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Bovine ovarian hyperstimulation induced changes in expression profile of circulatory miRNA in follicular fluid and blood plasma

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Dedicated to my family and wife; could always count on for love and supports in all my educational stages.

Abstract

Circulatory noncoding small RNAs (miRNAs), which are present in various body fluids, are reported to be potentially used as biomarkers for disease and pregnancy. The present study was conducted to investigate the effect of ovarian hyperstimulation on the expression pattern of circulatory miRNA in follicular fluid and blood plasma. For this, Simmental heifers (n=12) were synchronized using a standard synchronization protocol and six of them were hyperstimulated using FSH. Following this, whole blood samples were collected at day 0 (onset of oestrous), 3 and 7, follicular fluid samples were aspirated from dominant follicles at the day 0 from all animals by ovum pickup. Total RNA including miRNA was isolated from plasma samples of both groups at day 7 and follicular fluid at day 0. Subsequent expression profiling of miRNA was performed using the human miRCURY LNA™ Universal RT miRNA PCR array platform with 745 miRNA primer assays. Of the 24 miRNAs, which were differentially expressed in blood plasma between hyperstimulated and unstimulated animals, 9 miRNAs including miR-127-3p, miR-494, miR-147, miR-134 and miR-153 were down regulated and 15 miRNAs including miR-34a, miR-103, let-7g, miR-221 were found to be up regulated in the hyperstimulated animals. Similarly, 66 miRNAs were found to be differentially expressed in follicular fluid derived from hyperstimulated and unstimulated groups. Out of these, while 32 miRNAs were down regulated, 34 were up regulated in follicular fluid aspirated from hyperstimulated animals. Ingenuity pathway analysis (IPA) of potential target genes of candidate miRNAs, which are dysregulated due to ovarian hyperstimulation, revealed axonal guidance signaling and Wnt ß-catenin signaling pathways to be the dominant ones. In conclusion, this study revealed ovarian hyperstimulation resulted in changes in expression profile of circulatory miRNA in blood and follicular fluid.

Zusammenfassung

Zirkulierende nicht-kodierende micro RNAs (miRNAs), die in verschiedenen Körperflüssigkeiten vorhanden sind, sind möglicherweise potenzielle Biomarker für Krankheiten und Trächtigkeit. Die vorliegende Studie wurde durchgeführt, um die Wirkung einer ovarialen Überstimulation auf das Expressionsmuster von zirkulierenden miRNAs in der Follikelflüssigkeit und im Blutplasma zu untersuchen. Dazu wurden Fleckvieh-Färsen (n=12) mit einem Standard-Synchronisationsprotokoll synchronisiert und sechs von ihnen mit FSH überstimuliert. Die Probenentnahme beinhaltete Blutproben zum Zeitpunkt 0 (Beginn der Brunst), am 3. und am 7. Tag sowie die Follikelflüssigkeit von dominanten Follikel am Tag 0 von allen Tieren durch „Ovum pickup". Die Gesamt-RNA inklusive der miRNAs wurde aus den Plasmaproben von beiden Gruppen an Tag 7 und aus der Follikelflüssigkeit am Tag 0 isoliert. Das nachfolgende Expressionprofiling der miRNAs erfolgte unter Verwendung der Humanen miRCURY LNA ™ Universal-RT-PCR- miRNA-Array-Plattform mit 745 miRNA Primer-Assays. Von den 24 miRNAs, die im Blutplasma beim Vergleich zwischen hyperstimulierten und unstimulierten Tieren unterschiedlich exprimiert waren, zeigten 9 miRNAs, einschließlich miR-127-3p, miR-494, miR-147, miR-134 und miR-153, eine Runterregulation, während 15 miRNAs einschließlich miR-34a, miR-103 , let-7g, miR-221 eine erhöhte Expression in den hyperstimulierten Tieren aufwiesen. Des Weiteren, konnten beim Vergleich der Follikelflüssigkeit von hyperstimulierten und unstimulierten Tieren 66 differentiell exprimierte miRNAs identifiziert werden. Von diesen waren 32 miRNAs herunterreguliert, während 34 in der Gruppe der hyperstimulierten Tiere raufreguliert waren. Eine Ingenuity Pathway Analyse (IPA) der potentiellen Zielgene von Kandidaten miRNAs, die aufgrund von Überstimulation der Ovarien eine Fehlregulation zeigten, ergab als dominante Signalwege die axonale Führung sowie Wnt-ß-Catenin. Die Ergebnisse dieser Studie zeigte, dass eine Überstimulation der Ovarien zu Veränderungen im Expressionsprofil von zirkulierenden miRNAs im Blut und in der Follikelflüssigkeit führte.

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List of abbreviations

Abs	Apoptotic bodies
Acc. No	Gene bank accession number
aRNA	Amplified ribonucleic acid
Ago	Argonaute protein
AI	Artificial insemination
ART	Assisted reproductive technology
AMH	Anti-Müller-Hormon
ATP	Adenosine tri phosphate
BLAST	Basic local alignment search
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Bta	Bos taurus
CD	Cluster of differentiation protein
cDNA	complementary deoxy ribonucleic acid
CL	Corpus luteum
COCs	Cumulus oocyte complex
cRNA	Complementary ribonucleic acid
Ct	threshold cycle
COH	Controlled ovarian hyperstimulation
Cx	Connexin
CXCL	Chemokine (C-X-C Motif) ligand
Dcp	Dipeptidyl carboxypeptidase
ddH2O	Deionised and demineralised millipore water
DE	Differentially expressed
DEPC	Diethylpyrocarbonate
DGCR8	DiGeorge syndrome critical region gene 8
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECs	Endothelial cells

EDTA	Ethylenediaminetetraacetic acid
ES	Embryonic stem cell
Exo	Exosome
FF	Follicular fluid
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulosa cell
GH	Growth hormone
GVBD	Germinal vesicle break down
hr	Hour
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HGF	Hepatocyte growth factor
ICM	Inner cell mass
ILV	Intraluminal vesicles
IPA	Ingenuity pathway analysis
IVF	In vitro fertilization
IVM	In vitro maturation
LOS	Large Offspring Syndrome
LH	Luteinizing hormone
MI	Metaphase I
MII	Metaphase II
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
mg	Milligrams
MHC	Major histocompatibility class
min	Minute
miRNA	Micro RNA
MPs	Micro particles
MOET	Multiple ovulation and embryo transfer
mRNA	Messenger ribonucleic acid
MVBs	Multi vascular bodies

NCBI	National center for biotechnological information
nt	Nucleotides
OCS	Oestrus cow serum
OPU	Ovum pick up
P4	Progesterone
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGF2α	Prostaglandin F2α
POF	Premature ovarian failure
Pre-miRNA	Precursor micro RNA
Pri-miRNA	Primary micro RNA
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RNAi	RNA interference
rpm	Revolution per minute
rRNA	Ribosomal RNA
SCNT	Somatic cell nuclear transfer
sec	Second
siRNA	Small interfering RNA
SMCs	Smooth muscle cells
SSC	Sodium chloride sodium citrate
TC	Theca cell
TCM	Tissue culture media
TE	Trophectoderm
TGF-ß	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNRC	Trinucleotide Repeat Containing
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Vps	Vacuolar protein sorting
ZP	Zona pelluciada
ZGA	Zygotic genome activation
°C	Degree centigrade

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1 Introduction

Controlled ovarian hyperstimulation (COH) stimulates the ovaries by supraphysiological levels of gonadotropins to growth multiple follicle in single ovulation species including humans and bovine. In bovine, in order to expand the number of offspring during the lifetime of an individual, female animals with high genetic value are typically stimulated with gonadotropins hormones to persuade the ovulation of a variable number of follicles. Similarly, women undergoing IVF treatment undergo COH to induce the development of multiple dominant follicles.

For several years, the most common use of embryo transfer has benefited from the establishment of newer, more efficient methods for the superovulation of donors, embryo retrieval/transfer by low-invasive methods as well as by the better, simpler and more effective cryopreservation methods in human and bovine. However, despite significant advances in assisted reproduction technology protocols in humans and bovine, pregnancy rates are still relatively low and have not increased significantly in the last decade (Andersen et al. 2007). On another hand an increase in chromosomal abnormalities has been reported in human embryos after conventional stimulation, mainly resulting from an increased incidence of chromosome segregation errors during the first embryonic divisions, and abnormalities were lowest in embryos which divided within the expected physiological time frame (Baart et al. 2009). The changes in the oviduct and uterine environment related to hormonal treatments and subsequent influence on the transcriptome profile of embryos have been investigated (Gad et al. 2011). The potential implications of circulating progesterone on the composition of oviduct and uterine fluid are interesting but data on the composition of these fluids are sparse (Hugentobler et al. 2007, Hugentobler et al. 2008). It is well known that controlled ovarian hyperstimulation leads to deviant oocyte maturation in the follicle (Hyttel et al. 1989) as well as an abnormal endocrine environment in the reproductive tract.

Follicle development in the ovary consists of the growth of competent follicles, the division of granulosa cells, the expansion of the follicular basal lamina, and finally, the formation of the follicular fluid in the follicular antrum (Fortune 1994). Follicular fluid consists of secretions from the granulosa and thecal cells combined with plasma components that cross the blood-follicular barrier via the thecal capillaries (Rodgers and Irving-Rodgers 2010). This fluid provides a very important microenvironment and contains regulatory molecules that are important for the maturation and quality of the oocytes (Revelli et al. 2009). Certain lipids, proteins, vitamins, and metabolites in the follicular fluid have been found to be associated with reproductive diseases (Kim et al. 2006), oocyte quality (Berker et al. 2009), embryo quality, and the outcome of in vitro fertilization attempts (Wallace et al. 2012, Wu et al. 2007).

Follicular development, a coordinated and bidirectional communication mediated by a large number of gene products and the expression of these genes must be meticulous and well timed. Gene expression is a highly complex process controlled at transcriptional and translational levels. MicroRNAs are noncoding small RNAs which are known to play roles in posttranscriptional regulation of genes involved in various physiological processes in female reproduction. MicroRNAs are endogenous small noncoding RNAs that regulate the activities of target mRNAs by binding at sites in the 3' untranslated region of the mRNAs (Ambros 2001, Bartel 2004).Many studies showed the role of miRNAs in follicular development, oocyte maturation, peri-implantation and pre-implantation periods (Lei et al. 2010, Mondou et al. 2012) by regulating gene expression at the transcriptional level. Currently 783 bovine miRNAs are registered (miRBase 2013) and more than 3000 validated targets (miRecords 2013).

Circulating RNAs have been shown to be useful biomarkers for multiple clinical endpoints including the diagnosis of preeclampsia (Freeman et al. 2008), the diagnosis and monitoring of diabetic retinopathy (Shalchi et al. 2008) and neuropathy (Sandhu et al. 2008), and as diagnostic or prognostic markers for multiple cancers (Dasi et al. 2001, Kopreski et al. 1999, Mitchell et al. 2008, Rabinowits et al. 2009, Taylor and Gercel-Taylor 2008). Cellular release of miRNA molecules into the circulation has been shown to occur through multiple mechanisms. Among passive processes, the release of cellular

miRNA has been shown following necrotic cell death (Rainer et al. 2004). Among active processes, miRNA molecules have been identified coupled by exosomes (Valadi et al. 2007), high-density lipoprotein (HDL) (Vickers et al. 2011), Ago2 complex (Arroyo et al. 2011), shedding vesicles (Cocucci et al. 2009) and apoptotic blebs (Halicka et al. 2000). The function of exosomes, HDL and shedding vesicles are believed to be cell-to-cell communication and can be used as platforms for multi signalling processes (Cocucci et al. 2009, Halicka et al. 2000, Vickers et al. 2011).

To our knowledge, effects of hormonal treatment on circulating miRNA profile have not been investigated yet therefore this study is novel study to find a key tophysiological changes induced by controlled ovarian hyperstimulation in steroid hormones target tissues. Also in this work, we addressed questions regarding, possible origin and biological function of circulating extracellular miRNA in bovine follicular fluid and blood plasma. Taking all promising information together, this experiment has been conducted to find effects of ovarian hyperstimulation on the expression profile of circulatory miRNAs in follicular fluid and blood plasma, investigation of temporal circulatory miRNA expression during oestrus days and characterization plasma and follicular fluid circulatory miRNA transportation with exosomes or the Ago2 complex.

2 Literature review

2.1 Folliculogenesis

The mammalian ovary is an extremely dynamic organ with sequential waves of follicular growth and regression, rupture of mature follicles at the adjacent ovarian wall during ovulation, repair of the ovulation wound and the formation of fully functional corpora lutea followed by its demise a few days later. This occurs within relatively short cycles and under tight hormonal regulation throughout a female's reproductive life (Donadeu et al. 2012). Folliculogenesis is the biological developmental process in which an activated primordial follicle develops to be a preovulatory follicle following the growth and differentiation of the oocyte and its surrounding granulosa cells (Figure 2.1) (Gougeon 1996, Knight and Glister 2001). During folliculogenesis, a follicle may be classified as primordial, primary, secondary or tertiary (Pedersen and Peters 1968). The large population of non-growing primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life (Eppig 2001). When granulosa cells surrounding the oocytes change in shape from flattened to cuboidal and the oocyte begin its extensive growth phase, the primordial follicles are transformed into primary follicles (van Wezel and Rodgers 1996).

The initial stages of folliculogenesis occur independently of gonadotrophic hormones. Antral follicles initially become responsive to and then dependent on FSH. There are continual transient increases in FSH in cattle during the oestrous cycle and anoestrus which cause the recurrent emergence and development of cohorts of follicles (Roche 1996). The transition to antral follicle is characterized by formation of antrum which is a fluid filled cavity surrounded by multiple layer of granulosa cell; this fluid contain important regulatory substance to be delivered to oocyte (Braw-Tal and Yossefi 1997, Eppig 2001). After antrum formation, granulosa cells are divided into two distinct subtypes, the cumulus granulosa surrounding and in intimate metabolic contact with the oocyte forming a structure called cumulus oocyte complex (COC) and the mural granulosa lining the follicle wall adjacent to the basal lamina. The mural granulosa cells nearest the antrum are called periantral granulosa cells (Eppig 2001).

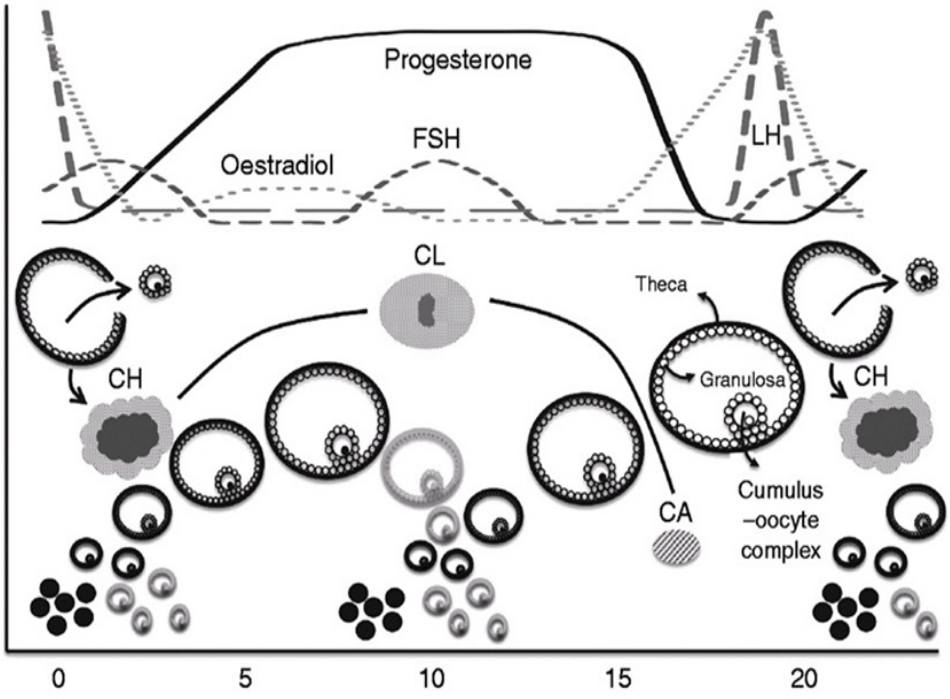

Figure 2.1 Schematic representation of ovarian events and associated changes in circulating hormone levels during an ovulatory cycle as exemplified by the bovine (Donadeu et al. 2012).

Finally, in response to preovulatory gonadotrophins surge, two marked events occur in cumulus oocyte complex:

A. The fully grown oocyte resume meiosis to complete first meiotic division via germinal vesicle break down, chromatin re-condensation, the pairs of homologous chromosomes are separated and half of them are expelled forming the first polar body and the mature oocyte is arrested again in metaphase of second meiotic division B. The cumulus cell surrounding the oocyte undergo expansion or mucification through secretion of hyaluronic acid, a nonsulphated glycosaminoglycan bound to the cumulus cells by linker proteins (Buccione et al. 1990, Chen et al. 1996, Eppig 1979, Salustri et al. 1989) and when the hyaluronic acid becomes hydrated, the spaces between cumulus cells become enlarged and the cells become embedded in a sticky mucified matrix. If cumulus expansion is suppressed, ovulation rate is greatly reduced (Chen et al. 1993).

After ovulation, the rest of follicle developed into corpus luteum which is a highly vascularised endocrine organ producing progesterone hormone which is essential for the maintenance of pregnancy.

2.2 Follicular fluid

Follicular fluid provides a very important microenvironment for the development of oocytes. Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of granulosa and thecal cells (Fortune 1994). It is reasonable to hypothesise that some biochemical characteristics of the follicular fluid circumfluenting the oocyte may play a critical role in oocyte quality index and the subsequent potential to achieve fertilization and embryo development. The analysis of follicular fluid components may also provide information on metabolic changes in blood serum, as the circulating biochemical milieu may be reflected in the composition of follicular fluid (Leroy et al. 2004). Follicular fluid is easily available as it is aspirated together with the oocyte at the time of oocyte pick up. In the last years, the studies of follicular fluid contents has been conducted by assaying one or more substances in the fluid derived from individual follicles and by relating it to the fate of the oocyte from that specific follicle (Revelli et al. 2009). In fact, the composition of follicular fluid is similar to serum with respect to low-molecular-weight components, with most electrolytes being at the same concentrations in fluid and serum (Gosden et al. 1988, Rodgers and Irving-Rodgers 2010, Shalgi et al. 1973).

2.3 Follicular fluid content and oocyte quality

The rate at which the follicular antrum expands and follicular fluid accumulates differs between follicles, particularly between dominant and subordinate follicles (Beg et al. 2001, Fortune et al. 1991). The proportion of a follicle that is follicular fluid at maximum size also varies from species to species. Generally, larger species such as ovine, equine, porcine, human, and bovine have larger follicles, with the fluid comprising a substantial proportion of the volume of the follicles at ovulation

(estimated at >95% in bovine) (Beg et al. 2001, Rodgers and Irving-Rodgers 2010). Smaller species such as rats and mice have smaller follicles with fractionally less follicular fluid (Rodgers and Irving-Rodgers 2010). A clear correspondence between specific follicular fluid biochemical characteristics and measurable oocyte quality linked, embryo-related variables has not been established to date. In the last years, the research in this area has progressed toward a more complex type of molecular analysis, metabolomics, which is the analysis of all substances contained in a biological fluid (Figure 2.2). The chemical constituents of follicular fluid have been grouped in the following categories: 1) hormones; 2) growth factors of the Transforming Growth Factor-beta superfamily; 3) other growth factors and interleukins; 4) reactive oxygen species; 5) anti-apoptotic factors; 6) proteins, peptides and amino-acids; 7) sugars; 8) prostanoids.

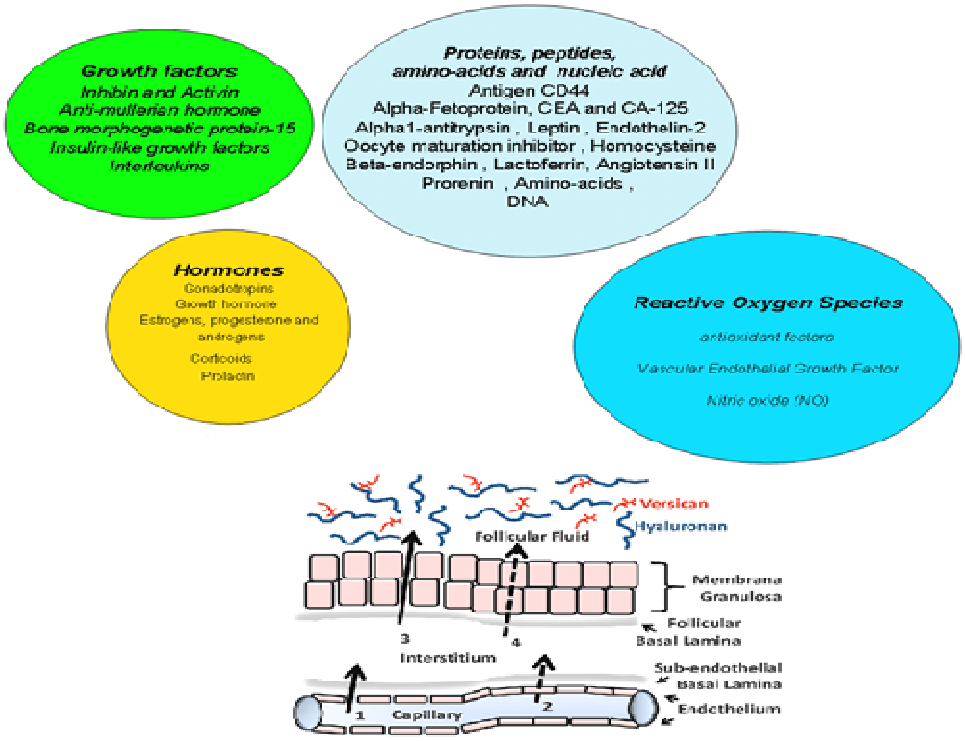

Figure 2.2 Drawing illustrating the routes fluid can take from the thecal capillary to the follicular fluid and the potential barriers of the endothelium, subendothelial basal lamina, interstitium, follicular basal lamina, and membrana granulosa. Routes 1 and 3 show movement of fluid between the cells (solid arrows), and routes 2 and 4 show transcellular routes (hatched arrows) that either involve aquaporins or transcytosis and follicular fluid biochemical modified from (Rodgers and Irving-Rodgers 2010).

2.3.1 Gonadotrophins

It becomes evident that gonadotropins play an important role in the secretion of several substances by granulosa cells (e.g. hyaluronic acid), in turn affecting oocyte development and maturation. They may also act synergistically with estradiol (E2) in enhancing cytoplasmic maturation of the oocyte and, via cyclic AMP (cAMP) secretion, control oocyte meiosis: higher levels of gonadotropins would improve these processes and lead to better oocytes, better embryos and improved pregnancy rate (Revelli et al. 2009). Notably, GH seems to play a similar (and may be synergic with gonadotropins) role (Mendoza et al. 2002). Intrafollicular titers of FSH and LH are affected by their circulating levels: in IVF cycles, the serum levels are determined by the amount of exogenously administered gonadotrophins and by the degree of pituitary suppression (relevantly reducing the endogenous gonadotropin secretion). High follicular fluid titers of FSH (Suchanek et al. 1988), hCG (Ellsworth et al. 1984), and LH (Cha et al. 1986) have been reported to promote oocyte maturation and to be associated with oocyte having a high chance of fertilization (Revelli et al. 2009). These data was confirmed by immunohistochemistry studies that stained granulosa cells for hCG: oocytes that subsequently had fertilized had, in fact, had significantly more granulosa cells immunobound to hCG than non-fertilizable oocytes (Enien et al. 1998). Follicular fluid LH was observed to be consistently higher in follicles containing oocytes that resulted in embryos leading to successful IVF attempts (Mendoza et al. 2002).

2.3.2 Steroid hormones

It is well known that a predominantly intrafollicular estrogenic environment is associated with good follicular growth and has anti-atresia effects(Revelli et al. 2009). In addition, estrogen enhances the cytoplasmic maturation of oocytes via a direct non-genomic action at the plasma membrane level, in turn inducing extracellular calcium influx into the cell and a specific pattern of Ca2+ oscillations (Tesarik and Mendoza 1997). Elevated estrogen and estrogen/progesterone ratio in follicular fluid indicate a more advanced stage of oocyte maturation and has been repeatedly found to be associated with a higher chance of achieving pregnancy (Botero-Ruiz et al. 1984, Lee et

al. 1987, Subramanian et al. 1988, Tarlatzis et al. 1985, Tarlatzis et al. 1993, Teissier et al. 2000). This observation, however, was not confirmed by other studies (Berger et al. 1987, Costa et al. 2004, Messinis and Templeton 1987, Rosenbusch et al. 1992, Suchanek et al. 1988) .

There is also conflicting evidence regarding the meaning of progesterone levels in follicular fluid. Several authors found that a high follicular fluid progesterone concentration (or a low E2/P ratio) was predictive of subsequent implantation and pregnancy (Basuray et al. 1988, Enien et al. 1995, Kobayashi et al. 1991, Vanluchene et al. 1991), and considered it as a reflection of progressive follicle luteinization and reduction of aromatase activity linked to the achievement of the final oocyte maturation. On the other side, however, oocytes from follicles having high follicular fluid P4 were frequently found in association with postmature oocytes that fertilized abnormally and gave rise to multipronuclear embryos (Ben-Rafael et al. 1987).

It seems that while an optimal exposure to progesterone has positive effects over oocyte characteristics, an excessive exposure leads to a rapid worsening of the cell's quality; a clear knowledge of the threshold at which P begins to damage the oocyte is presently lacking. Elevated follicular fluid androgen levels (testosterone) were associated with lower quality oocytes, and in particular with oocytes showing a trend toward lower cleavage rates after fertilization(Uehara et al. 1985). Estrogen/testosterone ratio was also reported to be higher in pregnancy-associated follicles (Andersen 1993, Xia and Younglai 2000). Taken together, these data seem to indicate that a low estrogen/androgen ratio in follicular fluid may be associated with early follicular atresia, which negatively affects the viability of the enclosed oocyte and limits the chances of fertilization and pregnancy. Indeed, the notion that a predominantly androgenic intrafollicular environment may lead to follicular atresia is well accepted, but at the same time it is widely accepted that a certain amount of intrafollicular androgens is needed to obtain optimal follicular growth(Revelli et al. 2009).

2.4 miRNAs in the embryo produced by assisted reproductive technologies

Assisted reproductive technology (ART) is the application of laboratory or clinical technology to gametes (oocyte or sperm) and/or embryos for the purposes of reproduction. These include artificial insemination (AI), embryo transfer (ET), embryo production in vitro (IVF), somatic cell nuclear transfer (SCNT and multiplication techniques (cloning) for the application of transgenesis animals. The female contribution to genetic progress was increased with the advent of embryo transfer and the associated techniques such as non-invasive surgery embryo collection, in vitro maturation, fertilization and culturing of bovine oocytes. Dams can be super-ovulated, artificial insemination and later the resulted embryos are collected by flushing. These embryos are then implanted in to synchronized heifers. This technique is referred as multiple ovulation and embryo transfer (MOET). Establishment of more efficient methods for the bovine ovarian hyperstimulation of donors, embryo retrieval／transfer by low-invasive methods as well as by the better, simpler and more effective cryopreservation methods made public for embryo transfer (Rodriguez-Martinez 2012).

The IVF and SCNT technology has opened the possibilities to manipulate and cultivate the embryo. However, it has also been linked to many abnormalities in embryo development. Well evident abnormalities in foetuses or calves following transfer of in vitro cultivated embryos includes lower pregnancy rate, increased abortion, oversized calves (Allen et al. 2006, Hashimoto et al. 2013, Schurmann et al. 2006, Shevell et al. 2005), musculoskeletal deformities and abnormalities of placental development, which are often described as “Large Offspring Syndrome” (LOS). LOS has been described for bovine (Farin et al. 2006), sheep (Sinclair et al. 1999) and mice et (Eggan et al. 2001). The abnormalities associated to the IVF, SCNT and in vitro culture of embryo are in principle due to aberrant or alteration of transcriptional activity at sub-cellular level (Schurmann et al. 2006). Several lines of evidence in mouse and cattle indicate that expression patterns of genes from in vitro-produced embryos are not necessarily representative of those of in vivo embryos (Niemann and Wrenzycki 2000). Connexin-43 , crucial for maintenance of compaction , has been found to be expressed by in vivo-derived bovine blastocysts, but not in their in vitro-produced counterparts (Niemann and

Wrenzycki 2000). The bovine leukemia inhibitory factor (bLIF) and LIF-receptor-R (LR-8) genes were found to be expressed by in vitro produced embryos, but not in their in vivo counterparts. The heat shock protein gene 70.1 (Hsp70.1) has been found upregulated by blastocysts produced in vitro compared to in vivo embryos, while the glucose transporter-l mRNA (Glut-l) is downregulated by morulae produced in vitro as compared to in vivo-derived morulae (Niemann and Wrenzycki 2000).

Candidate gene studies reveal that the failure of implantation may be due to aberrant expression of genes in the preimplantation cloned embryo, which are crucial for the early regulation and differentiation of the placenta (Hall et al. 2005). At the cellular level, a higher incidence of apoptosis (Park et al. 2004a) and aberrant allocation of inner cell mass (ICM) (Koo et al. 2002) is evident. At the sub-cellular level, aberrant DNA methylation patterns (Bourc'his et al. 2001) and the dysregulation of genes occurs (Humpherys et al. 2002). These abnormalities are thought to mainly be due to epigenetic defects (changes in chromatin structure, not involving a change in DNA base sequence) which occur during cell reprogramming, where the donor cell DNA is reprogrammed by the oocyte cytoplasm to an embryonic state (Schurmann et al. 2006).
Recent global gene expression profiling study has also evidenced aberrant regulation of gene expression either by genetic or epigenetic modification due to manipulation and culture of preimplantation embryos (Aston et al. 2009, Gad et al. 2011, Zhou et al. 2008). According to the nature and extent of regulatory mechanisms it could be considered that miRNAs are playing pivotal roles in such aberrant transcriptional processes. Since, miRNA has been appeared as first and foremost epigenomic tool or modifier that regulate gene expression epigenetically at the post-transcriptional or transcriptional level and were found to play important roles during mammalian development (Ambros and Lee 2004, Bartel 2004, Kloosterman and Plasterk 2006). They were found to be targeted by epigenetic modification and eventually controlling epigenetics and some imprinted miRNAs were found to undergo subsequent epigenetic reprogramming in mouse embryos (Cui et al. 2009, Kircher et al. 2008, Williams et al. 2007). Among them, one has revealed the disregulated expression of several miRNAs in bovine cloned elongated embryos using a heterologous microarray (Castro et al. 2010). miRNAs expression profiling in elongated cloned and in vitro-fertilized bovine embryos has suggested that the different state of reprogramming of miRNAs occurred in cloned

bovine elongated embryos (Castro et al. 2010). Among the most notable downregulated miRNAs found in their study were miR-30d and miR-26a. Both of these miRNAs interacted with TKDP, which is involved in maternal recognition of pregnancy in cattle (Lagos-Quintana et al. 2001).

2.5 Circulating miRNA in the biological fluids

Cellular release of RNA molecules into the biological fluids circulation has been demonstrated to occur through multiple mechanisms (Figure 2.3). Among passive processes, the release of cellular messenger RNA (mRNA) and miRNA has been shown following necrotic cell death (Melkonyan et al. 2008, Rainer et al. 2004). After first reports of extracellular RNA published in 1970s (Kamm and Smith 1972, Stroun et al. 1977), most investigators doubted that extracellular RNA could survive in the blood because of the presence of potent ribonucleases (El-Hefnawy et al. 2004). Though, more recent studies have published the presence of circulating extracellular RNA in plasma or serum, and it has also been demonesterd that this RNA is protected from plasma RNase activity (El-Hefnawy et al. 2004, Tsui et al. 2002). Circulating RNAs have been shown to be useful biomarkers for multiple clinical endpoints including mortality in acute trauma patients (Rainer et al. 2004), the diagnosis of preeclampsia (Freeman et al. 2008), the diagnosis and monitoring of diabetic retinopathy and neuropathy (Shalchi et al. 2008) and as diagnostic or prognostic markers for multiple cancers (Dasi et al. 2001, Kopreski et al. 1999, Mitchell et al. 2008, Rabinowits et al. 2009, Taylor and Gercel-Taylor 2008).

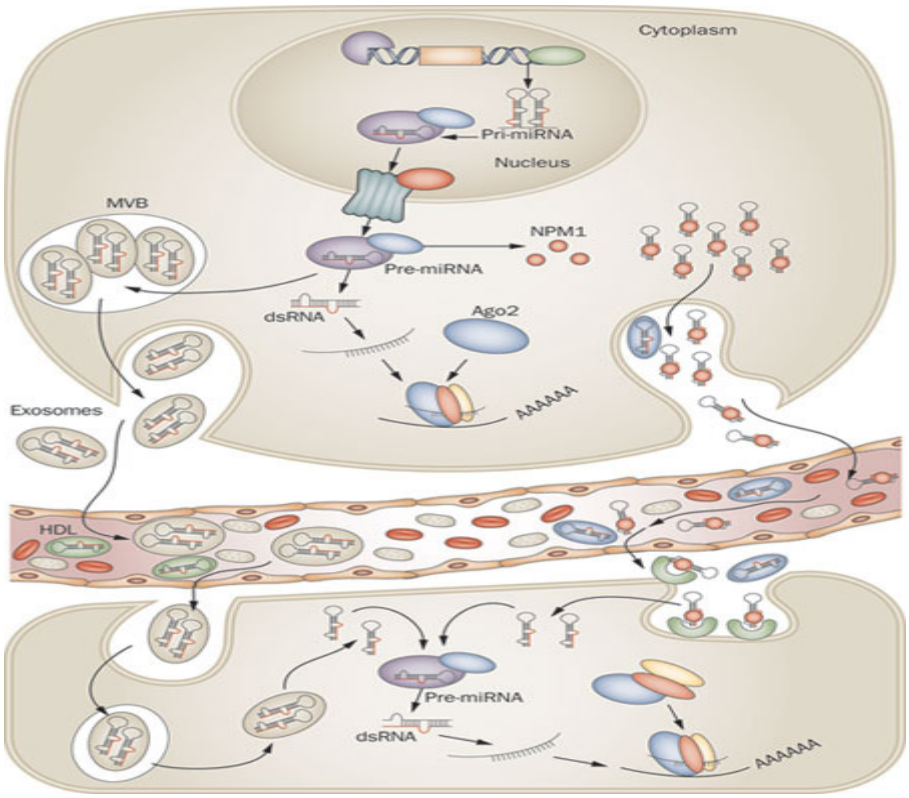

Figure 2.3 After being transcribed in the nucleus, pre-miRNA molecules can be processed further by Dicer in the cytoplasm. In addition, based on recent findings (Arroyo et al. 2011; Kosaka et al. 2010; Pigati et al. 2010; Valadi et al. 2007; Vickers et al. 2011; Wang et al. 2010), there are at least two ways that pre-miRNAs can be packaged and transported using exosomes and MVBs or other (not fully explored) pathways together with RNA-binding proteins. After fusion with the plasma membrane, MVBs release exosomes into the circulating compartments and bloodstream. Likewise, pre-miRNA inside the donor cell can be stably exported in conjunction with RNA-binding proteins, such as NPM1 (Wang et al. 2010) and Ago2 (Arroyo et al. 2011) or by HDL (Vickers et al. 2011). Circulating miRNAs enter the bloodstream and are taken up by the recipient cells by endocytosis or, hypothetically, by binding to receptors present at the recipient cellular membrane capable of recognizing RNA-binding proteins. More studies are necessary to elucidate how miRNAs are loaded into exosomes and how they can be internalized by recipient cells. Exosomal miRNAs are processed by the same machinery used in miRNA biogenesis and thus have widespread consequences within the cell by inhibiting the expression of target protein-coding genes (Cortez et al. 2011).

2.6 Characterization of miRNAs in biological fluids

Serum and plasma contain a large number of miRNAs, serum miRNAs remained stable after being subjected to severe conditions, such as boiling, very low or high pH, extended storage, and 10 freeze-thaw cycles, conditions that would normally degrade most RNAs (Chen et al. 2008). Many theories have attempted to explain the possible biological mechanisms by which RNA is preserved from plasma RNase activity digestion. One theory explained that RNAs may anneal with DNA, which would render them resistant to both RNase and DNase activity (Sisco 2001). Though, evidence showed that RNA present in plasma is protected from degradation not by binding to DNA, but probably by inclusion in lipid or lipoprotein complexes (El-Hefnawy et al. 2004).

The RNA molecules enter the circulation are either associated with cellular debris or in naked form (Rainer et al. 2004). Among active processes, mRNA and miRNA molecules have been identified (Figure 2.4) within membrane-encapsulated vesicles released by cells. These include shedding vesicles (Cocucci et al. 2009), exosomes (Valadi et al. 2007), high density lipoprotein (HDL) (Vickers et al. 2011) and apoptotic blebs (Halicka et al. 2000). Exosomes are small vesicles (40-100 nm) that are formed by inward budding of endosomal membranes (Pan and Johnstone 1983). The vesicles are packaged within larger intracellular multivesicular bodies that release their contents to the extracellular environment through exocytosis. Shedding vesicles (<200 nm) are released from live cells through direct budding from the plasma membrane (Cocucci et al. 2009), whereas apoptotic blebs (100 to >1,000 nm) bud directly from the plasma membrane upon cell death (Simpson et al. 2009). After release from the cell, exosomes, shedding vesicles, and apoptotic blebs circulate in the extracellular space, where most are broken down within minutes due to the display of phosphatidylserine on the external side of the membrane (Cocucci et al. 2009, Fadok et al. 1992). A fraction of the vesicles moves by diffusion into the circulation and appear in biological fluids. The function of exosomes and shedding vesicles are believed to be cell-to-cell communication and platforms for multisignaling processes (Cocucci et al. 2009, Simpson et al. 2009). Although exosomes and shedding vesicles are released in healthy

individuals, many pathological conditions and cellular perturbations stimulate further release of the particles (Cocucci et al. 2009, Simpson et al. 2009).

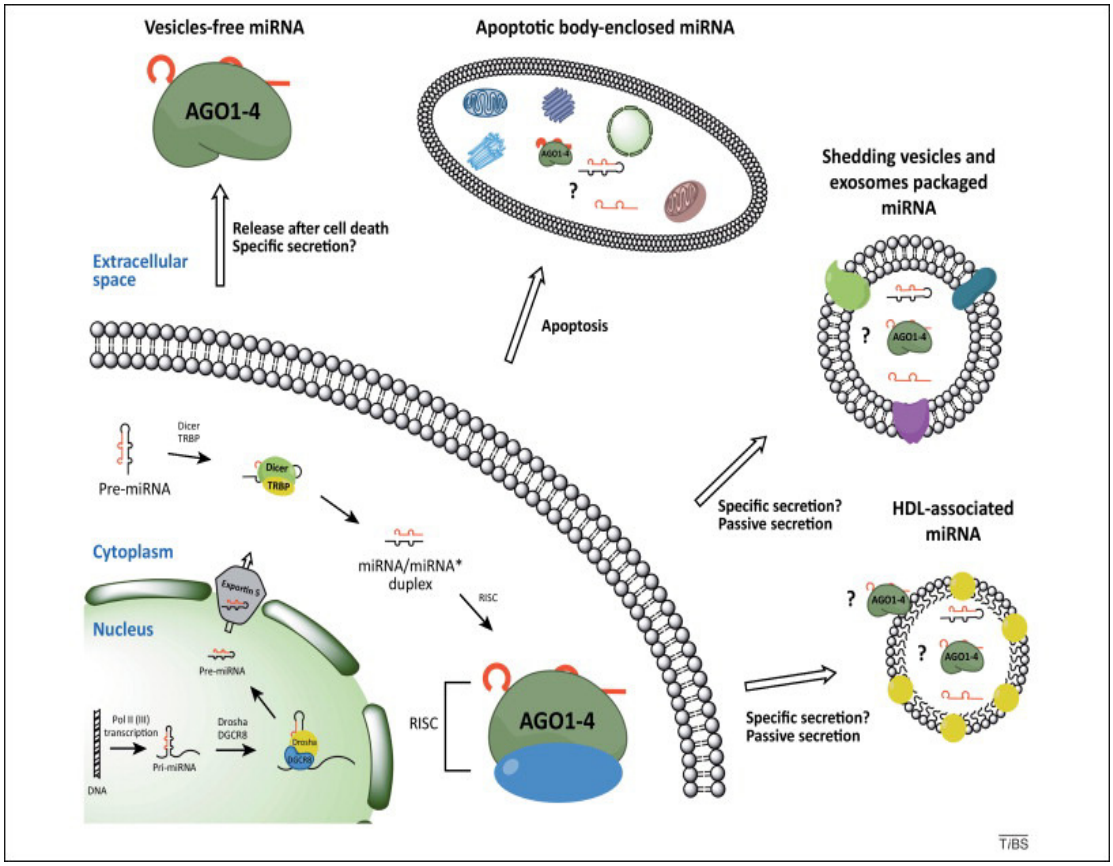

Figure 2.4 The three types of membranous vesicles that contain extracellular miRNA are apoptotic bodies, shedding vesicles, and exosomes (Hunter et al. 2008, Valadi et al. 2007, Zernecke et al. 2009). Besides these, extracellular miRNA can also be vesicle free and associated with either AGO proteins alone or be incorporated into HDL particles (Arroyo et al. 2011, Turchinovich et al. 2011, Vickers et al. 2011). Apoptotic bodies can contain various cellular organelles including mitochondria and nuclei. Shedding vesicles and exosomes both belong to the class of microvesicles and are limited by a lipid bilayer. It remains unknown whether microvesicles can also contain pre-miRNA or protein-free mature miRNA (question marks). It is also not clear whether miRNA is incorporated inside high-density lipoprotein particles or adsorbed on their surface (question marks)(Turchinovich et al. 2012).

Additionally, it has been hypothesised that miRNAs, mRNAs and proteins are transferred by exosomal signaling in the nervous system (Smalheiser 2007) and in embryonic stem cells microvesicles *in vitro* (Ratajczak et al. 2006, Yuan et al. 2009). Other authors demonstrated that miRNA contained in tumor exosomes is functional and can suppress the mRNA that encodes signal transduction components within T-cells (Taylor and Gercel-Taylor 2008). The same study reported an miRNA signature of circulating ovarian cancer exosomes that had a high correlation with primary tumor miRNA expression (Taylor and Gercel-Taylor 2008). They found, these miRNAs were identified at lower levels coupled with exosomes from women with benign disease and were not detected in normal controls. As well as, Rabinowits *et al*. demonstrated a significant difference in exosomal miRNA expression levels between patients with lung adenocarcinoma and patients without this disease (Rabinowits et al. 2009). Also, they showed a similarity in miRNA signatures between circulating exosomal miRNA and originating tumor cells (Rabinowits et al. 2009). In addition, exosomes released by glioblastoma cells containing mRNA, miRNA, and angiogenic proteins are taken up by normal recipient cells, such as brain microvascular endothelial cells.

Consistent with these observations, these results indicate that cancer patients present elevated levels of tumor-derived exosomes in plasma compared with controls. Although normal cells within the peripheral circulation can contribute to exosome population, the primary source of circulating exosomes in cancer patients is the tumor (Resnick et al. 2009). Messages delivered by tumor-derived exosomes are translated by recipient cells and stimulate proliferation of a human glioma cell line (Skog et al. 2008). Further, the tumor-specific epidermal growth factor receptor vIII was detected in serum exosomes from 7 of 25 glioblastoma patients (Skog et al. 2008). miR-21, known to be overexpressed in glioblastoma tumors (Chan et al. 2005), was elevated in serum microvesicles from glioblastoma patients. Moreover, mRNA and miRNA containing tumor-derived exosomes can affect biological processes inside of recipient cells. Still, little is known about the mechanisms in which miRNAs are generated in plasma and the biological impact of these molecules in distant sites of the body. Studies suggest that RNA molecules associated to specific types of exosomes can be released in the circulating compartment on fusion of multivesicular bodies (MVB) with the plasma membrane and may be internalized by recipient cells by endocytosis (Figure 2.5)

(Lotvall and Valadi 2007, Valadi et al. 2007). In addition, studies demonstrated the possibility to analyze miRNA expression using serum and plasma directly without any RNA extraction or serum filtration procedure (Chen et al. 2008). Therefore, lysed cells might contribute to the composition of miRNAs in the plasma. Nonetheless, additional studies are necessary to elucidate the mechanism in which miRNAs reach the bloodstream and the physiological impact of exosomal miRNA in global cellular processes.

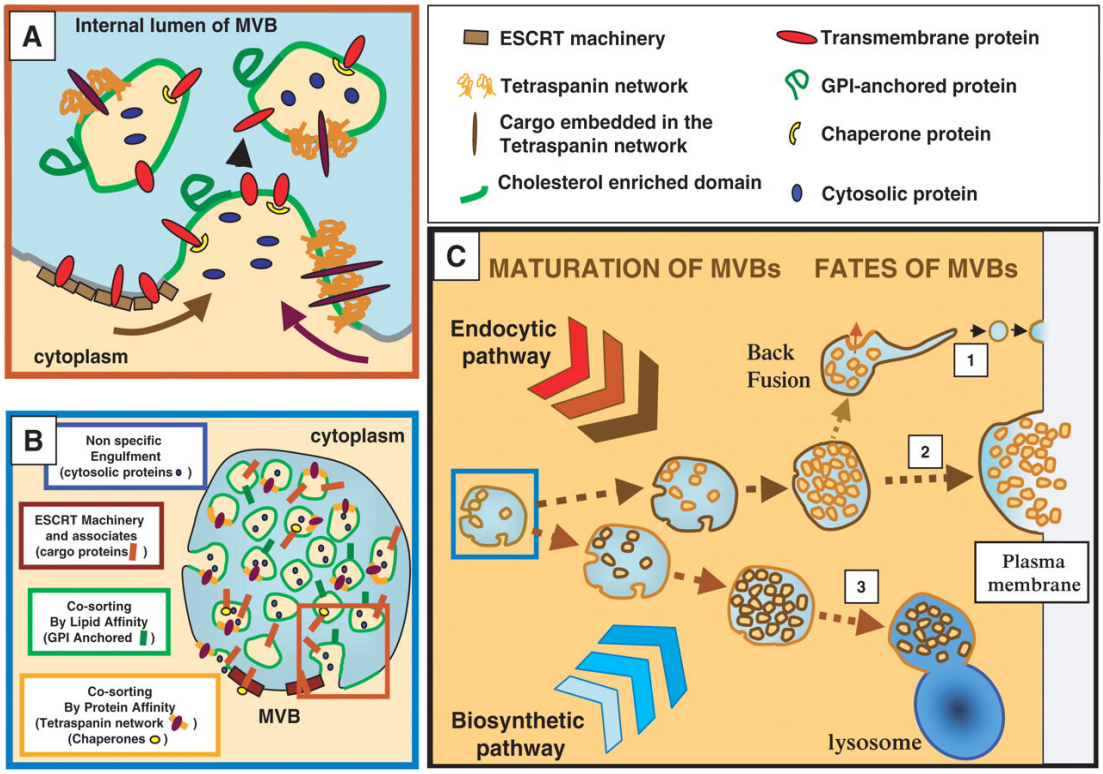

Figure 2.5 Biogenesis of exosomes; A: At the limiting membrane of MVBs, several mechanism act jointly to allow specific sorting of transmembrane, chaperones, membrane associated and cytosolic proteins on the forming ILVs. B: Presence of several sorting mechanisms may induce heterogeneity in the population of ILVs in single MVBs by acting separately on different domains of the limiting membrane. C: Receiving lipids and proteins from the endocytic and the biosynthetic pathway, different subpopulations of MVBs may be generated whose composition confers them different fate: (1) back fusion of the ILVs with the limiting membrane. During this process molecules previously sequestered on the ILVs are recycled to the limiting membrane and to the cytosol. Change in the composition of the limiting membrane may be

responsible for the tubulation allowing plasma membrane expression of endosomal proteins. (2) Unknown mechanism may lead MVBs toward the plasma membrane where proteins such as SNAREs and synaptogamins would allow their fusion and the consequent release of the ILVs in the extracellular medium as exosomes. (3) Similarly, the composition of the limiting membrane would preferentially induce fusion of MVBs with lysosomes leading to the degradation of the molecules sorted on ILVs (van Niel et al. 2006).

Although the presence of miRNAs in exosomes could explain their stability in serum, other possibilities include protection by chemical modifications or association with protein complexes. Nevertheless, there is a lack of an established endogenous miRNA control to normalize for plasma or serum miRNA levels measured by commonly used techniques as qRT-PCR. Usually, qRT-PCR data are normalized to an endogenous control gene, which is ideally stably expressed across the analyzed samples to reduce measurement errors, which may be due to technical variations (Davoren et al. 2008, Peltier and Latham 2008).

To date, there are very few reports of validated controls that are used to normalize miRNA levels measured in serum or plasma. U6 small nuclear RNA (RNU6B), a control commonly used to normalize miRNA qRT-PCR data (Corney et al. 2007, Shell et al. 2007) was found to be less stably expressed than miR-93, miR-106a, miR-17 – 5p, and miR-25 in serum (Peltier and Latham 2008). Moreover, another study reported that RNAU6B and 5S ribosomal RNA were degraded in serum samples (Chen et al. 2008). In another study to identify stable controls for normalization, (Resnick et al. 2009) identified 2 of 21 miRNAs (miR-142-3p and miR-16) from a previous expression profile study with cycle threshold (Ct) differences of four cycles or greater between ovarian cancer patients and healthy controls. In another study, a robust normalization protocol was identified by using synthetic versions of Caenorhabditis elegans miRNAs (Mitchell et al. 2008).

The exact process of how HDL is loaded with miRNAs and what proteins if any facilitate this association are not known. However, small RNAs (25 nucleotides) have previously been shown to complex with zwitterionic liposomes, specifically

phosphatidylcholine (Lu and Rhodes 2002). Previous biophysical studies indicate that HDL could simply bind to extracellular plasma miRNAs through divalent cation bridging. Interactions between DNA molecules and zwitterionic phosphatidylcholines resulted in conformational shifts in phosphatidyl- choline head groups and subsequent altered orientations of aliphatic chains, thus facilitating the incorporation of the DNA molecules into the protected space (Gromelski and Brezesinski 2006). In the case of HDL, this could lead to a tighter association with miRNAs, and could possibly shield bound miRNAs from external RNases (Mitchell et al. 2008).

2.7 Hormonal regulation of microRNA biogenesis

Steroid hormones are known to regulate target genes at both transcriptional and posttranscriptional levels when bound to specific nuclear receptors. MicroRNA biogenesis involves a nuclear processing event catalyzed by the microprocessor complex, which generates a short stem-loop structure known as a pre-miRNA from the primary miRNA (pri-miRNA) transcript. The core microprocessor complex consists of Drosha, an RNase III enzyme, and the DiGeorge syndrome critical region gene 8 protein (DGCR8). In addition, other proteins can associate with the microprocessor, including the DEAD box helicases p68 and p72 and a number of hnRNP proteins (Gregory et al. 2004). Biogenesis of some miRNAs is regulated at the level of microprocessor and/or Dicer processing, as shown by the identification of RNA-binding proteins such as Lin28, hnRNP A1, and KSRP that can regulate the production of specific pre- and mature miRNAs (Heo et al. 2008; Macias et al. 2009; Michlewski et al. 2008; Trabucchi et al. 2009). However knowledge, about the signaling cascades that regulate miRNA biogenesis is little, but interesting details are beginning to emerge as findings have demonstrated that the transforming growth factor beta (TGF-b) and bone morphogenetic protein (BMP) signalling pathways positively regulate the Drosha-mediated processing of miR-21, resulting in an induction of a contractile phenotype in human vascular smooth muscle cells (Davis et al. 2008). Pervious report of Kato laboratory has shown that the microprocessor-associated RNA helicases p68 and p72 are required for the processing of a subset of miRNAs (Fukuda et al. 2007).

Progesterone control of miRNA production appears to be essential for normal development of numerous tissues, including the female reproductive tract. Conditional inactivation of Dicer in the Müllerian duct resulted in infertile female mice with small oviducts and uterine horns (Cochrane et al. 2012; Gonzalez and Behringer 2009). The increase in Dicer and Exportin-5 coincides with an increase in uterine expression of miR-451 (Nothnick et al. 2010). Dicer1 is higher in severe versus mild endometriosis (Aghajanova and Giudice 2011; Cochrane et al. 2012), whereas Dicer is found to be decreased in aggressive breast cancers (Cochrane et al. 2010; Grelier et al. 2009) and ovarian cancers (Faggad et al. 2010; Pampalakis et al. 2009). Alterations of components of the miRNA biogenesis pathway by P4, suggests that P4 treatment could have a global impact on miRNA expression. Progesterone control of miRNA production miRNAs appear to be essential for normal development of numerous tissues, including the female reproductive tract.

In the other hand, the Kato lab described a specific example of how estrogens can indeed affect a posttranscriptional event by negatively regulating miRNA production (Yamagata et al. 2009). miRNA profiling result showed up regulation of a small subset of miRNAs in female mice deficient in estrogen receptor a (ERa). Conversely, estradiol (E2) treatment of ovariectomized female mice showed downregulation of some miRNAs in the uterus, an estrogen target organ. Altogether, this strongly suggests that ERa bound to E2 inhibits the production of a subset of miRNAs. The previous observation that ER a interacts with p68 and p72 raised the question how estrogen signals to the miRNA processing machinery (Watanabe. et al. 2001). Interestingly, Yamagata and colleagues have found the interactory E2-bound ERa with Drosha, which requires the presence of p68/72. The increased level of VEGF transcription and mRNA stability have been affected by estrogen (Ruohola et al. 1999), and its 3´UTR harbors binding sites for estrogen-regulated miRNAs, such as miR-125a and miR-195. Therefore, VEGF regulation by these miRNAs provides a useful system to study the physiological relevance of E2-mediated miRNA regulation. Using luciferase reporters fused to the VEGF 3´UTR, Yamagata et al. showed that E2 treatment of MCF-7 cells caused elevated levels of the VEGF reporter, and this upregulation was mediated by the E2-mediated reduction in the levels of the corresponding miRNAs. This mechanism could be generally applicable to other E2/ERa target genes whereby the mRNA stability

of target genes is increased via inhibition of the processing of miRNAs that downregulate them (Figure 2.6).

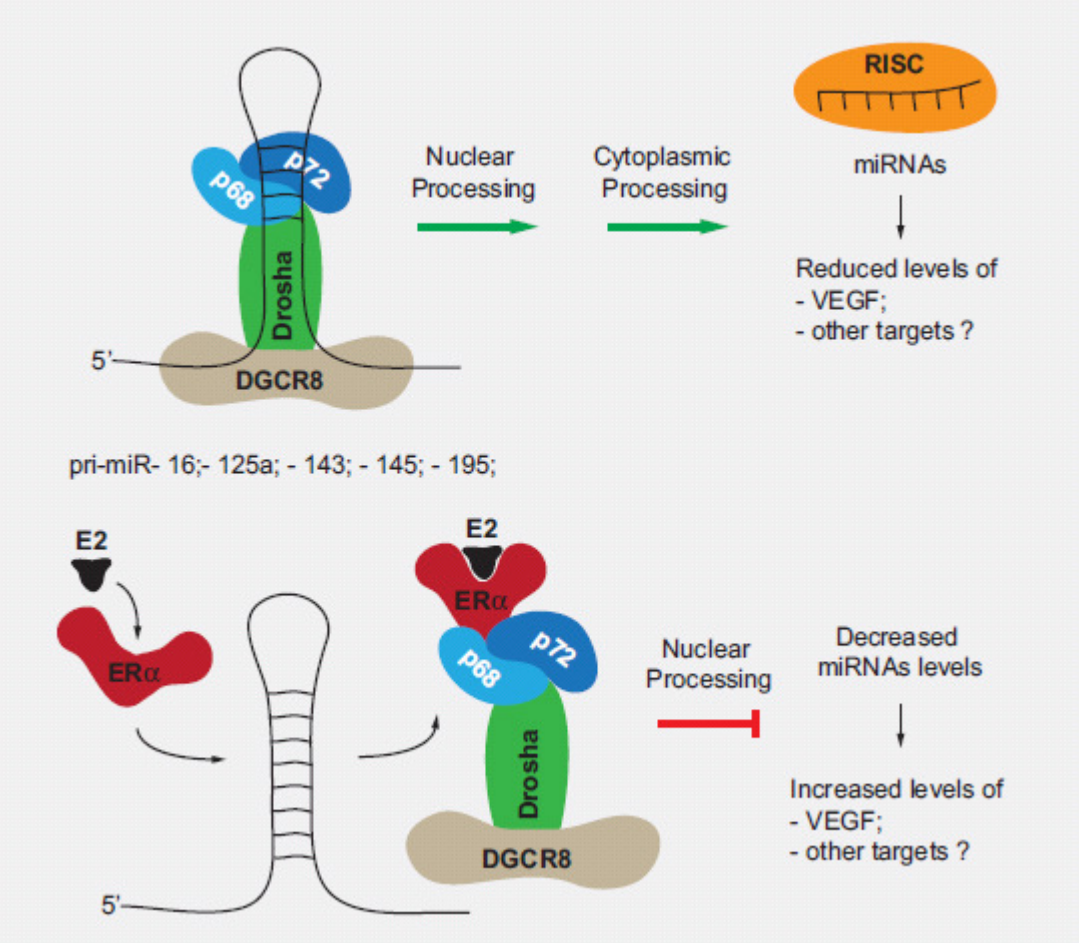

Figure 2.6 Estrogen Receptor-Mediated Inhibition of miRNA processing in the presence of estradiol (E2), ERa blocks Drosha processing of a subset of p68/p72-dependent miRNAs (Macias et al. 2009).

2.8 MicroRNA and follicular development

For folliculogenesis and subsequent successful ovulation, a coordinated and bidirectional communications mediated by a large number of gene products is required (Albertini and Barrett 2003, Assou et al. 2006). The expression of these genes must be precise and timely and any alteration in the expression of these genes could lead to oocyte developmental abnormalities, apoptosis, and poor cellular communication with

the supporting somatic cells affecting normal follicle recruitment and development (Toloubeydokhti et al. 2008).

To demonstrate the importance of miRNAs in folliculogenesis, studies uncovered an essential function for Dicer, in regulating mouse oogenesis (Murchison et al. 2007, Tang et al. 2007). Disruption of Dicer specifically in growing oocytes resulted in their inability to complete meiosis, with defects in meiotic spindle organization and chromosome congression (Murchison et al. 2007). In wild-type mice, most Dicer expression was discovered in fully grown germinal vesicle (GV) oocytes, a time when their development is largely driven by regulated use of maternally stored mRNAs (Murchison et al. 2007, Schier 2007, Tang et al. 2007). The down regulation of Dicer, which is initiated with the onset of oocyte maturation, is common to many maternal mRNAs (Murchison et al. 2007, Schier 2007, Tang et al. 2007). These observations further point out the importance of Dicer in meiotic maturation of oocytes. During the course of meiotic maturation, the oocyte is transcriptionally quiescent, which persists through fertilization and pronucleus formation until the embryonic genome becomes active (Murchison et al. 2007, Schier 2007, Tang et al. 2007). More miRNAs are discovered in oocytes throughout the development and are considered to function as key regulators of maternally derived transcripts (Amanai et al. 2006, Murchison et al. 2007, Tang et al. 2007, Watanabe et al. 2006). Scientific data supporting the regulatory role of miRNAs in oocytes comes from two studies they show direct evidence for the role of Dicer and requirement for miRNA processing in mature oocytes and the zygotes (Murchison et al. 2007, Tang et al. 2007), The biological implication of these miRNAs in human oogenesis is speculative, nevertheless nearly all the miRNAs identified are conserved in closely related species, or many have homologs in distant species, implying that their functions are conserved throughout the evolution (Bartel 2004, Farh et al. 2005). As such, it is possible that identical or similar maternally derived miRNAs also participate in regulation of oocyte and maternal-to-zygotic transcripts in humans. Whether alteration in maternally derived miRNA results in oocyte developmental failure in humans as seen in conditionally altered Dicer gene in mice, is unknown. However, transcriptional profiling of oocytes from polycystic ovary syndrome and from women with normal ovarian function (Liu et al. 2001, Luque-Ramirez et al. 2006, Park et al. 2007, Rajkovic 2007) implies a potential association between the level of

expression of these transcripts and regulatory function of miRNAs. Currently, the regulatory function of miRNAs in gene expression stability of granulosa and theca cells under physiologic and disease states remains to be elucidated.

2.9 MicroRNA in the peri-implantation period

The oviduct and the fluid contained in it provide a beneficial environment for gamete maturation, gamete transportation, fertilization and early embryonic development. These events are key processes in mammalian reproduction which are under the control of steroids (Murray et al. 1995) and prostaglandins (Lim et al. 1997). One possibility is that superstimulation accelerates embryo transport rate, resulting in their premature entry into the uterus, an environment in which O_2 availability is limited (Hawk 1988). Studies in mice, bovine and humans have shown that superstimulation can lead to improper genomic imprinting and decreased embryo quality (Fauque et al. 2007, Gad et al. 2011, Rossignol et al. 2006, Sato et al. 2007), suggesting that ovarian hormonal stimulation may affect embryo development and gene expression in many different ways. In addition, an increase in chromosomal abnormalities has been reported in human embryos after conventional stimulation, mainly due to an increased incidence of chromosome segregation errors during the first embryonic divisions which was lowest in embryos divided in the expected time frame (Baart et al. 2009).

During the oestrus cycle, inflammation-like processes occur aiming to prepare the immunological receptivity of the endometrium for implantation. These processes are controlled by several proteins, enzymes and angiogenic factors which are differentially expressed and tightly regulated (Morales Prieto and Markert 2011). Altered endometrial expression of these molecules seems to be responsible for inappropriate tissue regeneration, resulting in dysfunctional uterine bleeding, failed embryo implantation, and other endometrial disorders. Current reports have demonstrated that miRNAs participate in regulating dynamic changes in uterine gene expression patterns by controlling genes associated with the inflammatory response (Chakrabarty et al. 2007, Pan and Chegini 2008). A number of miRNAs are specifically expressed during the peri-implantation and pre-implantation periods in mice. Among the 32 miRNAs identified as up regulated during the receptive phase, miR-101 and miR-199a* were shown to target the cyclooxygenase-2 (Cox2) gene, which is known for its critical role

in implantation and for its abilities to promote inflammation and tumorigenesis (Chakrabarty et al. 2007, Morales Prieto and Markert 2011).

The significant rise in expression profiles obtained for mature miR-21 and miR-130a from one to eight cell stage embryo; they unexpected patterns could indicate an activity of miRNAs on mRNAs carrying a seed sequence in their 3′UTR during the first segmentations of the embryo (Mondou et al. 2012). In another study, the quantification of miR-10 and miR-424 in early bovine development showed a steady expression level from the GV oocyte until the 16-cell embryo (Tripurani et al. 2010). Other profile results were presented for miR-125, miR-127 and miR-145, which showed an increased expression level by the 4- and 8-cell embryo (Tesfaye et al. 2009). Moreover, miR-196a increases steadily from the 2- to the 8-cell embryo (Tripurani et al. 2011).All these results could propose an implication for these specific miRNAs during the maternal-to-embryonic transition (MET). Additionally, this previous study showed the potential evidence of a direct negative regulation of a maternal transcript, the NOBOX element, by miR-196a. The first report that some miRNA could originated from de novo transcription during the bovine pre-MET phase (Mondou et al. 2012).

In all these cases, a decreased expression level of these miRNAs was observed at the blastocyst stage and emphasized the expression period of the mature miRNAs during or before the MET(Mondou et al. 2012). Those profiles were interesting because the expression level increased from the 1- to 8-cell embryo stage instead of decreasing like total mRNA profiles (Mondou et al. 2012). As mentioned earlier for the trout and the zebrafish, these specific augmentations of certain miRNAs during the MET suggest a putative role in the destruction of the maternal target mRNAs at the MET (Giraldez et al. 2006, Ramachandra et al. 2008). The calculated ratios of mature-to-precursor forms can additionally illustrate this particular expression pattern at the MET, especially with miR-130a where the mature form decreased to an equal concentration to the precursor form at the blastocyst stage. Consequently, this marked decrease of the mature form at the blastocyst stage suggests a role for the miRNA during the MET. With these differential values obtained between the mature and the precursor forms, a recent study illustrates the importance of quantifying the corresponding precursor stem-loop sequence (Mondou et al. 2012).

Development of transvaginal endoscopy in cattle has allowed unrivalled access to the bovine oviduct for the recovery or transfer of embryos at different developmental stages, and offers an excellent tool for studying the early kinetics of embryo development as well as the effects of hormonal stimulation on embryo physiology (Besenfelder et al. 2001, Besenfelder et al. 2008). As a result of progress in optimization of this nonsurgical endoscopic access to the oviduct, there has been a steady increase in pregnancy rate after transfer of early cleavage stage embryos to the oviduct (Besenfelder et al. 2010, Gad et al. 2011). To our knowledge, the changes in the oviduct and uterine environment due to hormonal treatments and subsequent influence on transcriptome profile of embryos have not been investigated. It is well known that superstimulation leads to deviant oocyte maturation in the follicle (Hyttel et al. 1989) as well as an abnormal endocrine environment in the reproductive tract. Therefore this study aimed to separate these two processes and examine the effect of an abnormal endocrine environment on embryo development to Day 7 and its influence on the blastocyst transcriptome. To do this, a state-of-the-art nonsurgical endoscopy method was used to recover 2- to 4-cell stage embryos from superstimulated donors and to transfer them to non-stimulated single-ovulating recipients. The proportion of embryos developed to the blastocyst stage and the transcriptome of the resulting blastocysts was compared. These data have relevance not only for cattle embryo production but also for assisted human reproduction where oocytes derived from women undergoing ovarian stimulation are routinely replaced in the uterus of the same women during the same cycle, in contrast to the situation in cattle, where transfer to unstimulated surrogate recipients is the norm. This is highlighted by the fact that high-quality embryos transferred into women involved as embryo recipients in a surrogacy procedure have a higher likelihood of implanting than if they are transferred back into the donors (Check et al. 1992, Stafford-Bell and Copeland 2001) .

3 Materials and methods

3.1 Materials for laboratory analysis

In this section, materials used in this study such as chemicals, kits, all other biological materials, reagents, media, equipments, softwares and their sources are mentioned.

3.1.1 Chemicals, kits, biological and other materials

Chemicals, kits, biological and other materials	Manufacturer/Supplier
10x PCR buffer	Promega, WI, USA
2x SYBR Green master mix, Universal RT	Exiqon, Vedbaek, Denmark
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
5x First-Strand buffer	Invitrogen Life Technologies, Karlsruhe, Germany
Acetic acid	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ammonium acetate	Sigma-Aldrich Chemie GmbH, Munich, Germany
BME (essential amino acids)	Gibco BRL, life technologies, Karlsruhe, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, Germany
Brilliant cresyl blue stain (BCB)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Calcium chloride dihydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Chloroform	Roth , Karlsruhe, Germany

Citric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Roth , Karlsruhe, Germany
dNTPs	Roth , Karlsruhe, Germany
Dithiothreitol (DTT)	Invitrogen Life Technologies, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylenediaminetetra acetic acid (EDTA)	Roth , Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Fetal bovine serum (FBS)	Gibco, Karlsruhe, Germany
Gentamycin sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hemi-calcium lactate	Promega, WI, USA
HEPES	Sigma-Aldrich Chemie GmbH, Munich,Germany
Hyaluronidase	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hydrochloric acid	Roth, Karlsruhe, Germany
Hydroxylamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Igepal	Roth, Karlsruhe, Germany
Ionomycin	Sigma-Aldrich, Chemie GmbH, Munich, Germany
Isopropyl -D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
L-Glutamine	Sigma-Aldrich, Germany

Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Medium 199	Sigma-Aldrich Chemie GmbH, Munich, Germany
Methanol	Roth, Karlsruhe, Germany
MicroRNA LNA™ PCR primer set	Exiqon, Vedbaek, Denmark
MicroRNA Ready-to-Use PCR Panels	Exiqon, Vedbaek, Denmark
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
MiRNA PCR array	Exiqon, Vedbaek, Denmark
MiRNeasy® mini kit	QIAGEN, Hilden, Germany
Nuclease free water	Exiqon, Vedbaek, Denmark
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
Oligo (dT) 23	Promega, WI, USA
Penicillin G	Sigma-Aldrich Chemie GmbH, Munich, Germany
Peptone	Roth, Karlsruhe, Germany
Phenol red solution (5% in D-PBS)	Sigma-Aldrich Chemie GmbH , Munich, Germany
PKH67 Green Fluorescent Cell Linker mini kit	Sigma-Aldrich Chemie GmbH, Munich, Germany
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Proteinase K	Roth, Karlsruhe, Germany
QIAquick PCR Purification Kit	QIAGEN, Hilden, Germany
Random primer	Promega, WI, USA
Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA

RNA later	Sigma-Aldrich, MI, USA
Ribo-nuclease inhibitor (Rnasin)	Promega, WI, USA
RNA 6000 Nano LabChip® kit	Agilent Technologies Inc, CA, USA
Roti PAGE gradient (4-20%)	Carl Roth GmbH, Karlsruhe, Germany
Rotiphorese Gel 30 (37, 5:1)	Carl Roth GmbH, Karlsruhe, Germany
RQ1 Rnase-free Dnase	Promega, WI, USA
Sample Loading Solution (SLS)	Beckman Coulter, Krefeld, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium bicarbonate	Sigma-Aldrich chemie, Steinheim, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Sodium citrate	Roth, Karlsruhe, Germany
Sodium hydrogen carbonate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium dihydrogen phosphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium hydrogen sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium pyruvate	Sigma-Aldrich Inc, MO, USA
Streptomycin	Sigma-Aldrich, Deisenhofen, Germany
Streptomycin sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Superscript II reverse transcriptase	Invitrogen, CA, USA

SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific, Rockford, U.S.A.
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
TCM-199	Sigma-Aldrich Chemie GmbH, Munich, Germany
TEMED	Roth, Karlsruhe, Germany
Tris	Roth, Karlsruhe, Germany
Trypsin-EDTA	Sigma-Aldrich, Chemie GmbH, Munich, Germany
Tween-20	Roth, Karlsruhe, Germany
Universal cDNA synthesis kit	Exiqon, Vedbaek, Denmark

3.1.2 Reagents and media preparation

During this experiment, the following reagents and media formulation were used. All solutions used in this study were prepared with deionised and demineralised millipore water (ddH2O) and when necessary, the pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl). In addition, the solutions or buffers were subsequently filtered through 0.2 µ filter and autoclaved at 120°C for 20 minutes when it is necessary.

Name of the buffer/media	Constituents	Volume / amount
Physiological saline solution	NaCl	9.0 g
(NaCl 0.9%)	ddH2O added to	1000.0 ml
Manipulation medium	TCM-199	1500.0 g
	Gentamycine sulfate	0.050 g
	Sodium pyruvate	0.022 g
	NaHCO3	0.350 g
	BSA	1.00 g
70% Ethanol	Ethanol (100%)	700 ml
	ddH2O added to	300 ml
10x CMF (Calcium manesium free PBS)	Sodium chloride (NaCl)	8 gm
	Potasium chloride (KCl)	0.2 gm
	Sodium hydrogen phosphate (Na_2HPO_4)	1.15
	Potasium hydrogen phosphate (KH_2PO_4)	0.2 gm
	ddH2O added to	1000 ml

10x PBS	NaCl	8.77 g
	Na2HPO4	1.50 g
	NaH2PO4	2.04 g
	ddH2O added to	1000.0 ml
1x PBS	10x PBS	100 ml
	DEPC-treated water upto	1000 ml
3% BSA in PBS	BSA	30g
	10x PBS : added to	1,000.0 ml
3M Sodium acetate, pH 5.2	Sodium acetate	123.1 g
	ddH_2O added to	500 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	ddH2O added to	1000 ml
dNTP solution	dATP (100 mM)	10 µl
	dGTP (100 mM)	10 µl
	dTTP (100 mM)	10 µl
	ddH_2O added to	400 µl
EDTA 100 Mm (pH 2.0)	EDTA	37.224 g
	Water up to	1000.0 ml

4 % paraformaldehyde	Paraformaldehyde	4.0 g
	1X PBS	100.0 ml
Guanidin-Ethanol solution (50 ml)	Guanidin	1.43 gm
	95% Ethanol up to	50 ml
Proteinase K solution	Proteinase K	20 mg
	DEPC-treated H_2O	1 ml
Lysis buffer	Igepal (0.8%)	0.8 µl
	40U RNasin	5.0 µl
	DTT	5.0 µl
	ddH_2O added to	100.0 µl
Resolving solution	Rotiphorese gel	2 ml
	Trenngel buffer	1.25 ml
	ddH_2O added to	1.65 ml
	APS (10%)	25 µl
	TEMED	5 µl
1x Blotting Buffer (500 ml)	10x blotting buffer	50 ml
	Methanol	100 ml
	ddH_2O added to	350 ml
Fixation solution	Methanol	500 ml
	Glacial acetic acid	100 ml
	ddH_2O added to	1000 ml
Coomassie staining solution	Coomassie Blue R-250	2 ml
	Methanol	500 ml
	Glacial acetic acid	100 ml
	ddH_2O added to	1000 ml

Stacking Solution	Rotephorese gel	0.725 ml
	Sammel gel buffer	0.5 ml
	ddH_2O added to	2.825 ml
	APS (10%)	20 µl
	TEMED	5 µl
Stripping solution	Glycin	15 gm
	SDS	1 gm
	Tween 20	10 ml
	ddH_2O added to	1000 ml
Urea-DTT solution	Urea	3 gm
	ddH_2O added to	3 ml
	Stock DTT	250 µl

3.1.3 Equipments used in the study

Equipment	Manufacturer
ABI *StepOnePlus*™	Applied Bio Systems, CA, U.S.A.
ABI 7900 HT real time PCR system	Applied Bio Systems, CA, U.S.A.
Agilent 2100 Bioanalyzer	Agilent Technologies , CA, USA
ApoTome microscope	Carl Zeiss Microlmaging, Germany
Centrifuge	Hermel, Wehingen, Germany
CEQ™ 8000 Genetic Analysis	Beckman Coulter, Krefeld, Germany
Confocal laser scanning microscope-510	Carl Zeiss, Germany
CO2-incubator (MCO-17AI)	Sanyo, Japan
ECL plus western blotting detection system	GE Healthcare, Freiburg, Germany
Eight well chamber slide	Lab-Tek™, Thermo Scientific, US

Electrofusion machine CFA 400	Kruess Hamburg, Germany
Electrophoresis unit	BioRad, Munich, Germany
Four well culture dishes	Thermo Fisher Scientific, Nunc, Denmark
Inverted fluorescence microscope DM IRB	Leica, Germany
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Millipore apparatus	Millipore Corporation, USA
MyCycler Thermal Cycler	Bio-Rad Laboratories, CA, USA
MicroAmp® optical 96-well reaction plate with barcode	Applied Bio Systems
Stereomicroscope SMZ 645	Nikon, Japan
StepOnePlus real-time PCR system	Applied Bio Systems
Tweenty four well culture dishes	Thermo Fisher Scientific, Nunc, Denmark
Ultra centrifugation machine	Beckman Coulter, Krefeld, Germany
Ultra-low freezer (-80^{o}C)	Labotect GmbH, Göttingen, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.4 List of software programs and statistical packages used

Programs (soft wares) and statistical packages	Source
BLAST program	http://www.ncbi.nlm.nih.gov/Blast/
EndNote X1	Thomoson
ENSEMBL genome browser	http://www.ensembl.org/
Entrez Gene	http://www.ncbi.nlm.nih.gov/
GraphPad prism v.5	GraphPad software, Inc.
Ingenuity's pathway analysis	www.ingenuity.com
MiRBase v.19	http://www.mibase.org/
miRDB	http://www.mirdb.org/
MicroRNA.org-Target and Expression	http://www.microrna.org/
miRecords	http://mirecords.biolead.org/
PicTar	http://www.pictar.org/
Primer Express® software v.2.0	Applied Biosystems, Foster City, CA, USA
RT^2 PCR Array Data Analysis Version 3.5	http://pcrdataanalysis.sabiosciences.com/
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/
Prism for windows (ver.5.0)	GraphPad software, Inc.
TargetScan 5.1	http://www.targetscan.org/
Weight to molar quantity (for nucleic acids) converter	http://www.molbiol.ru/eng/scripts/01_07.html

3.2 Methods

3.2.1 Experimental design

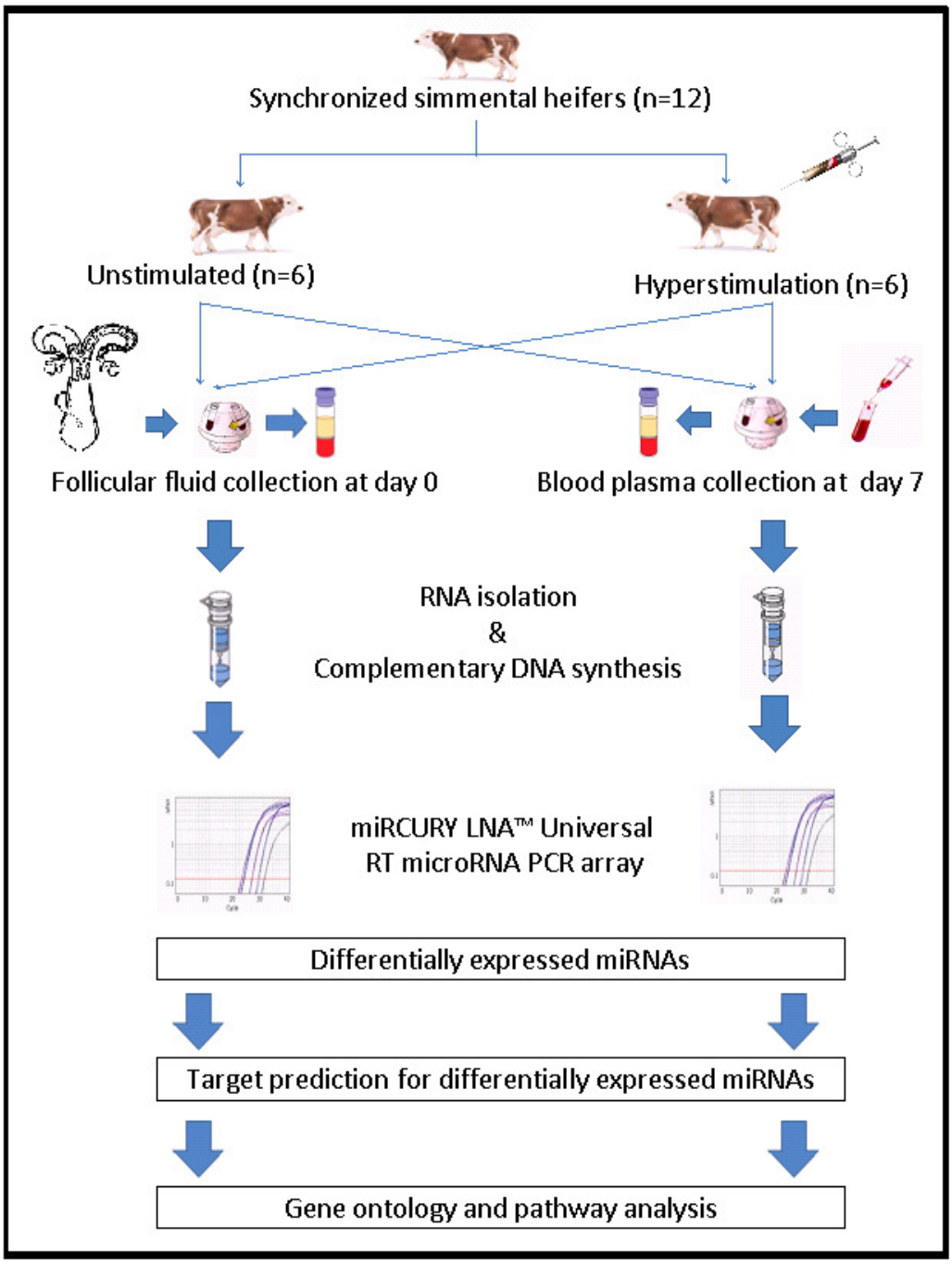

Figure 3.1 Overview of the present study : All experimental animals were handled according to the animal protection law of Germany. Simmental heifers (n =12) aged between 15 and 17 months and weighing between 380 and 450 kg is used in this study. All animals were kept under identical farm conditions within the same herd. Blood samples for miRNA detection were collected by EDTA tubes from the both group animals on day zero, day three and day 7 after ovulation and stored at -80ºC. In addition, Follicular fluid were collected from the both group animals on day zero was stored at -80ºC.Total RNA from blood plasma and follicular fluid were isolated by using the miRNeasy kit (Qiagen, Hilden, Germany). Reverse transcription will be performed by using universal cDNA syntheses Kit (Exiqon, Vedbeck, Denmark) according to the manufacture's protocol. Identification and expression profiling of miRNA were performed by PCR based array technology. We used miRCURY LNA™ Ready-to-Use PCR.Samples were profiled for candidate miRNAs by using miRNA RT-PCR individual assay.

3.2.2 Animal treatments and sample collection

All experimental animals were handled according to the animal protection law of Germany. Simmental heifers (n =12) aged between 15 and 17 months and weighing between 380 and 450 kg were used in this study. All animals were kept under identical farm conditions within the same herd. Pre-synchronization was performed by i.m. administration of 500 mg of cloprostenol (PGF2a, Estrumatew; Essex Tierarznei, Munich, Germany) twice within 11 days. Two days after each of the PGF2a treatments animals received 10 mg of GnRH (Receptalw; Intervet, Boxmeer, the Netherlands). Twelve days after the last GnRH injection, heifers received the first of eight consecutive FSH-injections over 4 days in decreasing doses (in total 300–400 mg of FSH equivalent according to the body weight; Stimufol, University of Liege, Belgium). Two PGF2a treatments were performed 60 and 72 h after the initial FSH injection. Finally, 48 h after the first PGF2a application, ovulation was induced by simultaneous administration of 10 mg of GnRH with the first of three artificial inseminations within a 12-h interval. The time of the second insemination (60 h after the first PGF2a application) was defined as Day onset of oestrus (D0)(Figure 3.2). The estrous cycles of recipient animals were

synchronized as mentioned above, but were not inseminated. Follicular contents (follicle ≥35 mm) were collected by transvaginal, ultrasound-guided follicular aspirations(figure . Follicular fluid was collected using a 12-gauge needle, centrifuged at 1500xg for 15 min, and later stored at -80°C and also blood samples were collected from each animal from D0, day 3 (D3) and day7 (D7) by tail venipuncture. Blood plasma for miRNA detection was collected by EDTA tubes from the both group animals and stored at -80ºC until processed for microvesicles/ exosomes, RNA, or protein isolation. Blood samples were refrigerated at 4°C for 12–24 h before being centrifuged at 1500g at 4°C for 15 min. Serum was separated and stored at -20°C until assayed to determine progesterone concentration.

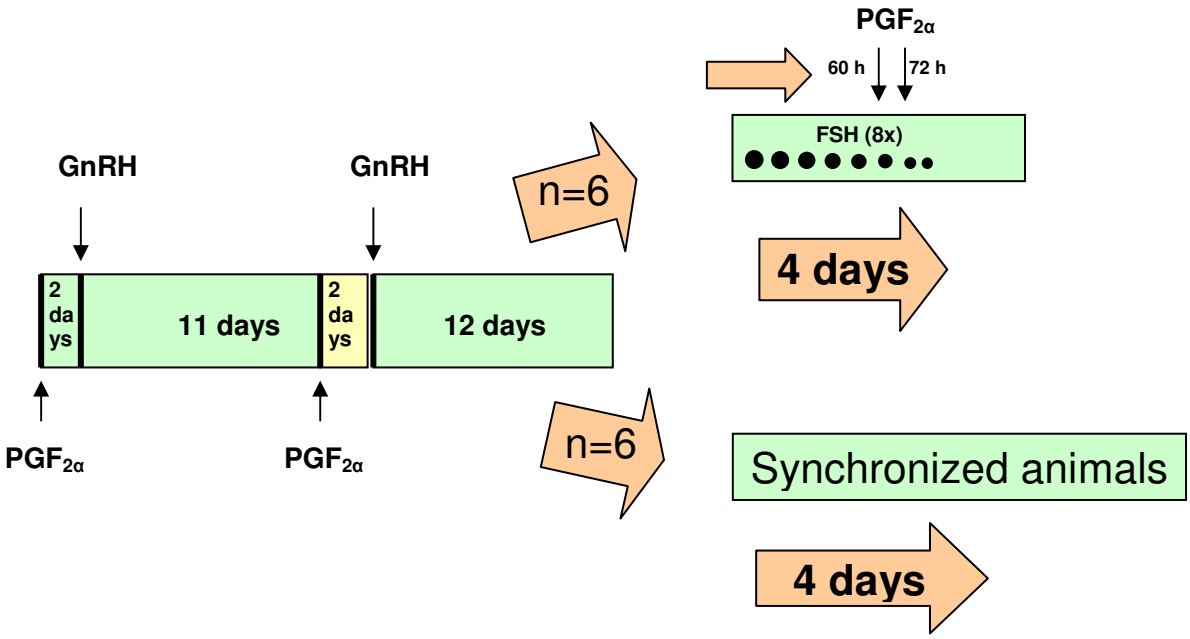

Figure 3.2 Overview of the ovarian hyperstimulation protocol

3.2.3 Progesterone assay

Progesterone concentrations were measured with commercially available assay kits. In hyperstimulated (n=6) and unstimulated heifers (n=6) at day 0 to day7 of oestrous. Progesterone concentration was determined by time-resolved fluoroimmunoassay using

an Auto DELFIA ™ Progesterone kit (Perkin Elmer, Wallac Oy, Turku, Finland). The sensitivity of the assay was 0.01ng/ml.

3.2.4 Total RNA isolation

The total RNA enriched with miRNAs was isolated from the experimental samples using miRNeasy® Mini kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions with the following modifications. Liquid samples (follicular fluid and blood plasma), ultracentrifugation pellets and immunoprecipitation pellets were resuspended in 800 µL of QIAzol (Qiagen, Hilden, Germany) by vortexing for 1 min then incubated at room temperature for 5 min. Two hundreds µL chloroform was added to the homogenate, the tube shacked vigorously for 15 sec and incubated at room temperature for 2 min and centrifuged for 15 min at 12000 x g at 4°C in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany). The upper aqueous phase was transferred carefully to a new 2 ml collection tube and 1.5 volume of 100% ethanol was added. After mixing, 700 µl of the sample was pipetted onto RNeasy Mini spin column and centrifuged for 15 sec at room temperature. The flow-through was discarded and the previous step was repeated with the remaining samples. Following this, the spin column was washed by 700 µl RWT buffer and centrifuged for 15 sec at 10000 rpm. After discarding the flow-through, 500 µl RPE buffer was added to the spin column followed by 15 sec centrifugation of 10000 rpm. This step was repeated for the second time and centrifuged at the same speed for 2 min. The spin column was then transferred to a new 2 ml collection tube and centrifuged at 14000 rpm for 1 min to eliminate any possible carryover of RPE buffer and residual flowthrough remained on the spin column. To elute total RNA, the RNeasy spin column was transferred into new 1.7 ml collection tube to which 30 µl of RNase-free water was added to the centre of the RNeasy spin column membrane and centrifuged at a speed of 10000 rpm for 1 min. Isolated total RNA was stored in -80°C until the further step.

3.2.5 Reverse transcription

A reverse transcription reaction was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Denmark) according to the manufacturer's instructions. In brief, approximately a total of 100 ng of total RNA, including small RNA, were anchor-tailed with a poly(A) sequence at their 3'end and then reverse transcribed into cDNA using a universal poly(T) primer with a 3'end degenerate anchor and a 5' end universal tag. For this, 5x reaction buffer and nuclease free water was gently thawed and immediately placed on ice. RNA spike-in was resuspended by adding 40 µl of nuclease free water and mixed by vortexing prior to use. The RT master mix was prepared by adding 5x reaction buffer, water, enzyme mix and spike-in RNA in the proportion indicated in the following table. The RT master mix was dispended into nuclease free tube and then the total RNA template was added to each tube and mixed by gentle vortexing or pipetting. The mix was incubated for 60 min at 42°C and 5 min at 95°C. After the incubation period samples were immediately cooled by placing in ice. The cDNA products were subsequently diluted 100 fold.

Table 3.1 Reverse transcription reaction setup

Reagent	Volume (µl), RT reaction
5x Reaction buffer	4
Nuclease-free water	9
Enzyme mix	2
Synthetic RNA spike ins, optional replace with H2O if omitted	1
Template total RNA	4
Total volume	20

3.2.6 Quality control of isolated RNA

For all the experiments, the resulting total RNAs were checked for their concentration and integrity (based on 260/280 nm measurement) using nanodrop 8000

spectrophotometer (Thermo Scientific, Germany) and subsequent analysis by using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Therefore, an alternative way of monitoring yield and quality control of the sample has to be used. The amount of RNA that can be extracted as well as the amount of inhibitors left after extraction can vary from sample to sample. Before committing RNA samples to profiling on microRNA qPCR panels, it is very important to ascertaining the purification yield and absence of PCR inhibitors. So, for this we perform real time PCR using different RNA sample input amounts in the cDNA synthesis reaction, for instance 0.5 µL, 1.0 µL, 2.0 µL, and 4.0 µL in a 10 µL reaction. Corresponding cDNA samples were analysed by qRT-PCR using miR-16 and miR-191.

3.2.7 miRNA Profiling

Samples were profiled for the relative abundance of 750 miRNAs by using miRNA Ready-to-Use PCR, Human Panel I &II I, V2.M qRT-PCR arrays (Exiqon, Vedbaek, Denmark) in ABI Prism® 7900 HT SDS (Applied Biosystems, Foster City, CA). To empirically determine the limit of linear quantification, the limit of detection, and the PCR efficiency for each miRNA assay, we used dilution analysis of plasma circulating miRNA for reverse transcription with the miRCURY LNA Universal RT miRNA PCR kit (Exiqon, Vedbaek, Denmark). As directed reverse transcription reaction products were combined with SYBR Green master mix (Exiqon, Vedbaek, Denmark) and loaded into the 384-well qRT-PCR arrays as directed. Quantitative PCR was performed on a ABI Prism® 7900 HT (Applied Biosystems, Foster City, CA). Data from all miRNA qRT-PCR arrays were imported into SDS Enterprise Software (V2.2.2; Applied Biosystems), and CT values were calculated by using automated assay-specific baseline and threshold settings. Plasma and follicular fluid datasets were subsequently analyzed independently. MicroRNAs were first normalized using global mean normalization across all miRNA arrays to achieve the same mean Ct (determined using only miRNAs passing detection threshold (Ct<32) for each array). To minimize potential noise introduced by measurements below detection threshold, miRNAs with Ct>35 in all groups were filtered out.

3.2.8 Exosomes isolation

Exosome isolation from independent follicular fluid samples was also performed by ultracentrifugation using previously described methods (Arroyo et al. 2010, Valadi et al. 2007). Briefly, follicular fluids were subjected to differential centrifugation at 300x*g* and then 4000x*g* each for 10 min to remove cells and cellular debris. The follicular fluid samples were then filtered through 0.22 µm screen to remove particles whose size is more than 200 nm (apoptotic bubbles and microvesicles portion). After that, samples were subjected to centrifugation at the 25,000×*g* for 30 minutes to remove further microparticles, and the microvesicles fraction remained in samples after filtration. Follicular fluids, obtained from 25,000×*g* centrifugation steps, were centrifuged at 120,000×*g* for 70 minutes at 4°C in a Beckman SWTi55 rotor. The resulting exosome pellet was resuspended in DPBS and re-centrifuged at 120,000×g for 70 min at 4°C and then resuspended in DPBS and stored until further use for exosome labeling and the uptake experiment.

3.2.9 AGO 2 immunoprecipitation

A total of 200 µL of plasma was diluted with 200 µL of PBS solution (pH 7.4). For each sample, 200 µL of Magna Bind goat anti-mouse IgG Magnetic Bead slurry (Thermo Scientific, Rockford , USA) were washed with PBS solution and incubated with 10 µg of mouse monoclonal anti- Ago2 mouse normal IgG (Santa Cruz Biotechnology, Heidelberg, Germany) antibodies for 2 h at 4 °C. The preincubated beads and antibody were then added to the 400 µL of diluted plasma and incubated overnight at 4 °C. Beads were washed three times with 1% Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA. Each sample was eluted in 700 µL of QIAzol and processed for RNA isolation.

3.2.10 Protein isolation and Western blot analysis

Exosomal proteins and the Ago2 complex were isolated from organic phenol portion during total RNA isolation using Qiagen miRNeasy mini kit following optimized protocol and resuspended in 8M urea. Briefly, after complete removal of aqueous phase 300 µl of 100% ethanol was added, mixed carefully and incubated at room temperature

for 2-3 min. Following centrifugation at 2000 × g at 4°C, supernatant containing the protein frection was transferred to a new cap lock tube, 1.5 ml of isopropanol added , and incubated for 10 min at room temperature. Samples were centrifuged at 12,000 × g for 10 min at 4°C, the supernatant was removed and 2 ml of guanidin-ethanol solution was added and incubated for 20 min followed by centrifugation at 7500 × g at room temperature. After removing the supernatant 2 ml of 100% ethanol was added, vortexed and incubated at room temperature for 20 min. Then 500 µl of urea-DTT solution was added and incubated at 95°C for 3 min. The solution containing protein was transferred into a new tube and centrifuged at 10,000 × g for 10 min. The supernatant containing protein was then transferred into new tube and the quantity was measured using nanodrop. Fifty µg of protein from each sample were loaded and resolved in 12% SDS-PAGE polyacrylamide gels (Bio-Rad, Corp., Hercules, CA, USA) and then transferred to nitrocellulose membranes (Biotrace NT, Pall Life Sciences, Pensacola, FL, USA). Membranes blocked (5% non-fat dried milk in TBST) for 1 h at room temperature and incubated separately with antibodies raised against CD63 (ExoAB Antibody, SBI, CA, USA) and EIF2C2 (0.4 µg/ml; Santa Cruz Biotechnology Inc, USA) overnight at 4°C. After subsequent washing, membranes preincubated with the respective primary antibody were further incubated with horseradish peroxidase conjugated anti-goat, anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology Inc., USA), respectively. After additional washes, membranes were incubated for 5 min in chemiluminescent substrate (Thermo Scientific, Waltham, USA), and immunoreactive proteins were visualized with a Chemidoc XRS (Bio-Rad) instrument.

3.2.11 Quantitative real time PCR analysis of selected microRNAs

The expression profiling of selected miRNAs in granulosa cells, theca cells and oocyte cumulus complexs have been examined using LNA^{TM} PCR miRNA qPCR. Individual primer set was used. Complementary DNA was synthesized from total RNA of the exosomal portion, Ago 2 complex portion, blood plasma and follicular fluid according to the previously mentioned protocol using Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark). The resulting cDNA was diluted 40x using nuclease free water. The qRT PCR master mix was prepared using the 4 µl of diluted cDNA template, 1µl LNA^{TM} PCR miRNA qPCR assay primer set and 5 µl of 2x SYBR Green master mix

(Exiqon, Vedbaek, Denmark). The reaction was done by using ABI prism 7000 real time PCR apparatus with a thermal program of initial heating at 95°C for 10 min followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. The relative quantification of miRNAs expression was calculated using a comparative threshold cycle (Ct) method as described before (Tesfaye et al. 2009).

Where, relative abundance (Fold change) = $2^{-\Delta\Delta Ct}$

ΔCt = average Ct target miRNA - average Ct of normalizer

$\Delta\Delta Ct = \Delta Ct$ target miRNA - ΔCt of the calibrator

The calibrator was the one with the highest ΔCt value among the groups

3.2.12 Target prediction and pathway analysis

Target Scan 6.2, PicTar and miRecords were used to predict target genes of the deregulated miRNAs identified. A list of the target predicted genes was uploaded into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com) to filter targeted genes and also to identify relationships between the genes of interest and to uncover common processes and pathways. Networks of the genes were then algorithmically generated based on their connectivity. The 'Functional Analysis' tool of the IPA software was used to identify the biological functions that were most significant to the data set. Canonical pathway analysis was also utilized to identify the pathways from the IPA library of canonical pathways that were most significant to the data set. Fisher's exact test was used to calculate a p-value determining the probability that each biological function or canonical pathway assigned to the data set. In addition, the significance of the association between the data set and the canonical pathway was calculated as the ratio of the number of genes from the data set that mapped to the pathway divided by the total number of genes that mapped to the canonical pathway.

4 Results

4.1 Effect of hyperstimulation on progesterone profile

The progesterone profiles of blood serum from hyperstimulated and unstimulated heifers were shown in (Figure 1). The mean progesterone concentration at day 7 was 19.59±8.1 and 2.11±1.10 ng/ml for the hyperstimulated and unstimulated heifers, respectively.

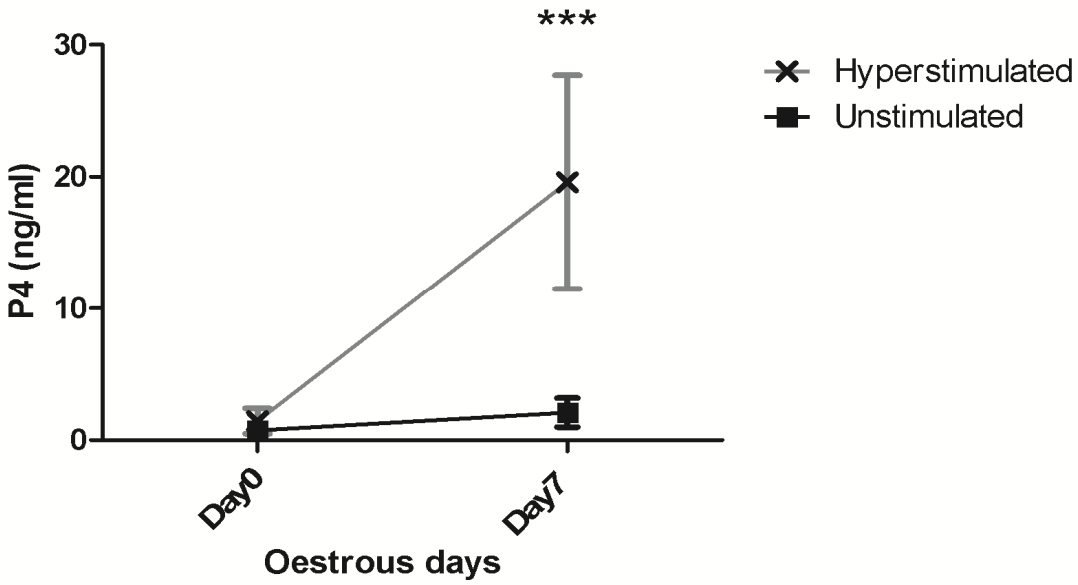

Figure 4.1 Progesterone (P4) profile of hyperstimulated (n=6) and unstimulated heifers (n=6) at day 0 onset of oestrous to day7. The plasma P4 concentration (SD) is indicated as Mean ± SEM and *** designate significant a level with $p<0.0001$.

4.2 Quality control of total RNA

Reliable results in real-time RT-PCR array and microarray analysis depend on the quality of the RNA sample. Standard methods for measurement of the RNA yield and quality are inappropriate for use with biofluid samples. The presence of carrier RNA in these samples makes measuring the low levels of endogenous RNA by OD260 impossible. Even if carrier was not included during the isolation, the RNA

concentration in the eluate would still be too low for reliable OD260 quantification on a NanoDrop or other spectrophotometers. Therefore, an extensive alternative quality control of total RNA has been implemented to secure technical soundness. For this total RNA from exosomes and non-exosomal fraction of follicular fluid was synthesized to cDNA with different inpute volume (i.e. 0.5 µl, 1 µl, 2 µl and 4 µl) and subsequently examined by quantitative real time PCR using miR-103 and miR-109. Following this, a correlation analysis was performed between the input volume of total RNA and threshold cycle value (Ct) of specific miRNA. The results revealed that miR-103 generated a linear curve for the total RNA samples from follicular fluid and blood plasma (Figure 4.2). On the other hand, the expression of miR-191 in exosome samples showed a linear pattern, for follicular fluid and blood plasma the deviation from linearity was acceptable (Figure 4.3).

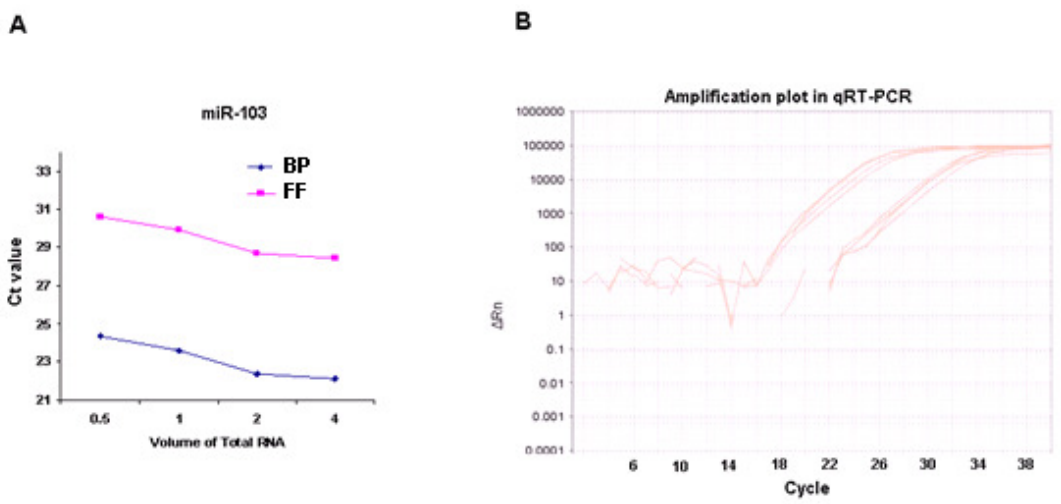

Figure 4.2 Expression of miR-103 in follicular fluid and blood plasma across the input volume (A) and amplification graph in qRT-PCR (B). Where Ct is the threshold cycle value and Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. (B) Amplification plot shows the variation of log (ΔRn) with PCR cycle number.

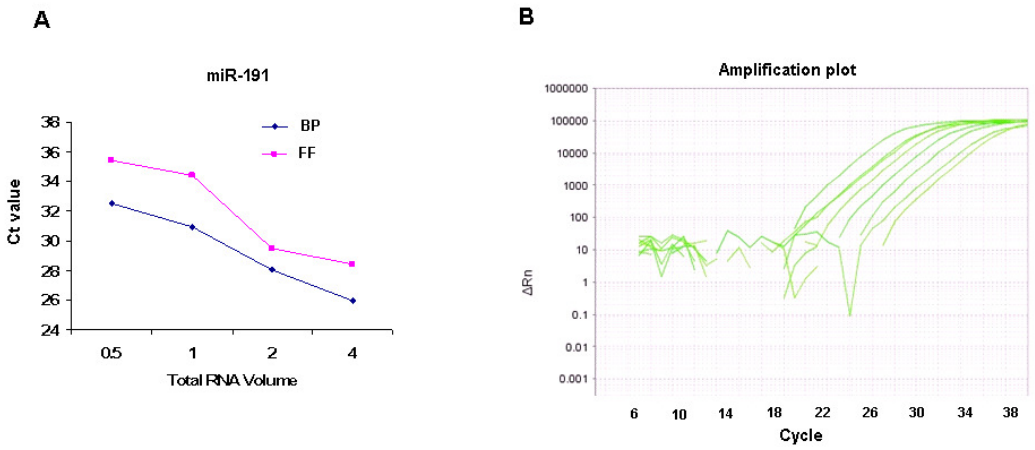

Figure 4.3 Expression of miR-191 in follicular fluid and blood plasma across the input volume (A) with amplification graph in qRT-PCR (B). Where Ct is the threshold cycle value and Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. (B) Amplification plot shows the variation of log (ΔRn) with PCR cycle number.

4.3 Expression profile of circulating miRNA

Real time PCR based miRNA arrays were used to measure the abundance of 742 miRNAs in the follicular fluid and blood plasma at day 7 from a single animal. Identification of differentially expressed miRNAs at first, we made hyperstimulated group-versus-control group (unstimulated) comparisons (i.e., 4 vs. 4). This analysis revealed that both in follicular fluid and blood plasma contain a large array of miRNAs. Among the 748 miRNAs profiled, 510 and 407 miRNAs were detected in follicular fluid and blood plasma, respectively. From the detected miRNAs, a total of 377 miRNAs were commonly detected in both follicular fluid and blood plasma. However, 143 and 30 miRNAs were detected only in follicular fluid and blood plasma (Figure 4.4).

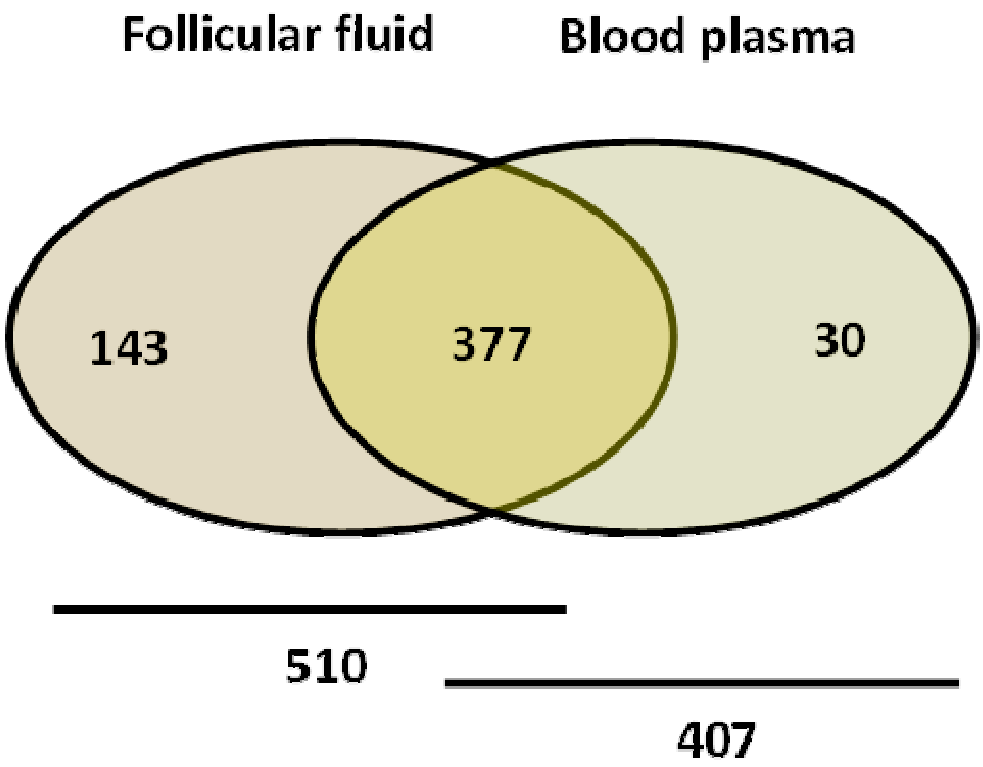

Figure 4.4 Venn diagram showing the number of detected miRNAs in bovine follicular fluid and blood plasma at day of oestrus. From a total of 748 miRNAs used in the PCR panel 510 and 407 miRNAs were detected (with threshold cycle value of ≤35 in real time PCR analysis) in bovine follicular fluid and blood plasma respectively.

In follicular fluid 66 miRNAs were found differentially expressed at least two-fold differential expression at the significance level of $p < 0.05$ (Figure 4.5). Compared with differentially expressed miRNAs derived from two groups, 34 miRNAs are up regulated and 32 miRNAs down regulated in hyperstimulated group comprised to unstimulated group. In other hand circulatory miRNA expression of blood plasma of day 7 profile of 26 miRNAs with at least two-fold differential expression at the significance level of $p < 0.05$ (Fig. 4.6). Compared with the differentially expressed miRNAs derived from two groups, 15 miRNAs are up regulated and nine miRNAs down regulated in hyperstimulated group in comparison-unstimulated group.

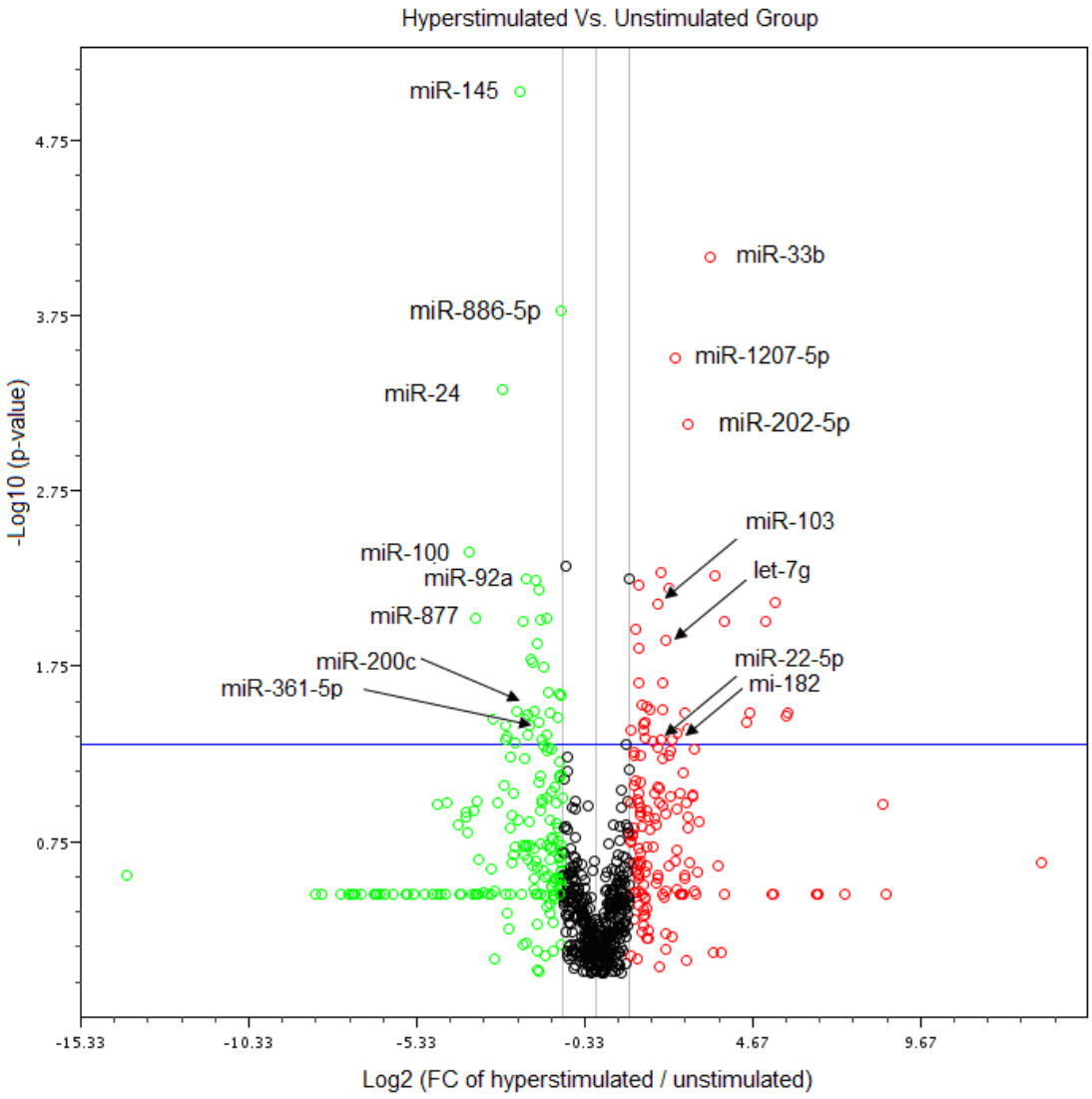

Figure 4.5: The volcano plot of differentially expressed circulatory miRNAs in follicular fluid collected at day 0 (onset of oestrus) from hyperstimulated and unstimulated heifers. Respectively red and green colours represent up & down regulated miRNA, respectively with fold change ≥ 2; $P \leq 0.05$(Points above the blue line).

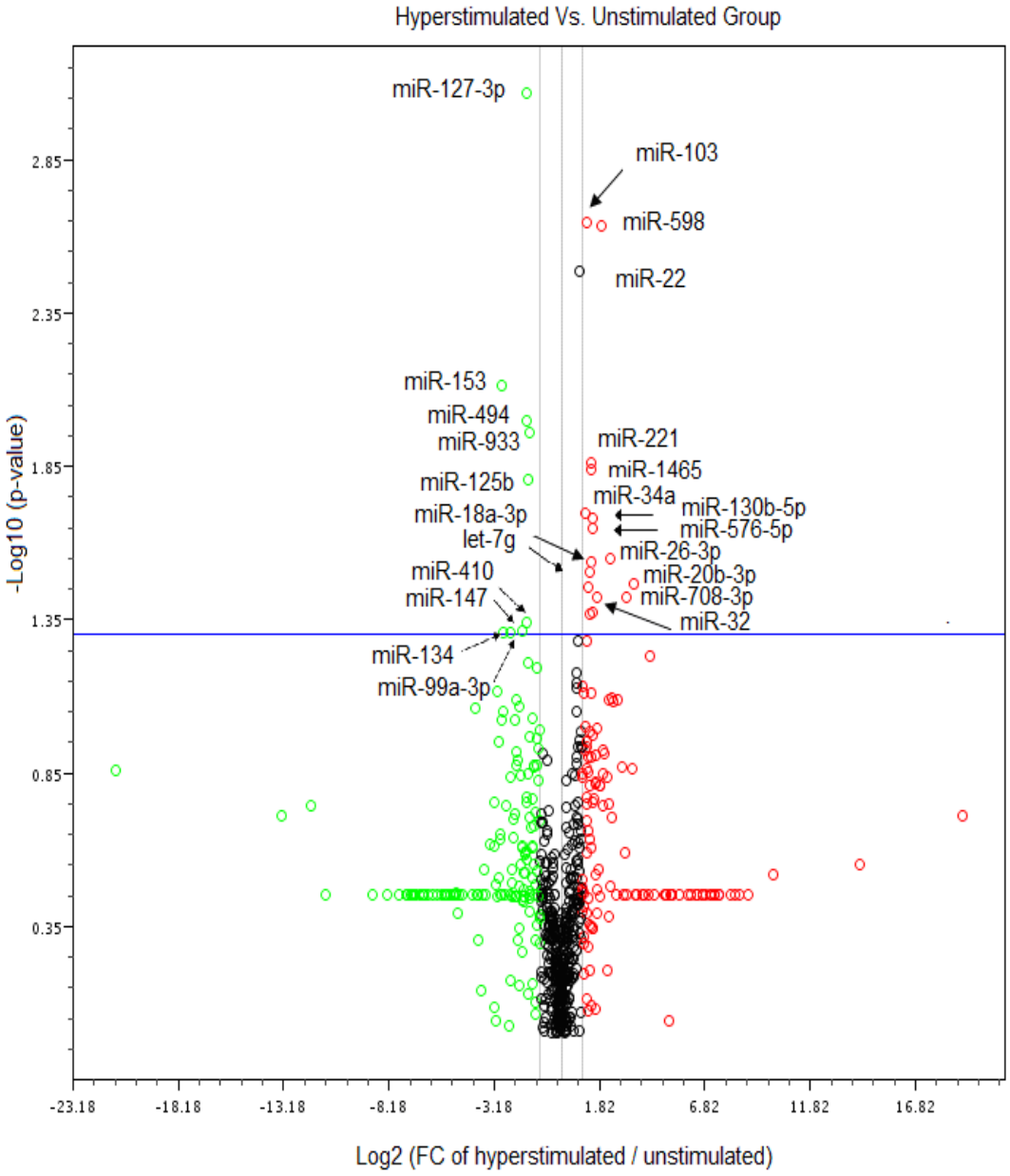

Figure 4.6 The volcano plot of differentially expressed circulatory miRNAs in blood plasma collected at day 7 (onset of oestrus) respectively from hyperstimulated and unstimulated heifers. Respectively red and green colours represent up & down regulated miRNAs, respectively with fold change ≥ 2; $P \leq 0.05$ (Points above the blue line).

4.4 Target prediction of different regulated miRNA

miRNAs can post-transcriptionally regulate a large number of target genes and many target prediction tools based on various algorithms are available for target predicting miRNAs. TargetScan 6.2, PicTar and miRecords were used to predict gene targets of the deregulated miRNAs identified in this study. The list of top predicted target genes by miRNAs from follicular fluid and blood plasma analysis are listed in tables 4-1 &4-2 respectively.

Table 4.1: The list of differentially regulated miRNA of follicular fluid between hyperstimulated and unstimulated heifers with top predicted target genes (Experimentally validated targets are underlined)

miRNA ID.	Fold Change	Predicted targets (Target scan /miRecords)
miR-643	51.9	KALRN, ZFP106, MEGF9, ANK3, PDZD7, SPTY2D1
miR-212	40.6	ARHGAP32, CAPN8, MECP2, MMP9, RB1, TJP1
miR-148b-5p	33.5	Not available
miR-548j	24.1	ERBB2, MTA1,CCNB1, NFAT5, RALA, GULP1
miR-132	22.7	RFX4, RICS , FGD4, MED28, ITCH, SAP30L
miR-224-3p	14.1	Not available
miR-129-3p	11.5	SEC14L1, TMEM136, SEC63, GLT25D2, PTAR1, VTI1A
miR-33b	10.7	ABCA1, ABCA1, CROT, CCNYL1, PCDH18, AKAP2
miR-202-5p	6.6	Not available
miR-155-3p	6.6	Not available
miR-720	6.3	DCUN1D4, DNMT3A, ACVR1B, SAMD4B, FOXG1, QKI
miR-374a	5.3	ACVR2B, N4BP2, MMP14, PARP8, SYNE1, ATAD2
miR-1207-5p	5.2	CSRP1, FAM155B, PHF15, NACC1, ARC, CBX5

miR-182	4.9	ADCY6, IGF1R, MITF, RARG, RGS17, MITF
miR-103	4.5	PLAG1, RAB1B, BACE1, CDCA4 , CRKL , EIF2C2
let-7g	4.3	ACP1, BCL7A, BMP2K,DICER1, EIF2C4, EIF3J
miR-106b	4	APP, BCL2, BMPR2, CRIM1, E2F2, BMPR2
miR-191	4	Ccl9, CRP, IL6, TLR3, TMOD2, ZBTB34
miR-542-5p	3.8	TBR1, HSPG2, NETO1, RCE1, MYLK2, RC3H1
miR-22-5p	3.8	Not available
miR-505-5p	3.6	Not available
miR-550a	3.2	ADCY1, SHISA2, TMEM151B, ADPRH, GJC1, MEF2A
miR-107	3.1	DICER1, ANO3, NF1, EIF5, ARIH2, FOXP1
miR-595	2.8	SLC30A8
miR-33a-3p	2.8	Not available
miR-384	2.8	ASXL2, C18orf25, HDGF, VPS53, METAP2, MRE11A
miR-374b	2.7	ACVR2B, N4BP2, MMP14, PARP8, SYNE1, ATAD2
miR-30b	2.6	ACVR1, AP2A1, BECN1, BCL6, DOCK7, ELMOD2
miR-31	2.6	CASR, CDKN2A, FOXP3, FZD3, HIF1A, MPRIP
miR-106b-3p	2.5	Not available
miR-940	2.4	SCN4A, STMN2, PDS5A, SEMA3F, NEDD4, UBE2F
miR-495	2.3	ONECUT1, DDIT4, TNFRSF1B, ASB5, TNC, S1PR3, CTNND2
miR-494	2.1	FGF16, HMOX1, PTEN, SCN3A, VSNL1, ONECUT1
miR-550a-3p	2	Not available
miR-452	-2.1	FAM8A1, DACT1, CASD1, PURA, IMMP2L, STAM2
miR-24	-2.1	AURKB, CCNA2, CDKN2A, FEN1, MAPK14, SMAD3

miR-300	-2.1	HOOK3, LCORL, FILIP1L, VASH2, PARD6B, FNIP1
miR-151-5p	-2.2	WNT1, SEZ6L, NTRK2, N4BP1, PHF15, CASZ1
miR-320a	-2.6	HSPB6, MCL1, TAGLN, VIM
miR-302e	-2.7	CD83, CD44, DKK1,GBP3, BAZ1A,HERPUD1
miR-525-5p	-2.7	MAP3K13, DIRAS2, FAM102B, GNB5, YTHDF3, SCN3B
miR-125a-3p	-2.8	MEAF6, MTFR1, NIT1, POP1, P2RX7, SLC22A23
miR-449a	-2.9	DLL1, MYC, SIRT1, VEGFA, JAG1, E2F5
miR-513c	-3.1	SEPSECS, GXYLT1, PDE6A, CNTNAP2, NUFIP2, YTHDC2
miR-23a	-3.2	ATAT1, LMNB1, MET, SMAD4, TRIM63, TRPS1
miR-206	-3.2	CAP1, CCSAP, CERS2, CHSY1, CNOT6, KCNE1
miR-623	-3.3	ACSM2A, ILDR2, RNF169, MTMR7, ELAVL2, AP3M2
miR-139-5p	-3.4	FOXO1, TMF1, USP6NL, TBX1, SCAPER, NDRG2, DCBLD2
miR-27b	-3.4	ADORA2B, CTNNBIP1, CYP1B1, FOXO1, GCA, PEX7
miR-125b-2-3p	-3.6	Not available
miR-378	-3.7	TOB2, HSPA12A, KCNIP2, KIAA1522,TSPAN17, PDIA4
miR-125b	-3.8	ABTB1, ADAMTS1, ANAPC16, ATP6AP1L, B3GALT4, CBX7
miR-141	-4	BAP1, CLOCK, CTBP2, CTNNB1, DLX5, ELMO2
miR-23b	-4.1	ATAT1, FBXO32, HES1, IL6R,LMNB1, MDH2
miR-621	-4.1	KLHL15, NFIB, CNOT6, ALDH1A2, ZC3H12B, VAMP7
miR-190	-4.2	ERG, NEUROD1, BACH2, CEBPA, OSBPL6, SMAD2
miR-382	-4.4	CAPN8, CNTN4, SEPT3 , TAGLN, VIM

miR-92a	-4.5	VSNL1, MYLIP, MAP2K4, ITGA5, HIPK3, ENPP6
miR-145	-4.7	ACBD3, CBFB, CCDC25, CCNA2, CLINT1, FBXO28
miR-361-5p	-5.2	RANBP17, DCTN6, ELOVL7, LACTB, SLC24A2, WNT7A
miR-200a-5p	-6.3	Not available
miR-29b-2-5p	-6.3	Not available
miR-200c	-6.4	ERBB2IP, ERRFI1, KLHL20, MARCKS, PLCG1, PPM1F
miR-659	-8.3	GRN, RANBP17, DCTN6, ELOVL7, LACTB, SLC24A2
miR-877	-12	TRIM10, TP53INP2, COL6A3, PDPN, NDUFA4, SORBS3
miR-100	-13.9	FGF16 , FGFR3, IGF1R, MTOR, PLK1,RPTOR

Table 4.2 The list of miRNA differentially regulated of blood plasma at day 7 onset of oestrus between hyperstimulated and unstimulated heifers with top predicted target genes (Experimentally validated targets are underlined)

miRNA ID	Fold change	Predicted targets (Target scan /miRecords)
miR-20b-3p	11	Not available
miR-708-3p	8.4	Not available
miR-26b-3p	5.1	KLHDC5, TET2, STRADB, CHORDC1, FAM98A, B3GNT5
miR-598	3.8	Not available
miR-32	3.2	ENPP6, VSNL1,BMPR2,CDKN1C,HIPK3,ITGA5
miR-423-3p	2.9	RAP2C, PABPC1, PANX2, BCORL1,
miR-130b-3p	2.8	Not available

miR-576-5p	2.8	Not available
miR-221	2.7	BM, FOS, MMP1, PPP2R2A, SOD2,TBK1
miR-1468	2.7	FUBP3, ST3GAL4, CUX1, STK40, PCBP2, TNRC6B
miR-18a-3p	2.6	KRAS
miR-24-2-5p	2.5	SLITRK1
miR-181c	2.5	AICDA, CD69, ESR1, GATA6, VSNL1, NLK
miR-103	2.4	PLAG1, RAB1B, BACE1, CDCA4 , CRKL , EIF2C2
miR-34a	2.2	AXIN2, JAG1, MAP2K1, MYB, MYCN, WISP2
miR-22	2.1	GRM5, CCDC67, MAX, FUT9, ESR1, BMP7,
let-7g	2	ACP1, BCL7A, BMP2K, DICER1, EIF2C4, EIF3J,
miR-933	-2.8	BDNF, MEF2A , RAP2B, HCN4,COL12A1, KPNA1
miR-125b	-3	ADAMTS1, ANAPC16, B3GALT4, ERBB2, SGPL1, IGFBP3
miR-410	-3.1	CPEB4, LCORL, LMTK2, TMEFF2, TMEM108, MKLN1
miR-127-3p	-3.1	BCL6, PRDM1, RTL1, XBP1,SETD8, FJX1
miR-494	-3.2	FGF16, HMOX1, SCN3A, VSNL1, PTEN, FAM169A
miR-147	-3.5	VEGFA, ZNF677, C13orf36, NF1, MAP4K3, PDPK1
miR-99a-3p	-5.4	Not available
miR-134	-6.8	PUM2, GNAO1, RAB27A, ANKRD55, STAT5B, TCF21
miR-153	-7	BCL2, FOXO1, MCL1, CLCN5, ANGPT1, HEY2

4.5 Characteristics of differentially expressed miRNAs on base of carrier

To determine the extent to which circulatory miRNAs are associated with exosomes in follicular fluid and blood plasma one day 7 we used differential ultracentrifugation to purify circulating exosomes from cell free, platelet-poor of follicular fluid and blood prepared from the same animals. The specificity of those isolation procedures were characterized at the protein level by the presence of CD63, a membrane protein, for exosomes, Ago2 protein in the non-exosomal fraction of the follicular fluid and blood plasma for both fractions. We confirmed recovery of exosomes consistent by CD 63 protein blotting (Fig. 4.7). In mammalian cells, mature miRNAs are coupled with Argonaute (Ago) complexes that mediate messenger RNA silencing activity (Kim et al. 2009). We hypothesized that these miRNAs are present within circulating Ago 2 complexes. To determine if Ago 2 proteins are present in plasma, we performed immunoprecipitation followed by Ago2 protein blotting (Fig. 4.8).

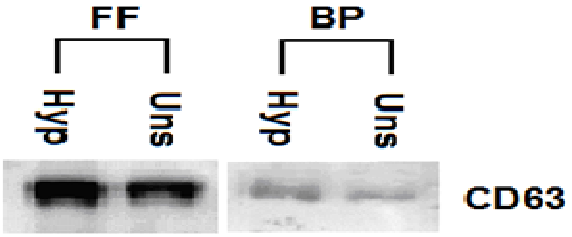

Figure 4.7 A representative western blot analysis of CD63 protein from purified exosomes by density gradient ultracentrifugation of follicular fluid (FF) and blood plasma (BP) collected at day 7 from hyperstimulated (Hyp) and unstimulated (Uns) heifers.

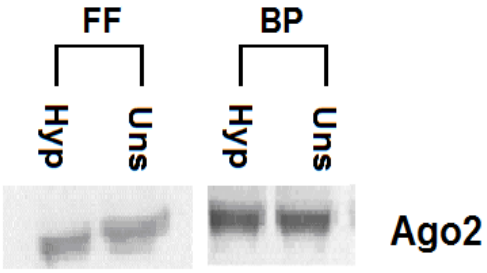

Figure 4.8 A representative western blot analysis of Ago2 protein from immunoprecipitated samples of follicular fluid (FF) and blood plasma (BP) collected at day 77 from hyperstimulated (Hyp) and unstimulated (Uns) heifers.

Expression pattern of candidate miRNAs in both fractions of follicular fluid and blood plasma was measured by qRT-PCR and the data are presented as raw Ct value in figure 4.9 and 4.10 (follicular fluid) and figure 4.11(blood plasma). Expression of miR-182 was significantly higher in follicular fluid exosomal fraction of hyperstimulated heifers than unstimulated heifers, whereas other miRNAs showed no significant expression differences between treatment groups. While miR-182 (follicular fluid), miR-127-3P and miR-103 (blood plasma) were undetected in Ago2 fraction derived from both treatment groups, the expression of let-7g was not significant between the treatment groups. Expression analysis of underexpressed candidate miRNAs in exosomal fraction of blood plasma revealed that all miRNAs were detected in both treatment groups. Although there is no significant expression difference between treatment groups, all miRNAs had lower average Ct value in unstimulated heifers than hyperstimulated heifers. In case of Ago2 fraction of blood plasma, miR-134 and miR-147 had lower average Ct value in unstimulated heifers. While all miRNAs had significantly higher expression in exosomal fraction, interestingly miR-134 had average higher expression in Ago2 fraction of blood plasma.

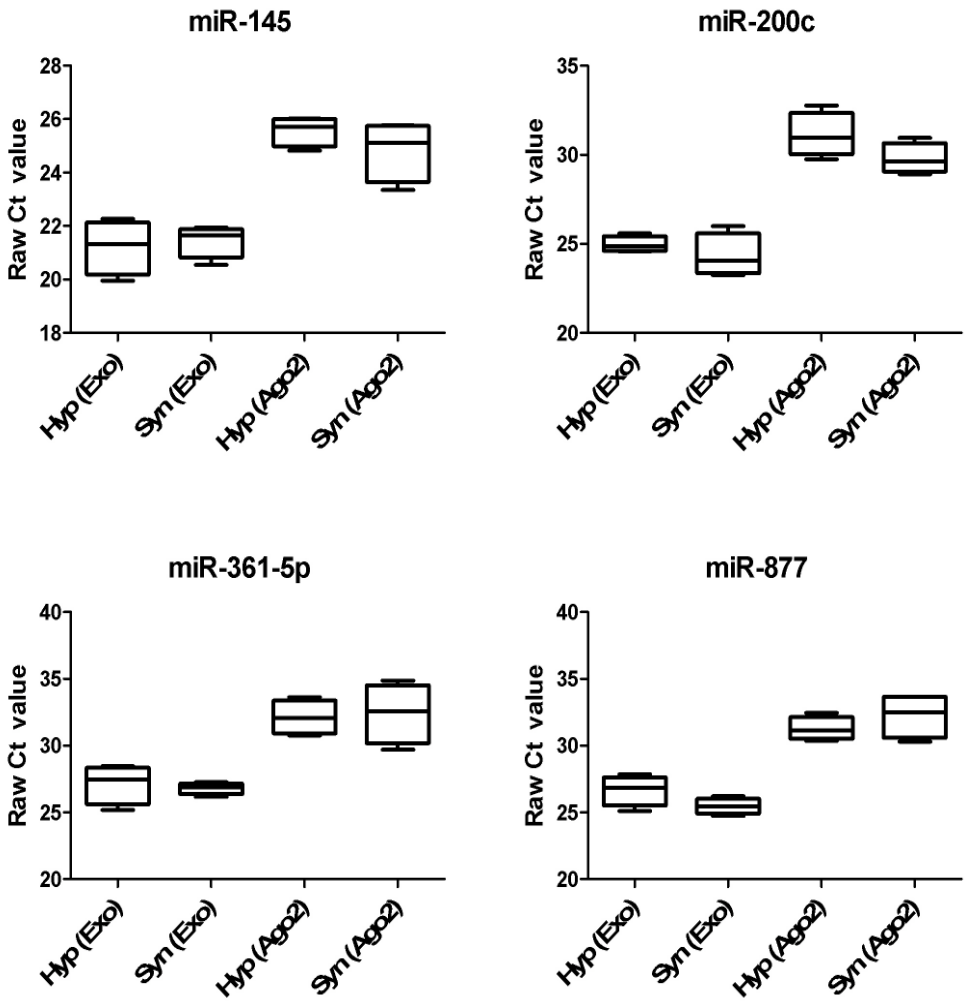

Figure 4.9 Expression levels of miR-145, miR-200c, miR-361-5p and miR-877 in bovine follicular fluid coupled with exosomes (Exo) and Ago2 complex (Ago2) from hyperstimulated (Hyp) and unstimulated (Uns) heifers (n = 4) are shown in raw Ct value. Ct values of more than 35 were considered as undetected.

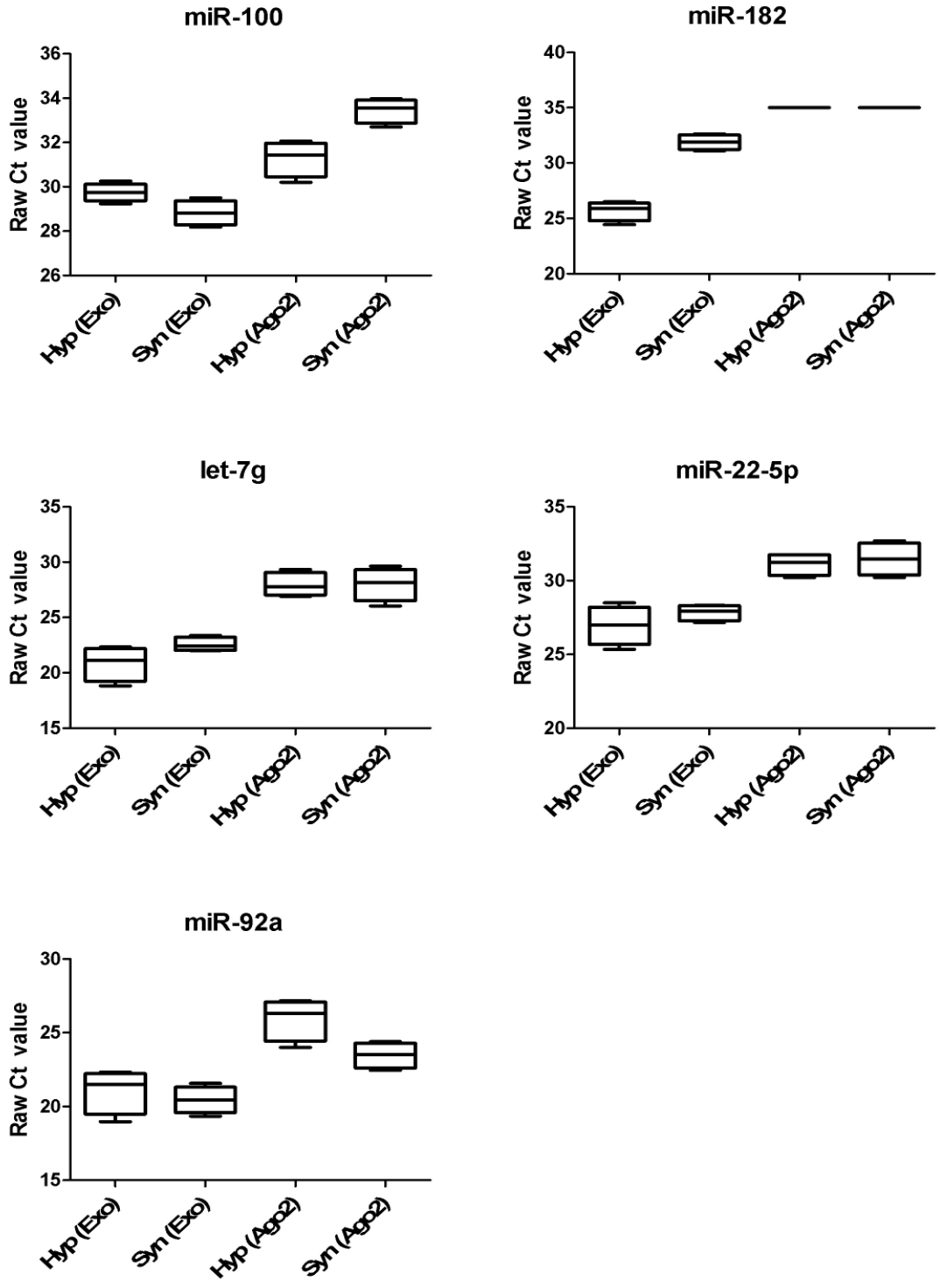

Figure 4.10 Expression levels of miR-100, miR-182, miR-22-5p, miR-92a and let-7g in bovine follicular fluid coupled with exosomes (Exo) and Ago2 complex (Ago2) from hyperstimulated (Hyp) and unstimulated (Uns) heifers (n = 4) are shown in raw Ct value. Ct values of more than 35 were considered as undetected.

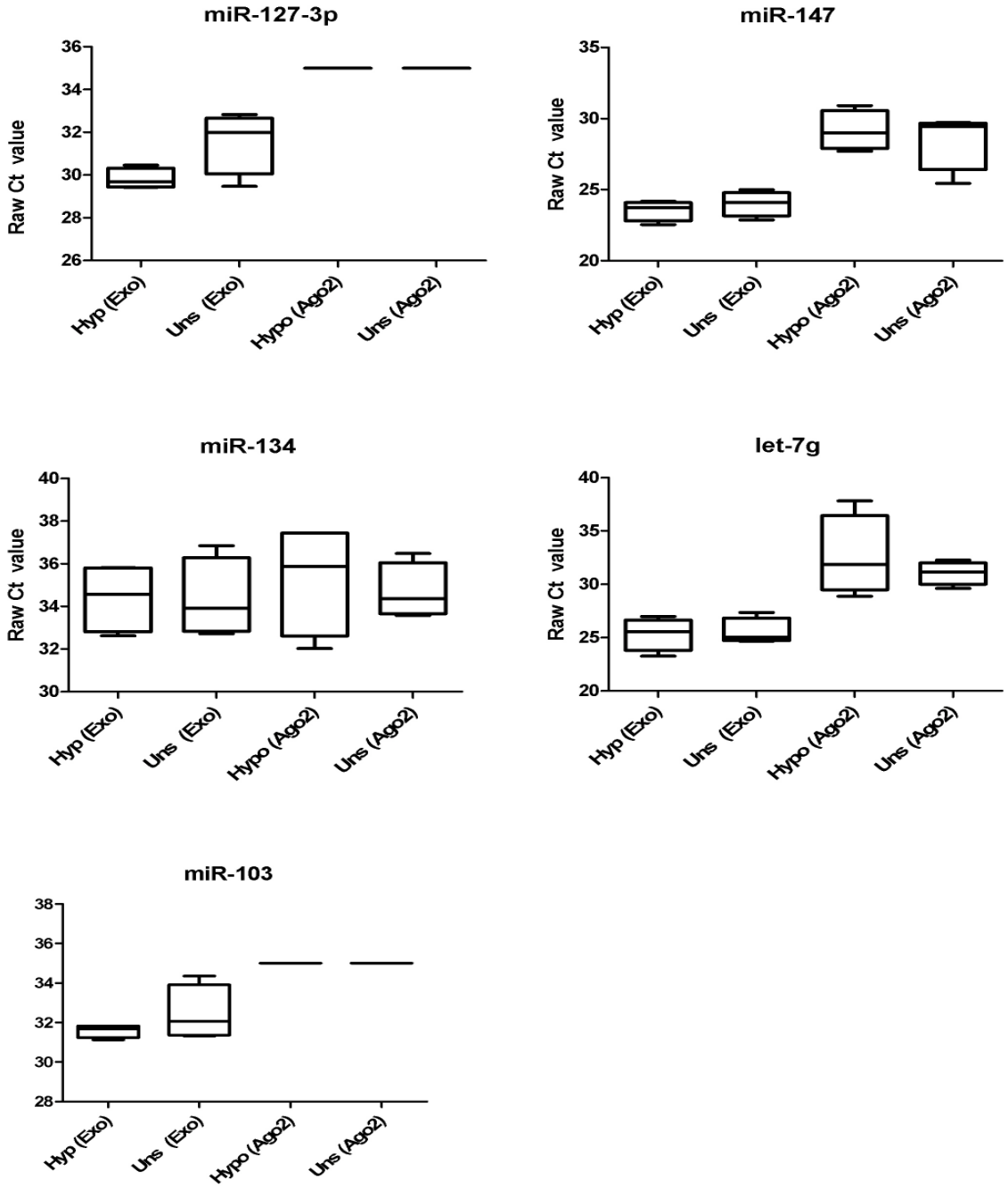

Figure 4.11 Expression levels of miR-147, miR-127-3p, miR-134, miR-103 and let-7g in bovine blood plasma coupled with exosomes (Exo) and Ago2 complex (Ago2) from hyperstimulated (Hyp) and unstimulated (Uns) heifers (n = 4) are shown in raw Ct value. Ct values of more than 35 were considered as undetected.

4.6 Temporal differences in miRNA expression

To evaluate the temporal expression of the selected miRNAs, we examined the temporal changes in expression level of candidate set of miRNAs in blood plasma at day 0, 3 and 7 after the onset of oestrus in hyperstimulated and unstimulated. The results (Fig. 4.12 and Fig. 4.13) showed that the expression was significantly changed by the time points within the groups. Most miRNAs have similar expression pattern during oestrus period between the hyperstimulated and unstimulated group. The expression of miR-32 and miR-134 were down regulated on day 3 has significant different expression pattern compare to hyperstimulated group.

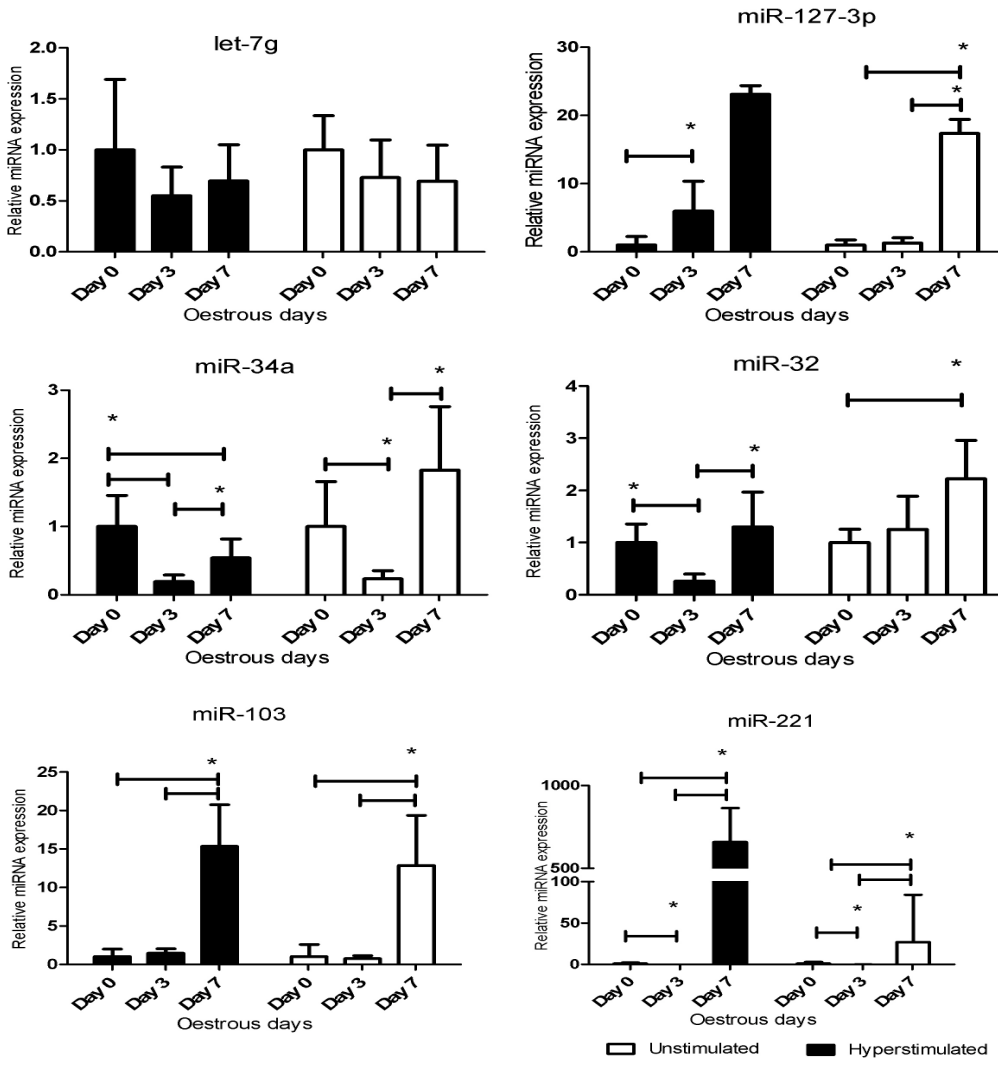

Figure 4.12 Relative abundance of circulatory miRNAs in blood plasma at different time points after the onset of oestrus. *, $P \leq 0.05$.

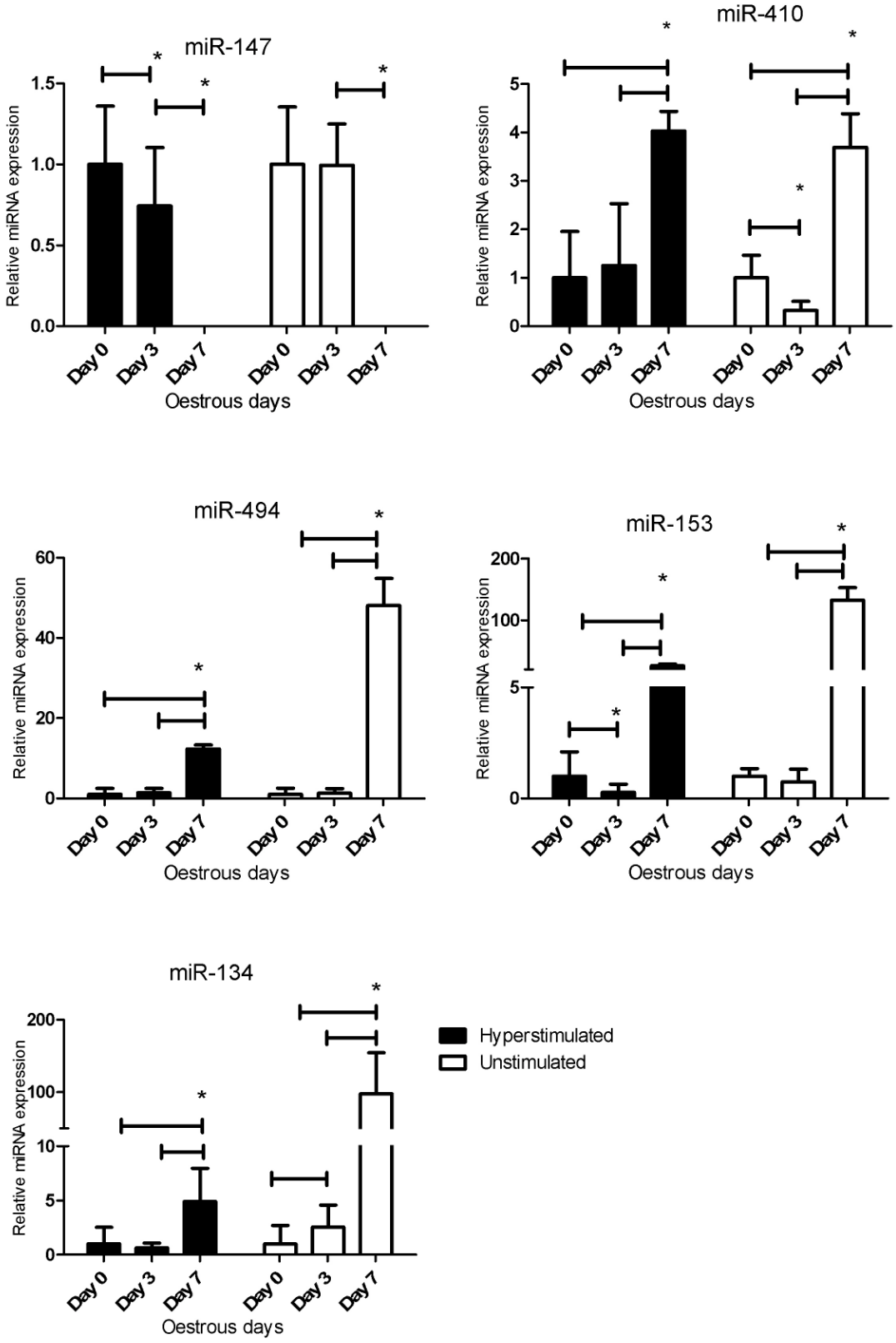

Figure 4.13 Relative abundance of circulatory miRNAs in blood plasma at different time points after the onset of oestrus. *, $P \leq 0.05$.

4.7 Functional annotation

miRNAs can post-transcriptionally regulate a large number of target genes and many target predication tools based on various algorithms are available for predicting the targets of selected miRNAs. To obtain an overview on the functional role of differentially expressed selected miRNAs, the predicted targets genes were then uploaded into the Ingenuity pathway analysis system (IPA) to identify the molecular networks (biological functions) and pathways based on the information in the IPA data base. IPA is a knowledgebase that contains approximately 3,000,000 biological and chemical interactions and functional annotations with definite scientific evidence, curated by expert biologists (Satoh 2012) . By uploading the list of target gene ID into the Core Analysis tool, the network-generation algorithm identifies focused genes integrated in a global molecular network. IPA calculates the score p-value that reflects the statistical significance of association between the genes and the networks by the Fisher's exact test (Satoh 2012). Networks analysis data showed as many interactions between molecules in a given dataset and how they might work together at the molecular level. Networks are assembled based on gene/molecule connectivity with other gene/molecules by the target predicted genes of miRNAs up regulated in follicular fluid, down regulated in follicular fluid, up regulated in blood plasma and down regulated in blood plasma, respectively (Table . 4.3, 4.4, 4.5, 4.6).

The analysis identified the most prominent canonical pathways related to the involved by the target pridicted genes of miRNAs up regulated in follicular fluid, down regulated in follicular fluid, up regulated in blood plasma and down regulated in blood plasma, respectively (Figure 4.14, 4.15, 4.16 and 4.17). Among these, cellular assembly and organization, cell cycle, carbohydrate metabolism, molecular transport, cellular development, cell signaling, cell death, cellular growth and proliferation, reproductive system development and function, embryonic development, amino acid metabolism, small molecule biochemistry, gene expression and ell to cell was dominated molecular networks.

Table 4.3 : Molecular networks involved by target genes of up regulated miRNAs in follicular fluid

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	ABCC9, ACAD9, Ahr-aryl hydrocarbon-Arnt Esr1, ANKRD52, ARSB, CHD2, CSNK1G2, DDX17, DDX18, DSCR3, ELOVL2, ESR1, GATAD2B, GJB2, HNRNPA3, KCNJ1, KCNJ16, Kcnj, MBNL1, NACC1, PHF5A, PPP6C, PPP6R2, RAB10, RAP2C, REEP6, SALL1, SF3A1, SUN2, U2SURP, WHSC1, ZBTB6	43	33	Cellular Assembly and Organization, Cellular Function and Maintenance, RNA Post-Transcriptional Modification
2	ARIH2, CBX6, ENSA, FAF2, GATAD2A, LONRF1, MARCH3, MGA, MSX1, OS9, PCGF2, PCGF5, PEG10, PLEKHF2, Ras, RFWD3, RNF2, RNF38, RNF125, RNF144B, SCML1, SMARCE1, TFCP2L1, TLE4, UBE2, UBE2J1, UBE2K, UBE2L6, UBE2Q2, UBE2R2, UBE2W, UBE2Z, UBR3, YAF2, ZMIZ2	43	33	Post-Translational Modification, Connective Tissue Development and Function, Embryonic Development
3	ABCF2, BRWD1, C16orf72, CD3, CDR2L, CUL4A, DCAF5, DCAF10, DCUN1D4, ELAVL1, FAM120AOS, FAM73A, HIPK2, HNRNPA2B1, HS2ST1, KIAA0247, KIAA1199, NKTR, PGAM1, PRPF39, SCAF11, Shc, snRNP, SNRPD3, STK4, TANGO2, TMTC4, TSPAN9, TTPAL, UBE4A, VSIG10, WDR61, ZKSCAN1, ZNF362, ZNF562	40	32	Carbohydrate Metabolism, Drug Metabolism, Small Molecule Biochemistry

4	ABCE1, Aconitase, AGO1, AGO2, AGO3, AGO4, CENPBD1, CNOT6L, DCP1A, DDX6, Eif2, EIF5, EIF1AX, EIF2A, EIF2AK3, ISCA2, Jnk, MIRLET7, PPAT, RC3H1, RNASEL, RNF13, RSU1, SFRP2, SFRP5, SH3BP5, SIX4, SIX5, TNRC6A, TNRC6B, UPF2, UPF3A, VANGL1, VANGL2, XRN1	37	31	RNA Damage and Repair, Infectious Disease, Protein Synthesis
5	AFF4, ARC, ARHGAP29, BEGAIN, CAB39, CCP110, CHD1, CRTC3, DUB, FLOT2, GRIA4, HERC2, KIAA0408, KIAA0947, LAPTM4A, LRP1B, MED13, MED20, MED29, mediator, NAV2, Pka, Rap, RAP2A, RAPGEF6, SIK2, TADA2B, TRAP/Media, TUBB4A, USP8, USP25, USP42, USP45, USP47, ZNF275	36	30	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cell Cycle

Table 4.4 Molecular networks involved by target genes of down regulated miRNAs in follicular fluid

ID	Molecules in Network	Scores	Focus Molecules	Top Functions
1	ANO6, AP1AR, BNC2, C19orf55, C1orf198, C3orf52, C5orf51, C6orf62, CCNJ, CDR2L, DRAM2, EFCAB14, ELAVL1, ERBB2, FAM102B, FAM73A, GUCD1, HECA, HERPUD2, LACTB, MBD6, PHTF2, PQLC1, RB1, RBAK, SKIDA1, SLC41A1, SLC48A1, TBC1D22B, TMEM209, TTPAL, VSIG10, ZFAND1, ZNF362, ZNF529	35	35	Cancer, Cell Cycle, Cell Death and Survival

2	ATP5G3, BCL11A, BRSK2, CCNYL1, CD84, CD5L, CDK16, CENPBD1, CORO2A, CRTAM, DCP1A, HINFP, HIPK3, IGF1R, INTERLEUKIN, KDM5A, KIF2A, MIF4GD, NFATC2IP, NPAT, SCAF11, SMARCD2, SMG1, SREK1, SRSF1, SRSF4, TBL1XR1, TFEC, TNRC6A, TRA2B, XRN1, YTHDC1, ZBTB33, ZMYM3, ZRANB2	34	34	RNA Post-Transcriptional Modification, Cellular Development, Connective Tissue Development and Function
3	BAI3, CALCRL, CCR8, CELSR2, CHRM5, ELTD1, EMR3, FZD3, FZD5, FZD10, GABBR2, Gpcr, GPR3, GPR37, GPR56, GPR85, GPR107, GPR124, GPR126, GPR155, GPR158, GPR160, GPR180, GPR137C, GPRC5A, LGR4, LPAR4, LPAR6, LPHN1, LYST, PCNX, PTGFR, SLC52A1, SSTR1, SSTR3	34	34	Cell-To-Cell Signaling and Interaction, Cancer, Endocrine System Disorders
4	60S ribosomal subunit, AARS2, ANKRD26, BRCA1, C7orf26, CDK4, CUX2, EML3, ERG, FOXD2, GDF10, HDGF, ING2, KIAA1109, LIMD2, MAP2K4, MBLAC2, MET, NKX2-4, PAXBP1, ROCK2, RPL15, RPL37, SIRT5, SMAD2, SMAD3, SMAD4, SMO, TMEM219, TRMT10C, TSEN54, YWHAQ, ZCCHC14, ZDHHC5, ZNF280D	34	34	Connective Tissue Development and Function, Embryonic Development, Organ Development
5	AEBP2, CDK2-CyclinE, COL14A1, DDX52, EPC2, FIBP, GMNN, HES2, HOXC8, IGF2BP1, IMPA2, IMPDH1, JARID2, KANSL2, MOB1B, MORC3, MSL3, MTUS2, MYC, OGT, PDZD2, PERP, PGAP1, PHF20, PHF20L1, PLSCR4, PMP22, RARRES1, SEC31B, STK38L, TBC1D1, TBC1D4, TMEM33, UTP15, ZXDB	33	34	Dermatological Diseases and Conditions, Hereditary Disorder, Organismal Injury and Abnormalities

Table 4.5: Molecular networks involved by target genes of up regulated miRNAs in blood plasma on day 7 onset of oestrus

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	alpha-N-acetylneuraminate alpha-2, 8-sialyltransferase, ANK1, ANO3, B3GNT5, CBX7, CCDC50, DBT, EDA, FRYL, KLF3, KPNA3, KPNA4, MEFV, MGA, NFkB (complex), NUP50, OLFM4, OTUD7B, PCGF2, PCGF5, PELI2, PHC3, PTPLAD1, RAB31, ST8SIA1, ST8SIA3, ST8SIA4, SYT6, SYT10, UBE4A, UMOD, VOPP1, YAF2, ZDHHC3, ZFAND5	46	33	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
2	ADNP, AGO1, AGO2, AGO3, AGO4, AKAP8, BAZ2A, C6, CNTNAP1, DICER1, DSG1, Eif2, EIF5, EIF1AX, FMR1, GNS, ING3, MAP4, NCK1, NCKAP5, OTUD4, P38 MAPK, PARP11, PHACTR2, PPM1A, RNase A, RUNX1T1, SALL1, SEPT6, SNX3, STAU2, SUN2, TBKBP1, TFCP2L1, TRIM33	42	32	Infectious Disease, Cell Death and Survival, Embryonic Development
3	Ahr-aryl hydrocarbon-Arnt-Esr1, ANKRD52, CDK8, CPNE3, CSNK1G2, DDX18, DUB, EPB41L5, ESR1, FAF2, G3BP2, HNRNPA3, MAML1, MED20, mediator, NUFIP2, OS9, PHF5A, PJA2, PPP6C, PPP6R2, RAP2C, RDX, SEL1L, THRAP3, TMX1, TRAP/Media, U2SURP, UBXN4, USP14, USP29, USP42, USP46, USP47, ZNF275	41	31	RNA Trafficking, Post-Translational Modification, Cancer

4	AChR, AFF4, BMP3, BTRC, Calmodulin, CAST, CLOCK, CYP26A1, ELL2, FBXW11, GLIS3, HCFC1, Hedgehog, IHH, IRF2BP2, KIAA0947, MAP9, MSL2, NECAB1, NEUROD1, NPAS2, NUP62CL, OGT, PCSK2, PER3, PHF20, Proinsulin, RIMBP2, RNF19A, STXBP4, SUFU, TIP60, TRIM26, VAMP1, VPS4A	38	30	Endocrine System Development and Function, Tissue Morphology, Cellular Development
5	ARIH2, BTN1A1, CALD1, CAPZA2, CCSER2, CDC14A, Cytoplasmic Dynein, DISC1, DYNC1LI1, ENSA, FEZ1, FNDC3A, FOXN2, Gamma tubulin, GLUD1, HERC2, KATNAL1, MZT1, NDEL1, PAIP2, PAPSS2, SERBP1, SLC31A1, SMEK2, SP4, TAF1D, TRIB2, trypsin, TUBGCP3, UBE2, UBE2J1, UBE2R2, Vegf, ZBTB10, ZNF197	37	30	Cellular Movement, Nervous System Development and Function, Cardiovascular Disease

Table 4.6: Molecular networks involved by target genes of down regulated miRNAs in blood plasma on day 7 onset of oestrus

ID	Molecules in Network	Score	Focus Molecules	Top Functions

1	AFF2, ANK3, ATP10D, BAZ2A, CEBPG, CREB5, DNM3, Dynamin, ENAH, GRB2, GTPBP2, Hd-perinuclear inclusions, ITSN2, KIAA1549L, LNPEP, MYO18A, NFASC, PSTPIP2, RAB22A, RAB8B, RCOR1, SEPT11, SH3BP4, SLC6A15, SLCO2A1, SMARCD2, SNX18, TEF, TES, TFAP2B, UNKL, WDR1, ZBTB37, ZNF217, ZNF609	42	33	Cardiovascular Disease, Developmental Disorder, Cell Cycle
2	ANKRD50, APP, C6orf106, CCDC149, DIRAS1, EBF4, FAM213A, HDDC3, HIC2, LURAP1L, NAV1, NECAB3, NPL, OLFML2A, PCDHGA12, PCDHGC5, PRRC1, PYROXD1, RABL3, RAPGEFL1, RASL12, RASL10B, RIMS4, SERAC1, SERTM1, SLAIN2, TBC1D20, TET3, TMCC2, TMEM180, TSPAN12, VASH2, ZFYVE1, ZNF343, ZNF483	41	35	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Nervous System Development and Function
3	ANK1, ANO3, cacn, CACNB1, CACNB3, CD300LF, CGREF1, FAM46A, GFPT2, HDLBP, INTERLEUKIN, KCNB1, KCNS3, KCTD6, KLF3, MFHAS1, N-type Calcium Channel, NFkB (complex), NKIRAS2, PHF21A, POU4F1, RHEBL1, RUSC2, SLC1A5, SLC2A12, SLC6A17, SMEK1, SNAP25, SPTB, ST18, SYN3, Syntaxin, TBX4, VAMP2, ZNF385A	36	30	Neurological Disease, Skeletal and Muscular Disorders, Cell-To-Cell Signaling and Interaction

4	ARID3A, ARID3B, ARID4A, ASXL1, CBX7, Cdc2, CDC42SE1, CEP164, DAAM2, DOCK3, DVL3, E2F2, E2F3, E2f, HERC3, HNF4α dimer, HOXB3, ING2, Jnk, MAP4K3, MAP4K5, MATN2, PCGF6, PHC2, RAPGEF5, RNF144A, RNF144B, RYBP, SCML1, SCML2, Stat1/3, THAP2, UBE2L3, VANGL1, VANGL2	36	30	Cellular Development, Connective Tissue Development and Function, Cell Cycle
5	ABTB1, BCL11A, C17orf59, CBFB, CD5L, CORO2A, Ctbp, DNMT3A, DYNLT3, ETV6, FAM65B, GATAD2A, histone-lysine N-methyltransferase, IGF1R, IKZF1, IKZF2, IKZF4, KDM5A, MAPK1IP1L, Mi2, MUL1, PERP, Pias, SATB1, SETD7, SETD8, SLC4A10, SUV39H1, TDG, TFEC, Top2, TSHZ1, UBE2I, VPS37B, VPS37C	36	30	Gene Expression, Cell Morphology, Cellular Assembly and Organization

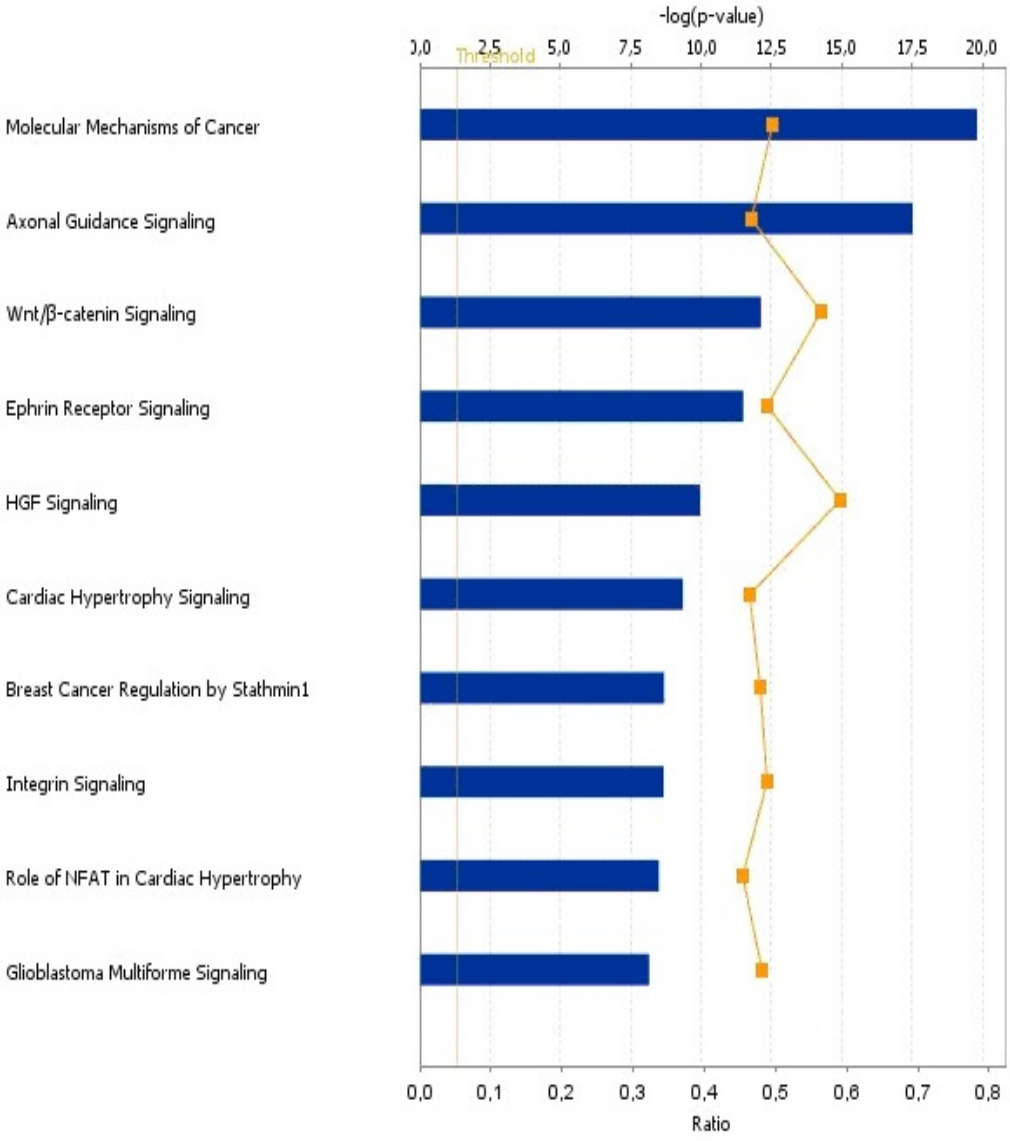

Figure 4.14 The most prominent canonical pathways related to the involved genes targeted by up regulated miRNA in follicular fluid. The bars represent the p-value for each pathway. The orange irregular line is a graph of the ratio (genes from the data set/total number of genes involved in the pathway) for the each pathway.

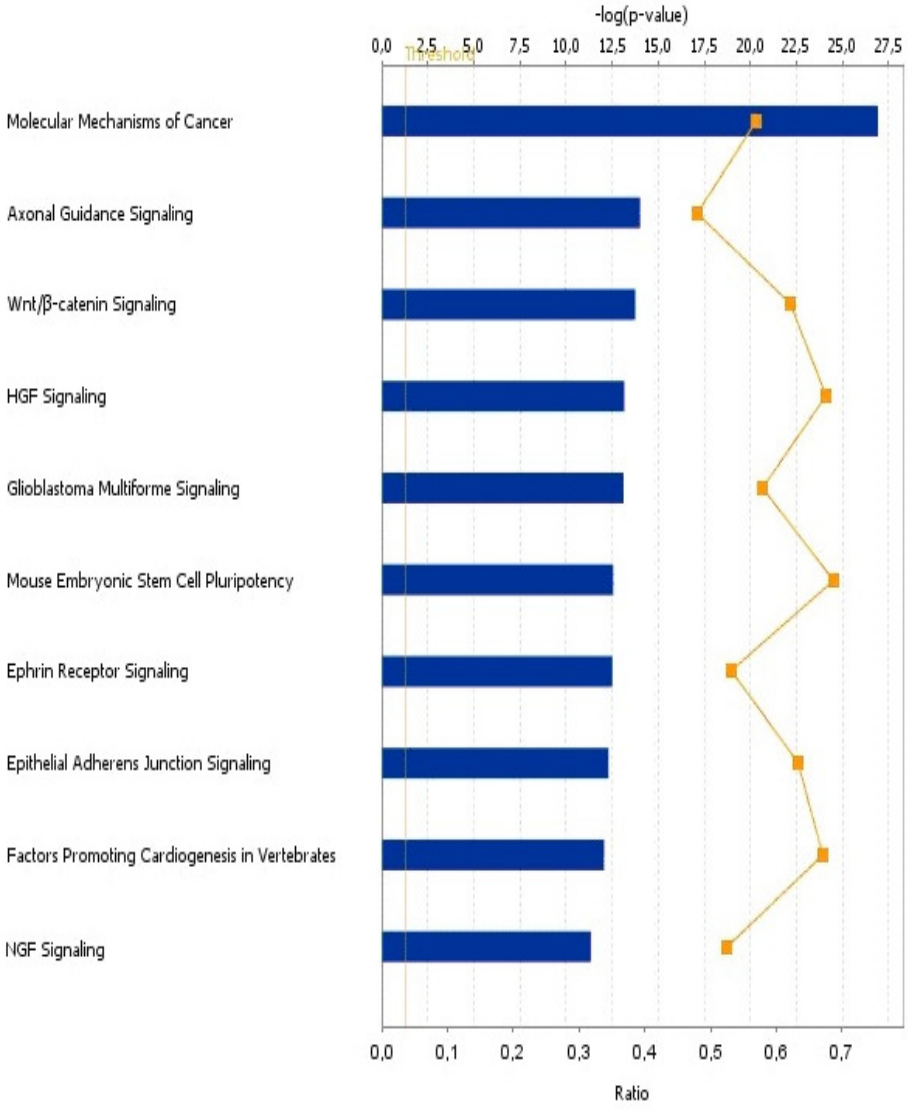

Figure 4.15 The most prominent canonical pathways related to the involved genes targeted by down regulated miRNA in follicular fluid. The bars represent the p-value for each pathway. The orange irregular line is a graph of the ratio (genes from the data set/total number of genes involved in the pathway) for the each pathway.

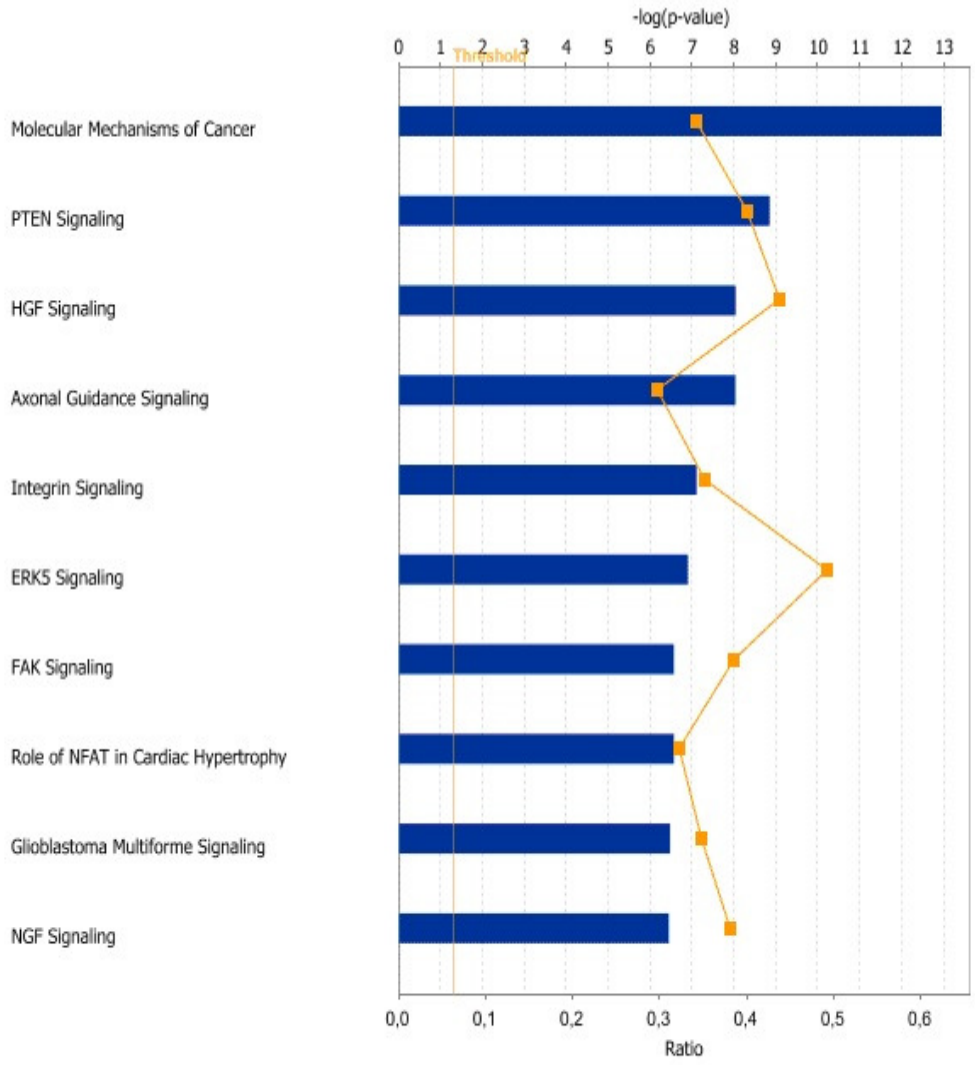

Figure 4.16 The most prominent canonical pathways related to the involved by genes targeted by up regulated miRNA in blood plasma collected at day 7. The bars represent the p-value for each pathway. The orange irregular line is a graph of the ratio (genes from the data set/total number of genes involved in the pathway) for each pathway.

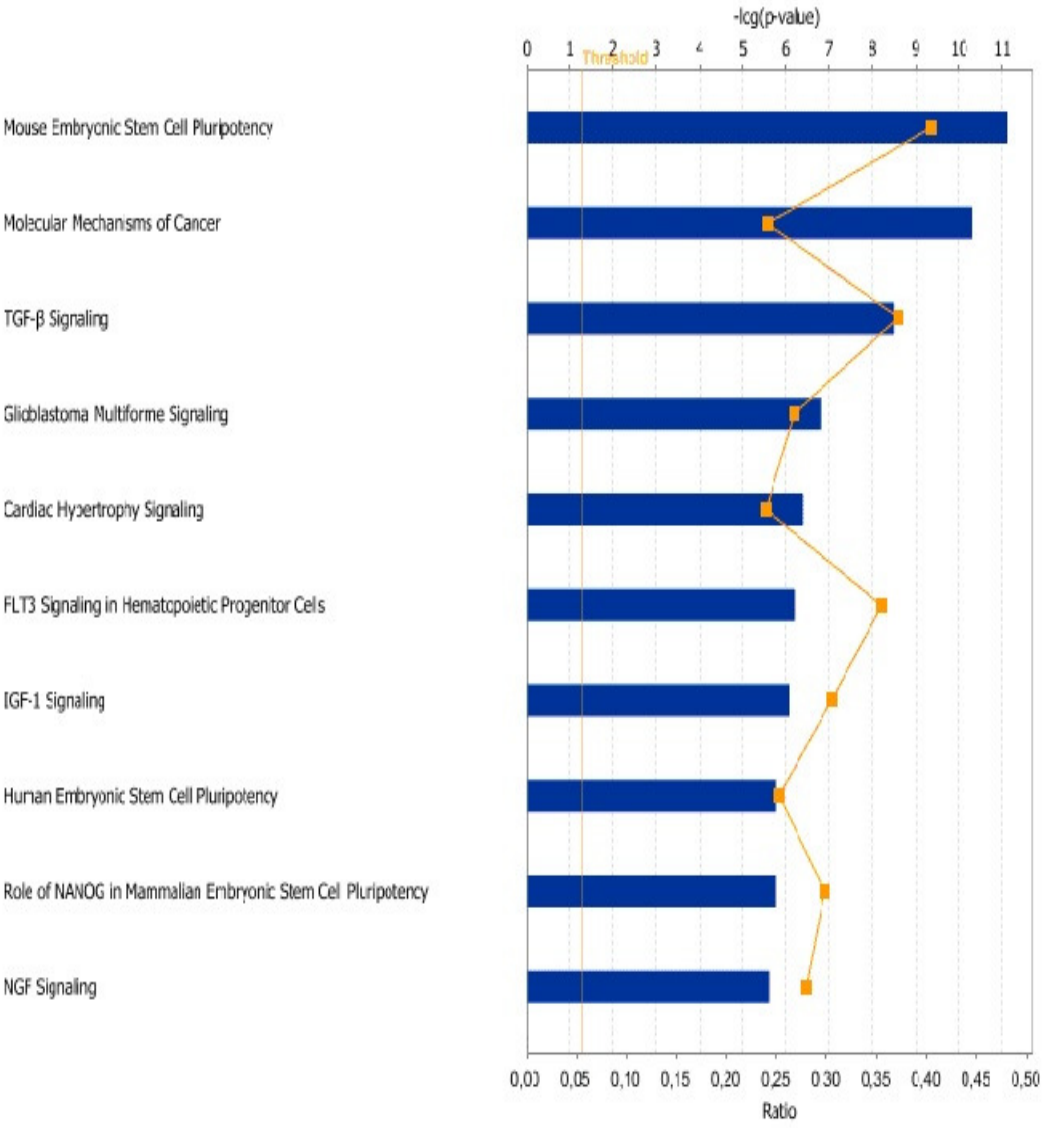

Figure 4.17 The most prominent canonical pathways related to the involved by genes targeted by down regulated miRNA in blood plasma collected at day 7. The bars represent the p-value for each pathway. The orange irregular line is a graph of the ratio (genes from the data set/total number of genes involved in the pathway) for each pathway.

5 Discussion

5.1 Polymerase chain reaction inhibitors in body fluid

A possible source of variation in miRNA array data from body fluids such as blood serum or plasma is the RNA isolation procedure and the presence of inhibitors that affect the cDNA synthesis and/or real time PCR reactions (Blondal et al. 2013). As cell-free miRNA concentrations are low in these samples, it is tempting to try to maximize the amount of sample used per reaction. In practice increasing the amount of RNA input could also lead to an increased concentration of PCR enzyme inhibitors such as heparin which may be derived from the bio-fluid or introduced via reagent carry-over during sample preparation and suboptimal RNA isolations. Therefore reliable results in real-time RT-PCR array and microarray analysis depend on the quality of the RNA sample. Traditionally, RNA integrity is determined by denaturing agarose gel electrophoresis, where intact RNA is indicated by a 2:1 ratio of the bands for 28S and 18S rRNA. Generally RNA purity is determined by spectrophotometrically. The ratio of absorbances at 260 nm and 280 nm (A260:A280) determines the degree of protein contamination and the A260:A230 ratio is used to identify any contamination by organic solvents. These ratios should be >1.8. Standard methods for measurement of the RNA yield and quality are inappropriate for use with bio-fluid samples. The presence of carrier RNA in these samples makes measuring the low levels of endogenous RNA by OD260 impossible. Even if the carrier was not included during the isolation, the RNA concentration in the eluate would still be too low for reliable OD260 quantification on a NanoDrop or other spectrophotometer. Therefore, an extensive alternative quality control of total RNA has been implemented to secure technical soundness. In order to check the quality of total RNA extracted from follicular fluid and blood plasma, we investigated two crucial parameters- presence of typical miRNAs which are abundantly present in bio-fluids and absence of inhibitors of cDNA synthesis and the PCR enzyme. These two parameters were investigated using a small set of single qPCR assays where cDNA was synthesized with different total RNA input (e.g. 0.5 µl, 1.0 µl, 2.0 µl, and 4.0 µl in a 10 µl reaction) to make a dilution curve. This pre-examination allowed us to discard these samples which fail to show linear amplification from depending on the RNA input.

5.2 Extracellular miRNAs expression profile in follicular fluid induced changes by COH

Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of granulosa and thecal cells (Fortune 1994). The follicular fluid is rich in metabolites, proteins, cytokines, vitamins, growth factors, and hormones. Some studies have also shown that the follicular fluid contains essential substances involved in follicle growth, oocyte fertilization and spontaneous abortion. The metabolite composition of the follicular fluid is correlated with the developmental competence of the human oocyte (Wallace et al. 2012), and some proteins in the follicular fluid that play roles in glucose metabolism, lipoprotein metabolism, cell proliferation, and insulin resistance have been found to be associated with polycystic ovary syndrome (Dai and Lu 2012). For example, increased ghrelin levels in the follicular fluid negatively affect human oocyte quality and in vitro embryo development (Li et al. 2011a).

The present study investigated for the first time, the effects of FSH-hyperstimulation treatment on expression profile of circulatory miRNA in cell-free bovine ovarian follicular fluid and blood plasma during estrus days. The primary objective of this study was to comprehensively define the dynamic changes in expression of circulatory miRNA in follicular fluid induced by COH during oestrus days. Our data showed COH induced change of circulatory miRNA expression profile in follicular fluid as microenvironment of oocyte maturation. Growth of the follicle encompasses enlargement of the oocyte, replication of follicular cells, and formation and expansion of a central follicular antrum or cavity. In vivo, follicle-stimulating hormone (FSH) in particular stimulates follicular growth. COH stimulates the ovaries by supraphysiological levels of gonadotrophins (FSH, GnRH) to induce multiple follicle in single ovulation species including humans and bovine.

Moreover it has been suggested that specific miRNAs may have crucial roles in the initiation and/or progression of bovine fertility through their effects on various molecular pathways. A better understanding of miRNA expression in different follicular stage may uncover novel molecular pathways, or novel mechanisms of activation for

known pathways associated with folliculogenesis. Role of gonadotrophins to activate or deactivate of steroid hormones production in theca and granulosa cells is a well established fact (Whirledge and Cidlowski 2010). miRNA profiling revealed the upregulation of a small subset of miRNAs in female mice deficient in estrogen receptor α(ERα) and conversely, estradiol treatment of ovariectomized female mice showed downregulation of some miRNAs in the uterus, which is an estrogen target organ (Macias et al. 2009).

Functional analysis in a human granulosa cell tumors -derived cell line cells showed that miR-24, miR-132, miR-320, miR-222, miR-520c-3p, miRNA-193b, and miRNA-483-5p regulated hormone production in granulosa cell (Sang et al. 2013). Their reported reveals the existence of miRNAs in human cell-free follicular fluid and demonstrates that some highly expressed miRNAs many have key functions in steroidogenesis in vitro and are associated with PCOS in vivo (Sang et al. 2013).

Studies have shown that miRNAs exist in serum (Mitchell et al. 2008), urine (Chan et al. 2003), saliva (Chan et al. 2003), milk (Kosaka et al. 2010) and semen (Li et al. 2012) and that these circulating miRNAs can be used as biomarkers for various cancers (Chen et al. 2008, Hu et al. 2010, Yu et al. 2012), cardiovascular diseases (Ai et al. 2010, Li et al. 2011b), and metabolic diseases (Zampetaki et al. 2010, Zhao et al. 2011). Until now, however, no studies have been carried out on the expression miRNAs in bovine follicular fluid under COH. In this study, we have used real time PCR assay to identify several miRNAs in the follicular fluid that appear to have important functions in a variety of cellular processes. Previous studies demonstrated that circulating miRNAs in serum and other body fluids were found to exist in both microvesicles and AGO2 complex (Arroyo et al. 2011, Turchinovich et al. 2011). Consistent with these studies, we evidenced expression of the follicular fluid miRNA being coupled in microvesicles and AGO2 protein complex.

5.3 Circulatory miRNAs expression affected in blood plasma by hyperstimulation

Plasma miRNAs are a promising source of potential biomarkers for non-invasive diagnosis of many diseases. Most plasma miRNA biomarkers associated with different pathologies that have been discovered so far are also found to be expressed at high levels in blood cells. The alterations in blood cell counts and specimen handling procedures may have an impact on the candidate circulating cell-free miRNA biomarker specificity, as the changes observed may be caused by physiological changes rather than the disease (Pritchard et al. 2012, Rekker et al. 2013) .

Considering the temporal miRNA expression profile miRNAs (miR-125a, miR-199a-3p, miR-125b, miR-99a, let-7c, miR-145, miR-31, miR-202 and miR-27b) with decreased expression and miRNAs (miR-503, miR-21, miR-29b, miR-142-3p, miR-34a, miR-152, miR-25 and miR-130a) with increased expression between the follicular and luteal stages in ovine ovarian tissues were descried (McBride et al. 2012). On the other hand a recent study reported no differences in plasma miRNA expression levels between the menstrual cycle time-points. Based on this finding, that miRNA levels detectable in plasma of healthy women are not significantly altered by the processes occurring during the menstrual cycle. It is probable that miRNA expression changes in tissues are necessary for the local gene expression regulation but the fluctuations are insufficient to alter the miRNA patterns detectable in circulation (Rekker et al. 2013).

miRNAs regulate many cellular processes that also occur during the cyclic changes in the endometrium and other tissues. Several studies have demonstrated differential expression of miRNAs in endometrium during the estrus cycle and have emphasised their role in endometrial differentiation into its receptive state (Altmae et al. 2013; Kuokkanen et al. 2010; Sha et al. 2011). These studies reported up to seven fold differences in miRNA expression levels between receptive (LH day 7) and non-receptive (cycle day 12 or LH day 2) endometrium, where miR-30d, miR-30b, miR-31, miR-193a-5p, miR-203 showed up-regulation and miR-503 down-regulation in receptive endometrium (Altmae et al. 2013; Kuokkanen et al. 2010; Sha et al. 2011).

Our result first time showed after COH significant changes of the circulatory miRNA profile in blood plasma, induced by the supraphysiological progesterone concentrations associated with ovulation induction. We hypothesise circulatory miRNAs expression affected in blood plasma on day 7 past estrus by hyperstimulation on basis of physiological changes in tissue that effected by hyperstimulation(figure 4.6). The supra-physiological concentrations of steroid hormones, secreted by a higher number of corporalutea during the early luteal phase, seems to be the main cause of luteal phase defect in hyperstimulated IVF cycles in human and bovine that in term directly inhibit LH release via negative feedback actions at the hypothalamic–pituitary axis level (Fauser and Devroey 2003). Indeed, expression levels of miRNAs seem to be more important in the preimplantation embryos, around the MET rather than at the maturing oocyte stages. Recent studies have demonstrated that changes in global miRNA expression pattern in circulating body fluids and cells are influenced by several demographic and normal physiological variables. Age (Sredni et al. 2011), gender (Duttagupta et al. 2011) have been shown to be some of the factors contributing to miRNA expression level differences (Rekker et al. 2013) .

Meanwhile, different expression profiles have been obtained for other miRNAs during early embryo development. Some of them showed higher levels in the oocyte while others showed a very weak expression level throughout early development and an augmentation only at the blastocyst stage (Tripurani et al., 2010). A negative effect of hyperstimulation on embryo development rate was reported, as evidenced by the greater proportion of morula than blastocysts flushed from the hyperstimulated heifers at day 7 compared to unstimulated heifers (Gad et al. 2011). Otherwise oviducts were unable to support migration of oocytes and were therefore non-functional and the uterus had a decrease for epithelium compared to normal uteri (Gonzalez and Behringer 2009). On other studies in the mouse uterus, progesterone treatment significantly increased the expression of Dicer1 and Exportin-5, two components of the miRNA biogenesis pathway (Cochrane et al. 2012; Nothnick et al. 2010). This suggests developmental retardation of embryos developing under hyperstimulation conditions. In addition, the increasing expression level of the immature forms of miR-21 (primary and precursor) at the 2-cell embryo, together with its sensitivity to a-amanitin treatment, indicates the

possibility that they represent a portion of the minor transcriptional activation (Mondou et al. 2012) and pregnancy stage (Gilad et al. 2008).

5.4 Expression pattern of candidate circulatory miRNAs in exosomes and Ago2 fraction

In this work, we addressed questions regarding, the possible origin and biological function of circulating extracellular miRNA in bovine follicular fluid and blood plasma. Cells release microvesicles and exosomes that can transfer biological materials over long distances when released into the bloodstream, and potentially modulate cell function at target cells. Microvesicles and exosomes are present in a variety of body fluids in health and disease (urine, blood, and ascites fluid), suggesting that they have a role in normal homeostasis and are released under disease conditions (da Silveira et al. 2012).

As major tool for the characterization of miRNAs on basis of carrier differential ultracentrifugation and Ago2 immunoprecipitation were used to isolate cell-secreted exosomes or miRNA/Ago2 complexes from ovarian follicular fluid and blood plasma. We utilized individual miRNA assays for three selected miRNAs .These miRNAs were all detected as differentially regulated miRNA in bovine follicular fluid and blood plasma. Once we confirmed the efficient recovery of exosomes and Ago2 protein complex from follicular fluid, we investigated the expression pattern of candidate miRNAs in both fractions (figure 4.7 and 4.8). By using a systemic approach, we have revealed that there are at least two populations of circulating miRNAs in follicular fluid and blood plasma, namely exosomes and Ago2 complex associated. The specificity of exosomes isolation were confirmed by the presence of CD63 in isolated exosomes (Gallo et al. 2012; Logozzi et al. 2009; Valadi et al. 2007). It has been well documented that platelet-derived exosomes are enriched in CD63. However, this tetraspan protein is also found in exosomes from other cell types. Tetraspan proteins have been implicated in adhesive as well as costimulatory and signaling functions. To support the hypothesis that along with exosomes Argonaute2 protein complex also carry a sub set of miRNA in follicular fluid, we checked the presence of Ago2 protein abundance in non-exosomal portion of follicular fluid. Recent reports have suggested that a common source of

unwanted bias in miRNA profiles in serum or plasma samples is cellular contamination of the samples with various cell types from blood or their donor cells (McDonald et al. 2011). Expression pattern of candidate miRNAs was measured by qRT-PCR and resulting data are shown as raw Ct value in Figure 4.9, 4.10 and 4.11. Our data, demonstrated miR-182 in follicular fluid, miR-127-3p and miR-103 was not detected in Ago2 complex portion, that conduct shows that not all extracellular miRNA are associated with the Ago2 complex. On the other hand, miR-34a, miR-32 miR-221, miR-410, miR-494 and miR-153 were not detected in both portions of exosomal and Ago2 complex in blood plasma. Expression of these candidates in Ago2 fraction of follicular fluid shows aberrant pattern.

Expression data showed not all selected miRNAs were present in the Ago2 complex portion and exosomal portion. We validated exosomes mediated cell-cell communication within follicular microenvironment; we examined uptake of exosomes and resulting increase of endogenous miRNA level and subsequent alteration of mRNA levels in follicular cells form of exosome between follicular cells. We have investigated the ability of granulosa cells to take up exosomes isolated from follicular fluid *in vitro*. The presence of exosome or non-exosome mediated transfer of miRNA in the bovine follicular fluid, and oocyte growth dependent variation in extra-cellular miRNA signatures in the follicular environment was shown (Sohel et al. 2013). Similar studies have also shown the stability of circulating serum miRNAs (Gilad et al. 2008), breast milk exosomal miRNA at room temperature and other multiple freeze-thaw cycle (Zhou et al. 2012).

We hypothesize that miRNAs absent in exosomes and Ago2/miRNA complex are carried by HDL transport within the developing follicle. This may be critical to normal oocyte development and emphasizes the need for further studies to elucidate the mechanisms that regulate intrafollicular circulatory miRNA transport during folliculogenesis. A paradigm of autocrine and paracrine miRNA signalling through exosomes cannot be excluded. Recent studies demonstrate circulatory miRNA are associated with HDL (Vickers et al. 2011). The remarkable study provided the first clue to an important relationship that exists between HDL particles and the ovarian follicle

(Trigatti et al. 1999). This study was designed before the novel finding that circulatory miRNA are associated with HDL.

The physiological role of circulating miRNAs coupled with HDL particles in the developing follicle and embryo has remained obscure. HDL appears to be the dominant lipoprotein particle in follicular fluid during human folliculogenesis (Fujimoto et al. 2010) . The physiological role of HDL in the developing follicle and continuing pregnancy is well established that both HDL and low-density lipoprotein (LDL) deliver cholesterol to the corpus luteum as a substrate for progesterone synthesis (Azhar et al. 1998, Reaven et al. 2000, Roy and Belanger 1992).

Oxidative stress caused by using ART method is main open issue. It is also well established that HDL particles can moderate reactive free radicals, thereby limiting oxidative damage to cells and other lipoproteins (Decossin et al. 1995, Klimov et al. 1993, Kunitake et al. 1992, Mackness and Durrington 1995, Parthasarathy et al. 1990). Numerous animal models illustrating the effects of HDL metabolism on reproductive outcomes suggest that HDL cholesterol metabolism may be important in human and bovine reproduction (Aiello et al. 2003, Bujo et al. 1994, Christiansen-Weber et al. 2000, Fujimoto et al. 2010, Miettinen et al. 2001, Nimpf et al. 1989, Trigatti et al. 1999, Yesilaltay et al. 2006). Browne et al., demonstrated by the scavenger receptor class B type I (SR-BI KO) mouse model, the role of serum and follicular fluid HDL particles on the reproductive potential of the human oocyte using embryo morphologic parameters as surrogate biomarkers for oocyte quality during IVF. Their findings showed negative correlations between follicular fluid HDL cholesterol and Apo AI levels with embryo fragmentation evaluated after 72 h of IVF culture (Browne et al. 2008).

5.5 Predicted target genes and network analysis

Bioinformatics analysis revealed that several important target genes of the differently expressed miRNAs are related to PTEN signaling, neurotrophin signaling pathway, HGF signaling pathway, VEGF signaling pathway, TGF-β signaling pathway, axonal

guidance signaling and Wnt ß-catenin signaling pathways to be the dominant ones (Figure 4.14, 4.15, 4.16 and 4.17).

As previously mentioned, miR-132, miR-24, miR-320 and miR-520c-3p were found to regulate steroidogenesis in vitro (Sang et al. 2013). miRNA-132 is known to lead to the activation of nuclear factor κB and to the transcription of IL-8 and monocyte chemoattractant protein-1 (MCP-1) in both primary human preadipocytes and differentiated adipocytes (Strum et al. 2009). In addition, serum miRNA-132 levels are significantly decreased in gestational diabetes mellitus, suggesting that miRNA-132 may be involved insulin secretion (Zhao et al. 2011). miRNA-132 is also known to have key roles in immune system development, metabolic pathways and neurodevelopment (White and Giffard 2012). First identified a partial amino acid sequence from transforming growth factor beta 1 (TGFB1) purified from blood platelets, numerous studies have highlighted the role of this peptide as a modulator of cell growth, proliferation and differentiation in many types of cells and tissues (Gilchrist and Ritter 2011, Nagamatsu et al. 2012). It is also thought that TGF proteins are involved in the regulation of oocyte and embryonic growth and development (Juengel and McNatty 2005).

Vascular endothelial growth factor (VEGF) has been reported to be involved in oocyte maturation as it enhances vascularisation via stimulation of the vascular endothelial growth factor receptor (VEGFR) leading to migration and differentiation of blood vessels, thereby increasing the availability of serum factors such as follicle stimulating hormone (FSH) and LH. The EGF-like growth factor amphiregulin (AREG), functions to mediate the LH induction of ovulation in pre-ovulatory follicles and granulosa cell differentiation (Ashkenazi et al. 2005, Hsieh et al. 2007, Park et al. 2004b) Another important factor is growth differentiation factor 9 (GDF9), which is a member of the TGF-β family and has been shown to be expressed by mammalian oocytes throughout ovulation beginning in pre-antral follicles and is involved in the selection of primordial follicles for further development beyond the primary follicle stage (Aaltonen et al. 1999) .

Other signalling factors which have been shown to impact ovarian cell function and organization are members of the Wnt and Frizzled family of signalling molecules. Wnts are secreted signalling molecules that act to control many processes influencing cell proliferation and differentiation. Wnts act by binding to G-protein-coupled receptors of the Frizzled family stimulating signalling cascades that impact the development and function of reproductive organs. Wnt-4 provides an example of the importance of such signalling factors, as mice null for Wnt-4 have sex reversed ovaries and express genes characteristic of testes development such as Müllerian inhibiting substance (Vainio et al. 1999). Wnt-4 is expressed in primary follicle granulosa cells and has been shown to have increased expression following the LH surge. Although it is known that the Wnt/Frizzled signalling pathway is important for the growth and development of ovarian follicles, the regulatory mechanisms of these molecules as well as other potential downstream targets needs to be further investigated (Richards et al. 2002).

6 Summary

Controlled ovarian hyperstimulation (COH) stimulates the ovaries by supraphysiological levels of gonadotropins to induce the growth of multiple follicle in single ovulation species including humans and bovine. In bovine, in order to increase the number of offspring during the lifetime of the individual, female animals with high genetic value are typically stimulated with gonadotropin hormones to persuade the ovulation of a variable but many number of follicles. For several years, the most common use of embryo transfer has benefited from the establishment of newer, more efficient methods for the superovulation of donors, embryo retrieval / transfer by low-invasive methods as well as by the better, simpler and more effective cryopreservation methods in human and bovine. However despite significant advances in assisted reproduction technology protocols in humans and bovine, pregnancy rates are still relatively low and have not increased significantly in the last decade.

MicroRNAs are post transcriptional regulators that influences expression of hundreds of genes in numerous biological processes including development. Circulatory noncoding small RNAs (miRNAs), which are present in various body fluids, are reported to be potentially used as biomarkers for disease and pregnancy. The present study was preformed to investigate the effect of ovarian hyperstimulation on the expression pattern of circulatory miRNA in follicular fluid and blood plasma. For this, Simmental heifers (n=12) were synchronized using a standard synchronization protocol and six of them were hyperstimulated using FSH. Following this, while blood samples were collected at 0 (onset of oestrous), 3rd and 7th day, follicular fluid samples were aspirated from dominant follicles at the day 0 from all animals by ovum pickup. Total RNA isolation was done using miRNeasy® Mini kit. From each follicular fluid and blood plasma portion, 100 ng of total RNA was reverse transcribed using Universal cDNA synthesis kit. The resulted cDNA from total RNA was subsequently diluted 100 fold and the human miRCURY (Human Panel I+II, V2.M) Ready-to-Use PCR array (Exiqon) consisting of 748 miRNAs, was used for the expression profile of circulatory miRNA in both portion of follicular fluid and blood plasma. The qPCR data was

analyzed using a comparative threshold cycle (ΔCT) method after normalization by global normalization method. To minimize potential noise, miRNAs with a Ct value of > 35 were filtered out. Subsequent expression profiling of miRNA was performed using the human miRCURY LNA™ Universal RT miRNA PCR array platform with 745 miRNA primer assays. Of the 24 miRNAs, which were differentially expressed in blood plasma between hyperstimulated and unstimulated animals, 9 miRNAs including miR-127-3p, miR-494, miR-147, miR-134 and miR-153 were down regulated and 15 miRNAs including miR-34a, miR-103, let-7g, miR-221 were found to be up regulated in the hyperstimulated animals. Similarly, 66 miRNAs were found to be differentially expressed in follicular fluid derived from hyperstimulated and unstimulated groups. Out of these, while 32 miRNAs, were down regulated, while 34 were up regulated in follicular fluid aspirated from hyperstimulated animals.

To determine the extent to which circulatory miRNAs are associated with exosomes in follicular fluid and blood plasma on day 7 we used differential ultracentrifugation to purify circulating exosomes from cell free, platelet-poor follicular fluid and blood plasma at day 7 prepared from same animals. To determine if Ago 2 proteins are present in plasma, we performed immunoprecipitation followed by Ago2 protein blotting. By using quantitative RT-PCR, we assayed expression levels of a candidate set of miRNAs in the vesicle pellets and the immunoprecipitated Ago2 for well characterized circulating miRNAs of follicular fluid and blood plasma in hyperstimulated and unstimulated heifers. To evaluate the expression of the selected miRNAs we examined temporal changes in expression levels of a candidate set of miRNAs in blood plasma at day 0, 3 and 7 the onset of oestrus of hyperstimulated as compared to unstimulated in normal conditions.

Ingenuity pathway analysis (IPA) of potential target genes of candidate miRNAs, which were dysregulated due to ovarian hyperstimulation, revealed that the genes targeted by the miRNAs were mainly involved in PTEN signaling , neurotrophin signaling pathway, and HGF signaling pathway, VEGF signaling pathway, TGF-β signaling pathway, axonal guidance signaling and Wnt ß-catenin signaling pathways to be the dominant ones.

In conclusion, the present study reported for the first time bovine ovarian hyperstimulation to induce changes in expression profiles of circulatory miRNA coupled with exosomes or non-exosomes structures (i.e. Ago2, HDL) in follicular fluid and blood. This data highlight a number of differentially expressed circulatory miRNAs in bovine follicular fluid and blood plasma in relation to oocyte development competence and early bovine embryo development. Moreover, this dissertation also documented the expression profile of circulatory miRNA in follicular fluid and blood which considers the extra cellular miRNA role in the cell-cell communication. Our result is basis of the hypothesis that circulatory miRNA affect development competence of oocyte and early bovine embryo development. Although this data open a new area of extra-cellular miRNA research regarding microenvironment such as follicular and oviductal fluid, in-depth functional studies will be required to understand the functional contribution of differentially expressed miRNAs to acquire follicular and early embryo development under supraphysiological levels of gonadotropins.

7 Zusammenfassung

Durch eine kontrollierte ovarielle Hyperstimulation (COH) der Eierstöcke mittels eines supraphysiologischen Gonadotropin-Spiegels wird das Wachstum mehrerer Follikel angeregt. Wertvolle Zuchtrinder werden ebenfalls mit Gonadotropin stimuliert um ihre Nachkommenzahl zu erhöhen. Fortschritte wurden bei der Superovulation und der Anwendung wenig invasiver Methoden bei der Embryogewinnung und dem Transfer sowie bei der Embryo-Tiefgefrierung gemacht. Doch trotz des erheblichen Fortschritts in den assistierten Reproduktionstechnologien beim Mensch und Rind, sind die Entwicklungsraten relativ niedrig und haben nicht wesentlich in den letzten zehn Jahren zugenommen.

MicroRNAs (miRNAs) sind Posttranskriptionsregulatoren, die die Expression von hunderten von Genen in zahlreichen biologischen Prozessen, einschließlich der Entwicklung, beeinflussen. Zirkulierende nicht-kodierende miRNAs sind in verschiedenen Körperflüssigkeiten vorhanden und können möglicherweise potenzielle Biomarker für Krankheiten und Trächtigkeiten sein. Die vorliegende Studie wurde durchgeführt, um die Wirkung einer ovarialen Hyperstimulation auf das Expressionsmuster von zirkulierenden miRNAs in der Follikelflüssigkeit und im Blutplasma zu untersuchen. Dazu wurden Fleckvieh-Färsen (n = 12) mit einem Standard-Synchronisationsprotokoll synchronisiert und sechs von ihnen mit FSH hyperstimuliert. Die Probenentnahme beinhaltete Blutproben zum Zeitpunkt 0 (Beginn der Brunst), am 3. und am 7. Tag sowie die Entnahme von Follikelflüssigkeit von dominanten Follikeln am Tag 0 von allen Tieren durch Ovum pickup.

Die Gesamt-RNA Isolierung erfolgte mit dem miRNeasy ® Mini-Kit. 100 ng Gesamt-RNA aus der Follikelflüssigkeit und dem Blutplasma wurden mit dem Universal cDNA-Synthese-Kit transkribiert. Die resultierende cDNA wurde anschließend 100-fach verdünnt. Mittels des Human miRCURY (Human Panel I + II, V2.M) Ready-to-Use-PCR-Array (Exiqon), bestehend aus 748 miRNAs, wurde von den zirkulierenden miRNAs aus der Follikelflüssigkeit und dem Blutplasma ein Expressionsprofil erstellt. Die Analyse der qPCR-Daten erfolgte durch einen Vergleich der Schwellenzyklen

(ΔCt-Methode) nach einer Normalisierung durch das globale Normierungsverfahren. Um mögliche Störungen zu minimieren, wurden miRNAs mit einem Ct-Wert von > 35 herausgefiltert.

Die nachfolgende Expressionprofilierung der miRNAs erfolgte unter Verwendung der Humanen miRCURY LNA ™ Universal- RT -PCR- miRNA -Array -Plattform mit 745 miRNA Primer -Assays. Von den 24 miRNAs, die im Blutplasma beim Vergleich zwischen hyperstimulierten und unstimulierten Tiere unterschiedlich exprimiert waren, zeigten 9 miRNAs, einschließlich, miR- 127- 3p, miR- 494, miR- 147, miR- 134 und miR -153, eine Runterregulation während 15 miRNAs einschließlich miR- 34a, miR -103 , let- 7g, miR -221 eine erhöhte Expression in den hyperstimulierten Tiere aufwiesen. Des Weiteren konnten beim Vergleich der Follikelflüssigkeit von hyperstimulierten und unstimulierten Tieren 66 differentiell exprimierte miRNAs identifiziert werden. Von diesen waren 32 miRNAs herunterreguliert, während 34 in der Gruppe der hyperstimulierten Tiere raufreguliert waren.

Um das Ausmaß der Assoziation der zirkulierenden miRNAs mit Exosomen aus Follikelflüssigkeit und Blutplasma an Tag 7 festzustellen, wurde mittels Dichtegradientenzentrifugation von denselben Tieren die zirkulierenden Exosomen von der zellfreien, plättchenarmen der Follikelflüssigkeit und dem Blutplasma am Tag 7 gereinigt. Um festzustellen ob Ago 2 Proteine im Plasma vorhanden sind, führten wir eine Immunpräzipitation durch, dieser folgte ein Ago 2 Protein –Blot. Mittels quantitativer RT-RCR analysierten wir eine Reihe gut charakterisierter miRNAs aus Follikelflüssigkeit und Blutplasma in hyperstimulierten und unstimulierten Färsen. Temporale Expressionsprofile der miRNAs wurden an den Tagen 0, 3 und 7 des Zyklus zwischen stimulierten und unstimulierten Färsen erhoben und verglichen. Die Ergebnisse zeigten signifikante Expressionsveränderungen der meisten miRNAs zwischen den verschiedenen Zyklustagen, während innerhalb der gleichen Zyklustage ähnliche Expressionsmuster zwischen stimulierten und unstimulierten Färsen zu beobachten waren. Lediglich miR-32 war am Tag 3 bei unstimulierten Färsen im Vergleich zu hyperstimulierten herunterreguliert. Eine Ingenuity Pathway Analyse (IPA) der potentiellen Zielgene von Kandidaten miRNAs, die aufgrund von Hyperstimulation der Ovarien eine Fehlregulation zeigten, ergab als dominante

Signalwege die PTEN Signalisierung, den Neurotrophin Signalweg, den HGF Signalweg, den VEGF Signalweg, den TGF-ß Signalweg sowie den Wnt-ß-Catenin Signalweg.

Die Ergebnisse der vorliegenden Studie berichten zum ersten Mal über die Veränderungen der Expressionsprofile von zirkulierenden miRNAs aus der Follikelflüssigkeit und dem Blutplasma von Rindern mit einer induzierten Hyperstimulation der Ovarien sowie von einer möglichen Kopplung der zirkulierenden miRNAs mit Exosomen oder nicht Exosomen Strukturen (z.B. Ago 2, HDL). Es wurden eine Reihe von differenziell exprimierten zirkulierenden miRNAs in Follikelflüssigkeit und Blutplasma in Bezug auf die Entwicklungskompetenz der Eizelle und frühen Rinderembryoentwicklung identifiziert. Diese Dissertation dokumentiert das Expressionsprofil extrazellulärer zirkulatorischer miRNAs in Follikelflüssigkeit und Blut. Die Daten unterstützen die Hypothese, dass miRNAs sowohl an der Oozyten- als auch der frühen Embryonalentwicklungskompetenz beteiligt sind. Weitergehende funktionelle Unternehmungen werden notwendig sein, um die an dem Follikelwachstum unter Hyperstimulationsbedingungen beteilige Genregulation näher abzuklären.

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