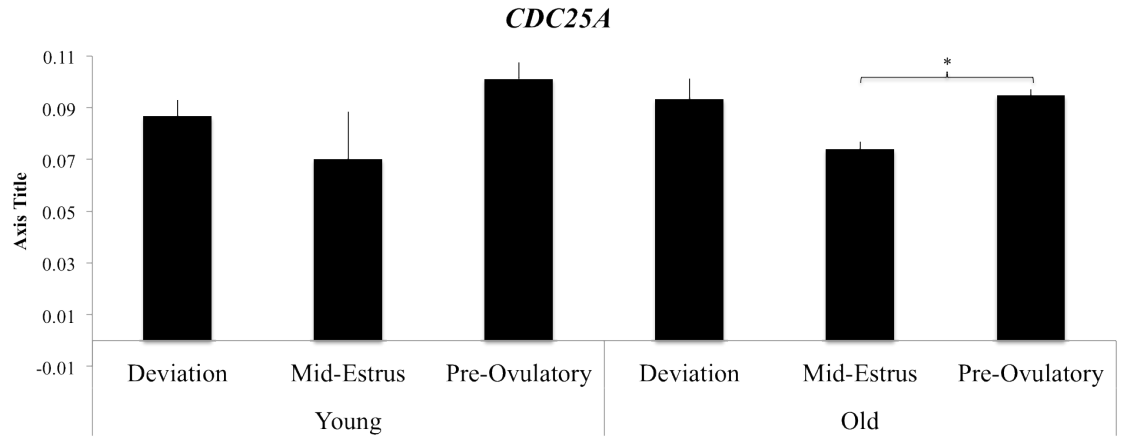
Significant differences are represented by *P<0.05

a= significant difference between deviation and mid-estrous ($P < 0.05$)

b= significant difference between deviation and pre-ovulatory ($P < 0.05$)

c= significant difference between mid-estrous and pre-ovulatory ($P < 0.05$)

A)



B)

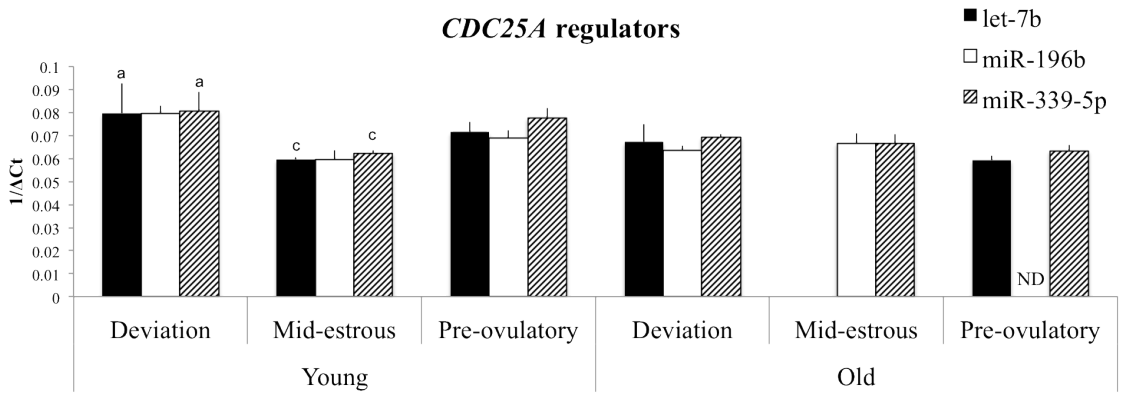


Figure IV.7: TGFβ signaling member *CDC25A* and its miRNA regulator. A) Relative levels of *CDC25A*. B) Relative levels of predicted miRNAs regulators of *CDC25A*.

Significant differences are represented by *P<0.05

a= significant difference between deviation and mid-estrous (P < 0.05)

b= significant difference between deviation and pre-ovulatory (P < 0.05)

c= significant difference between mid-estrous and pre-ovulatory (P < 0.05)

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GENERAL DISCUSSION

This dissertation focused on the role of cell-secreted vesicles during equine ovarian follicle development and the effects of aging. Three questions where addressed in this dissertation: **1)** Are cell-secreted vesicles present in ovarian follicular fluid? **2)** Do exosomes regulate TGF-β signaling during ovarian follicle development? **3)** Are exosomal miRNAs and TGF-β signaling members levels altered during follicle development in old compared to young mares?

In the first manuscript, we demonstrated the presence of cell-secreted vesicles (microvesicles and exosomes) within the follicular fluid of pre-ovulatory follicles. Presence of cell-secreted vesicles their morphology and size agree with data available in the literature (Raposo, 2013; Théry, 2006). Furthermore, we demonstrated the presence of miRNAs and proteins in exosomes. The presence of miRNAs and specific proteins was important to further confirm the presence of exosomes according to their expected profile (Raposo, 2013; Mathivanan, 2011; Simpson, 2012). MiRNA profiling of microvesicles and exosomes demonstrated the presence of unique miRNAs in microvesicles or exosomes isolated from ovarian follicular fluid, suggesting different roles for two vesicular populations. We also verified differences in exosomal miRNAs between follicular fluid from young and old mares, suggesting its use as a biomarker for aging and fertility. Finally, we demonstrated that cell-secreted vesicles could be taken up by granulosa cell in vitro and in vivo. Together these data suggest that cell-secreted vesicles mediate cell communication within the ovarian follicle, and can lead to the use of these vesicles for association studies related to fertility and biomarker discovery.

In the second manuscript, we analyzed the effects of exosome treatment on TGF-β signaling in granulosa cells in vitro. Initially, bioinformatics analysis of the exosomal miRNAs predicted regulation of TGF-β signaling members. Analysis of selected TGF-β members demonstrated changes in mRNA levels and protein levels between immature and mature granulosa cells. Importantly, treatment of mature granulosa cells with exosomes from immature follicles caused decrease in *ID2* and increase in ACVR1 at 24 h after treatment. Analysis of exosomes content from immature follicles demonstrated the high levels of miR-27b and ACVR1. These data suggest that exosomes are responsible for transferring miR-27b, a regulator of *ID2*, causing a decrease in *ID2* transcript, and also for transfer of ACVR1 to recipient cells. Inhibition of *ID2* and increase in ACVR1 signaling are necessary for final differentiation and acquisition of FSHr and LHr (Aoki, 2001; Song, 2012). Together these results suggest that exosomes play an important role regulating granulosa cell differentiation during the transition from immature to mature follicles by decreasing in *ID2* and increasing of ACVR1.

In the third manuscript, we evaluated the correlation between exosomal content and TGF-β signaling members from deviation, mid-estrous and pre-ovulatory follicles collected from young and old mares. Initially, we verified significantly different exosomal miRNAs between the different follicular stages, in young mares (48 different miRNAs) and in old mares (37 different miRNAs). Bioinformatics analysis reveals TGF-β signaling pathway as a frequently targeted pathway by exosomal miRNAs. This correlation between exosomal miRNAs and TGF-β signaling pathway was because a similar association previously observed (Manuscript #1; Manuscript #2). Analysis of selected TGF-β members demonstrated *TGFb1*, *STAT1* were significantly up-regulated at the pre-ovulatory stage, and *ID2* was significantly down-regulated at the pre-ovulatory stage compared to mid-estrous follicles. This expression pattern was

conserved between young and old mares, suggesting that these are important factors involved in final follicular maturation. Interestingly, *ACVR1*, *BMPR2*, *MYCBP* and *CDC25A* were significantly different between follicular stages in old mares, and similar changes were not observed in granulosa cells from young mares. These findings are suggesting that these genes are involved in mediating the effects of aging during follicular development in old mares. Based on bioinformatics analysis, we identified miRNAs predicted to regulate selected TGF-β members, and compared the levels of validated miRNAs and their predicted targets. Relative levels of the selected exosomal miRNAs were inversely correlated with their predicted mRNA target. Similar correlation between miRNAs and targets was not observed in young mares indicating that these predicted TGF-β targets were not altered during follicle development in young mares. Based on this we postulated that exosomal miRNAs are playing a role in helping to rescue and reestablish "normal levels" of important intracellular signaling members during follicle development in old mares. This last finding could help explain the irregularity of ovulations and differences in response to gonadotropin stimulation observed in old mares and old women.

In conclusion, cell-secreted vesicles are present in follicular fluid and need to be studied in more detail to clarify their entire function in follicle development and function. Here we only began to address some of the characteristics and function of these vesicles. Recently, the use of cell-secreted vesicles for diagnostic and therapeutic use as drug delivery agents opens new and exciting avenues to explore their use in follicular development. Therefore, cell-secreted vesicles can become important biomarkers or therapeutic agents involved with diagnostic of follicle development problems and treatment of infertility mediating the rescue of ovarian function in POF and cancer patients.

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APPENDIX I

Supplemental material chapter 2: ***Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: A possible new form of cell communication within the ovarian follicle.***

Supplemental Table II.1. Mature miRNAs assayed using the Human miRNome Profiling plate. Listed are the human mature miRNA sequences and corresponding equine mature miRNA sequences.

Human mature miRNA ID	Human microRNA Mature Sequence	# bases	Equine mature miRNA ID	Equine microRNA Mature Sequence	# bases
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	22	**eca-let-7a**	UGAGGUAGUAGGUUGUAUAGUU	22
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU	22	**eca-let-7b**	No sequence available	
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU	22	**eca-let-7c**	UGAGGUAGUAGGUUGUAUGGUU	22
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU	22	**eca-let-7d**	AGAGGUAGUAGGUUGCAUAGUU	22
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU	22	**eca-let-7e**	UGAGGUAGGAGGUUGUAUAGUU	22
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU	22	**eca-let-7f**	UGAGGUAGUAGAUUGUAUAGUU	22
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU	22	**eca-let-7g**	UGAGGUAGUAGUUUGUACAGUU	22
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU	22	**eca-let-7i**	No sequence available	
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU	22	**eca-mir-1**	UGGAAUGUAAAGAAGUAUGUAU	22
hsa-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	**eca-mir-7**	UGGAAGACUAGUGAUUUUGUUGU	23
hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA	23	**eca-mir-9a**	UCUUUGGUUAUCUAGCUGUAUGA	23
hsa-miR-10a	UACCCUGUAGAUCCGAAUUUGUG	23	**eca-mir-10a**	UACCCUGUAGAUCCGAAUUUGUG	23
hsa-miR-10b	UACCCUGUAGAACCGAAUUUGUG	23	**eca-mir-10b**	UACCCUGUAGAACCGAAUUUGUG	23
hsa-miR-15a	UAGCAGCACAUAAUGGUUUGUG	22	**eca-mir-15a**	UAGCAGCACAUAAUGGUUUGUG	22
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA	22	**eca-mir-15b**	UAGCAGCACAUCAUGGUUUACA	22
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	22	**eca-mir-16**	UAGCAGCACGUAAAUAUUGGCG	22
hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG	23	**eca-mir-17**	CAAAGUGCUUACAGUGCAGGUAG	23
hsa-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG	23	**eca-mir-18a**	UAAGGUGCAUCUAGUGCAGAUAG	23
hsa-miR-18b	UAAGGUGCAUCUAGUGCAGUUAG	23	**eca-mir-18b**	UAAGGUGCAUCUAGUGCAGUUAG	23
hsa-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	23	**eca-mir-19a**	UGUGCAAAUCUAUGCAAAACUGA	23
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	23	**eca-mir-19b**	UGUGCAAAUC**U**AUGCAAAACUGA	23
hsa-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	23	**eca-mir-20a**	UAAAGUGCUUAUAGUGCAGGUAG	23
hsa-miR-20b	CAAAGUGCUCAUAGUGCAGGUAG	23	**eca-mir-20b**	CAAAGUGCUCAUAGUGCAGGUAG	23
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	22	**eca-mir-21**	UAGCUUAUCAGACUGAUGUUGA	22
hsa-miR-22	AAGCUGCCAGUUGAAGAACUGU	22	**eca-mir-22**	AAGCUGCCAGUUGAAGAACUGU	22
hsa-miR-23a	AUCACAUUGCCAGGGAUUUCC	21	**eca-mir-23a**	AUCACAUUGCCAGGGAUUUCC	21
hsa-miR-23b	AUCACAUUGCCAGGGAUUACC	21	**eca-mir-23b**	AUCACAUUGCCAGGGAUUACC	21
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG	22	**eca-mir-24**	UGGCUCAGUUCAGCAGGAACAG	22
hsa-miR-25	CAUUGCACUUGUCUCGGUCUGA	22	**eca-mir-25**	CAUUGCACUUGUCUCGGUCUGA	22
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGCU	22	**eca-mir-26a**	UUCAAGUAAUCCAGGAUAGGCU	22
hsa-miR-26b	UUCAAGUAAUUCAGGAUAGGU	21	**eca-mir-26b**	No sequence available	

hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC	21	**eca-mir-27a**	UUCACAGUGGCUAAGUUCCGC	21
hsa-miR-27b	UUCACAGUGGCUAAGUUCUGC	21	**eca-mir-27b**	UUCACAGUGGCUAAGUUCUGC	21
hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA	22	**eca-mir-28-3p**	CACUAGAUUGUGAGCUCCUGGA	22
hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG	22	**eca-mir-28-5p**	AAGGAGCUCACAGUCUAUUGAG	22
hsa-miR-29a	UAGCACCAUCUGAAAUCGGUUA	22	**eca-mir-29a**	UAGCACCAUCUGAAAUCGGUUA	22
hsa-miR-29b	UAGCACCAUUUGAAAUCAGUGUU	23	**eca-mir-29b**	UAGCACCAUUUGAAAUCAGUGUU	23
hsa-miR-29c	UAGCACCAUUUGAAAUCGGUUA	22	**eca-mir-29c**	UAGCACCAUUUGAAAUCGGUUA	22
hsa-miR-30a	UGUAAACAUCCUCGACUGGAAG	22	**eca-mir-30a**	No sequence available	
hsa-miR-30b	UGUAAACAUCCUACACUCAGCU	22	**eca-mir-30b**	UGUAAACAUCCUACACUCAGCU	22
hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC	23	**eca-mir-30c**	UGUAAACAUCCUACACUCUCAGC	23
hsa-miR-30d	UGUAAACAUCCCCGACUGGAAG	22	**eca-mir-30d**	UGUAAACAUCCCCGACUGGAAG	22
hsa-miR-30e	UGUAAACAUCCUUGACUGGAAG	22	**eca-mir-30e**	UGUAAACAUCCUUGACUGGAAG	22
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU	21	**eca-mir-31**	AGGCAAGAUGCUGGCAUAGCU	21
hsa-miR-32	UAUUGCACAUUACUAAGUUGCA	22	**eca-mir-32**	UAUUGCACAUUACUAAGUUGCA	22
hsa-miR-33a	GUGCAUUGUAGUUGCAUUGCA	21	**eca-mir-33a**	GUGCAUUGUAGUUGCAUUGCA	21
hsa-miR-33b	GUGCAUUGCUGUUGCAUUGC	20	**eca-mir-33b**	GUGCAUUGCUGUUGCAUUGC	20
hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU	22	**eca-mir-34**	UGGCAGUGUCUUAGCUGGUUGU	22
hsa-miR-34b	CAAUCACUAACUCCACUGCCAU	22	**eca-mir-34b**	No sequence available	
hsa-miR-34c-3p	AAUCACUAACCACACGGCCAGG	22	**eca-mir-34c-3p**	No sequence available	
hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC	23	**eca-mir-34c-5p**	No sequence available	
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU	22	**eca-mir-92a**	UAUUGCACUUGUCCCGGCCUGU	22
hsa-miR-92b	UAUUGCACUCGUCCCGGCCUCC	22	**eca-mir-92b**	UAUUGCACUCGUCCCGGCCUCC	22
hsa-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	23	**eca-mir-93**	CAAAGUGCUGUUCGUGCAGGUAG	23
hsa-miR-95	UUCAACGGGUAUUUAUUGAGCA	22	**eca-mir-95**	UUCAACGGGU**C**UUUAUUGAGCA	22
hsa-miR-96	UUUGGCACUAGCACAUUUUUGCU	23	**eca-mir-96**	UUUGGCACUAGCACAUUUUUGCU	23
hsa-miR-98	UGAGGUAGUAAGUUGUAUUGUU	22	**eca-mir-98**	UGAGGUAGUAAGUUGUAUUGUU	22
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG	22	**eca-mir-99a**	AACCCGUAGAUCCGAUCUUGUG	22
hsa-miR-99b	CACCCGUAGAACCGACCUUGCG	22	**eca-mir-99b**	CACCCGUAGAACCGACCUUGCG	22
hsa-miR-100	AACCCGUAGAUCCGAACUUGUG	22	**eca-mir-100**	AACCCGUAGAUCCGAACUUGUG	22
hsa-miR-101	UACAGUACUGUGAUAACUGAA	21	**eca-mir-101**	UACAGUACUGUGAUAACUGAA	21
hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA	23	**eca-mir-103**	AGCAGCAUUGUACAGGGCUAUGA	23
hsa-miR-103-as	UCAUAGCCCUGUACAAUGCUGCU	23	**eca-mir-103-as**	No sequence available	
hsa-miR-105	UCAAAUGCUCAGACUCCUGUGGU	23	**eca-mir-105**	UCAAAUGCUCAGACUCCUGUGGU	23
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG	23	**eca-mir-106a**	**C**AAAGUGCUUACAGUGCAGGUAG	23
hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU	21	**eca-mir-106b**	UAAAGUGCUGACAGUGCAGAU	21

hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA	23	**eca-mir-107b**	AGCAGCAUUGUACAGGGCUAUCA	23
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG	22	**eca-mir-122**	UGGAGUGUGACAAUGGUGUUUG	22
hsa-miR-124	UAAGGCACGCGGUGAAUGCC	20	**eca-mir-124**	UAAGGCACGCGGUGAAUGCC	20
hsa-miR-125a-3p	ACAGGUGAGGUUCUUGGGAGCC	22	**eca-mir-125a-3p**	ACAGGUGAGGUUCUUGGGAGCC	22
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	24	**eca-mir-125a-5p**	UCCCUGAGACCCUUUAACCUGUGA	24
hsa-miR-125b	UCCCUGAGACCCUAACUUGUGA	22	**eca-mir-125b**	UCCCUGAGACCCUAACUUGUGA	22
hsa-miR-126	UCGUACCGUGAGUAAUAAUGCG	22	**eca-mir-126-3p**	UCGUACCGUGAGUAAUAAUGCG	22
hsa-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	22	**eca-mir-127**	UCGGAUCCGUCUGAGCUUGGCU	22
hsa-miR-127-5p	CUGAAGCUCAGAGGGCUCUGAU	22	**eca-mir-127-5p**	No sequence available	
hsa-miR-128	UCACAGUGAACCGGUCUCUUU	21	**eca-mir-128**	UCACAGUGAACCGGUCUCUUU	21
hsa-miR-129-3p	AAGCCCUUACCCCAAAAAGCAU	22	**eca-mir-129a-3p**	AAGCCCUUACCCCAAAAAG**U**AU	22
hsa-miR-129-5p	CUUUUUGCGGUCUGGGCUUGC	21	**eca-mir-129a-5p**	CUUUUUGCGGUCUGGGCUUGC	21
hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU	22	**eca-mir-130a**	CAGUGCAAUGUUAAAAGGGCAU	22
hsa-miR-130b	CAGUGCAAUGAUGAAAGGGCAU	22	**eca-mir-130b**	CAGUGCAAUGAUGAAAGGGCAU	22
hsa-miR-132	UAACAGUCUACAGCCAUGGUCG	22	**eca-mir-132**	UAACAGUCUACAGCCAUGGUCG	22
hsa-miR-133a	UUUGGUCCCCUUCAACCAGCUG	22	**eca-mir-133a**	UUUGGUCCCCUUCAACCAGCUG	22
hsa-miR-133b	UUUGGUCCCCUUCAACCAGCUA	22	**eca-mir-133b**	UUUGGUCCCCUUCAACCAGCUA	22
hsa-miR-134	UGUGACUGGUUGACCAGAGGGG	22	**eca-mir-134**	UGUGACUGGUUGACCAGAGGGG	22
hsa-miR-135a	UAUGGCUUUUUAUUCCUAUGUGA	23	**eca-mir-135a**	UAUGGCUUUUUAUUCCUAUGUGA	23
hsa-miR-135b	UAUGGCUUUUCAUUCCUAUGUGA	23	**eca-mir-135b**	UAUGGCUUUUCAUUCCUAUGUGA	23
hsa-miR-136	ACUCCAUUUGUUUUGAUGAUGGA	23	**eca-mir-136**	ACUCCAUUUGUUUUGAUGAUGG_	**22**
hsa-miR-137	UUAUUGCUUAAGAAUACGCGUAG	23	**eca-mir-137**	UUAUUGCUUAAGAAUACGCGUAG	23
hsa-miR-138	AGCUGGUGUUGUGAAUCAGGCCG	23	**eca-mir-138**	AGCUGGUGUUGUGAAUCAGGCCG	23
hsa-miR-139-3p	GGAGACGCGGCCCUGUUGGAGU	22	**eca-mir-139-3p**	GGAGACGCGGCCCUGUUGGAGU	22
hsa-miR-139-5p	UCUACAGUGCACGUGUCUCCAG	22	**eca-mir-139-5p**	UCUACAGUGCACGUGUCUCCAG	22
hsa-miR-140-3p	UACCACAGGGUAGAACCACGG	21	**eca-mir-140-3p**	UACCACAGGGUAGAACCACGG	21
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	22	**eca-mir-140-5p**	CAGUGGUUUUACCCUAUGGUAG	22
hsa-miR-141	UAACACUGUCUGGUAAAGAUGG	22	**eca-mir-141**	UAACACUGUCUGGUAAAGAUGG	22
hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA	23	**eca-mir-142-3p**	UGUAGUGUUUCCUACUUUAUGGA	23
hsa-miR-142-5p	CAUAAAGUAGAAAGCACUACU	21	**eca-mir-142-5p**	CAUAAAGUAGAAAGCACUACU	21
hsa-miR-143	UGAGAUGAAGCACUGUAGCUC	21	**eca-mir-143**	UGAGAUGAAGCACUGUAGCUC	21
hsa-miR-144	UACAGUAUAGAUGAUGUACU	20	**eca-mir-144**	UACAGUAUAGAUGAUGUACU	20
hsa-miR-145	GUCCAGUUUUCCCAGGAAUCCCU	23	**eca-mir-145**	GUCCAGUUUUCCCAGGAAUCCCU	23
hsa-miR-146a	UGAGAACUGAAUUCCAUGGGUU	22	**eca-mir-146a**	UGAGAACUGAAUUCCAUGGGUU	22

hsa-miR-146b-3p	UGCCCUGUGGACUCAGUUCUGG	22	**eca-mir-146b-3p**	UGCCCU**AG**GGACUCAGUUCUGG	22
hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU	22	**eca-mir-146b-5p**	UGAGAACUGAAUUCCAUAGGCU	22
hsa-miR-147	GUGUGUGGAAAUGCUUCUGC	20	**eca-mir-147**	No sequence available	
hsa-miR-147b	GUGUGCGGAAAUGCUUCUGCUA	22	**eca-mir-147b**	GUGUGC**C**GAAAUGCUUCUGCUA	22
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU	22	**eca-mir-148a**	UCAGUGCACUACAGAACUUUGU	22
hsa-miR-148b	UCAGUGCAUCACAGAACUUUGU	22	**eca-mir-148b-3p**	UCAGUGCAUCACAGAACUUUGU	22
hsa-miR-149	UCUGGCUCCGUGUCUUCACUCCC	23	**eca-mir-149**	UCUGGCUCCGUGUCUUCACUCCC	23
hsa-miR-150	UCUCCCAACCCUUGUACCAGUG	22	**eca-mir-150**	UCUCCCAACCCUUGUACCAGUG	22
hsa-miR-151-3p	CUAGACUGAAGCUCCUUGAGG	21	**eca-mir-151-3p**	No sequence available	
hsa-miR-151-5p	UCGAGGAGCUCACAGUCUAGU	21	**eca-mir-151-5p**	UCGAGGAGCUCACAGUCUAGU	21
hsa-miR-152	UCAGUGCAUGACAGAACUUGG	21	**eca-mir-152**	No sequence available	
hsa-miR-153	UUGCAUAGUCACAAAAGUGAUC	22	**eca-mir-153**	UUGCAUAGUCACAAAAGUGAUC	22
hsa-miR-154	UAGGUUAUCCGUGUUGCCUUCG	22	**eca-mir-154**	UAGGUUAUCCGUGUUGCCUUCG	22
hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU	23	**eca-mir-155**	UUAAUGCUAAUCGUGAUAGGGGU	23
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU	23	**eca-mir-181a**	AACAUUCAACGCUGUCGGUGAGU	23
hsa-miR-181b	AACAUUCAUUGCUGUCGGUGGGU	23	**eca-mir-181b**	AACAUUCAUUGCUGUCGGUGGGU	23
hsa-miR-181c	AACAUUCAACCUGUCGGUGAGU	22	**eca-mir-181c**	No sequence available	
hsa-miR-181d	AACAUUCAUUGUUGUCGGUGGGU	23	**eca-mir-181d**	No sequence available	
hsa-miR-182	UUUGGCAAUGGUAGAACUCACACU	24	**eca-mir-182**	UUUGGCAAUGGUAGAACUCACACU**G**	25
hsa-miR-183	UAUGGCACUGGUAGAAUUCACU	22	**eca-mir-183**	UAUGGCACUGGUAGAAUUCACU	22
hsa-miR-184	UGGACGGAGAACUGAUAAGGGU	22	**eca-mir-184**	UGGACGGAGAACUGAUAAGGGU	22
hsa-miR-185	UGGAGAGAAAGGCAGUUCCUGA	22	**eca-mir-185**	No sequence available	
hsa-miR-186	CAAAGAAUUCUCCUUUUGGGCU	22	**eca-mir-186**	CAAAGAAUUCUCCUUUUGGGCU	22
hsa-miR-187	UCGUGUCUUGUGUUGCAGCCGG	22	**eca-mir-187**	UCGUGUCUUGUGUUGCAGCCGG	22
hsa-miR-188-3p	CUCCCACAUGCAGGGUUUGCA	21	**eca-mir-188-3p**	CUCCCACAUGCAGGGUUUGCA	21
hsa-miR-188-5p	CAUCCCUUGCAUGGUGGAGGG	21	**eca-mir-188-5p**	CAUCCCUUGCAUGGUGGAGGG	21
hsa-miR-190	UGAUAUGUUUGAUAUAUUAGGU	22	**eca-mir-190**	UGAUAUGUUUGAUAUAUUAGGU	22
hsa-miR-190b	UGAUAUGUUUGAUAUUGGGUU	21	**eca-mir-190b**	UGAUAUGUUUGAUAUUGGGUU	21
hsa-miR-191	CAACGGAAUCCCAAAAGCAGCUG	23	**eca-mir-191**	CAACGGAAUCCCAAAAGCAGCUG	23
hsa-miR-192	CUGACCUAUGAAUUGACAGCC	21	**eca-mir-192**	CUGACCUAUGAAUUGACAGCC	21
hsa-miR-193a-3p	AACUGGCCUACAAAGUCCCAGU	22	**eca-mir-193a-3p**	AACUGGCCUACAAAGUCCCAGU	22
hsa-miR-193a-5p	UGGGUCUUUGCGGGCGAGAUGA	22	**eca-mir-193a-5p**	UGGGUCUUUGCGGGCGAGAUGA	22
hsa-miR-193b	AACUGGCCCUCAAAGUCCCGCU	22	**eca-mir-193b**	AACUGGCCC**A**CAAAGUCCCGCU	22
hsa-miR-194	UGUAACAGCAACUCCAUGUGGA	22	**eca-mir-194**	UGUAACAGCAACUCCAUGUGGA	22

hsa-miR-195	UAGCAGCACAGAAAUAUUGGC	21	**eca-mir-195**	UAGCAGCACAGAAAUAUUGGC	21
hsa-miR-196a	UAGGUAGUUUCAUGUUGUUGGG	22	**eca-mir-196a**	UAGGUAGUUUCAUGUUGUUGGG	22
hsa-miR-196b	UAGGUAGUUUCCUGUUGUUGGG	22	**eca-mir-196b**	UAGGUAGUUUCCUGUUGUUGGG	22
hsa-miR-197	UUCACCACCUUCUCCACCCAGC	22	**eca-mir-197**	UUCACCACCUUCUCCACCCAGC	22
hsa-miR-198	GGUCCAGAGGGGAGAUAGGUUC	22	**eca-mir-198**	No sequence available	
hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA	22	**eca-mir-199a-3p**	ACAGUAGUCUGCACAUUGGU**AG**	22
hsa-miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC	23	**eca-mir-199a-5p**	CCCAGUGUUCAGACUACCUGUUC	23
hsa-miR-199b-3p	ACAGUAGUCUGCACAUUGGUUA	22	**eca-mir-199b-3p**	ACAGUAGUCUGCACAUUGGUUA	22
hsa-miR-199b-5p	CCCAGUGUUUAGACUAUCUGUUC	23	**eca-mir-199b-5p**	CCCAGUGUUUAGACUAUCUGUUC	23
hsa-miR-200a	UAACACUGUCUGGUAACGAUGU	22	**eca-mir-200a**	UAACACUGUCUGGUAACGAUGU	22
hsa-miR-200b	UAAUACUGCCUGGUAAUGAUGA	22	**eca-mir-200b**	UAAUACUGCCUGGUAAUGAUGA	22
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA	23	**eca-mir-200c**	UAAUACUGCCGGGUAAUGAUGGA	23
hsa-miR-202	AGAGGUAUAGGGCAUGGGAA	20	**eca-mir-202**	No sequence available	
hsa-miR-203	GUGAAAUGUUUAGGACCACUAG	22	**eca-mir-203**	No sequence available	
hsa-miR-204	UUCCCUUUGUCAUCCUAUGCCU	22	**eca-mir-204b**	UUCCCUUUGUCAUCCUAUGCCU	22
hsa-miR-205	UCCUUCAUUCCACCGGAGUCUG	22	**eca-mir-205**	UCCUUCAUUCCACCGGAGUCUG	22
hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG	22	**eca-mir-206**	UGGAAUGUAAGGAAGUGUGUGG	22
hsa-miR-208a	AUAAGACGAGCAAAAAGCUUGU	22	**eca-mir-208a**	AUAAGACGAGCAAAAAGCUUGU	22
hsa-miR-208b	AUAAGACGAACAAAAGGUUUGU	22	**eca-mir-208b**	AUAAGACGAACAAAAGGUUUGU	22
hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA	22	**eca-mir-210**	No sequence available	
hsa-miR-211	UUCCCUUUGUCAUCCUUCGCCU	22	**eca-mir-211**	UUCCCUUUGUCAUCCUU**U**GCCU	22
hsa-miR-212	UAACAGUCUCCAGUCACGGCC	21	**eca-mir-212**	UAACAGUCUCCAGUCACGGCC	21
hsa-miR-214	ACAGCAGGCACAGACAGGCAGU	22	**eca-mir-214**	ACAGCAGGCACAGACAGGCAGU	22
hsa-miR-215	AUGACCUAUGAAUUGACAGAC	21	**eca-mir-215**	AUGACCUAUGAAUUGACAGAC	21
hsa-miR-216a	UAAUCUCAGCUGGCAACUGUGA	22	**eca-mir-216a**	UAAUCUCAGCUGGCAACUGUGA	22
hsa-miR-216b	AAAUCUCUGCAGGCAAAUGUGA	22	**eca-mir-216b**	AAAUCUCUGCAGGCAAAUGUGA	22
hsa-miR-217	UACUGCAUCAGGAACUGAUUGGA	23	**eca-mir-217**	UACUGCAUCAGGAACUGAUUGGA	23
hsa-miR-218	UUGUGCUUGAUCUAACCAUGU	21	**eca-mir-218**	UUGUGCUUGAUCUAACCAUGU	21
hsa-miR-219-1-3p	AGAGUUGAGUCUGGACGUCCCG	22	**eca-mir-219-1-3p**	No sequence available	
hsa-miR-219-2-3p	AGAAUUGUGGCUGGACAUCUGU	22	**eca-mir-219-2-3p**	No sequence available	
hsa-miR-219-5p	UGAUUGUCCAAACGCAAUUCU	21	**eca-mir-219-5p**	UGAUUGUCCAAACGCAAUUCU	21
hsa-miR-220a	CCACACCGUAUCUGACACUUU	21	**eca-mir-220a**	No sequence available	
hsa-miR-220b	CCACCACCGUGUCUGACACUU	21	**eca-mir-220b**	No sequence available	
hsa-miR-220c	ACACAGGGCUGUUGUGAAGACU	22	**eca-mir-220c**	No sequence available	

hsa-miR-221	AGCUACAUUGUCUGCUGGGUUUC	23	**eca-mir-221**	AGCUACAUUGUCUGCUGGGUUUC	23
hsa-miR-222	AGCUACAUCUGGCUACUGGGU	21	**eca-mir-222**	AGCUACAUCUGGCUACUGGGU	21
hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA	22	**eca-mir-223**	UGUCAGUUUGUCAAAUACCCCA	22
hsa-miR-224	CAAGUCACUAGUGGUUCCGUU	21	**eca-mir-224**	CAAGUCACUAGUGGUUCCGUU	21
hsa-miR-296-3p	GAGGGUUGGGUGGAGGCUCUCC	22	**eca-mir-296**	GAGGGUUGGGUGGAGGCU**U**UCC	22
hsa-miR-296-5p	AGGGCCCCCCCUCAAUCCUGU	21	**eca-mir-296-5p**	No sequence available	
hsa-miR-297	AUGUAUGUGUGCAUGUGCAUG	21	**eca-mir-297**	No sequence available	
hsa-miR-298	AGCAGAAGCAGGGAGGUUCUCCCA	24	**eca-mir-298**	No sequence available	
hsa-miR-299-3p	UAUGUGGGAUGGUAAACCGCUU	22	**eca-mir-299**	UAUGUGGGAUGGUAAACCGCUU	22
hsa-miR-299-5p	UGGUUUACCGUCCCACAUACAU	22	**eca-mir-299-5p**	No sequence available	
hsa-miR-300	UAUACAAGGGCAGACUCUCUCU	22	**eca-mir-300**	No sequence available	
hsa-miR-301a	CAGUGCAAUAGUAUUGUCAAAGC	23	**eca-mir-301a**	CAGUGCAAUAGUAUUGUCAAAGC	23
hsa-miR-301b	CAGUGCAAUGAUAUUGUCAAAGC	23	**eca-mir-301b-3p**	CAGUGCAAUGAUAUUGUCAAAGC	23
hsa-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	23	**eca-mir-302a**	UAAGUGCUUCCAUGUUUU**A**GUGA	23
hsa-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	23	**eca-mir-302b**	UAAGUGCUUCCAUGUUUUAGUAG	23
hsa-miR-302c	UAAGUGCUUCCAUGUUUCAGUGG	23	**eca-mir-302c**	UAAGUGCUUCCAUGUUUCAGUGG	23
hsa-miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	23	**eca-mir-302d**	UAAGUGCUUCCAUGUUUUAGUGU	23
hsa-miR-302e	UAAGUGCUUCCAUGCUU	17	**eca-mir-302e**	No sequence available	
hsa-miR-302f	UAAUUGCUUCCAUGUUU	17	**eca-mir-302f**	No sequence available	
hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA	22	**eca-mir-320a**	No sequence available	
hsa-miR-320b	AAAAGCUGGGUUGAGAGGGCAA	22	**eca-mir-320b**	No sequence available	
hsa-miR-320c	AAAAGCUGGGUUGAGAGGGU	20	**eca-mir-320c**	No sequence available	
hsa-miR-320d	AAAAGCUGGGUUGAGAGGA	19	**eca-mir-320d**	No sequence available	
hsa-miR-323-3p	CACAUUACACGGUCGACCUCU	21	**eca-mir-323-3p**	CACAUUACACGGUCGACCUCU	21
hsa-miR-323-5p	AGGUGGUCCGUGGCGCGUUCGC	22	**eca-mir-323-5p**	AGGUGGUCCGUGGCGCGUUCGC	22
hsa-miR-324-3p	ACUGCCCCAGGUGCUGCUGG	20	**eca-mir-324-3p**	**CC**ACUGCCCCAGGUGCUGCUGG	22
hsa-miR-324-5p	CGCAUCCCCUAGGGCAUUGGUGU	23	**eca-mir-324-5p**	CGCAUCCCCUAGGGCAUUGGUGU	23
hsa-miR-325	CCUAGUAGGUGUCCAGUAAGUGU	23	**eca-mir-325**	No sequence available	
hsa-miR-326	CCUCUGGGCCCUUCCUCCAG	20	**eca-mir-326**	CCUCUGGGCCCUUCCUCCAGC	20
hsa-miR-328	CUGGCCCUCUCUGCCCUUCCGU	22	**eca-mir-328**	CUGGCCCUCUCUGCCCUUCCGU	22
hsa-miR-329	AACACACCUGGUUAACCUCUUU	22	**eca-mir-329**	AACACACCU**A**GUUAACCUCUUU	22
hsa-miR-330-3p	GCAAAGCACACGGCCUGCAGAGA	23	**eca-mir-330-3p**	No sequence available	
hsa-miR-330-5p	UCUCUGGGCCUGUGUCUUAGGC	22	**eca-mir-330**	UCUCUGGGCCUGUGUCUUAGGC	22
hsa-miR-331-3p	GCCCCUGGGCCUAUCCUAGAA	21	**eca-mir-331**	GCCCCUGGGCCUAUCCUAGAA	21

hsa-miR-331-5p	CUAGGUAUGGUCCCAGGGAUCC	22	**eca-mir-331-5p**	No sequence available	
hsa-miR-335	UCAAGAGCAAUAACGAAAAAUGU	23	**eca-mir-335**	UCAAGAGCAAUAACGAAAAAUGU	23
hsa-miR-337-3p	CUCCUAUAUGAUGCCUUUCUUC	22	**eca-mir-337-3p**	CUCCUAU**GA**GAUGCCUUUCCUC	22
hsa-miR-337-5p	GAACGGCUUCAUACAGGAGUU	21	**eca-mir-337-5p**	GAACGGCUUCAUACAGGAGCU	21
hsa-miR-338-3p	UCCAGCAUCAGUGAUUUUGUUG	22	**eca-mir-338-3p**	UCCAGCAUCAGUGAUUUUGUUG	22
hsa-miR-338-5p	AACAAUAUCCUGGUGCUGAGUG	22	**eca-mir-338-5p**	AACAAUAUCCUGGUGCUGAGUG	22
hsa-miR-339-3p	UGAGCGCCUCGACGACAGAGCCG	23	**eca-mir-339-3p**	No sequence available	
hsa-miR-339-5p	UCCCUGUCCUCCAGGAGCUCACG	23	**eca-mir-339-5p**	No sequence available	
hsa-miR-340	UUAUAAAGCAAUGAGACUGAUU	22	**eca-mir-340-5p**	UUAUAAAGCAAUGAGACUGAUU	22
hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU	23	**eca-mir-342-3p**	UCUCACACAGAAAUCGCACCCGU	23
hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA	21	**eca-mir-342-5p**	AGGGGUGCUAUCUGUGAUUGA**G**	22
hsa-miR-345	GCUGACUCCUAGUCCAGGGCUC	22	**eca-mir-345-5p**	GCUGACUCCUAGUCCAGUGCUC	22
hsa-miR-346	UGUCUGCCCGCAUGCCUGCCUCU	23	**eca-mir-346**	UGUCUGCCCGCAUGCCUGCCUCU	23
hsa-miR-361-3p	UCCCCCAGGUGUGAUUCUGAUUU	23	**eca-mir-361-3p**	UCCCCCAGGCGUGAUUCUGAUUU	23
hsa-miR-361-5p	UUAUCAGAAUCUCCAGGGGUAC	22	**eca-mir-361-5p**	UUAUCAGAAUCUCCAGGGGUAC	22
hsa-miR-362-3p	AACACACCUAUUCAAGGAUUCA	22	**eca-mir-362-3p**	AACACACCUAUUCAAGGAUUCA	22
hsa-miR-362-5p	AAUCCUUGGAACCUAGGUGUGAGU	24	**eca-mir-362-5p**	AAUCCUUGGAACCUAGGUGUGAGU	24
hsa-miR-363	AAUUGCACGGUAUCCAUCUGUA	22	**eca-mir-363**	AAUUGCACGGUAUCCAUCUGUA	22
hsa-miR-365	UAAUGCCCCUAAAAAUCCUUAU	22	**eca-mir-365**	UAAUGCCCCUAAAAAUCCUUAU	22
hsa-miR-367	AAUUGCACUUUAGCAAUGGUGA	22	**eca-mir-367**	AAUUGCACUUUAGCAAUGGUGA	22
hsa-miR-369-3p	AAUAAUACAUGGUUGAUCUUU	21	**eca-mir-369-3p**	AAUAAUACAUGGUUGAUCUUU	21
hsa-miR-369-5p	AGAUCGACCGUGUUAUAUUCGC	22	**eca-mir-369-5p**	AGAUCGACCGUGU**C**AUAUUCGC	22
hsa-miR-370	GCCUGCUGGGGUGGAACCUGGU	22	**eca-mir-370**	GCCUGCUGGGGUGGAACCUGGU	22
hsa-miR-371-3p	AAGUGCCGCCAUCUUUUGAGUGU	23	**eca-mir-371-3p**	AAGUGCCGCCAU**U**UUUUGAGUGU	23
hsa-miR-371-5p	ACUCAAACUGUGGGGGCACU	20	**eca-mir-371-5p**	ACUCAAACUGUGGGGGCACU	20
hsa-miR-372	AAAGUGCUGCGACAUUUGAGCGU	23	**eca-mir-372**	No sequence available	
hsa-miR-373	GAAGUGCUUCGAUUUUGGGGUGU	23	**eca-mir-373**	No sequence available	
hsa-miR-374a	UUAUAAUACAACCUGAUAAGUG	22	**eca-mir-374a**	UUAUAAUACAACCUGAUAAGUG	22
hsa-miR-374b	AUAUAAUACAACCUGCUAAGUG	22	**eca-mir-374b**	AUAUAAUACAACCUGCUAAGUG	22
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA	22	**eca-mir-375**	No sequence available	
hsa-miR-376a	AUCAUAGAGGAAAAUCCACGU	21	**eca-mir-376a**	AUCAUAGAGGAAAAUCCACGU	21
hsa-miR-376b	AUCAUAGAGGAAAAUCCAUGUU	22	**eca-mir-376b**	AUCAUAGAGGAAAAUCCAUGU_	**21**
hsa-miR-376c	AACAUAGAGGAAAUUCCACGU	21	**eca-mir-376c**	AACAUAGAGGAAAUUCCACGU	21
hsa-miR-377	AUCACACAAAGGCAACUUUUGU	22	**eca-mir-377**	AUCACACAAAGGCAACUUUUGU	22

hsa-miR-378	ACUGGACUUGGAGUCAGAAGG	21	**eca-mir-378**	ACUGGACUUGGAGUCAGAAGG	21
hsa-miR-379	UGGUAGACUAUGGAACGUAGG	21	**eca-mir-379**	UGGUAGACUAUGGAACGUAGG	21
hsa-miR-380	UAUGUAAUAUGGUCCACAUCUU	22	**eca-mir-380**	UAUGUAAUAUGGUCCAC**G**UCUU	22
hsa-miR-381	UAUACAAGGGCAAGCUCUCUGU	22	**eca-mir-381**	UAUACAAGGGCAAGCUCUCUGU	22
hsa-miR-382	GAAGUUGUUCGUGGUGGAUUCG	22	**eca-mir-382**	GAAGUUGUUCGUGGUGGAUUCG	22
hsa-miR-383	AGAUCAGAAGGUGAUUGUGGCU	22	**eca-mir-383**	AGAUCAGAAGGUGAUUGUGGCU	22
hsa-miR-384	AUUCCUAGAAAUUGUUCAUA	20	**eca-mir-384**	AUUCCUAGAAAUUGUUCACA	20
hsa-miR-409-3p	GAAUGUUGCUCGGUGAACCCCU	22	**eca-mir-409-3p**	GAAUGUUGCUCGGUGAACCCCU	22
hsa-miR-409-5p	AGGUUACCCGAGCAACUUUGCAU	23	**eca-mir-409-5p**	AGGUUACCCGAGCAACUUUGCAU	23
hsa-miR-410	AAUAUAACACAGAUGGCCUGU	21	**eca-mir-410**	AAUAUAACACAGAUGGCCUGU	21
hsa-miR-411	UAGUAGACCGUAUAGCGUACG	21	**eca-mir-411**	UAGUAGACCGUAUAGCGUACG	21
hsa-miR-412	ACUUCACCUGGUCCACUAGCCGU	23	**eca-mir-412**	__UUCACCUGGUCCACUAGCCG_	**20**
hsa-miR-421	AUCAACAGACAUUAAUUGGGCGC	23	**eca-mir-421**	**GGCCU**CA**UUAAAU**G**U**UUG**UUG**	21
hsa-miR-422a	ACUGGACUUAGGGUCAGAAGGC	22	**eca-mir-422a**	No sequence available	
hsa-miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU	23	**eca-mir-423-3p**	AGCUCGGUCUGAGGCCCCUCAGU	23
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	23	**eca-mir-423-5p**	UGAGGGGCAGAGAGCGAGACUUU	23
hsa-miR-424	CAGCAGCAAUUCAUGUUUUGAA	22	**eca-mir-424**	CAGCAGCAAUUCAUGUUUUGAA	22
hsa-miR-425	AAUGACACGAUCACUCCCGUUGA	23	**eca-mir-425**	No sequence available	
hsa-miR-429	UAAUACUGUCUGGUAAAACCGU	22	**eca-mir-429**	UAAUACUGUCUGGUAA**UG**CCG_	**21**
hsa-miR-431	UGUCUUGCAGGCCGUCAUGCA	21	**eca-mir-431**	UGUCUUGCAGGCCGUCAUGCA**GG**	23
hsa-miR-432	UCUUGGAGUAGGUCAUUGGGUGG	23	**eca-mir-432**	UCUUGGAGUAGGUCAUUGGGUGG	23
hsa-miR-433	AUCAUGAUGGGCUCCUCGGUGU	22	**eca-mir-433**	AUCAUGAUGGGCUCCUCGGUGU	22
hsa-miR-448	UUGCAUAUGUAGGAUGUCCCAU	22	**eca-mir-448**	UUGCAUAUGUAGGAUGUCCCAU	22
hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU	22	**eca-mir-449a**	UGGCAGUGUAUUGUUAGCUGGU	22
hsa-miR-449b	AGGCAGUGUAUUGUUAGCUGGC	22	**eca-mir-449b**	No sequence available	
hsa-miR-450a	UUUUGCGAUGUGUUCCUAAUAU	22	**eca-mir-450a**	UUUUGCGAUGUGUUCCUAAUAU	22
hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA	22	**eca-mir-450b-3p**	UUGGGA**A**CAUUUUGCAUCCAUA	22
hsa-miR-450b-5p	UUUUGCAAUAUGUUCCUGAAUA	22	**eca-mir-450b-5p**	UUUUGCAAUAUGUUCCUGAAUA	22
hsa-miR-451	AAACCGUUACCAUUACUGAGUU	22	**eca-mir-451**	AAACCGUUACCAUUACUG**U**GUU	22
hsa-miR-452	AACUGUUUGCAGAGGAAACUGA	22	**eca-mir-452**	No sequence available	
hsa-miR-453	AGGUUGUCCGUGGUGAGUUCGCA	23	**eca-mir-453**	No sequence available	
hsa-miR-454	UAGUGCAAUAUUGCUUAUAGGGU	23	**eca-mir-454**	UAGUGCAAUAUUGCUUAUAGGGU	23
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC	21	**eca-mir-455-3p**	No sequence available	
hsa-miR-455-5p	UAUGUGCCUUUGGACUACAUCG	22	**eca-mir-455-5p**	No sequence available	

hsa-miR-483-3p	UCACUCCUCUCCUCCCGUCUU	21	**eca-mir-483-3p**	No sequence available	
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	22	**eca-mir-483-5p**	No sequence available	
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU	22	**eca-mir-484**	No sequence available	
hsa-miR-485-3p	GUCAUACACGGCUCUCCUCUCU	22	**eca-mir-485-3p**	GUCAUACACGGCUCUCCUCUCU	22
hsa-miR-485-5p	AGAGGCUGGCCGUGAUGAAUUC	22	**eca-mir-485-5p**	AGAGGCUGGCCGUGAUGAAUUC	22
hsa-miR-486-3p	CGGGGCAGCUCAGUACAGGAU	21	**eca-mir-486-3p**	CGGGGCAGCUCAGUACAGGAU	21
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	22	**eca-mir-486-5p**	UCCUGUACUGAGCUGCCCCGAG	22
hsa-miR-487a	AAUCAUACAGGGACAUCCAGUU	22	**eca-mir-487a**	AAUCAUACAGGGACAUCCAGUU	22
hsa-miR-487b	AAUCGUACAGGGUCAUCCACUU	22	**eca-mir-487b**	AAUCGUACAGGGUCAUCCACUU	22
hsa-miR-488	UUGAAAGGCUAUUUCUUGGUC	21	**eca-mir-488**	UUGAAAGGCUAUUUCUUGGUC	21
hsa-miR-489	GUGACAUCACAUAUACGGCAGC	22	**eca-mir-489**	GUGACAUCACAUAUACGGC**G**GC	22
hsa-miR-490-3p	CAACCUGGAGGACUCCAUGCUG	22	**eca-mir-490-3p**	CAACCUGGAGGACUCCAUGCUG	22
hsa-miR-490-5p	CCAUGGAUCUCCAGGUGGGU	20	**eca-mir-490-5p**	CCAUGGAUCUCCAGGUGGGU	20
hsa-miR-491-3p	CUUAUGCAAGAUUCCCUUCUAC	22	**eca-mir-491-3p**	CUUAUGCAAGAUUCCCUUCUAC	22
hsa-miR-491-5p	AGUGGGGAACCCUUCCAUGAGG	22	**eca-mir-491-5p**	AGUGGGGAACCCUUCCAUGAGG	22
hsa-miR-492	AGGACCUGCGGGACAAGAUUCUU	23	**eca-mir-492**	AGGA**G**CUGCGGGACAAGAUUCUU	23
hsa-miR-493	UGAAGGUCUACUGUGUGCCAGG	22	**eca-mir-493b**	UGAAGGUCU**U**C**C**GUGUGCCAGG	22
hsa-miR-494	UGAAACAUACACGGGAAACCUC	22	**eca-mir-494**	UGAAACAUACACGGGAAACCUC	22
hsa-miR-495	AAACAAACAUGGUGCACUUCUU	22	**eca-mir-495**	AAACAAACAUGGUGCACUUCUU	22
hsa-miR-496	UGAGUAUUACAUGGCCAAUCUC	22	**eca-mir-496**	UGAGUAUUACAUGGCCAAUCUC	22
hsa-miR-497	CAGCAGCACACUGUGGUUUGU	21	**eca-mir-497**	CAGCAGCACACUGUGGUUUGU	21
hsa-miR-498	UUUCAAGCCAGGGGGCGUUUUUC	23	**eca-mir-498**	No sequence available	
hsa-miR-499-3p	AACAUCACAGCAAGUCUGUGCU	22	**eca-mir-499-3p**	AACAUCACAGCAAGUCUGUGCU	22
hsa-miR-499-5p	UUAAGACUUGCAGUGAUGUUU	21	**eca-mir-499-5p**	UUAAGACUUGCAGUGAUGUUU	21
hsa-miR-500	UAAUCCUUGCUACCUGGGUGAGA	23	**eca-mir-500**	UAAUCCUUGCUACCUGGGUGAGA	23
hsa-miR-501-3p	AAUGCACCCGGGCAAGGAUUCU	22	**eca-mir-501-3p**	No sequence available	
hsa-miR-501-5p	AAUCCUUUGUCCCUGGGUGAGA	22	**eca-mir-501**	_AUCCUU**C**GUCCCUGGGUGAGA	**21**
hsa-miR-502-3p	AAUGCACCUGGGCAAGGAUUCA	22	**eca-mir-502-3p**	AAUGCACCUGGGCAAGGAUUCA	22
hsa-miR-502-5p	AUCCUUGCUAUCUGGGUGCUA	21	**eca-mir-502-5p**	AUCCUUGCUAUCUGGGUGCUA	21
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG	23	**eca-mir-503**	UAGCAGCGGGAACAGU**A**CUGCAG	23
hsa-miR-504	AGACCCUGGUCUGCACUCUAUC	22	**eca-mir-504**	AGACCCUGGUCUGCACUCUAUC	22
hsa-miR-505	CGUCAACACUUGCUGGUUUCCU	22	**eca-mir-505**	CGUCAACACUUGCUGGUUUCCU	22
hsa-miR-506	UAAGGCACCCUUCUGAGUAGA	21	**eca-mir-506**	No sequence available	
hsa-miR-507	UUUUGCACCUUUUGGAGUGAA	21	**eca-mir-507**	**A**UU**G**GCACCU**C**UU**A**GAGUGAA	21

hsa-miR-508-3p	UGAUUGUAGCCUUUUGGAGUAGA	23	**eca-mir-508-3p**	UGAUUGU**CA**CCUUUUGGAGUAGA	23
hsa-miR-508-5p	UACUCCAGAGGGCGUCACUCAUG	23	**eca-mir-508-5p**	UACUCCAGAGGG**U**GUCA**U**UCA**CA**	23
hsa-miR-509-3-5p	UACUGCAGACGUGGCAAUCAUG	22	**eca-mir-509-3-5p**	No sequence available	
hsa-miR-509-3p	UGAUUGGUACGUCUGUGGGUAG	22	**eca-mir-509-3p**	No sequence available	
hsa-miR-509-5p	UACUGCAGACAGUGGCAAUCA	21	**eca-mir-509-5p**	UACUGCAGACAGUGGCAAUCA	21
hsa-miR-510	UACUCAGGAGAGUGGCAAUCAC	22	**eca-mir-510**	No sequence available	
hsa-miR-511	GUGUCUUUUGCUCUGCAGUCA	21	**eca-mir-511**	No sequence available	
hsa-miR-512-3p	AAGUGCUGUCAUAGCUGAGGUC	22	**eca-mir-512-3p**	No sequence available	
hsa-miR-512-5p	CACUCAGCCUUGAGGGCACUUUC	23	**eca-mir-512-5p**	No sequence available	
hsa-miR-513a-3p	UAAAUUUCACCUUUCUGAGAAGG	23	**eca-mir-513a-3p**	No sequence available	
hsa-miR-513a-5p	UUCACAGGGAGGUGUCAU	18	**eca-mir-513a-5p**	No sequence available	
hsa-miR-513b	UUCACAAGGAGGUGUCAUUUAU	22	**eca-mir-513b**	No sequence available	
hsa-miR-513c	UUCUCAAGGAGGUGUCGUUUAU	22	**eca-mir-513c**	No sequence available	
hsa-miR-514	AUUGACACUUCUGUGAGUAGA	21	**eca-mir-514**	AUUGACAC**C**UCUGUGAGU**G**GA	21
hsa-miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU	22	**eca-mir-515-3p**	No sequence available	
hsa-miR-515-5p	UUCUCCAAAAGAAAGCACUUUCUG	24	**eca-mir-515-5p**	No sequence available	
hsa-miR-516a-3p	UGCUUCCUUUCAGAGGGU	18	**eca-mir-516-3p**	No sequence available	
hsa-miR-516a-5p	UUCUCGAGGAAAGAAGCACUUUC	23	**eca-mir-516-5p**	No sequence available	
hsa-miR-516b	AUCUGGAGGUAAGAAGCACUUU	22	**eca-mir-516b**	No sequence available	
hsa-miR-517a	AUCGUGCAUCCCUUUAGAGUGU	22	**eca-mir-517a**	No sequence available	
hsa-miR-517b	UCGUGCAUCCCUUUAGAGUGUU	22	**eca-mir-517b**	No sequence available	
hsa-miR-517c	AUCGUGCAUCCUUUUAGAGUGU	22	**eca-mir-517c**	No sequence available	
hsa-miR-518a-3p	GAAAGCGCUUCCCUUUGCUGGA	22	**eca-mir-518a-3p**	No sequence available	
hsa-miR-518a-5p	CUGCAAAGGGAAGCCCUUUC	20	**eca-mir-518a-5p**	No sequence available	
hsa-miR-518b	CAAAGCGCUCCCCUUUAGAGGU	22	**eca-mir-518b**	No sequence available	
hsa-miR-518c	CAAAGCGCUUCUCUUUAGAGUGU	23	**eca-mir-518c**	No sequence available	
hsa-miR-518d-3p	CAAAGCGCUUCCCUUUGGAGC	21	**eca-mir-518d-3p**	No sequence available	
hsa-miR-518d-5p	CUCUAGAGGGAAGCACUUUCUG	22	**eca-mir-518d-5p**	No sequence available	
hsa-miR-518e	AAAGCGCUUCCCUUCAGAGUG	21	**eca-mir-518e**	No sequence available	
hsa-miR-518f	GAAAGCGCUUCUCUUUAGAGG	21	**eca-mir-518f**	No sequence available	
hsa-miR-519a	AAAGUGCAUCCUUUUAGAGUGU	22	**eca-mir-519a**	No sequence available	
hsa-miR-519b-3p	AAAGUGCAUCCUUUUAGAGGUU	22	**eca-mir-519b-3p**	No sequence available	
hsa-miR-519b-5p	CUCUAGAGGGAAGCGCUUUCUG	22	**eca-mir-519b-5p**	No sequence available	
hsa-miR-519c-3p	AAAGUGCAUCUUUUUAGAGGAU	22	**eca-mir-519c-3p**	No sequence available	

hsa-miR-519c-5p	CUCUAGAGGGAAGCGCUUUCUG	22	**eca-mir-519c-5p**	No sequence available	
hsa-miR-519d	CAAAGUGCCUCCCUUUAGAGUG	22	**eca-mir-519d**	No sequence available	
hsa-miR-519e	AAGUGCCUCCUUUUAGAGUGUU	22	**eca-mir-519e**	No sequence available	
hsa-miR-520a-3p	AAAGUGCUUCCCUUUGGACUGU	22	**eca-mir-520a-3p**	No sequence available	
hsa-miR-520a-5p	CUCCAGAGGGAAGUACUUUCU	21	**eca-mir-520a-5p**	No sequence available	
hsa-miR-520b	AAAGUGCUUCCUUUUAGAGGG	21	**eca-mir-520b**	No sequence available	
hsa-miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU	22	**eca-mir-520c-3p**	No sequence available	
hsa-miR-520c-5p	CUCUAGAGGGAAGCACUUUCUG	22	**eca-mir-520c-5p**	No sequence available	
hsa-miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU	22	**eca-mir-520d-3p**	No sequence available	
hsa-miR-520d-5p	CUACAAAGGGAAGCCCUUUC	20	**eca-mir-520d-5p**	No sequence available	
hsa-miR-520e	AAAGUGCUUCCUUUUUGAGGG	21	**eca-mir-520e**	No sequence available	
hsa-miR-520f	AAGUGCUUCCUUUUAGAGGGUU	22	**eca-mir-520f**	No sequence available	
hsa-miR-520g	ACAAAGUGCUUCCCUUUAGAGUGU	24	**eca-mir-520g**	No sequence available	
hsa-miR-520h	ACAAAGUGCUUCCCUUUAGAGU	22	**eca-mir-520h**	No sequence available	
hsa-miR-521	AACGCACUUCCCUUUAGAGUGU	22	**eca-mir-521**	No sequence available	
hsa-miR-522	AAAAUGGUUCCCUUUAGAGUGU	22	**eca-mir-522**	No sequence available	
hsa-miR-523	GAACGCGCUUCCCUAUAGAGGGU	23	**eca-mir-523**	No sequence available	
hsa-miR-524-3p	GAAGGCGCUUCCCUUUGGAGU	21	**eca-mir-524-3p**	No sequence available	
hsa-miR-524-5p	CUACAAAGGGAAGCACUUUCUC	22	**eca-mir-524-5p**	No sequence available	
hsa-miR-525-3p	GAAGGCGCUUCCCUUUAGAGCG	22	**eca-mir-525-3p**	No sequence available	
hsa-miR-525-5p	CUCCAGAGGGAUGCACUUUCU	21	**eca-mir-525-5p**	No sequence available	
hsa-miR-526a	CUCUAGAGGGAAGCACUUUCUG	22	**eca-mir-526a**	No sequence available	
hsa-miR-526b	CUCUUGAGGGAAGCACUUUCUGU	23	**eca-mir-526b**	No sequence available	
hsa-miR-527	CUGCAAAGGGAAGCCCUUUC	20	**eca-mir-527**	No sequence available	
hsa-miR-532-3p	CCUCCCACACCCAAGGCUUGCA	22	**eca-mir-532-3p**	CCUCCCACACCCAAGGCUUGCA	22
hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU	22	**eca-mir-532-5p**	CAUGCCUUGAGUGUAGGACCGU	22
hsa-miR-539	GGAGAAAUUAUCCUUGGUGUGU	22	**eca-mir-539**	GGAGAAAUUAUCCUUGCUGUGU	22
hsa-miR-541	UGGUGGGCACAGAAUCUGGACU	22	**eca-mir-541**	UGGUGGGCACAGAAUCCAGUCU	22
hsa-miR-542-3p	UGUGACAGAUUGAUAACUGAAA	22	**eca-mir-542-3p**	UGUGACAGAUUGAUAACUGAAA	22
hsa-miR-542-5p	UCGGGGAUCAUCAUGUCACGAGA	23	**eca-mir-542-5p**	**C**UCGGGGAUCAUCAUGUCACGA__	**22**
hsa-miR-543	AAACAUUCGCGGUGCACUUCUU	22	**eca-mir-543**	AAACAUUCGCGGUGCACUUCUU	22
hsa-miR-544	AUUCUGCAUUUUUAGCAAGUUC	22	**eca-mir-544b**	AUUCUGCAUUUUUA**A**CAAGUUC	22
hsa-miR-545	UCAGCAAACAUUUAUUGUGUGC	22	**eca-mir-545**	UCA**A**CAAACAUUUAUUGUGUGC	22
hsa-miR-548a-3p	CAAAACUGGCAAUUACUUUUGC	22	**eca-mir-548a-3p**	No sequence available	

hsa-miR-548a-5p	AAAAGUAAUUGCGAGUUUUACC	22	**eca-mir-548a-5p**	No sequence available	
hsa-miR-548b-3p	CAAGAACCUCAGUUGCUUUUGU	22	**eca-mir-548b-3p**	No sequence available	
hsa-miR-548b-5p	AAAAGUAAUUGUGGUUUUGGCC	22	**eca-mir-548b-5p**	No sequence available	
hsa-miR-548c-3p	CAAAAAUCUCAAUUACUUUUGC	22	**eca-mir-548c-3p**	No sequence available	
hsa-miR-548c-5p	AAAAGUAAUUGCGGUUUUUGCC	22	**eca-mir-548c-5p**	No sequence available	
hsa-miR-548d-3p	CAAAAACCACAGUUUCUUUUGC	22	**eca-mir-548d-3p**	No sequence available	
hsa-miR-548d-5p	AAAAGUAAUUGUGGUUUUUGCC	22	**eca-mir-548d-5p**	No sequence available	
hsa-miR-548e	AAAAACUGAGACUACUUUUGCA	22	**eca-mir-548e**	No sequence available	
Human U6 snRNA	CGCAAGGAUGACACGCAAAUUC	22	**Human U6 snRNA**	No sequence available	
RNU43 snoRNA	CUUAUUGACGGGCGGACAGAAAC	23	**RNU43 snoRNA**	No sequence available	
Hm/Ms/Rt U1 snRNA	CGACUGCAUAAUUUGUGGUAGUGG	24	**Hm/Ms/Rt U1 snRNA**	No sequence available	

Supplemental Table II.2. MiRNAs enriched in microvesicle preparations isolated from follicular fluid of young mares.

miRNA	Average Ct values	miRNA	Average Ct values
miR-17	35.40 ±0.41	*miR-222*	35.12 ±1.14
miR-19a	33.69 ±0.86	*miR-296-3p*	34.28 ±2.08
miR-19b	34.90 ±1.10	*miR-320a*	33.77 ±1.05
miR-21	32.79 ±0.56	*miR-320b*	35.98 ±1.51
miR-24	35.57 ±0.29	*miR-323-5p*	33.20 ±0.68
miR-25	33.85 ±1.56	*miR-324-3p*	34.15 ±0.80
miR-27a	35.21 ±0.49	*miR-324-5p*	35.99 ±0.73
miR-28-3p	35.45 ±1.57	*miR-326*	34.30 ±2.15
miR-30a	34.62 ±0.38	*miR-328*	35.28 ±1.34
miR-30e	35.39 ±0.12	*miR-331-5p*	11.39 ±1.01
miR-31	36.84 ±0.04	*miR-342-3p*	36.17 ±0.50
miR-34c-3p	33.41 ±1.42	*miR-345*	34.33 ±1.16
miR-92a	34.51 ±1.34	*miR-346*	33.47 ±1.48
miR-92b	31.68 ±0.97	*miR-362-5p*	24.52 ±1.72
miR-106a	35.76 ±0.75	*miR-370*	34.42 ±0.14
miR-106b	35.87 ±0.67	*miR-372*	36.28 ±0.53
miR-124	35.62 ±0.32	*miR-373*	34.91 ±1.52
miR-125a-5p	34.24 ±0.83	*miR-374a*	36.44 ±0.56
miR-127-3p	35.11 ±0.77	*miR-375*	32.16 ±0.37
miR-129-3p	34.43 ±0.20	*miR-377*	10.82 ±1.81
miR-129-5p	34.53 ±2.19	*miR-409-3p*	34.48 ±0.43
miR-132	24.88 ±0.58	*miR-409-5p*	29.46 ±11.24
miR-134	35.22 ±0.71	*miR-411*	18.71 ±2.50
miR-135a	31.74 ±1.22	*miR-421*	15.00 ±0.69
miR-135b	31.27 ±0.88	*miR-455-5p*	36.06 ±0.37
miR-139-5p	34.96 ±1.07	*miR-485-3p*	32.88 ±1.06
miR-149	33.21 ±0.61	*miR-486-5p*	35.14 ±1,98
miR-181a	26.84 ±2.45	*miR-488*	36.03 ±0.71
miR-181b	34.59 ±2.25	*miR-493*	33.53 ±1.25
miR-181c	24.03 ±1.84	*miR-494*	31.62 ±0.11
miR-187	32.61 ±2.23	*miR-503*	11.73 ±0.17
miR-191	13.19 ±0.73	*miR-512-3p*	31.18 ±1.83
miR-192	33.31 ±0.96	*miR-513c*	34.79 ±0.23
miR-193b	35.40 ±1.43	*miR-515-3p*	36.58 ±0.44
miR-195	34.63 ±0.07	*miR-520d-3p*	33.51 ±2.06
miR-197	34.24 ±1.94	*miR-523*	30.54 ±1.20
miR-202	34.16 ±4.33	*miR-532-3p*	34.52 ±2.46
miR-210	34.98 ±1.37	*miR-539*	33.54 ±1.02

miR-212	28.12 ±2.68	*miR-548b-5p*	21.67 ±0.82
miR-214	34.73 ±1.47		

*The RNA imput was ~500 total per reaction, and only Ct velues <37 cycles were considered

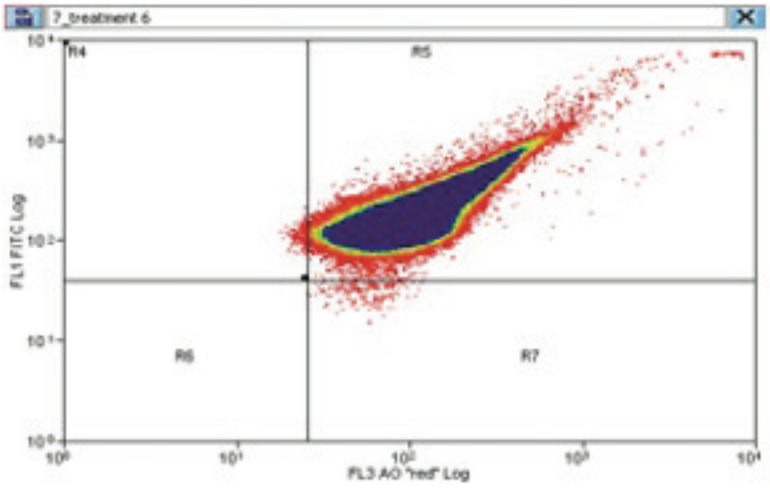
Average Ct values are reported ± standard deviation

Supplemental Table II.3. MiRNAs enriched in exosome preparations isolated from follicular fluid from young mares.

miRNA	Average Ct values	miRNA	Average Ct values
miR-17	35.17 ±1.78	*miR-181c*	25.45 ±0.62
miR-19b	35.28 ±1.50	*miR-191*	34.92 ±0.76
miR-20a	34.73 ±2.04	*miR-192*	32.26 ±2.09
miR-20b	34.50 ±0.53	*miR-195*	33.83 ±1.71
miR-21	33.80 ±2.64	*miR-197*	33.53 ±0.57
miR-22	35.32 ±0.39	*miR-215*	33.17 ±0.48
miR-23a	33.61 ±0.41	*miR-223*	33.02 ±0.15
miR-23b	35.00 ±0.54	*miR-320a*	33.58 ±0.26
miR-24	32.97 ±0.41	*miR-323-5p*	32.03 ±0.21
miR-25	33.50 ±1.76	*miR-362-5p*	25.76 ±0.78
miR-30a	34.36 ±1.16	*miR-375*	33.17 ±0.14
miR-30b	34.26 ±1.07	*miR-378*	35.31 ±0.76
miR-92a	32.20 ±1.42	*miR-421*	23.41 ±11.60
miR-92b	33.49 ±1.06	*miR-433*	36.38 ±0.13
miR-93	35.80 ±1.66	*miR-483-3p*	33.97 ±1.75
miR-101	34.89 ±1.74	*miR-485-3p*	34.46 ±1.64
miR-132	33.50 ±0.51	*miR-486-5p*	32.00 ±3.70
miR-135a	33.72 ±0.86	*miR-499-3p*	34.54 ±1.70
miR-135b	33.90 ±0.24	*miR-513a-3p*	34.84 ±0.24
miR-152	35.07 ±0.52	*miR-523*	35.05 ±0.35
miR-181a	31.99 ±0.34		

*The RNA imput was ~500 total per reaction, and only Ct velues <37 cycles were considered

Average Ct values are reported ± standard deviation



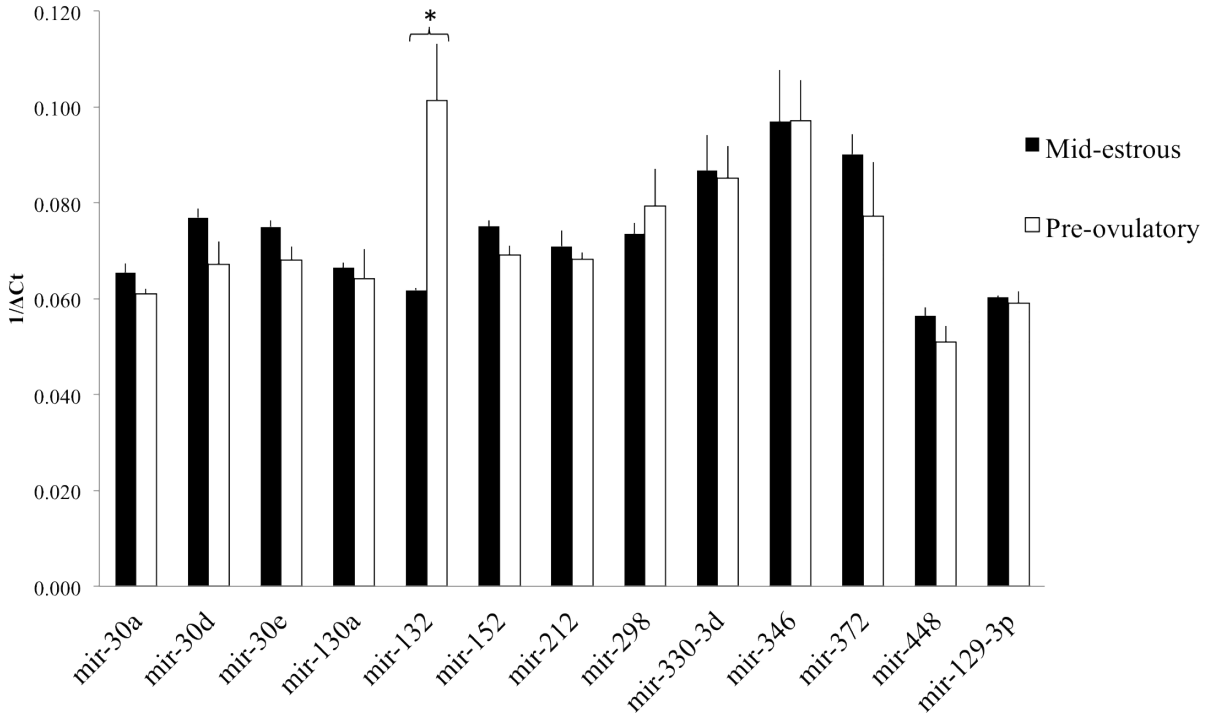
Supplemental Figure II.1. Flow cytometric analysis reveals the presence of CD63-positive vesicles (exosomes) containing RNA (AO-positive) in blood serum.

APPENDIX II

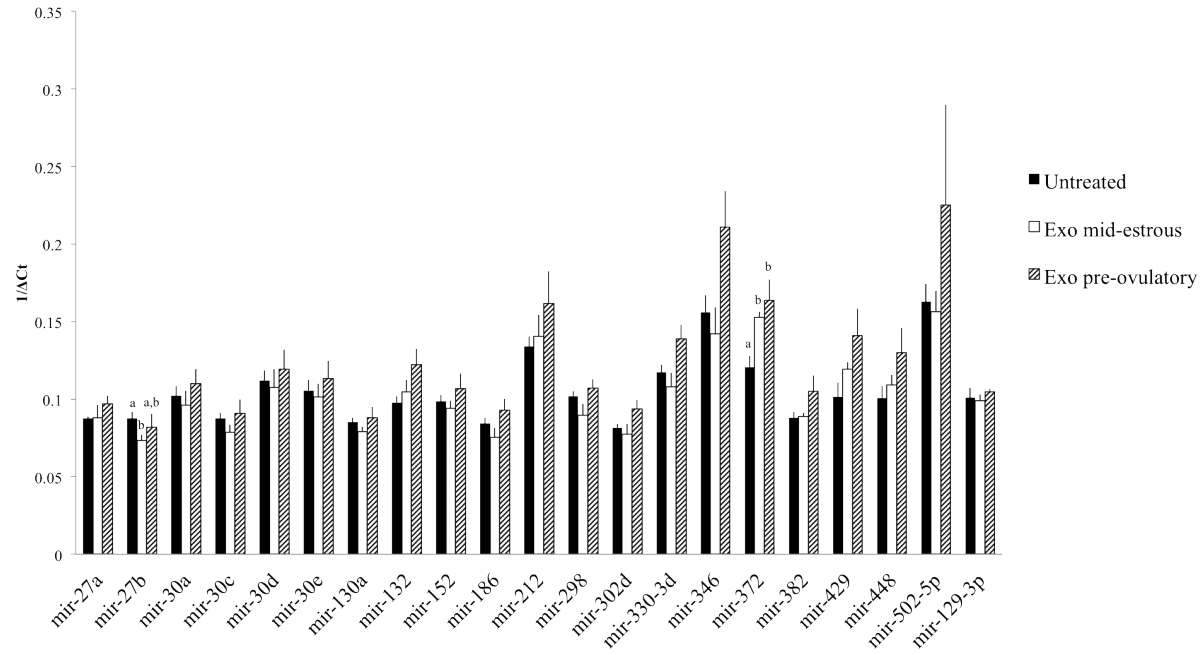
Supplemental material chapter 3: Exosome regulation of TGFβ signaling during follicle maturation.

Supplemental Table III.1: MicroRNAs target prediction analysis.

KEGG Pathway	Pathway ID	# of Genes (Union)	-ln(p-value) (Union)
Axon guidance	hsa04360	64	21.78
MAPK signaling pathway	hsa04010	109	21.36
Adherens junction	hsa04520	41	17.55
Regulation of actin cytoskeleton	hsa04810	89	16.71
Focal adhesion	hsa04510	83	15.8
Colorectal cancer	hsa05210	45	15.64
Chronic myeloid leukemia	hsa05220	41	15.13
Glioma	hsa05214	36	15.04
Oxidative phosphorylation	hsa00190	7	14.93
Renal cell carcinoma	hsa05211	37	13.52
Non-small cell lung cancer	hsa05223	31	13.17
TGF-beta signaling pathway	hsa04350	44	12.44
ErbB signaling pathway	hsa04012	43	12.17
Wnt signaling pathway	hsa04310	63	11.9



Supplemental Figure III.1: Relative levels of miRNAs predicted to regulate *ACVR1* and *ID2* in granulosa cells collected from equine ovarian follicles. Values on y-axis indicate normalized (Δ) Ct values relative to miR-99b. * = $P < 0.05$



Supplemental Figure III.2: Relative levels of miRNAs predicted to regulate *ACVR1* and *ID2* in granulosa cells after exposure to exosomes isolated from follicular fluid of mid-estrus and pre-ovulatory follicles. Values on y-axis indicate normalized (Δ) Ct values relative to miR-99b.

Exo mid-estrous = exosomes isolated from follicular fluid of mid-estrous follicles

Exo pre-ovulatory = exosomes isolated from follicular fluid of pre-ovulatory follicles

Different letters $P < 0.05$, same letters $P > 0.05$