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Exosomal and non-exosomal circulatory miRNAs in bovine follicular fluid: potential role of exosomal miRNAs in oocyte development

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Dedicated to my Mom and Dad, the two people I could always count on for love and support. I would also like to dedicate this work to my lovely wife, Masreka Khan, the angel who held my hand through this educational journey.

Exosomal and non-exosomal circulatory miRNAs in bovine follicular fluid: potential role of exosomal miRNAs in oocyte development

Growth and development of bovine follicle and oocyte is the result of series of complex and coordinated processes that involves extensive cell-to-cell communication in the follicle. This phenomenon involves enormous complex and heterogeneous biochemical substances existing in the oocytes and its surrounding cells and the follicular fluid. Cell-cell communication within the follicle involves many signaling molecules, and this process may be mediated by secretion and uptake of exosomes that contain several bioactive molecules including extra-cellular miRNAs. The molecular mechanism of oocytes development and interaction between oocyte and follicular cells in the follicular micro-environment remains vague. Follicular fluid and cells from individual follicles of cattle were grouped based on Brilliant Cresyl Blue Pro staining of the corresponding oocytes. Both Exoquick™ precipitation and differential ultracentrifugation were used to separate the exosome and non-exosomal portion of follicular fluid. Following miRNA isolation from both fractions, the human miRCURY LNA™ Universal RT miRNA PCR array system was used to profile miRNA expression. Western blot analysis against specific protein and electron microscopy imaging confirms the efficient separation of exosomal and non-exosomal fraction of follicular fluid. The real time qPCR array analysis revealed that a handful number of miRNAs are present in both exosomal and non-exosomal portion of bovine follicular fluid. Results revealed 25 miRNAs differentially expressed (16 up and 9 down) in exosomes and 30 miRNAs differentially expressed (21 up and 9 down) in non-exosomal portion of follicular fluid in comparison of BCB- versus BCB+ oocyte groups. Expression of selected miRNAs was detected in theca, granulosa and cumulus cells that may indicate the origin of extra-cellular miRNAs in follicular fluid. To further explore the potential roles of these extra-cellular miRNAs in follicular fluid, the potential targets were predicted using in silico based analysis, and functional annotation and pathway analysis revealed most of these pathways are known regulators of follicular development and oocyte growth. In order to validate exosome mediated cell-cell communication within follicular microenvironment, we demonstrated uptake of exosomes and resulting increase of endogenous miRNA level and subsequent alteration of mRNA levels in follicular cells *in vitro*. The present study demonstrates for the first time, 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.

Exosomale und non-exosomale zirkulierende miRNAs in der Follikelflüssigkeit beim Rind: Die Rolle von exosomalen miRNAs in der Oozytenentwicklung

Wachstum und Entwicklung der Rinder Follikel und Eizellen ist das Ergebnis einer Reihe von komplexen und koordinierten Prozessen, die umfangreiche Zell-Zell-Kommunikation innerhalb der Follikel beinhaltet. Diese Interaktion beruht auf komplexen und heterogenen biochemischen Stoffen sowohl in den Eizellen, den benachbarten Zellen als auch in der Follikelflüssigkeit. Die Zell-Zell-Kommunikation innerhalb der Follikel ist geprägt durch viele Signalmoleküle. Dieser Prozess kann durch Sekretion und Aufnahme von Exosomen die mehrere bioaktive Moleküle einschließlich extrazellulärer miRNAs enthalten, vermittelt werden. Bisher jedoch, sind die molekularen Mechanismen der Eizellenentwicklung und der Interaktion zwischen Eizellen und Follikelzellen in der follikulären Umgebung noch nicht voll aufgeklärt. Follikelflüssigkeit und Zellen aus einzelnen Rinder Follikeln wurden auf der Grundlage einer Vitalfärbung der entsprechenden Eizellen mittels Brillant Cresyl blau Pro gruppiert. Sowohl eine Exoquick-Präzipitation als auch differentielle Ultrazentrifugation wurde verwendet, um die exosomalen und non-exosomalen Bestandteile der Follikelflüssigkeit zu trennen. Nach miRNA Isolation von beiden Fraktionen wurde mittels des humanen miRCURY LNA™ Universal RT miRNA PCR-Array-System, ein miRNA Expressionsprofil erstellt. Durch Western-Blot-Analysen und Elektronen-Mikroskopie konnte die effiziente Trennung von exosomalen und non-exosomalen Bestandteilen in der Follikelflüssigkeit bestätigt werden. Mittels Echtzeit-qPCR-Array-Analysen konnte einige miRNAs im exosomalen und non-exosomalen Teil der Rinder Follikelflüssigkeit nachgewiesen werden. Von diesen waren 25 miRNAs im exosomalen Teil differentiell exprimiert (16 rauf- und 9 runter reguliert) und 30 miRNAs waren im non-exosomalen Teil der Follikelflüssigkeit differentiell exprimiert (21 rauf- und 9 runter reguliert) im Vergleich zu den BCB- versus BCB+ Oozyten Gruppen. Im Anschluss daran, wurde die Expression ausgewählter miRNAs in Thecazellen, Granulosa- und Kumuluszellen ermittelt. Das Ergebnis könnte ein Hinweis auf die Herkunft der extrazellulären miRNAs in der Follikelflüssigkeit sein. Für eine genauere Erklärung der möglichen Funktion dieser extrazellulären miRNAs in der Follikelflüssigkeit wurden potenziellen Zielgene mit in silico Analyse, funktioneller Annotation und Pathway-Analysen untersucht. Dies ergab, dass die miRNAs überwiegend an Signalwegen der Regulation der follikulären Entwicklung und des Eizellen Wachstums beteiligt sind. Um die Ergebnisse in Bezug auf die exosomal vermittelte Zell-Zell-Kommunikation in der follikulären Mikroumgebung zu überprüfen, konnten wir nachweisen, dass aus der Aufnahme des Exosoms in die Zelle eine Erhöhung des miRNA-Levels mit nachträglicher Veränderung des mRNA-Niveaus von Targetgenen in Follikel-Zellen in vitro resultiert. Die vorliegende Studie zeigt zum ersten Mal, den von exosomalen oder non-exosomalen Bestandteilen vermittelten Transfer von miRNA in die bovine Follikelflüssigkeit. Weiterhin ist das Wachstum der Eizellen von den Variationen der extrazellulären miRNA Signaturen in der follikulären Umgebung abhängig.

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List of abbreviation

A	Adenine
Abs	Apoptotic bodies
ACC. No	Gene bank accession number
aRNA	Amplified ribonucleic acid
Ago	Argonaute protein
AI	Artificial insemination
Alix	ALG-2 interacting protein X
AMH	Anti-Müller-Hormon
AMI	Acute myocardial infarction
ATP	Adenosine tri phosphate
BCB	Brilliant cresyl blue
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
CHX	Cycloheximide
CL	Corpus luteum
COCs	Cumulus oocyte complex
cRNA	Complementary ribonucleic acid
cTnl	Cardiac troponin l
Cx	Connexin
CXCL	Chemokine (C-X-C Motif) ligand
Dcp	Dipeptidyl carboxypeptidase
DCs	Dendritic cells
ddH2O	Deionised and demineralised millipore water
DE	Differentially expressed
DEPC	Diethylpyrocarbonate
DGCR8	DiGeorge syndrome critical region gene 8
DMAP	Dimethylaminopurine
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EE	Early endosome
ES	Embryonic stem cell
ESCRT	Endosomal sorting complex required for transport
ER	Endoplasmic reticulum
Exo	Exosome
FBs	Fibroblast cells
FDR	False discovery rate
FF	Follicular fluid
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
G6PDH	Glucose 6 phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GEO	Gene expression omnibus
GH	Growth hormone
GO	Gene ontology
GVBD	Germinal vesicle breakdown
hr	Hour
HCC	Hepatocellular carcinoma
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HEK	Human Embryonic Kidney
hES-MSC	Stem cell-derived mesenchymal stem cells
HF	Heart failure
HGF	Hepatocyte growth factor

ICI	Intra-cytoplasmic injection
ICM	Inner cell mass
ILV	Intraluminal vesicles
IPA	Ingenuity pathway analysis
IVF	In vitro fertilization
IVM	In vitro maturation
IVT	In vitro transcription
IVP	In vitro production
iPS	Induced pluripotent stem cells
kDa	Kilo dalton
KGF	Keratinocyte growth factor
KL	Kit ligand
KO	Knock out
LH	Luteinizing hormone
LNA	Locked nucleic acid
MI	Metaphase I
MII	Metaphase II
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MeOH	Methanol
mg	Milligrams
MHC	Major histocompatibility class
min	Minute
miRNA	Micro RNA
MPs	Micro particles
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
P-bodies	Processing bodies

PBS	Phosphate buffer saline
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
SLS	Sample loading solution
SMCs	Smooth muscle cells
SSC	Sodium chloride sodium citrate
TC	Theca cell
TCM	Tissue culture media
TE	Trophectoderm
TGF-ß	Transforming growth factor beta
THP-1 cells	Human monocytic leukemia cell line
TICs	Theca-interstitial cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNRC	Trinucleotide Repeat Containing
TRBP	TAR RNA binding protein
tRNA	Transfer ribonucleic acid
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

Growth and development of bovine follicle and subsequent maturation of oocyte is the result of series of complex and coordinated processes that involves functional and morphological changes in different types of follicular cells and extensive cell-to-cell communication within follicular micro-environment (Eppig 2001). The close physical association of two cell types in follicular microenvironment, namely somatic cells and gonadal cells, indicated this to early biologists, however, physiological evidence was first presented by Pincus and Enzmann (1935). The authors found that fully grown oocytes removed from antral follicles underwent a spontaneous, gonadotrophin-independent resumption of meiosis in culture, and concluded that follicular somatic cells maintain oocytes in meiotic arrest. Subsequent studies demonstrated that follicular somatic cells promote the reinitiation of meiosis and its progression to metaphase II (nuclear maturation). Follicular somatic cells also promote oocyte competence to undergo fertilization and pre-implantation embryogenesis (cytoplasmic maturation) (Buccione et al. 1990) and granulosa cells participate in the global suppression of transcription in oocytes that occurs before nuclear maturation (De La Fuente and Eppig 2001). Efficient delivery of factors to and from oocyte at critical stages of development is essential for coordination of folliculogenesis and triggering of different signaling molecules: Kit ligand, members of TGFB, insulin & WNT signaling family (Harwood et al. 2008; Luo et al. 2010), growth factors: GDF9, BMP15 (Su et al. 2004), hormonal regulation FSH, LH (Patsoula et al. 2001) are also crucial for oocyte growth and developmental competence. A fully grown oocyte shows better competency than a growing oocyte in terms of in-vitro maturation and fertilization (Alm et al. 2005; Wang et al. 2012). Oocyte competence is acquired during folliculogenesis, and ultimately the coordinated communication and signaling between the different follicular cells and follicular cells-oocyte as well which is critical for the growth, maturation and release of an oocyte that can be fertilized and develop into an embryo. Oocyte developmental competence is defined as the ability of an oocyte to resume meiosis, to cleave following fertilization, to develop to the blastocyst stage, to induce a pregnancy and bring offspring to term with a good health (Krisher 2004; Sirard et al. 2006). This ability of oocytes to develop is possible to asses by measuring the activity of glucose-6-phosphate dehydrogenase (G6PDH) enzyme in response to Brilliant Cresyl Blue (BCB) stain

(Ericsson et al. 1993). Many factors including follicular fluid (FF) are involved in follicle development and subsequent oocyte developmental competence (Armstrong and Webb 1997).

Follicular fluid is a product of both the transfer of blood plasma constituents that cross the 'blood-follicle barrier' and of the secretory activity of granulosa and thecal cells (Gosden et al. 1988). Follicular fluid has been recognized as a reservoir of biochemical factors and theoretically represent an optimal source on non-invasive biochemical predictors of oocyte quality (Revelli et al. 2009). Follicular fluid provides an important microenvironment for oocyte maturation and contains hormones such as FSH, LH, GH, inhibin, activin, estrogens and androgens, pro-apoptotic factors including TNF and Fas-ligand, proteins, peptides, amino acids, and nucleotides (Revelli et al. 2009). Follicular fluid is at least partly responsible for subsequent embryo quality and development and has some important oocyte-related functions: maintenance of meiotic arrest (McNatty and Baird 1978), protection against proteolysis, extrusion during ovulation (Espey 1994) and acts as a buffer against adverse haematic influences (Gosden et al. 1988). As follicular fluids are derived from plasma and secretory results of granulosa and theca cells, thus, it is logical to think that products contained within follicular fluid may play a role in follicle growth and oocyte developmental competence. However, it is unclear if or how surrounding different follicular cells respond to these factors during follicular development and oocyte maturation.

Exosomes have been postulated to play an important role in cell–cell communication, other physiological and pathological processed and appear to affect target cells either by stimulating them directly by surface expressed ligands or by transferring molecules between cells (Valadi et al. 2007). However, the exosomes-cell interaction mode and the intracellular trafficking pathway of exosomes in their recipient cells remain unclear. Exosomes are small membrane vesicles that are released into the extra-cellular milieu upon the fusion of multivesicular bodies with the plasma membrane. Unlike other cell secreted vesicle, exosomes are more homogenous and smaller in size ranges from 40-100 nm in diameter. Exosomes contain a characteristic composition of proteins, and express cell recognition molecules on their surface that facilitates their selective targeting of and uptake by recipient cells (Mittelbrunn et al. 2011). Recent reports indicate that exosomes also harbor a variety of mRNAs and microRNAs (miRNA)

(Valadi et al. 2007), which can be transported over large distances through blood to recipient cells and modulate their function (da Silveira et al. 2011; Pegtel et al. 2010; Taylor and Gercel-Taylor 2008; Valadi et al. 2007). These findings have increased interest in the role of exosomes in cell-to-cell communication, and support the idea that exosomes might constitute an exquisite mechanism for local and systemic intercellular transfer not only of proteins but also of genetic information in the form of RNA (mRNA and miRNA). Currently a role of bovine follicular fluid exosomes and/or miRNAs in follicular growth and oocyte maturation is unknown.

During the dynamic phase of follicular development and oocyte maturation miRNAs plays an important role by coordinating the expression of genes in a spatial and temporal specific manner (Baley and Li 2012; Hossain et al. 2012). However, miRNAs are not only present in cells but also a considerable proportion of miRNAs are present in body fluids including plasma, serum, urine, saliva, milk and semen (Mitchell et al. 2008; Park et al. 2009; Weber et al. 2010) commonly termed as circulating or extra-cellular miRNA. Recent findings have shown that these extra-cellular miRNAs are remarkably stable in plasma despite high RNase activity in extra-cellular environment (Mitchell et al. 2008), suggesting circulating miRNAs may be wrapped with some protective manner to bypass this extra-cellular harsh conditions. Currently, the dominant model for circulating miRNA stability is that miRNAs are released from cells in membrane-bound vesicles, which protect them from high RNase activity in circulation (Simons and Raposo 2009; Valadi et al. 2007; Weber et al. 2010). In particular, a significant portion of circulating miRNA is also associated with Argonaute 2 (Ago2), the effector component of the miRNA-induced silencing complex that directly binds miRNAs and mediates messenger RNA repression in cells (Arroyo et al. 2011).

Therefore, the present study was conducted to investigate 1) the presence of extra-cellular miRNAs in exosomal and non-exosomal portion of follicular fluid, 2) differential expression of extra-cellular miRNAs follicular fluid derived from follicle containing growing and fully grown oocyte sources and finally 3) exosome mediated transfer of miRNAs in follicular microenvironment. In this study, we showed that microRNAs are present in bovine follicular fluid and most of the extra-cellular miRNAs are associated with exosomes and a significant portion of extra-cellular miRNAs are

also present in non exosomal fraction of follicular fluid being coupled with Ago2 protein, which is a part of RNA-induced silencing complex. Furthermore, fluorescent microscopy demonstrated that exosomes are taken up by surrounding follicular cells and exosomes mediated transfection of miRNAs can elevate the endogenous level of miRNA in cellular level.

2. Literature review

2.1 Ovarian follicle

The ovary, the primary organ in female reproductive system, is only the source of female germ cells called oocyte which is pre-requisite for sexual reproduction. The ovarian follicle is the fundamental unit of the ovary which contains the oocyte that may eventually ovulate, undergo fertilization and sustain embryo development. It also provides the essential steroids and protein hormones that is crucial for maintenance of the ovarian cycle, follicular development, the secondary sex characteristics and preparation of the uterus for implantation, and after ovulation, the corpus luteum provides the hormones essential for establishment and maintenance of pregnancy (Pohler et al. 2012). Follicle formation and folliculogenesis have been well documented for many mammalian species including cattle (Adams et al. 2008; van Wezel and Rodgers 1996), sheep (Evans et al. 2000; Seekallu et al. 2010), pig (Schwarz et al. 2008) and human (Gougeon 2010; van Dessel et al. 1996). However, the control of follicular reserves (Tilly and Rueda 2008) and entry of follicles into the growth path towards atresia or ovulation (Findlay et al. 2002) are not well understood. The adult ovary contains a reserve of inactive primordial follicles. Each contains a small non growing oocyte and a layer of non-dividing pre-granulosa cells encapsulated by the follicular basal lamina (Rodgers and Irving-Rodgers 2010b). Number of primordial follicles are selected and become active every day, and the oocyte commences growing while the granulosa cells start to divide. As the granulosa cells divide, the number of layers of cells (called the membrana granulosa or follicular epithelium) around the oocyte increases, and the follicular basal lamina expands (Cox 1997). Later in development, a fluid filled cavity or antrum forms and specialized stromal layers, the theca interna and externa, develop. Only follicles that reach the stage of having a large antrum, and in the follicular wave following regression of corpora lutea, can ovulate an oocyte in response to the surge release of LH (Rodgers and Irving-Rodgers 2010b). Following ovulation, the granulosa cells and thecal cells differentiate into the large and small luteal cells of the corpus luteum, and the vascular supply of the corpus luteum is derived from the capillaries of the theca interna. All non-ovulating follicles undergo atresia and regression (Rodgers and Irving-Rodgers 2010b).

Ideally a fully grown follicle (graafian follicle) is a three-dimensional structure with an antrum in centre and surrounded by a variety of different cell types. There are at least six distinct histologic components in the mature follicle, including the theca externa, theca interna, basal lamina, granulosa cells, oocyte, and follicular fluid (O'Shea 1981). Following paragraphs highlight the different components of a follicle and their functions.

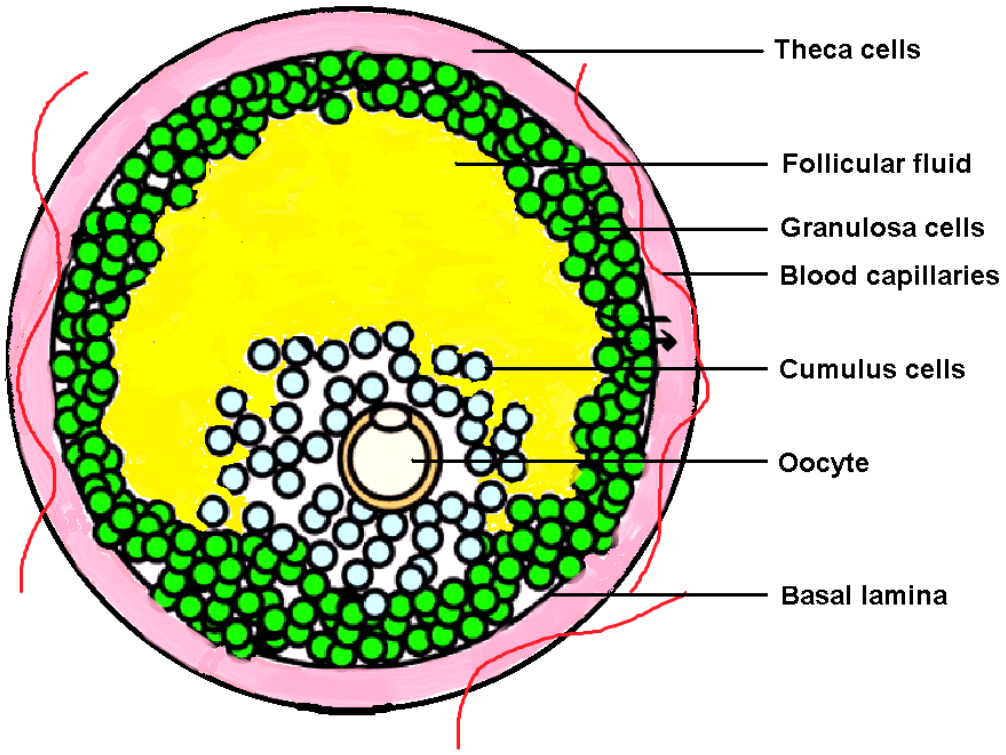


Figure 2.1: Schematic presentation of a fully grown follicle indicating different components

The remarkable characteristics of theca externa is the presence of smooth muscle cells (Amsterdam et al. 1977), which are innervated by autonomic nerves (Erickson et al. 1985). Although the physiologic significance of the theca externa remains unclear, there is evidence that it has certain function during ovulation and atresia (Moley and Schreiber 1995). The theca interna is composed of differentiated TICs located within a matrix of loose connective tissue and blood vessels. In all graafian follicle, LH is a key

regulatory hormone for TIC function, and its importance in regulating TIC androgen production *in vivo* and *in vitro* has been established (Erickson et al. 1985). Beginning at the very early stages of graafian follicle development, the TICs express their differentiated state as androgen (*i.e.* androstenedione-producing cells) (Erickson et al. 1985). The theca interna is richly vascularized and serves to deliver hormones (*e.g.* FSH, LH), nutrient molecules, vitamins, and cofactors required for the growth and differentiation of the oocyte and granulosa cells. The theca compartments (*i.e.* theca externa and interna) express their differentiated functions at the beginning of graafian follicle development (at cavitation) and appear to constitutively express a mature phenotype throughout the life and death of the graafian follicle.

Basal lamina is a specialized extracellular matrix sheets that provide mechanical support and important signals for growth and differentiation to cells with which they are associated (Yurchenco and Schittny 1990). It separates the granulosa cells from the surrounding stromal elements in primordial and pre-antral follicles, or from the specialized stromal theca layers in antral follicles (Irving-Rodgers and Rodgers 2006). Basal laminas are generally observed as a single layer aligned to the cell surface. However, a number of physiological and pathological conditions lead to different morphological appearances of basal laminas (Irving-Rodgers et al. 2009). The follicular basal lamina is believed to play a role in influencing granulosa cell proliferation and differentiation (Irving-Rodgers and Rodgers 2006). Additionally, it has been well documented that the components of basal lamina/basement membrane obtained from non-ovarian sources regulated the morphology and steroidogenesis in granulosa cells from rat (Furman et al. 1986b) and human (Furman et al. 1986a) ovaries.

In the antral follicle, granulosa cells represent the major category of follicular cells. The granulosa cells and oocyte exist as a mass of perfectly shaped and precisely positioned cells. The positional variation of granulosa cells creates at least four different cell layers or domains. These are the outermost domain is known as membrana granulosa, the inner most domain is the periantral, the intermediate domain is the cumulus oophorus, and the domain close together to the oocyte is the corona radiata (Amsterdam and Rotmensch 1987). A characteristic histologic property of the membrana domain is that it is composed of a pseudostratified epithelium of tall columnar granulosa cells, all of which are anchored to the basal lamina (Irving-Rodgers and Rodgers 2000). The

differentiation of a granulosa cell can be traced to its position within the cellular mass. For example, cells in the membrana domain stop proliferating before those in central domain (Hirshfield 1989). The ability of the granulosa cells to continue dividing in the inner domains of a follicle throughout graafian follicle development leads to conclude that they might be precursor cells (Hirshfield 1989). Cumulus oophorus may be defined as those granulose cells which are closely associated with oocyte (Tanghe et al. 2002). The fully developed cumulus oophorus perform three important biological functions- firstly, before ovulation it supports during oocyte maturation (Tanghe et al. 2002), secondly during ovulation it conducts the oocyte in to the oviduct (Mahi-Brown and Yanagimachi 1983) and finally shortly after ovulation it participate in the complex mechanisms that controlling the access of spermatozoa in to the oocyte (Tanghe et al. 2002).

In mammals, several studies have demonstrated that steroidal and nonsteroidal factors produced by granulosa and theca cells together influence proliferation and differentiation of both cell types on opposite sides of a basal membrane during folliculogenesis (Driancourt et al. 2000; Gougeon 1996; Monget et al. 2002). During preantral follicle development, LH receptors are found exclusively on theca cells and FSH receptors exclusively on granulosa cells. It has been well documented that LH stimulates theca cell androgen and growth factor production, while FSH induces aromatase expression and increases the conversion of theca cell androgen to estrogen, which is commonly known as two-cell two-gonadotropin theory (Hillier et al. 1980). Androgens also enhance FSH action in the follicles by increasing FSH receptor expression, FSH-induced granulosa cell aromatase activity and proliferation, and follicular growth (Pakarainen et al. 2005).

2.2 Follicular development

In present most widely accepted hypothesis of follicular reserve is “at birth the ovaries of primates and most domesticated animal species contain a finite number of primordial follicles whereas in rodents this ovarian reserve develops in the first few days postpartum” (Fortune 2003). In contrast, Johnaon *et al.* demonstrated the existence of mitotically active germ cells in juvenile and adult mouse ovaries, and claim stem cell-based renewal of follicle reserve in postnatal mammalian ovaries (Johnson et al. 2004).

Although some studies reported the presence of mitotically active germ cells in ovaries of some species of prosimian primate adults (*Loris tardigradus lydekkerianus* and *Nycticebus coucang*) (David et al. 1974; Duke 1967), to date the presence of germline stem cells in other mammalian species remains to be proven (Telfer 2004).

Follicular development within the ovary is a dynamic process which occurs throughout the estrous cycle and involves recruitment of follicles into the growing pool, physiological and morphological changes in the follicular cells, physiologic selection of an ovulatory follicle and ovulation or regression (Araki et al. 1996). Since it is a continuous process, therefore throughout the reproductive life, the mammalian ovary contains a mixed population of follicles in different stages of development. There is a large pool of primordial follicles in the bovine ovary (~150,000) in a resting phase, some of them are released in each estrous cycle and this process continues throughout the reproductive life and the number of follicles decrease to about 3,000 by the age of 15-20 years (Webb et al. 1992). Sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis these processes are repeated on a cyclical order within the ovary and resulting in the development of a number of ovulatory follicles (Hunter et al. 2004). All follicles start as a primordial follicle, among them some will eventually develop to the preovulatory stage. Morphologic studies of ovaries have shown that during ovarian follicular developmental process a primordial follicle continuously grow into primary follicles, preantral and antral follicles (van Wezel and Rodgers 1996). Only antral follicles are capable of releasing oocytes at a stage when the oocyte can be fertilized (Tomic et al. 2004). Thus, the continuous growth of primordial follicles to the antral stage is essential for female fertility. The development of a follicle begins with the transformation of the flattened pre-granulosa cells of the primordial follicle to cuboidal granulosa (follicular) cells, after which a follicle with a single layer of granulosa cells is termed a primary follicle (Eppig 2001). The granulosa cells in primary follicles undergo continuous proliferation, the oocyte enlarges and becomes surrounded by a zona pellucida. Gradually the follicles changes to secondary follicles and, when fibroblast cells in the inner thecal layer differentiate, the secondary follicle is defined as a preantral follicle (Nussey and Whitehead 2001). The early growth phase of a follicle is considered to be independent of gonadotropin stimulation (Braw-Tal and Roth 2005; Palma et al. 2012), nevertheless some studies has reported that the presence

of FSH receptors in these immature follicles (Ranta et al. 1984). It is poorly understood, why a few primordial follicles start to grow and how they are selected, but paracrine factors within the ovary such as cytokines and epidermal growth factor has shown to be involved in this process. In the early luteal phase of each menstrual cycle, cohorts of preantral follicles undergo further growth into antral follicles (Nussey and Whitehead 2001). At this time, the follicles enlarge, the thecal cells become richly supplied with blood vessels and a fluid-filled cavity (the antrum) forms (Fraser 2006). The oocyte itself becomes surrounded by several layers of granulosa cells known as the cumulus oophorus (Khamsi and Roberge 2001).

In bovine and several other mammalian species, the pattern of follicular development during the terminal stages of folliculogenesis (follicles ≥4 mm) has been characterized as wave-like pattern that include 2 or 3 consecutive waves, which refers to the periodic and synchronous growth of a group of follicles (Adams 1999; Webb et al. 1992). The wave-like pattern of follicular development in heifers was first proposed more than 50 years ago by Rajakoski using the histological data of ovaries collected from different heifers on different days of the estrous cycle (Rajakoski 1960). However, the proposed wave-like developmental pattern of follicles was validated using ultrasonographic real-time follicular images, collected over a time-period on the same follicles from the same set of animals (Pierson and Ginther 1984; Pierson and Ginther 1986; Savio et al. 1988; Sirois and Fortune 1988). During each wave of follicular growth, a group (normally 1 to 6) of follicles (primordial) 4 to 5 mm in diameter emerge and begin to grow, this phase is termed as recruitment. Initiation of each wave of follicular growth is preceded by a transient increase in FSH that begins about 2.5 day before initiation of the new wave of follicular growth and starts to decline about the time of appearance of the group of follicles in the wave. This subsequent decrease of circulating FSH is temporally associated with the selection of the dominant follicle (Adams et al. 1992). The group of recruited follicles grow over the next 36 to 48 h, after which one follicle (8 to 9 mm in diameter) is selected (selection) for further growth to become larger than the others, where as others stop growing and undergo atresia (Adams et al. 1992). The selected follicle achieves dominance over the other follicles in the cohort, which regress while the dominant follicle continues to increase in size.

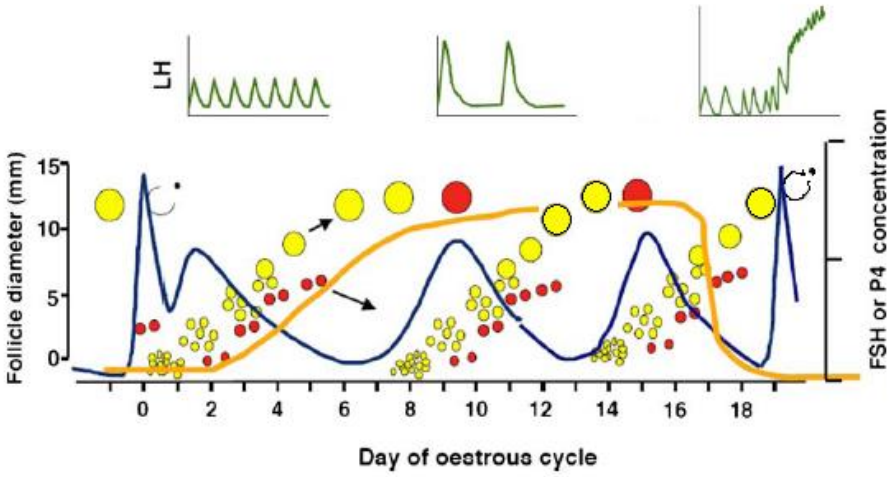


Figure 2.2: Schematic depiction of follicular wave dynamics in cattle in relation to hormones. In this figure the pattern of secretion of follicle-stimulating hormone (FSH; blue line), luteinizing hormone (LH; green lines), and progesterone (P4; orange line); and the pattern of growth of ovarian follicles during the estrous cycle in cattle was demonstrated (Mark and Michael 2013).

During this stage of follicular development, granulosa cells of large healthy, estrogen-secreting follicles also acquire LH receptors (Ireland and Roche 1983; Xu et al. 1995). It has been shown that the granulosa cells of large healthy antral follicles possess more LH receptors than smaller atretic follicles (Ireland and Roche 1983; Spicer et al. 1986) and the levels of mRNA for LH receptor in granulosa cells of a dominant follicles increase as follicular development advances. When the dominant follicle reaches its maximum size, it maintains that size for 3 to 6 day before regressing if the animal is in the luteal phase of the estrous cycle (Ginther et al. 1996). The fate of the dominant follicle that reaches maximum size during the luteal phase of the estrous cycle and of subordinate follicles that enter as cohorts during the initiation of each wave of follicular growth is atresia. If luteal regression occurs during the growing phase of the dominant follicle, the fate of dominant follicle follicle is ovulation (Kastelic et al. 1990).

2.3 Cell-cell communication during follicular development

Oocyte growth and follicular development occurs in an ovarian follicular microenvironment characterized by extensive cell-cell interaction mediated by gap junctional communication, as well as autocrine, paracrine and endocrine signaling. Gap junctions coupling between surface membranes of oocytes and their surrounding granulosa cells has been reported from the primordial stage in mice (Mitchell and Burghardt 1986) and secondary follicle stage in cattle (Fair et al. 1997). Gap junctions are specialized structures occurring between very close cell-cell contact; facilitate the transfer of amino acids, glucose metabolites and nucleotides to the growing oocyte (Eppig 1991). The proteins connexins is the principle component of gap junction. It has been demonstrated that connexins 32, 37, 43, 45 and 57 have been detected within growing and mature mouse follicles (Wright et al. 2001). However, in cattle, connexin expression shows in a stage specific manner; for instance, Cx26 is expressed in oocytes of primordial, primary and secondary follicles and in the granulosa of healthy antral follicles (Johnson et al. 1999). Furthermore, expression of Cx37 in preantral follicles is high, but expression decreases significantly at the onset of antral formation (Nuttinck et al. 2000). However, the reverse is true of Cx43, which is weakly expressed, in preantral follicles but increased significantly at the onset of antral cavity formation and become more intense with increase in follicular size in healthy antral follicle (Nuttinck et al. 2000). These collective data provides a plausible suggestion of differential regulation of Cx37 and Cx43 throughout folliculogenesis. Studies in mice (Wright et al. 2001), and pig (Itahana et al. 1996) indicating the expression of connexins is associated with granulosa cell proliferation. Connexin KO mice exhibit infertility due to failure of follicle growth past the late preantral stage. Furthermore, gonadotropin stimulation could not induce ovulation in these mice (Carabatsos et al. 2000). Studies has revealed that smaller gonads appear on Cx43 deficient mice in the neonates and impaired post natal folliclulogenesis; indicating the importance of requirement of Cx43 for both germ line development and early folliculogenesis (Juneja et al. 1999).

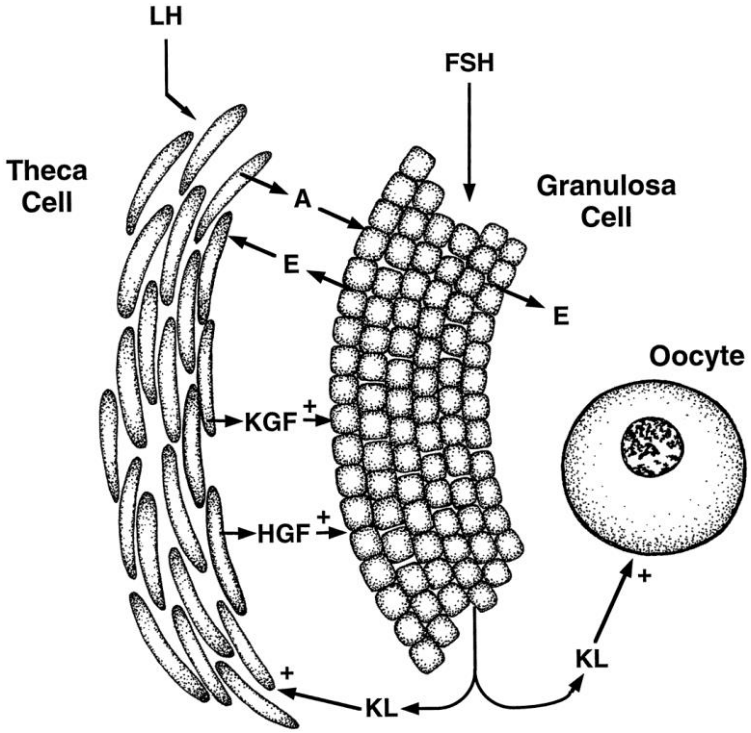


Figure 2.3: Proposed schematic of thecal cell - granulosa cell interactions during follicular development. Mesenchyme-derived thecal cells produce KGF and HGF that regulate epithelial-derived granulosa cell functions. Granulosa cell expression of KL is stimulated (+) by KGF and HGF. Granulosa cell-derived KL feedback on thecal cells to regulate thecal cell growth and differentiated functions. Thecal cell expression of KGF and HGF is stimulated (+) by KL. Also illustrated is the previously established positive effect (+) of KL on oocyte development. Adopted from Parrott and Skinner (1998c).

During follicular development, mesenchymal-derived thecal cells produce a number of growth factors that include KGF (keratinocyte growth factor) and HGF (hepatocyte growth factor) (Parrott and Skinner 1998c). Both KGF and HGF are mesenchymal-derived growth factors that act on adjacent epithelial cells in a number of tissues (Parrott and Skinner 1998c). During follicular development gene expression of KGF and HGF is developmentally and hormonally regulated in thecal cells (Parrott and

Skinner 1998a; Parrott and Skinner 1998b). Thecal cells have been shown to produce and secrete these growth factors (Parrott et al. 1994), furthermore, granulosa cells have also been shown to proliferate in response to KGF and HGF *in vitro* (Parrott et al. 1994). These observations strongly suggest that KGF and HGF may be important mediators of ovarian mesenchymal-epithelial cell interactions that promote folliculogenesis. Moreover, later investigations demonstrate that thecal cell-derived KGF and HGF also stimulate KL expression in granulosa cells (Parrott and Skinner 1998c). The actions of KGF and HGF on granulosa cell KL expression indicate that these growth factors also alter cellular parameters other than cell growth (Parrott and Skinner 1998c). As granulosa cell-derived KL is important for oocyte maturation, thecal cells may indirectly regulate oocyte function by influencing granulosa cell production of KL (Parrott and Skinner 1998c).

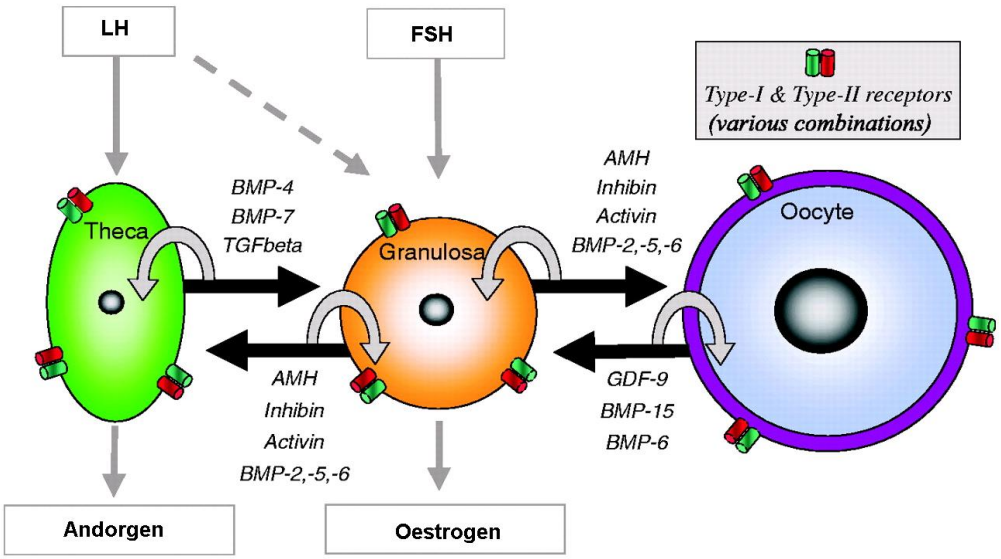


Figure 2.4: Members of the TGF-β super family feature prominently amongst the growing list of extra-cellular ligands implicated in the bi-directional communication between theca and granulosa cells, and granulosa cells and oocyte. Both autocrine (thick grey arrows) and paracrine (thick black arrows) signaling events are likely, depending on the expression of appropriate

combinations of type-I and type-II receptors on the cell surface (Knight and Glister 2006).

Several studies have demonstrated the importance of the gap junction network during oocyte growth, development and meiotic maturation (Simon et al. 1997; Vozzi et al. 2001). Denudation of preantral mouse oocytes prior to in vitro culture inhibits their growth, alone or in co-culture with granulosa cells (Eppig 1979). In addition, the growth rate of oocyte is directly correlated to the number of granulosa cells coupled to the oocyte (Herlands and Schultz 1984). It has been well documented that granulosa cells play a crucial role in oocyte development (Buccione et al. 1987), affecting the pattern of protein phosphorylation in oocytes in a stage specific manner (Cecconi et al. 1991). In mice, the network facilitates the transport of a paracrine factor which is commonly known as 'Cumulus expansion enabling factor' (Eppig et al. 1993). In addition, this paracrine factor appears to be essential for bringing about gonadotropin induced production of hyaluronic acid and cumulus expansion just before ovulation (Salustri et al. 1990; Vanderhyden et al. 1990). Gene targeting ablation of Cx37 resulted in the removal of all gap junctions from the mouse oocyte surface and compromised oocyte meiotic maturation (Simon et al. 1997). Similarly, exposition of bovine COCs to gap junction uncoupling agents during routine in vitro maturation reversibly blocked the resumption of meiotic maturation and anti-sense silencing of Cx43 expression in COCs with a recombinant adenovirus resulted in the inhibition of GVBD in 50% of the COCs (Vozzi et al. 2001).

2.4 Oocyte competence

Female fertility is determined to a large extent by the developmental competence of the oocyte. Oocyte competence is acquired gradually over a prolonged period of oocyte growth (i.e., oogenesis) and follicular development (i.e., folliculogenesis), and is reliant on complex bi-directional interactions between germ and follicular somatic cells beginning in the fetus and extending into adult life (Eppig 2001). A good quality oocyte or a competent oocyte is the prerequisite for high blastocyst rates. A good-quality oocyte is an oocyte at metaphase I (MI) or metaphase II (MII) with a clear zona pellucida, clear or moderately granular cytoplasm and a small perivitelline space, on the

other hand a poor-quality oocyte is at the germinal stage, is post-mature or has a fractured zona pellucida (Balaban and Urman 2006; Robker et al. 2009). Oocyte competence also refers to the completion of the first meiotic division and complementary processes essential for subsequent fertilization and embryo development. This process consists of two general components, cytoplasmic maturation and nuclear maturation; both must take place to achieve healthy and competent oocytes (Swain and Pool 2008). Many important factors play a crucial role in determining the final intrinsic developmental capacity and quality of the oocytes obtained. Most of these are donor-related, stage of the estrous cycle at the time of retrieval, follicular status related to oocyte growth and final maturation, donor condition and breed, hormonal stimulation prior to OPU and the number of oocyte collections within a specific time span. All of these can be considered as 'biological factors', acting separately or in combination for a specific donor (Bols 2005). In parallel, there is a group of more 'technical factors', intrinsically related to the retrieval procedure: follicle visualization, needles and aspiration vacuum used and cumulus oocyte complex (COC) processing immediately before IVP (Bols 2005).

Since the primary goal for a commercial IVP set up is to select oocytes with the highest developmental potential, preferentially before in vitro maturation process, oocytes with lower developmental competence should be discarded from the production system in the mean time. This will be undoubtedly beneficial for the development of the remaining oocytes as well for the efficiency of the production system. Non-invasive oocyte quality assessment techniques need to meet several specific criteria to be applicable in routine IVP systems. The ideal technique to assess quality parameters should be easy to perform, economically sound, quick, allow a high throughput of COCs, have an acceptable reliability and above all, be non-invasive, so that the oocyte's developmental capacity is not hampered by quality assessment (Bols 2005). When cumulus investment morphology and homogeneity of the cytoplasm is used as criteria to assess oocyte quality only 35-45% blastocyst can be produced (Bols 2005), which strongly suggests a need for additional oocyte quality parameters. The use of brilliant cresyl blue (BCB), to select competent oocytes for IVP, is substantially documented in several studies. One of the earliest reports demonstrates the use of BCB for the selection of pig oocytes for IVM and IVF (Ericsson et al. 1993). The basic idea behind using BCB staining is a

measure for the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that is synthesized by the oocyte during oogenesis as part of the pentose phosphate cycle (Alm et al. 2005). This enzyme is particularly active in growing oocytes with a clear decrease in its activity when the oocyte finishes its growth phase (Mangia and Epstein 1975). Brilliant cresyl blue is a blue dye that is converted to a colorless substance through the action of G6PDH. As fully-grown oocyte show a decreased or no G6PDH activity, their cytoplasm will remain blue following exposing and subsequent uptake of BCB. This specific characteristic makes BCB an indicator for G6PDH activity and therefore indirectly indicates the growth stage of the oocyte. Rodríguez-González *et al*. used BCB stain to assess more competent pre-pubertal goat oocytes for *in vitro* embryo production (Rodriguez-Gonzalez et al. 2002). After exposing the oocytes to BCB, they categorized them according to the color of their cytoplasm: oocyte with a blue cytoplasm or fully grown oocytes (BCB+) and oocyte with a colorless cytoplasm or growing oocytes (BCB-). Importantly, they showed that BCB+ oocytes were significantly larger than BCB- ones and there was a higher proportion of BCB+ oocytes reached the MII stages compared to their BCB- counterparts (Rodriguez-Gonzalez et al. 2002). Similar results were demonstrated by Pujol *et al*. in a study where they used the BCB test to assess the developmental competence of heifer oocytes. While a slightly modified oocyte quality grading system was employed, they showed that nearly 79% of the morphologically classified grade 1 oocytes were BCB+ and confirmed that BCB+ oocytes were larger and more competent for IVP than control heifer oocytes (Pujol et al. 2004).

2.5 Follicular fluid

Follicular fluid is composed partially of locally produced substances, which are related to the metabolic activity of the follicular cells and partly from the peripheral plasma by transudation across the follicular basal lamina and accumulates in the antrum formed by the coalescence of small pocket of fluid. Its composition reflects changes in the secretory processes of the granulosa layer and theca interna, and alterations in the components of plasma due to physiological or pathological processes (Ben-Rafael et al. 1987). The fluid can be collected easily, especially as the follicle enlarges, and there has

been prolonged interest in its composition and functions (Edwards 1974). The following sub-sections describe in brief the formation and function of follicular fluid.

2.5.1 Formation of follicular fluid

It was originally proposed in 1948 that follicular fluid was merely a simple transudate of blood that accumulated between the layers of the granulosa cells in growing follicles (Harter, 1948). It is now well established that follicular fluid contains a large variety of components of serum along with an array of secretions, which may be released by the specialized cells in the follicular microenvironment. As a result, the components of follicular fluid changes depending on the growth and expansion status of each follicle (Wise 1987). Follicular fluid is a slightly viscous solution with a pH reported to be 7.4, similar to that of plasma. Investigators have reported various elements of follicular fluid to be similar to those found in serum, with only a few differences (Edwards 1974; Greve et al. 1989; Grinsted et al. 1980; Luck et al. 2001). For instance, in case of bovine, while sodium and potassium were found to be higher in follicular fluid than serum, no differences were found in the concentration of zinc, magnesium, chloride, copper and inorganic phosphate (Menezo et al. 2011; Silberstein et al. 2009). To be transported into the follicular antrum fluid from the thecal capillaries obviously needs to cross the endothelium and sub-endothelial basal lamina before traversing the thecal interstitium, the follicular basal lamina, and the membrana granulosa. Changes in permeability of the thecal capillaries will lead to edema of the thecal tissue, as observed following the luteinizing hormone (LH) surge (Cavender and Murdoch 1988; Espey 1980), but additional mechanisms are needed for fluid to accumulate in the follicular antrum. In earlier literature, both a sodium pump and cleavage of glycosaminoglycans to raise pressure in the preovulatory follicle were considered as such mechanisms (Gosden et al. 1988). Even if the cells of the membrana granulosa constitute a stratified epithelium and can directionally secrete osmotically active molecules toward the center of the follicle, because these cells lack a network of tight junctions, it would not be possible to establish an osmotic gradient across the membrana granulosa with small molecules like sodium (Rodgers and Irving-Rodgers 2010a). In addition, an early review found the evidence for a role for sodium to be “inconclusive” (Gosden et al. 1988). 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 (Shalgi et al. 1972). However, for increasing sizes above 100 kDa, plasma proteins are found at progressively lower concentrations than in plasma (Andersen et al. 1976), suggesting that there is a nominal "blood-follicle barrier" at sizes above 100 kDa. This barrier probably exists at the level of the follicular basal lamina and additionally at the level of the thecal capillaries, especially for the larger molecules (Zhou et al. 2007). However, such a barrier may also exist in reverse in that large molecules produced by oocytes or granulosa cells cannot cross the membrana granulosa or follicular basal lamina, thereby establishing a potential osmotic gradient. This osmotic gradient could then be responsible for recruiting fluid to the center of the follicle.

2.5.2 Function of follicular fluid

Follicular fluid is an avascular compartment within the mammalian ovary, separated from the perifollicular stroma by the follicular wall that constitutes a 'blood-follicle barrier (Bagavandoss et al. 1983). Besides a contribution of serum, follicular fluid is partially composed of locally produced substances, which might be related to the metabolic activity of follicular cells (Gerard et al. 2002). Within the ovarian follicle, developing oocyte is surrounded by the follicular fluid. Besides meeting nutritional requirement of the growing oocyte, follicular fluid also maintains proper environment for growth and maturation of the oocyte (Romero-Arredondo and Seidel 1996). Follicular fluid contains numerous biochemical components that are essential for ovarian physiology, including steroidogenesis, follicular growth and maturation of oocytes, ovulation and transportation of oocyte to the oviduct for fertilization (Choi et al. 1998). In addition, it has been suggested that progesterone in follicular fluid could possibly play a role in the induction of the acrosome reaction in spermatozoa of stallions (Cheng et al. 1998; Rathi et al. 2003) and bulls (Lenz et al. 1982). However, follicular fluid does not play a major role in inducing the acrosome reaction in human sperm cells (Mortimer and Camenzind 1989). Another important function of follicular fluid is to inhibit the production of both progesterone and PGF2α (Shemesh 1979). Bovine follicular fluid from mid-cycle follicles inhibited prostaglandin synthetase as well as luteinization of follicles (Tsai and Wiltbank 2001). However, this inhibition was

not noted when follicular fluid from preovulatory follicles was used (Tsai and Wiltbank 2001). Follicular fluid also functions to provide nourishment to the oocyte and granulosa cells by facilitating transport of specialized nutrients from plasma (Lange-Consiglio et al. 2012). The dynamic constituents of this fluid reflect both biochemical and endocrinological activity of the follicle, thereby facilitating its role as a conductor of growth and development through the reproductive cycle (Edwards 1974). Like any other biological fluid, the physical characteristics and chemical composition of follicular fluid reflect physiological and/or pathological events that take place outside its environment. In a follicle, physiologic factors are influenced principally by the properties of the tissues, which separate follicular fluid from circulation. The permeability of the "blood-follicle barrier" to different biochemical components and water originating from blood can markedly alter the composition of follicular fluid (Fisch et al. 1990). In addition, the ability of granulosa cells to secrete both stimulatory and inhibitory factors as a result of metabolic processes can modify the composition of follicular fluid. These factors influence physical parameters such as osmolality, color, pH, viscosity and volume of the fluid originating within the developing follicle (Fisch et al. 1990).

2.6 Involvement of miRNAs in follicular development

The mammalian ovary is an extremely dynamic organ within which sequential waves of follicular growth and regression, rupture of mature follicles and 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 occur within relatively short cycles and under tight transcriptional regulation throughout a female's reproductive life (Hawkins and Matzuk 2010). Cyclic ovarian activity is key to reproductive success and the profound changes in tissue composition and function involved require exquisite spatio-temporal co-ordination of proliferation, apoptosis and differentiation of many different cell types within follicles (Donadeu et al. 2012). Recruitment of growing follicles, atresia, ovulation, and luteal tissue formation and regression are dynamically regulated events that regenerate on a cyclical basis in the ovary. These events involve dynamic changes in cellular growth, angiogenesis, steroidogenesis, cell cycle, and apoptosis and are accurately regulated at the endocrine

and tissue levels (Carletti and Christenson 2009). Deregulation in the regulatory network results in ovarian failure such as premature ovarian failure (POF) due to disruption of folliculogenesis, blockage of ovulation, and loss of oocytes *via* apoptosis (Yang et al. 2012). Small RNA populations have been identified by cloning-based or next-generation sequencing of normal ovarian tissues from human (Landgraf et al. 2007), cattle (Hossain et al. 2009; Huang et al. 2011; Tripurani et al. 2010), mice (Ahn et al. 2010; Mishima et al. 2008), pigs (Li et al. 2011), and sheep (McBride et al. 2012). Most of those studies involved analyses on whole ovaries rather than on specific ovarian tissue components, an approach that, although very useful for comprehensive identification of miRNA sequences, provides very limited insight into their functional relevance (McBride et al. 2012). For many miRNAs, cloning frequencies changed across developmental stages, and some miRNAs were clearly expressed differentially between follicular and luteal stages, this data suggests the involvement of miRNAs in the follicular–luteal transition period (McBride et al. 2012). Several studies have been precisely examined the involvement of specific miRNAs in different aspects of follicle development. Dynamic changes in the levels of different miRNAs have been reported during follicle development in mice (Lei et al. 2010; Yao et al. 2009), pigs (Xu et al. 2011) and sheep (McBride et al. 2012) and also in response to stimulation of murine follicular cells with gonadotropins (Yin et al. 2012) or growth factors (Yao et al. 2010). Treatment of granulosa cells from mouse pre-antral follicles with TGFβ1 in culture resulted in the upregulation of three miRNAs and downregulation of 13 miRNAs (Yao et al. 2010). A subsequent study (Yin et al. 2012) showed that miR-383, a miRNA downregulated by TGFβ1, positively regulated aromatase expression and oestradiol production by mouse granulosa cells in culture. These observations, together with the finding that miR-383 levels increased *in vivo* during equine chorionic gonadotropin (eCG)-induced follicle growth and decreased following administration of hCG, provide strong support for a role for miR-383 in physiologically regulating changes in oestradiol production during follicle development. Three recent *in vitro* studies have also proposed roles of miRNAs in regulating granulosa cell proliferation and apoptosis. First Yan *et al.* reported attenuation of activin-induced proliferation of mouse granulosa cells by miR-145 targeting of both activin receptor 1B and cyclin D2 (Yan et al. 2012). In another study, showed that miR-23a was pro-apoptotic in cultured human luteinised granulosa cells presumably by decreasing the levels of X-linked inhibitor of apoptosis

protein (XIAP) and increasing caspase-3 cleavage, although it was not clarified whether these were actually direct targets of miR-23a in granulosa cells (Yang et al. 2012). Using a microarray approach in porcine ovarian follicles, another study demonstrated that miR-26b expression increased during follicular atresia and further showed that this miRNA could induce granulosa cell death by directly targeting ataxia telangiectasia mutated (ATM), a gene involved in DNA repair (Lin et al. 2012). Overall, these studies illustrate how a key follicular function, oestradiol production, can be distinctly regulated, directly or indirectly, by different miRNAs.

2.7 Extra-cellular miRNAs

While the majority of miRNAs detected intracellularly, a considerable number of miRNAs, commonly known as circulating miRNA or extra-cellular miRNA, have been also detected outside cells, mainly in various bio-fluids (Gilad et al. 2008; Hunter et al. 2008; Mitchell et al. 2008; Weber et al. 2010). Recent studies have shown that miRNAs are not only present in serum (Chen et al. 2008; Chin and Slack 2008; Mitchell et al. 2008) or plasma (Arroyo et al. 2011; Chim et al. 2008; Shen et al. 2010) but also different extra-cellular body fluids including saliva urine, tears, seminal fluid, breast milk, colostrum, peritoneal fluid, bronchial lavage and cerebrospinal fluid (Weber et al. 2010). Moreover, the expression profile of extra-cellular miRNAs from different types of body fluids in relation to different physiological / pathological conditions shows a specific pattern which indicating that extra-cellular miRNAs are not only passively released from the necrotic or injured cells but also selectively released from the cells (Pigati et al. 2010).

2.7.1 Characteristics of extra-cellular miRNAs

In contrast to circulating miRNAs, when synthetic miRNAs spiked into human plasma, it shows rapid degradation (within few minutes). While denaturing solution inactivated RNase activity in plasma, the exogenous miRNAs get released from degradation (Mitchell et al. 2008). Thus, synthetic miRNA species are vulnerable and susceptible to quick degradation in plasma, where as circulating miRNAs are protected and more resistant to high endogenous RNase activity, indicating that circulating miRNAs are likely wrapped with some protective manner to bypass high RNase activity in the extra-

cellular environment. Moreover, studies have been demonstrated that circulating miRNAs in body fluid remain stable even they subjected to harsh conditions like boiling, high or low pH, prolonged storage time and multiple freeze-thaw cycles while most of cellular RNAs were degraded quickly (Gilad et al. 2008; Taylor and Gercel-Taylor 2008). In addition, recent studies have been demonstrated that miRNAs in serum gives specific expression pattern in quantitative PCR (qPCR) after being subjected to incubation at room temperature for 24 h (Mitchell et al. 2008) and maximum 10 freeze-thaw cycle (Chen et al. 2008). Chen et al. found that isolated serum miRNAs can survive the treatment of RNase A, compared to other endogenous RNAs such as 18s rRNA, 28s rRNA, GAPDH, β-actin and U6 (Chen et al. 2008). Most serum miRNAs maintain considerable expression levels after 3 hours or overnight RNase A treatment; however large RNAs were degraded following 3 hours of RNase A treatment (Chen et al. 2008).

The mechanism underlying the remarkable stability of circulating miRNAs in the RNase-rich environment of blood is not well understood. Many hypotheses have been proposed to explain the possible mechanisms through which RNAs and miRNAs are released and protected from endogenous RNase activity in circulation. One of the earliest theory suggested that RNAs might conjugated with protein which would later protected them from both DNase and RNase activity (Sisco 2001). However, later on it has been showed that RNA species present in plasma are protected from degradation probably due to inclusion in lipid or lipoprotein complexes, not by binding with DNA (El-Hefnawy et al. 2004). Another hypotheses is that miRNAs are wrapped with microvesicles (exosomes, microparticles and apoptotic bodies), which shaded miRNAs in circulation and protect from RNase activity (Valadi et al. 2007). On the other hand some other studies have shown that, after isolation of microvesicles using high-speed ultra centrifugation from culture media (Turchinovich et al. 2011; Wang et al. 2010c) or plasma, a handful miRNAs are still detectable in the microvesicles free fraction, suggested that the presence of non-vesicle associated miRNA (might be miRNA-protein or miRNA-lipid/lipoprotein complexes) in extra-cellular fluid .

2.7.2 Pathways to release circulatory miRNAs

There are arguments regarding how these miRNAs are released in extracellular body fluids, stability and serves cell-cell communications. Numbers of studies have been demonstrated that the transfer of protein, mRNA and miRNA in different body fluids can be mediated via exosomes, microvesicles apoptotic bodies and RNA-protein or RNA-lipoprotein complexes that are released from a variety of cell types to modulate angiogenesis, cell proliferation/ apoptosis, tumor cell invasion and cell-cell communication.

2.7.2.1 Transport of circulating miRNA through exosomes

Exosomes are homologous small vesicles (50-90 nm) and have an endosomal origin (Camussi et al. 2010; Heijnen et al. 1999; Thery et al. 2002). Exosomes are formed by invagination of the membrane of endosomes to produce intraluminal vesicles, thus rendering these organelles multivesicular bodies (Urbe et al. 2003). In response to cell stimulation, budding of endosomes occurs, a process dependent on calcium influx, calpain and cytoskeleton reorganization (Johnstone 2006). Currently, accumulating evidences suggest that these secretory vesicles can function as intercellular transmitters to convey their contents, in particular, miRNA (Rechavi et al. 2009; Skog et al. 2008; Valadi et al. 2007). A total of 121 miRNAs were identified in exosomes from mast cells and the expression pattern of certain miRNAs was higher in microvesicles than in the donor cells (Valadi et al. 2007). Valadi and colleague reported that exosomes that are released from human and murine bone marrow-derived mast cells contain mRNA and miRNA, which are transferable to other human or mouse mast cells. When exosomes from mouse mast cells transferred to human mast cells, they produce new mouse protein in recipient cells, indicating that the exosomal mRNAs are functional and they can be translated after entering into another cell (Valadi et al. 2007). Furthermore, it has been shown that embryonic stem cell derived microvesicles are miRNA enriched and they can transfer a subset of miRNAs to mouse embryonic fibroblasts in culture, suggesting that gene expression of neighboring cells might be affected by exosomal miRNA that released by embryonic stem cells (Yuan et al. 2009). It has been demonstrated that miRNA released in exosomes by Epstein-Barr virus (EBV)-infected cells can be taken

up by peripheral blood mononuclear cells and these EBV-miRNAs repressed confirmed EBV target genes (Pegtel et al. 2010).

2.7.2.2 Transport of circulating miRNA through microparticles

Microparticles are lipid vesicles that are more than 1 μm in diameter and are secreted into extracellula environment by different type of cells and platelets (Aharon et al. 2009; Chironi et al. 2009; Ratajczak et al. 2006b). Many cell types are known to secrete microparticles and these include some cancer cells (Castellana et al. 2009), neurons (Marzesco et al. 2005) and many of the vascular and hematopoietic cell types such as endothelial cells, dendritic cells and B cells (Shet 2008). Microparticles are larger than exosomes and form through plasma membrane budding and also contain miRNA (Yuan et al. 2009). Initially microparticles were considered as cell debris but experimental evidence suggests that microparticles influences diverse biological functions for example cardiovascular disorders, including atherogogenesis and thrombosis (Mack et al. 2000; Mause and Weber 2010; Shantsila et al. 2010). The presence of miRNAs in microvesicles has now been reported from different cells including mesenchymal stem cell (Chen et al. 2009), mast cells (Shefler et al. 2010; Valadi et al. 2007), cancer cells (Jaiswal et al. 2011; Taylor and Gercel-Taylor 2008), platelets (Hunter et al. 2008) and endothelial cells (Skog et al. 2008). Recently it has been shown that chemically modified miR-143 entrapped by microvesicles was significantly secreted from miR-143-transfected human monocytic leukemia THP-1 cells during incubation in serum-free media (Akao et al. 2011). These findings highly support that at least some extracellular miRNAs are used for cell-cell communication via microparticles but still thorough investigation need to understand the mechanisms how miRNAs are selected for secretion, recognized for uptake, and what information can be transmitted.

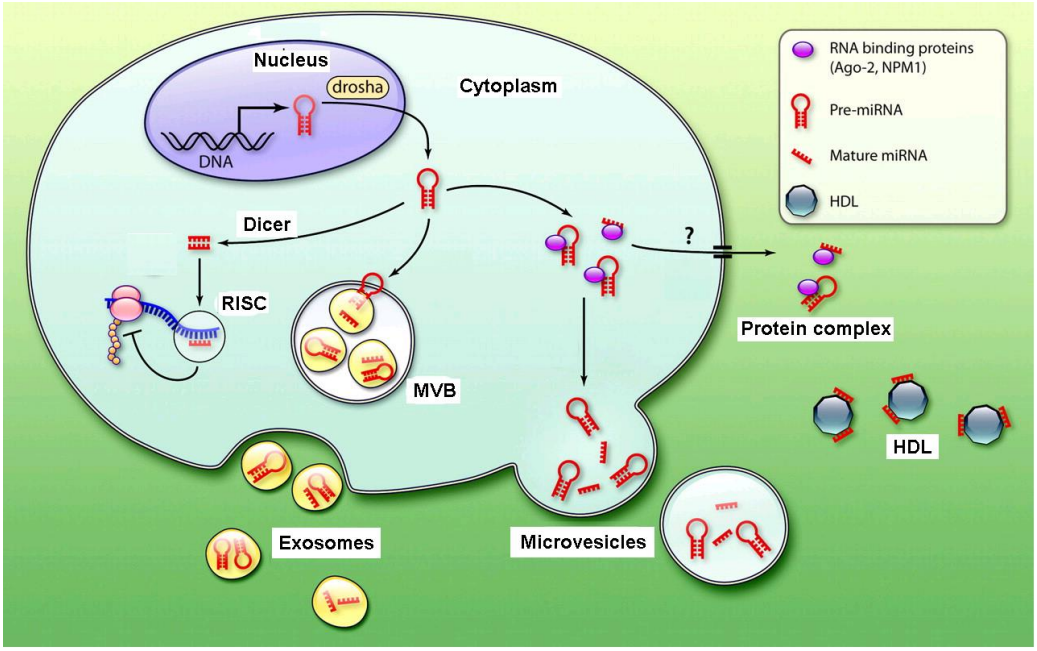


Figure 2.5: Extracellular transportation systems of miRNAs (Creemers et al. 2012).

2.7.2.3 Transport of Circulatory miRNA through apoptotic bodies

Apoptotic bodies (Abs) are small membranous particles released during programmed cell death (Hasselmann et al. 2001). The formation of ABs is a final outcome of apoptotic cell death. Budding of microparticles occurs mainly during early apoptosis, whereas ABs are formed in the late stages of this death process. Abs are larger than MPs and represent the compacted or condensed remnants of the shrinking apoptotic cells (Belting and Wittrup 2008; Beyer and Pisetsky 2009; Hristov et al. 2004). Recently it has been reported that ABs plays an important role in the repair of injured cells. In vitro experiment shows that endothelial progenitor cells (EPCs) engulf ABs released from endothelial cells (ECs) which trigger the secretion of cytokines and/or growth factors indicating that it may facilitate the repair of injured endothelial cells or somatic cells (Hristov et al. 2004). A microarray result shows that the presence of a panel of miRNAs in ABs derived from ECs where mir-126 is the most abundant one (Zernecke et al. 2009). Sprouty-related protein, SPRED1, and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) are the negative regulators/ intracellular inhibitors of the

vascular endothelial growth factor (VEGF) signaling pathway. Through repressing the expression of these negative regulators in ECs, mir-126 facilitates the pro-angiogenic actions of VEGF and fibroblast growth factor (FGF) and promotes blood vessel formation (Fish et al. 2008; Wang et al. 2008). Moreover, mir-126 knockout in mice resulted leaky vessels, and partial embryonic lethality, because of a loss of vascular integrity, defects in endothelial cell proliferation and angiogenesis (Wang et al. 2008). In damaged tissue, the CXC chemokine CXCL12 and its receptor CXCR4 counteract apoptosis and recruit progenitor cells. In case of atherosclerosis, endothelial cell-derived ABs are generated and convey paracrine signals to recipient vascular cells that trigger the production of CXCL12 in a miR-126 dependent manner (Zernecke et al. 2009). Though, these observations confirms that ABs may serve as a carrier of circulating miRNA, more studies need to determine how specific miRNA secreted, recognized for up-take via this process.

2.7.2.4 Transport of circulating miRNA through protein complex

One of the major components of miRNA silencing complex is Argonaut 2 protein (Ago2). Ago2-miRNA complexes were detected in different cell culture media and western blot immunoassay shows that extracellular miRNA ultrafiltrated together with the Ago2 protein, a part of RNA-induced silencing complex, not associated with microvesicles or exosomes (Turchinovich et al. 2011). Furthermore, it has been demonstrated that only 10% cell-free miRNAs released in plasma through micro vesicles whereas potentially 90% of the miRNAs in the circulation co-fractionated with ribonucleo-protein complexes (Arroyo et al. 2011). Size-exclusion chromatography has been used to exclude the microvesicle or exosome contamination from protein complexes and shows that most of the miRNA co-purified with non-vesicle-associated ribonucleo-protein complexes, only few miRNA, such as miR-16 and miR-92a associated predominantly with microvesicles (Arroyo et al. 2011). However, Ago2-miRNA is not only the protein complex that released in cell culture supernatant, mass spectrometry results revealed that a total of 197 proteins present in human fibroblast conditioned medium 2 h after serum starvation, of which 12 were known RNA-binding proteins (Wang et al. 2010b). Although, the role of other RNA-binding protein complexes, except Ago2, is presently unclear. Collectively these results indicate that

Ago2-protein complexes might be involved with the delivery of miRNA from donor cell to recipient cells and facilitate cell-cell communications.

2.7.2.5 Transport of circulatory miRNA through high density lipoprotein

Recently, Vickers *et al.* reported that purified fractions of HDL from healthy human plasma contain a number of miRNAs (Vickers et al. 2011). Highly purified HDL that is negative for exosomal marker proteins is rich in small RNA molecules that are 15 to 30 nucleotides in length but devoid of long mRNAs (Vickers et al. 2011). Total RNA extracted from HDL and exosomes isolated from the plasma of healthy individuals revealed that their miRNA profile is distinct (Vickers et al. 2011). A specific miRNA signature of HDL-miRNA complexes was identified in patients with familial hypercholesterolemia, including miR-22, miR-105, and miR-106a (Vickers et al. 2011). The authors further showed that direct delivery of miRNAs to recipient cells can also occur by HDL in a ceramide signaling pathway dependent manner (Vickers et al. 2011). Thus, native HDL is associated with miRNAs in a way that resembles artificial gene delivery vehicles, acting as a carrier or depot for circulating miRNAs in plasma and facilitating their transport and delivery to recipient cells. Finally, the study by Vickers *et al*. provides evidence that the miRNAs within HDL alter the cellular miRNA pool and functionally downregulate corresponding miRNA targets (Vickers et al. 2011), suggesting that the miRNA content of HDL is biologically relevant. Collectively, these results indicate that besides its classical role as a delivery vehicle for excess cellular cholesterol, HDL may also function as a transporter of endogenous miRNAs.

2.7.3 Potential role of extra-cellular miRNAs

Whether the extra-cellular forms of miRNAs are simply waste products from cells or have a biological function, such as participating in intercellular communication is not yet clear. There are reports showing increased level of miRNAs in blood upon organ toxicity (Laterza et al. 2009; Zhang et al. 2010), and this could of course represent waste products. Nevertheless, since the various forms of extracellular miRNAs are probably products of distinct cellular processes, they might play different roles, and therefore it is important to distinguish between them. Apoptotic bodies are by definition formed during apoptosis. miRNA bound to Ago2 may be released from cells upon

apoptosis or necrosis (Turchinovich et al. 2011), but it is not known if miRNA-Ago2 complexes also can be transported out of viable cells. This means that miRNAs bound to Ago2 proteins and miRNAs incorporated into apoptotic bodies might solely be by-products from dying cells or represent a way for dying cells to communicate with neighboring cells. They could represent a signal warning the organism about cellular dysfunction.

Shedding vesicles and exosomes are thought to be released by viable cells, though it is not ruled out whether these vesicles also are released by dying cells. Therefore, these vesicles have to a greater extent been suggested to play a role in intercellular signaling (Hunter et al. 2008; Valadi et al. 2007). Indeed, it has been shown that miRNAs can be transferred by exosomes from one cell to another *in vitro* and result in downregulation of target genes in the recipient cell (Kogure et al. 2011; Montecalvo et al. 2012). These findings indicate a role in intercellular communication which could have a huge impact. However, this remains to be shown *in vivo* conditions. Interestingly, it has been reported that injection of exosomes loaded with siRNA into mice can result in specific gene knockdown in certain cells (Alvarez-Erviti et al. 2011). It has been questioned whether the concentration of exosomes in biological fluids is high enough to play a role in intercellular communication, but this does not exclude a role in autocrine or paracrine signaling (Turchinovich et al. 2011). Exosomes probably exert their effect on neighboring cells, and thereby participate in creating a specific microenvironment. In this scenario, the exosomes found in body fluids would only be residual amounts, representing a secondary effect.

In addition to their conventional role in post-transcriptional gene regulation, a new role for miRNAs as signaling molecules has recently been described by two independent groups. Interestingly, extra-cellular let-7 was shown to activate Toll-like receptor 7 in neurons and induce neurodegeneration (Lehmann et al. 2012). By another group, exosomal miR-21 and miR-29a was shown to activate TLR 7 and 8 in immune cells, triggering a prometastatic inflammatory response that may lead to tumor growth and metastasis (Fabbri et al. 2012). Thus, extra-cellular miRNAs could be important regulators of tumor microenvironment as well as exacerbate CNS damage, through agonistic effect on TLR 7 and 8.

Another possible role for miRNAs in exosomes and microvesicles is that they might function together with the RNAi machinery. RISC proteins have been shown to be associated with MVBs and exosomes (Gibbings et al. 2009). Moreover, blocking MVB formation by depletion of ESCRT (endosomal sorting complex required for transport) components has been reported to result in impaired miRNA silencing, indicating a role in RNAi dynamics (Gibbings et al. 2009; Lee et al. 2009).

2.7.4 Potential application of extra-cellular miRNAs

Utility of minimally invasive serum biomarkers in the form of DNA and RNA fragments in serum for altered physiological conditions were well documented (Fleischhacker and Schmidt 2010). In 1977, Leon *et al.* first observed that concentrations of cell-free circulating DNA (cirDNA) in blood of cancer patients is higher as compared to normal subjects (Leon et al. 1977). Since then, various studies have demonstrated elevated level of cell-free DNA concentration in plasma/serum in various pathological conditions, including cancer, autoimmune disorders, pregnancy and trauma (Mitchell et al. 2008). Recent discoveries on miRNAs in serum have stirred the demanding biomarker discovery field to identify reliable markers for early disease detection and classification.

In general, the blood based biomarkers, including antigens, enzymes, and lipid components are very useful for prognosis, monitoring the effectiveness of treatments, prediction of recurrence risk, and also to monitor the recurrence of the disease. Currently there are very few blood-based biomarkers with respect to tumors and cancers. Some of the currently available serum/plasma biomarkers, such as carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA21-1) and alphafetoprotein (AFP) offer some promise to analyze tumors systematically without involving the tissue biopsies or surgery (Dbouk et al. 2007). However, clinical implementation of these biomarkers at a broader level faces some major challenges specially their low sensitivity and specificity.

The stability of miRNAs in plasma and the easier detection of these miRNAs in a quantitative manner by methods such as real-time PCR and microarrays have sparked great interest in the use of circulating miRNAs as clinical biomarkers for altered

physiological conditions. However, an ideal biomarker should satisfy a number of criteria, such as accessibility through noninvasive methods, a high degree of specificity and sensitivity, the ability to differentiate pathologies, allowing early detection, sensitivity to relevant changes in the disease, a long half-life within the sample, and the capability for rapid and accurate detection (Etheridge et al. 2011). Because of circulating miRNAs are able to fulfill a number of those criteria, since 2009, several groups have reported on the use of miRNAs as circulating biomarkers for diagnosis or prognosis of cardiovascular diseases such as myocardial infarction, HF, atherosclerosis and hypertension (Chen et al. 2008; Heneghan et al. 2010; Laterza et al. 2009; Wang et al. 2009a). Although many of those studies still require replication in multiple independent study populations, the picture emerges that some plasma miRNAs are quite specific for cardiovascular pathologies and not only may be useful for diagnostic and monitoring purposes but also may provide much needed intermediate end points for clinical trials (Creemers et al. 2012).

Several groups have studied the hypothesis regarding heart-specific miRNAs that leak into the circulation during an acute myocardial infarction (AMI) and can be used to detect and monitor myocardial injury (Corsten et al. 2010). MicroRNAs such as miR-208a, miR-499, miR-1 and miR-133 are found to be consistently highly abundant in plasma of AMI patients within hours after the onset of infarction (Cheng et al. 2010; Corsten et al. 2010; Wang et al. 2010b). Of these 4 miRNAs, miR-208a, which is encoded by an intron of the αMHC gene, is to the best of our knowledge the only heart-specific miRNA. The other 3 miRNAs (miR-499, miR-1, and miR-133), besides being highly expressed in the heart, are also expressed in skeletal muscle (Chen et al. 2006; van Rooij et al. 2009).

In the early phase after AMI (<3 hours), miR-1, miR-133a, and more particularly miR-208a may even be more sensitive than the classic biomarker cTnI, because these miRNAs achieve their peak before cTnI (Creemers et al. 2012). The miRNA elevated level of these miRNAs in this earlier stage of infection suggests a faster release of miRNAs in circulation than cTnI from damaged cardiomyocytes because of differential release kinetics (Creemers et al. 2012). In this regard, cTnI is mainly bound to myofibrils (Ng et al. 2009), whereas miRNAs are believed to be bound to protein complexes in the cytosol or wrapped by vesicles, latter facilitate a faster release from

damaged cells (Wang et al. 2010b). Interestingly, not all myocardial miRNAs leak into the circulation with the same kinetics (Tijsen et al. 2012). The slower release of miR-499 into the bloodstream compared with several other myocardial miRNAs may suggest the preferential release of miRNAs in the circulation (Creemers et al. 2012). Strikingly, miR-30c and miR-24 are strongly expressed in the heart, even more strongly than miR-208, but their levels in the bloodstream still failed to increase after AMI (D'Alessandra et al. 2010).

A significant reduction or elevation of specific miRNA level in serum/plasma of cancer patients as compared to healthy controls and their correlation with the surgical treatment of the tumor signifies the potential link between miRNA expression levels and primary tumors (Srivastava et al. 2011). Systematically analyzing the expression pattern of various miRNAs in human cancers can also provide significant understanding of origin of an unknown primary carcinoma (Barker et al. 2009). In one of the pioneer studies dealing with miRNA levels in serum samples, Lawrie *et al.* suggested that serum miR-21 levels are associated with relapse-free survival in patients with diffuse large B-cell lymphoma and therefore holds potential to be a diagnostic biomarker for the disease (Lawrie et al. 2008). Heneghan *et al*. showed that cancer-specific miRNAs are detectable and significantly altered (miR-195 and *let*-7a) in the circulation of breast cancer patients, and that increased systemic miR-195 levels in breast cancer patients were also reflected in breast tumors (Heneghan et al. 2010). Following that, they also correlate specific circulating miRNAs with various pathological variables such as nodal status, estrogen receptor status etc (Heneghan et al. 2010). Altered expression of *let*-7, miR-25 and miR-223 miRNAs in serum has been observed in lung cancer patients while miR-15/16 were found to be down regulated in leukemia patients (Calin et al. 2002; Carlsen et al. 2013). Furthermore, some miRNAs like miR-210, miR-200a and miR-200b have been shown to be overexpressed in patients with pancreatic cancer (Ho et al. 2010; Wang et al. 2009a). In the case of pancreatic ductal adenocarcinoma, a collective analysis of four differentially expressed miRNAs (miR-21, miR-210, miR-155, and miR-196a) in plasma has been suggested to discriminate patients from healthy individuals (Wang et al. 2009a). In tongue squamous cell carcinoma miR-184 has been shown to be upregulated in plasma of patients (Wong et al. 2008). Moreover, *let*-7a miR-21 and miR-195 were observed to be upregulated in the breast cancer (Wang et al.

2010a). In gastric cancer miR-17-5p, miR-21, miR-106a, miR-106b are known to be upregulated (Wang et al. 2009a) while *let*-7a expression was down regulated (Tsujiura et al. 2010). miR-155 was differentially expressed in the serum of women with hormone-sensitive as compared to hormone-insensitive breast cancer patients (Zhu et al. 2009). The level of miR-500 in the sera of the hepatocellular carcinoma (HCC) patients was highly elevated pre-surgery and which returned to normal level after the surgery, whereas lowered expression of miR-92a to miR-638 in plasma of HCC subjects was observed as compared to controls (Shigoka et al. 2010; Yamamoto et al. 2009). In colorectal cancer, miR-17-3p and miR-92 levels were elevated significantly in plasma of matched patient samples prior to surgery compared to post surgery (Ng et al. 2009). Decreased expression of miR-92a in plasma was also observed in acute leukemia patients (Tanaka et al. 2009). In ovarian cancer miR-21, -29a, -92, -93, and -126 were significantly overexpressed in serum from cancer patients as compared to healthy controls (Hausler et al. 2010; Taylor and Gercel-Taylor 2008). Also, miR-155, miR-210, and miR-21 were found to be elevated in the patients' serum of diffuse large B-cell lymphoma (DLBCL) as compared to healthy controls (Lawrie et al. 2008). In several studies, miRNA expression profiles have been shown to have signatures related to tumor classification, diagnosis, and disease progression. The realisation that miRNAs are deregulated in human cancers has generated considerable interest with regard to their potential as biomarkers. miRNAs have a number of desirable characteristics for such an application. Perhaps most importantly, miRNA expression profiles are often tissue, developmental, and disease specific.

2.7.5 Challenges in extra-cellular miRNA study

MicroRNAs have only been discovered within the last two decades. Although the assessment of the miRNA expression pattern in various cancers has revealed a plethora of potential miRNA biomarker candidates for cancer risk, diagnosis, prognosis and/or prediction and therapy, development of a reliable and reproducible miRNA clinical test still in infancy (Srivastava et al. 2011). In that respect, circulating miRNAs offer many features to make them an attractive class of biomarkers. They are stable; their sequences are evolutionarily conserved; miRNA expression is often tissue or pathology specific; and because they are detected by real-time PCR, assays can be highly sensitive and

specific. However, there are also challenges associated with the detection of circulating miRNAs that still need to be addressed. One of the challenges relates to the low amount of total RNA in blood, which makes it virtually impossible to measure the concentration and quality of the isolated RNA. As a consequence, it is of crucial importance to precisely normalize detected miRNA values for variances based on the amount of starting material and miRNA extraction. This has been tried by seeking a "housekeeping" circulating RNA. Some reports use U6 or other miRNAs (eg, miR-16) as a housekeeping RNA; however, the levels of these RNAs often change under pathological conditions. Mitchell *et al*. reported a spiked-in normalization approach in which 3 synthetic *Caenorhabditis elegans* miRNAs (without homology to human miRNAs) were added during the purification procedure and used for data normalization (Mitchell et al. 2008). This worked well in their hands; however, these synthetic miRNAs may be less stable than endogenous miRNAs when added to plasma. Cheng *et al*. (2010) reported that plasma volume is the best factor with which to standardize the amount of input miRNA. The amount of molecules per volume of plasma or serum is also used as the standard to evaluate blood levels of other molecules. Future studies are warranted to systematically characterize the different normalization methods to find the best way to reproducibly measure miRNAs in plasma. In this regard, it is very possible that plasma miRNAs from microparticles, HDL, or unbound miRNA require a different normalization procedure.

2.8 Exosomes

Exosomes are small nano sized (30–90 nm) extracellular vesicles and they correspond to the internal vesicles of multivesicular bodies (MVBs) and are released in the extracellular environment upon fusion of MVBs with the plasma membrane (Thery et al. 2002). Exosomes were first discovered in the process of eliminating transferrin receptors during reticulocyte (immature red blood cells) maturation, and thought to function as cellular waste products, thus received little attention and remained little studied. This "cellular waste" hypothesis was challenged in the 90's, when exosomes were shown to have an immunoregulatory effect, with the demonstration that B cell-derived exosomes could stimulate T cells (Raposo et al. 1996). The authors demonstrate that Epstein–Barr virus (EBV)-transformed B-lymphocytes secreted exosomes that bore

molecules essential for the adaptive immune response namely major histocompatibility class (MHC) II dimers bound to antigenic peptides. These exosomes were also shown to present the MHC-peptide complexes to specific T cells, suggesting that they could play a role in adaptive immune responses (Raposo et al. 1996). Two years later, the groups of Raposo, Amigorena, and Zitvogel demonstrated that dendritic cells (the immune cells that initiate adaptive immune responses by presenting MHC–peptide complexes to naïve T cells) also secrete exosomes bearing functional MHC–peptide complexes, which could promote induction of antitumor immune responses in mice *in vivo* (Zitvogel et al. 1998). These results provided the basis for the hypothesis that exosomes could play an active role in intercellular communication, at least in the immune system, and prompted the very first attempt at using them in the clinic, as a new type of anticancer therapy in humans (Thery 2011). This greatly increased the interest in the field. Today, exosomes have been found in a number of human body fluids.

Exosomes can be distinguished from other classes of cell-secreted vesicles such as microvesicles (MVs) and apoptotic bodies by several classification features including size, biological content, and most markedly, by biogenesis and mode of release from the cell. Exosomes range in size from 100 nm or less while MVs are typically larger at 100 to 1000 nm in diameter (Thery 2011). The secretion of exosomes can be spontaneous or induced depending on the cell type. Following the activation of a cell surface receptor, reticulocytes (Pan et al. 1985), T cells (Blanchard et al. 2002), mastocytes (Raposo et al. 1997) and resting B cells (Arita et al. 2008; Muntasell et al. 2007; Saunderson et al. 2008) secrete detectable levels of exosomes in the extra cellular milieu. In contrast, Epstein-Barr virus (EBV)-transformed B cells (Raposo et al. 1996), dendritic cells (Zitvogel et al. 1998) and macrophage (Bhatnagar et al. 2007) constitutively secrete exosomes *in vitro*, as do most tumor cell lines. Exosomes have also been purified in the absence of any mechanical dissociation from several body fluids, such as human plasma (Caby et al. 2005), serum (Taylor et al. 2006; Taylor and Gercel-Taylor 2008), broncho-alveolar fluid (Prado et al. 2008), urine (Pisitkun et al. 2004), tumoral effusions (Andre et al. 2002), epididymal fluid (Gatti et al. 2005), amniotic fluid (Asea et al. 2008) and milk (Admyre et al. 2007). These exosomes contain proteins that are expressed by epithelial, tumor and haematopoietic cells, which suggest that exosomes could be released from several cell types and tissues *in vivo*.

2.8.1 Biogenesis of exosomes

Exosome formation begins with initiation of the endocytotic pathway as material, such as protein, is engulfed by the cell at its surface. This process may be clathrin dependent, as is true in the case of the transferrin receptor, or may be clathrin-independent, as is the case with glycosylphosphatidylinositol (GPI)-anchored proteins, caveolae, or lipid raft mechanisms (Mayor and Riezman 2004). Once internalized, the endocytotic vesicles are delivered to early endosomes in which the mildly acidic pH (~6.2) causes an un-coupling of housekeeping receptors from their ligands, and then recycled back to the plasma membrane or transported to late endosomes together with other proteins and lipids (Maxfield and McGraw 2004). In late endosomes, which is spherical in shape and acidic in nature (pH 5.0 to 5.5), two sequential steps have been recognized for sorting of protein during MVBs formation. The first step include the lateral sorting or selection of proteins at the limiting membrane of endosomal lumen and the second step is the formation of inwardly budding vesicles, commonly known as intraluminal vesicles (ILV), with the concomitant incorporation of selected proteins, lipids, mRNAs and miRNAs. Once MVBs formed they can either fuse with lysosomes for degradation of proteins or direct interaction with the plasma membrane, resulting in the release of the ILVs as exosomes. Since two membrane inversion occur during the formation of an exosome from a membrane patch on the plasma membrane, it allow exosomes to present trans membrane cargo in the same orientation relative to the cell cytoplasm and with the same membrane protein on the outside as at the plasma membrane (Lakkaraju and Rodriguez-Boulan 2008).

The mechanisms underlying the formation of ILVs and the sorting of proteins and lipids into these vesicles, as well as docking and fusion of the MVBs with the plasma membrane are poorly understood, although the ESCRT have been shown to be involved in this process. However, it has been shown that protein can also be sorted independently of ESCRT (de Gassart et al. 2003). There are five distinct protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and Vps4, found to be involved in ESCRT machinery protein complexes, including which direct ubiquitin tagged cargo to endosomes where they are sorted into ILVs (Gould and Lippincott-Schwartz 2009; Henne et al. 2011). The process is started by ESCRT-0 which is made up of HRS (hepatocyte growth factor- regulated Tyr-kinase substrate) or Vps27 in the case of yeast,

and STAM (signal transducing adaptor molecule) that binds to ubiquitinated proteins (Katzmann et al. 2003). ESCRT-0 then binds with the ubiquitin binding protein Tsg101 (tumor susceptibility gene-101) via its N-terminal UEV domain, which is a component of ESCRT-I. Importantly, ESCRT-I also composed of Vps28 and one of four isotypes of Vps37 (Vps37A-D) (Teo et al. 2004). ESCRT-I recruits the next pathway complex, ESCRT-II, with the help of ESCRT adaptor protein Alix (ALG-2 interacting protein X), leads to the recruitment of ESCRT-III. ESCRT-III is composed of the most constituents and associated proteins, more than the other ESCRT complexes, including Vps2A,B, CHMP3 (charged MVB proteins or chromatin modifying protein-3), SNF7-1,-2,-3, and CHMP7, among others (Williams and Urbe 2007). The final step of ILVs formation also requires Vps4, which binds to ESCRT-III and also induces the ESCRT machinery's release from the endosomal membrane, thus enabling it to be recycled (Babst 2011; Hanson et al. 2009). Another theory for the underlying mechanism behind sorting of content into ILVs involves a ubiquitin-independent pathway while utilizing the ESCRT machinery, most likely by direct interference with ESCRT-I and –III (Babst 2005; de Gassart et al. 2004; van Niel et al. 2006).

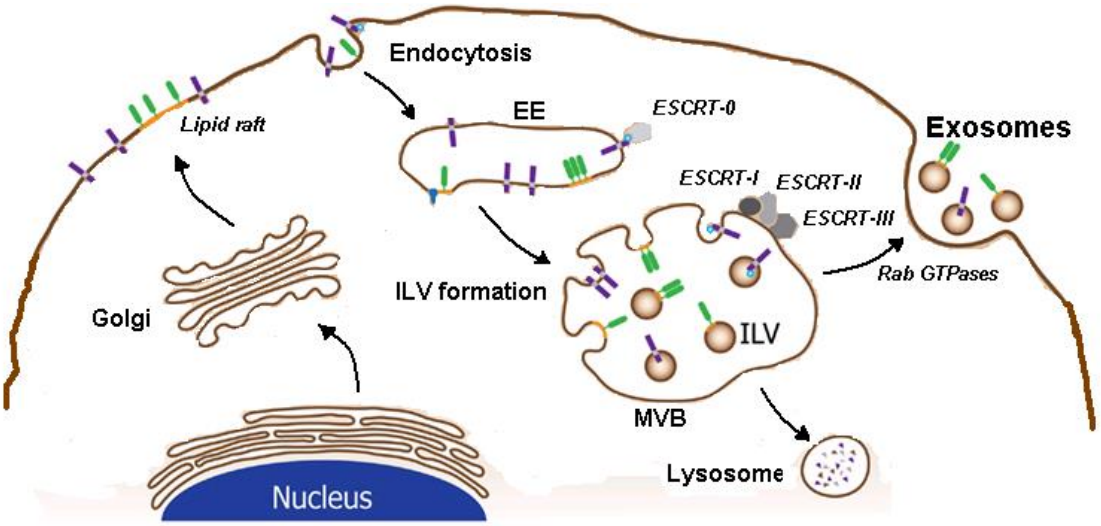


Figure 2.6: Exosome biogenesis. Membrane proteins (shown in purple and green) are internalized and delivered to early endosomes (EE). In early endosomes, the proteins can be recycled to the plasma membrane or delivered to late endosomes.

Intraluminal vesicles are formed by budding of the endosomal limiting membrane into the endosomal lumen, forming multivesicular bodies (MVB). MVBs either can fuse with lysosomes for protein degradation or they can also dock to and fuse with the plasma membrane. This results in the release of the intraluminal vesicles to the extracellular compartment. The vesicles are now called exosomes. Exospores display the same topology as the plasma membrane, with extracellular domains exposed on the outside and cytoplasm on the inside enclosed by a lipid membrane. Modified from (Bellingham et al. 2012).

In the absence of the ESCRT machinery, proteins can also be sorted independently of ESCRT. Under this circumstances cells may employ different mechanisms for the formation and protein sorting of MVBs, and consequently ILVs. These ESCRT-independent pathways seem to be driven by cholesterol, tetraspanins and lipids, such as sphingomyelin, that form specialised microdomains in the endosomal limiting membrane, called lipid rafts, which can bend inwards and consequently form ILVs (de Gassart et al. 2003; Wubbolts et al. 2003). Proteins can also be co-sorted into ILVs, by binding to membrane proteins or lipids (de Gassart et al. 2003). Additionally, engulfment of the cells cytoplasm during the vesicle formation can lead to random packaging of cytosolic proteins. Proteins and lipids are not the only content that is sorted into ILVs, as exosomes have been shown to also contain different RNA species (Valadi et al. 2007). The sorting of RNA into ILVs is an even a greater enigma, however the poor correlation between the mRNA content of exosomes and their donor cells (Mittelbrunn et al. 2011) suggests that the RNA is specifically packed into the vesicles, rather than randomly engulfed from the cells cytoplasm (Nazarenko et al. 2010; Valadi et al. 2007). Some studies have suggested that MVBs are the location of the assembly and loading of RISC and therefore miRNA (Gibbings et al. 2009).

In addition to an incomplete understanding of biogenesis and sorting, the mechanisms underlying the secretion of exosomes is also poorly understood. Secretion involves the docking and fusion of MVBs with the plasma membrane, with several different factors involved in the regulation of this process. Several members of the GTPase family Rab, regulators of membrane trafficking, have been shown to be involved in the docking and fusion of MVBs with the plasma membrane, and thus the release of exosomes. One member of the family, Rab11, has been shown to be involved in this process in a

calcium (Ca2+)-dependent manner (Savina et al. 2003). It was first suggested that an intracellular increase of Ca2+ stimulate exosome secretion (Savina et al. 2003). Later, it was shown that Rab11, together with Ca2+, are involved in the secretion of exosomes. An overexpression of Rab11, together with an increased intracellular Ca2+ concentration, promotes MVBs docking and fusion with the plasma membrane, and thus increases the secretion of exosomes (Savina et al. 2005). The regulation of exosome secretion is not only controlled by Rab proteins. Other proteins such as the p53 protein and the transmembrane protein TSAP6 have been shown to be involved in the secretion process (Amzallag et al. 2004; Yu et al. 2006). In addition, factors such as stress and pH are also involved in the secretion of exosomes (King et al. 2012). The release and the uptake of exosomes by recipient cells have been shown to increase at low pH (Parolini et al. 2009). Furthermore, stress such as hypoxia has also been shown to increase exosome secretion (King et al. 2012).

2.8.2 Isolation methods of exosomes

The accepted protocol for isolation of exosomes includes ultracentrifugation, often in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes (Thery et al. 2006). Isolation of membrane vesicles by sequential differential centrifugations is complicated by the possibility of overlapping size distributions with other microvesicles or macromolecular complexes. Furthermore, centrifugation to pelleting may prove insufficient means to separate vesicles based on their sizes. However, sequential centrifugations, when combined with sucrose gradient ultracentrifugation, can provide a high enrichment of exosomes.

Isolation of exosomes based on size, using alternatives to the ultracentrifugation routes, is an obvious option. Cheruvanky *et al*. reported successfully purifying exosomes using ultrafiltration procedures that are less time consuming than ultracentrifugation, and do not require use of special equipment (Cheruvanky et al. 2007). Similarly, Bioo Scientific launched a kit (ExomiR) that essentially removes all cells, platelets and cellular debris on one microfilter and captures all vesicles bigger than 30 nm on a second microfilter using positive pressure to drive the fluid. For this process, the exosomes are not reclaimed - their RNA content is directly extracted off the material caught on the second microfilter, which can then be used for PCR analysis. HPLC-

based protocols could potentially allow one to obtain highly pure exosomes, though these processes require dedicated equipment and are not trivial to scale up (Lai et al. 2010). The complication is, both blood and cell culture media contain a large number of nanoparticles (some non-vesicular) in the same size range as exosomes. For example, Wang *et al*. found that large number of miRNAs are contained within extracellular protein complexes rather than exosomes (biological roles for these are yet to be understood). As a consequence, the above methods are best described as allowing one to obtain exosome-enriched samples, rather than pure exosomes (Wang et al. 2010c).

Volume-excluding polymers such as PEGs are routinely used for precipitation of viruses and other small particles (Lewis and Metcalf 1988). This principle, or perhaps differential solubility in alternative solvents, could be used to precipitate exosomes (quite probably along with other macromolecular particles) from experimental samples. The precipitate can be isolated using either low-speed centrifugation or filtration. Recently, System Biosciences released a proprietary reagent named ExoQuick that can be added to serum, conditioned cell media or urine, and is claimed to precipitate the exosomes (Vlassov et al. 2012). However, although the process is very fast and straightforward, there is the inevitable lack of specificity toward exosomes, and the pellet from serum is rather difficult to resuspend (Vlassov et al. 2012).

In theory, a superior alternative for specific isolation of exosomes should be affinity purification with antibodies to CD63, CD81, CD82, CD9, EpCAM, and Rab5. These could be used by themselves or potentially in combination. For this application, the antibodies could be immobilized on a variety of media, including magnetic beads, chromatography matrices, plates and microfluidic devices (Chen et al. 2010; Thery et al. 2006). HansaBioMed is offering an array of products called ExoTest kits – featuring anti-CD63, -CD81 or -CD9 antibodies immobilized on 96 well plates – for exosome capturing and characterization (Vlassov et al. 2012). As with any young field, it has to be confirmed how well these systems work, and for researchers wanting a diverse exosome population, which of these proteins is (are) the best and most robust exosomal tag(s) for their needs.

In the same vein as antibodies, other affinity-capture methods could be used, such as lectins, which will bind to specific saccharide residues on the exosome surface. This

strategy has been proposed by Aethlon Medical (Vlassov et al. 2012) using a proprietary lectin that targets mannose residues. The convenient feature of this procedure is easy elution/release of the captured exosomes by free alpha-methyl-mannoside. However this approach is not specific to exosomes as a number of cells contain mannose on their surface; and it has yet to be proven if all exosome types can be captured this way. Multiple types of lectins are available, and these can be carefully investigated to select the best options.

2.8.3 Characteristics and composition of exosomes

Besides a characteristic morphology, exosomes are thought to be somewhat unique in their protein and lipid composition, providing additional traits for their identification. Due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, Annexins, flotillin), tetraspannins (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70, Hsp 90), proteins involved in multivesicular body biogenesis (Alix, TSG101), as well as lipid-related proteins and phospholipases (Conde-Vancells et al. 2008). Although these proteins are routinely used as positive markers, there is wide variation across exosomes from different sources. Beyond these membrane-associated proteins, over 4400 different proteins have been identified in association with exosomes, usually by mass spectrometry, presumably serving as cargo for inter-cell communication (Mathivanan and Simpson 2009). Although much of this variation has to do with the cells of origin, Carayon *et al*. reported that the protein repertoire in secreted exosomes also changed during erythrocyte maturation. The most widely used "markers" include tetraspannins, Alix, flotillin, TSG101, and Rab5b. Antibody-based techniques to detect these targets, such as Western or ELISA, are becoming popular for rapid confirmation of exosome presence (Carayon et al. 2011).

Besides proteins, exosomes are enriched in certain raft-associated lipids such as cholesterol (primarily B lymphocytes), ceramide (implicated in the differentiation of exosomes from lysosomes) other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains (Subra et al. 2007). Laulagnier *et al*. also found that, at least for mast and dendritic cells, there was an increase in phosphatidylethanolamines and that the rate of flipping between the two leaflets of the bilayer was higher than in

cellular membranes (Laulagnier et al. 2004). There are also indications that exosomes could serve to deliver prostaglandins to target cells (Subra et al. 2010).

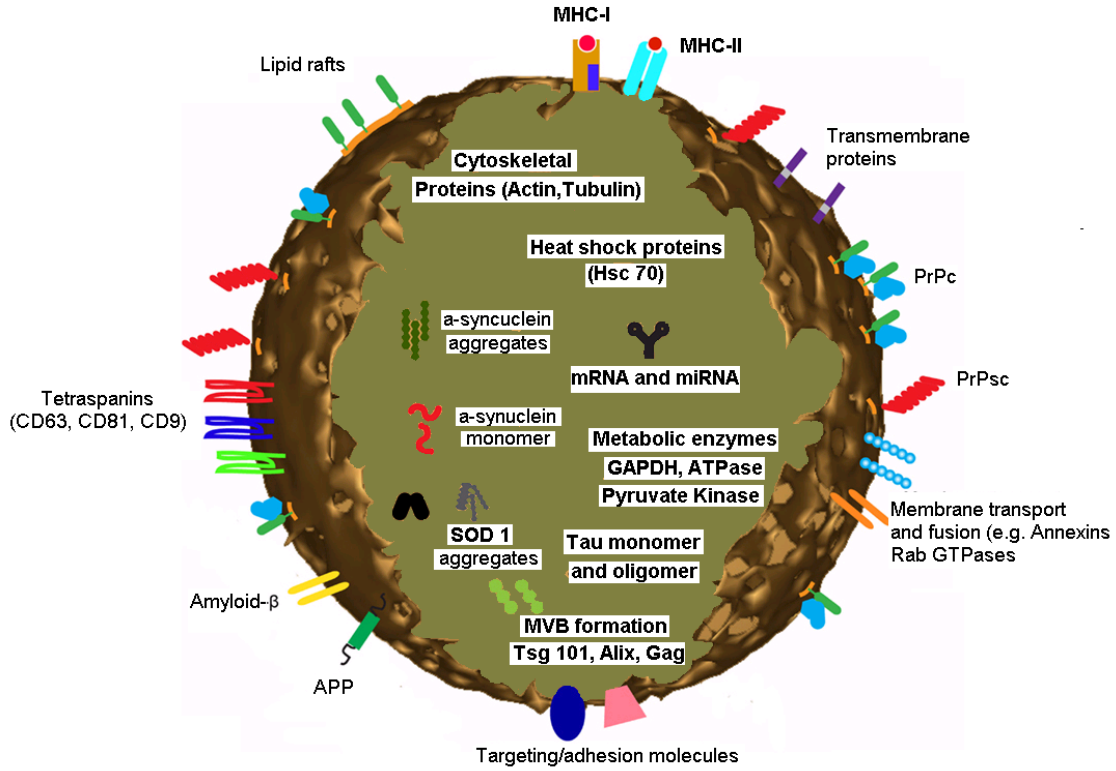


Figure 2.7: Composition of exosomes. Exosome contain mRNA and miRNA, and a vast array of different proteins depending on their host cell. In addition to generic proteins, proteins associated with neurodegenerative diseases such as Alzheimer's, Prion disease, and Parkinson's disease have been identified in exosomes. Adopted from Bellingham *et al.* (2012)

Exosomes also bear saccharide groups on their outer surface. This was investigated recently by Batista *et al.* and they found that these were enriched in mannose, polylactosamine, α-2, 6 sialic acid, and complex N-linked glycans (Batista et al. 2011). Exosomes have been reported to contain significant amounts of miRNA, other non-coding RNAs, as well as mRNA. Valadi *et al.* reported that, although the RNA appeared to be mostly degraded to less than 200 nt fragments, some full-length

molecules must also be present, since the extracted RNA could be used to generate identifiable full-length proteins using an in vitro translation system (Valadi et al. 2007).

Table 2.1: Molecular composition of exosomes, adopted from Denzer *et al.* (2000)

	Functions	**Reference for exosomal localization**
TfR	Iron transport	Davis et al., 1986; Johnstone et al., 1987
Integrin a4b1	Adhesion/co-stimulation	Rieu et al., 2000
Mac-1/integrin a chain	Adhesion to target cells	Théry et al., 1999
MFG-E8/lactadherin	"	Théry et al., 1999
GPI-anchored proteins		
AchE		Johnstone et al., 1987
DAF/CD55	Regulation of complement activation	Rabesandratana et al., 1998
MIRL/CD59	Protection from complement mediated lysis/signaling	Rabesandratana et al., 1998
LFA-3/CD58 (GPI-isoform)	Adhesion/co-stimulation	Rabesandratana et al., 1998
Immunoglobulin-supergene family		
MHC class II molecule	Antigen presentation	Raposo et al., 1996, 1997a; Zitvogel et al., 1998
MHC class I molecule	"	Zitvogel et al., 1998; Kleijmeer et al., 1998
CD86/B7.2	Co-stimulation	Escola et al., 1998
CD54/ICAM-1	Adhesion to target cells	Théry et al., 1999
Tetraspan protein family		
CD9	Signal transduction/adhesion/complex formation with MHC and integrins	Théry et al., 1999
CD37	"	Escola et al., 1998
CD53	"	Escola et al., 1998
CD63/Lamp-3	"	Escola et al., 1998; Heijnen et al., 1999
CD81	"	Escola et al., 1998
CD82	"+HLA-DM/DO	Escola et al., 1998
Cytosolic proteins		
Hsp70	Protein folding	Mathew et al., 1995
Hsc73	Uncoating ATPase/peptide transfer to MHC	Théry et al., 1999
Gi2 a subunit	Fusion	Théry et al., 1999
Annexin II	MVB formation	Théry et al., 1999
Lipids		
LBPA	Cholesterol transport	Denzer et al., 2000

Several papers indicate that the RNA "cargo" of exosomes is significantly different from the parental cell content, i.e. certain RNAs are present at significantly different levels compared to the total cell lysate from the originating cells (Mittelbrunn et al. 2011; Zomer et al. 2010). This runs counter to several authors working with cancer cells, who have noted that the miRNA content for their originating cancer cells is similar to that found in circulating exosomes, and they have postulated the feasibility of using this as a basis for diagnostic markers (Rabinowits et al. 2009). Since the primary current procedure to verify the presence of exosomes is through EM, a costly and time-consuming process, there is clearly an urgent need to develop simpler, more molecule-based tools and protocols for confirmation of exosomal presence. MicroRNA (miRNA) may provide this marker, although it has yet to be determined if any RNA molecules can serve as reliable generic exosomal markers.

2.8.4 Functions of exosomes

The function of exosomes most probably depends on the cells from and conditions under which they are produced, as this provides them with their characteristic composition. When exosomes were first discovered from reticulocytes, they were shown to function as a way to discard proteins, such as the transferrin receptor during the maturation process of reticulocytes into erythrocytes (Johnstone et al. 1987). Reticulocytes lack lysosomes, and it seems as if exosome release is an alternative to lysosome degradation. Another function of exosomes that was later described is as cell free messengers, which can be released from one cell and have an effect on another. This communication with other cells may occur either in the microenvironment, or over a distance (Fevrier and Raposo 2004). Since exosomes have been found in blood plasma (Caby et al. 2005), they may be transported between organs via the systemic circulation.

How this interaction occurs between exosomes and cells is not fully known. However, several mechanisms describing the interactions of exosomes and cells have been hypothesized. Exosomes can bind to cells through receptor-ligand interactions, similar to cell to cell communication, mediating for example antigen presentation (Admyre et al. 2006). Clayton *et al.* showed that B cell exosomes express functional integrines, which are capable of mediating adhesion to extra-cellular matrix components and activated fibroblasts. This adhesion was strong and resulted in an increase in

intracellular calcium (Clayton et al. 2004). Alternatively, exosomes can attach to or fuse with the target cell membrane, thus delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell (Denzer et al. 2000). MHCII positive exosomes have been shown to be attached to follicular DCs. These cells do not express MHCII themselves, and the exosomes provide them with new properties (Denzer et al. 2000). Finally, exosomes may be internalized by the recipient cells due to mechanisms such as endocytosis. Immature DCs have been shown to internalize and process exosomes for antigen presentation to CD4+ T cells (Morelli et al. 2004).

2.8.5 Functional delivery of miRNAs via exosomes

A recent breakthrough in exosome biology is that RNA molecules, in particular, miRNAs, are present in these vesicles. To date, studies have demonstrated that miRNAs in exosomes can influence target cell function (Kosaka et al. 2010). The transport of nucleic acids inside the exosomes can play an important role in functioning of a multicellular organism; moreover, the supposed specificity of this process can, on necessity, contribute to the directed transfection of an exogenous material (Mittelbrunn et al. 2011). In early 2008, Hunter *et al*. identified miRNAs that differentially expressed in circulating plasma microvesicles from diseased and normal subjects, creating the platform for future work for examination of the predictive role of peripheral blood miRNA signatures in human disease, as well as defining the biological processes regulated by particular miRNAs (Hunter et al. 2008). Advances in miRNA array techniques have provided significant advances in miRNA profiling of exosomes. Koh *et al*. demonstrated that miRNAs are also found in the extracellular environment of human embryonic stem cell-derived mesenchymal stem cells (hES-MSC). Significant differences in expression profile of miRNAs in the intracellular and extracellular environment of hES-MSC cultures were identified. Interestingly, the let-7 miRNA family is highly expressed in both intra- and extra-cellular samples of hES-MSC (Koh et al. 2010). It has been well documented that exosomes contain a substantial amount of small RNAs, but little or no ribosomal RNA compared to the levels observed in the donor cells (Valadi et al. 2007). Moreover, selective packaging of miRNAs into exosomes appears to occur, as the miRNA profiles in exosomes do not reflect the miRNA profiles observed in the parental cells (Mittelbrunn et al. 2011; Rabinowits et

al. 2009; Valadi et al. 2007). T cells, B cells, and dendritic cells derived exosomes had miRNA expression profiles unique from their donor cells. Furthermore, the authors also showed that there was antigen-driven unidirectional transfer of miRNAs from the T cell to the APC mediated by $CD63^+$ exosomes (Mittelbrunn et al. 2011). The mechanism for the selective packaging of RNAs into exosomes is still illusive. Alvares-Erviti *et al*. used exosomes of mice dendritic cells as a transfection vector for short interfering RNAs (Alvarez-Erviti et al. 2011). It was supposed that the use of exosomes produced by animal's own cells will lead to the decreased immunogenicity of this vector, here as inclusion into exosomes of a hybrid protein consisting of Lamp2b (a membrane protein of the exosomal fraction) and a neuron specific peptide RVG should result in a directed delivery of RNA into the cells of nervous tissue. The intravenous injection of exosomes electroporated with delivered RNAs resulted in a specific delivery of biologically active RNAs into neurons, microglia cells, and brain oligodendrocytes, but not into other tissues (liver, spleen, kidneys, etc.). The exosome mediated transfection allowed the authors to obtain significant decrease of the levels of the target mRNA and of the protein encoded by it (by 60 and 62%, respectively). In this case the authors showed not only the possibility of using short RNAs inside exosomes as a transfecting system capable of inhibiting the target gene expression in brain tissues (i.e. of penetrating across the blood–brain barrier), but the principle of *in vivo* functioning of exosomes was demonstrated, which is of no less importance (Alvarez-Erviti et al. 2011).

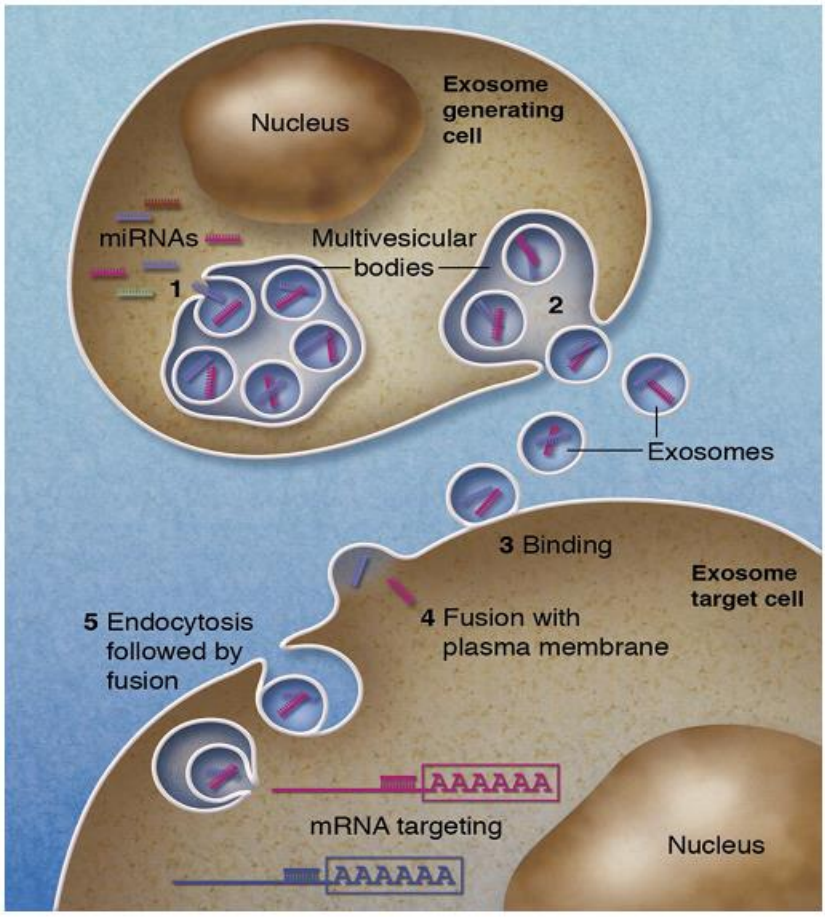


Figure 2.8: Scheme of microRNA transfer by exosomes. (1) microRNAs are selectively incorporated into the intraluminal vesicles of a multivesicular body. (2) Multivesicular bodies fuse with the plasma membrane, therewith secreting their intraluminal vesicles into the extracellular milieu. (3) Exosomes may bind to the plasma membrane of a target cell. Recruited exosomes may either fuse directly with the plasma membrane (4) or first be endocytosed and then fuse with the delimiting membrane of an endocytic compartment. (5) Both pathways result in the delivery of the exosomal microRNA to the cytosol of the target cell where it may associate with and silence corresponding mRNA (Stoorvogel 2012).

Regulation of the immune system can be an important function of released miRNAs. Both T and B lymphocytes and also dendritic cells of the immune system are known to secrete miRNAs as components of exosomes (Mittelbrunn et al. 2011; Naslund et al.

2013). It has also been demonstrated that the interaction of T-lymphocytes with antigen presenting cells may be associated with antigen-induced directed transfer of miRNAs. Exosomal miRNAs are transferred into antigen-presenting cells during formation of the immune synapse with T-lymphocyte, and they can modulate the transcriptional function of the recipient cell (Mittelbrunn et al. 2011). The involvement of exosomes in the transfer of biologically active RNAs from mother to child during pregnancy and breast feeding might represent a unique example of immunological functions of exosomes. By using real time PCR and in situ hybridization the authors demonstrated that specific placental miRNAs were also in exosomes of the villiferous trophoblast (Luo et al. 2009). The exchange of exosomal contents between the fetus and mother's tissues is supposed to provide adaptation of the two organisms to one another during the progression of pregnancy. Breast milk also contains a significant amount of immuno-relevant miRNAs, and at least some of them are inside the exosomes (Luo et al. 2009). These miRNAs might play an important role in development of the child's immune system.

2.8.6 Transfer of infectious agents via exosomes

Exosomes have not only been shown to be involved in the metastasis of cancers but have also been implicated in the spread of infectious agents. Prior to the knowledge about exosomes, HIV particles were demonstrated to accumulate in late endosomes of macrophages (Pelchen-Matthews et al. 2003) and the fact that retroviruses and exosomes had biochemical similarities (Nguyen et al. 2003) led to the "The Trojan exosome hypothesis" (Gould et al. 2003). "The Trojan exosome hypothesis" proposes that retroviruses can hi-jack the exosomal biogenesis pathway for their replication and release, and consequently escape the host's immune system (Gould et al. 2003). Contentiously, despite the similarities between retroviruses and exosomes, differences between the two have been demonstrated that argue against the "The Trojan exosome hypothesis" (Gould et al. 2003). Furthermore, exosomes have been suggested to transfer the infectious prion proteins and β-amyloid peptides between cells (Fevrier et al. 2004; Wakim and Bevan 2011).

2.8.7 Role of exosomal RNA in cell-cell communication

Exosomes have many different functions, which depend on both their cellular origin as well as the current state of that cell, as it influences the cargo of the released exosomes. However, these functions have primarily been associated with the exosomal proteins. The novel finding that exosomes contain RNA (Valadi et al. 2007) has prompted many researchers to address the biological role of exosomal RNA, which further increases the complexity of the understanding of cell-to-cell communication. Valadi *et al.* did not only demonstrate the presence of RNA in exosomes, but also demonstrated that the exosomal RNA could be transferred to recipient cells. This was demonstrated by culturing mouse mast cells with radioactive uridine prior to exosome isolation, thus incorporating radioactive RNA into the exosomes released by these cells. Radioactive RNA could be measured in recipient cells following co-culture with the exosomes, demonstrating RNA transfer from exosome to cell. Since exosomes themselves do not contain the complete machinery to produce proteins, the functionality of the RNA content was shown by using an *in vitro* translation assay, where mouse mast cell-derived exosomes were used as templates. Subsequently, after adding mouse mast cell-derived exosomes to human mast cells, newly produced mouse proteins could be identified in the human cells. These proteins were shown to be present only as mRNA, not as proteins, in the mouse mast cell-derived exosomes. Thus, this translation demonstrated the functionality of the exosomal mRNA (Valadi et al. 2007).

The fact that exosomal mRNA can travel a long distance to recipient cells, subsequently affect the protein production of recipient cells (Valadi et al. 2007), and that it has been well documented that a single miRNA can interfere with 100-200 mRNA (Lim et al. 2005), suggests a potential biological role for exosomal RNA. Keeping this fact in mind, numerous studies have been conducted and published describing RNAs in exosomes from different cellular origin (Gibbings et al. 2009; Kogure et al. 2011; Mittelbrunn et al. 2011; Montecalvo et al. 2012; Palanisamy et al. 2010) and functional delivery of exosomal RNAs from donor cells to recipient cells (Kosaka et al. 2010; Montecalvo et al. 2012; Palanisamy et al. 2010; Pan et al. 2012; Tomasoni et al. 2012) in various research fields including cell biology, reproduction, immunology and cancer. In order to demonstrate that the exosomal RNAs to be functional, exosomes must not only be internalized by the recipient cell, but it is important that the exosomal RNA

must also be transported to the cytosol where the mRNA can be translated and miRNA function as an inhibitor of translation. The functionality of mRNA has been further shown in other studies by the use of luciferase reporter genes. One study conducted by Skog *et al.* revealed that the exosomal mRNA derived from glioblastoma cells could be transferred and subsequently expressed in recipient cells (Skog et al. 2008). The authors demonstrated this by transducing glioblastoma cells with a lentivirus vector encoding a luciferase reporter gene. The resulting exosomes from donor cells contain luciferase activity. These exosomes were then added to endothelial recipient cells, where they can measure the luciferase activity. Furthermore, they also showed that this activity increased over time, indicating an ongoing translation (Skog et al. 2008). A similar study confirmed that exosomes could transfer mRNA coding for a luciferase reporter gene, leading to a luciferase activity in recipient cells. Furthermore, they conclude that this activity was dose dependent (Kogure et al. 2011). Montecalvo *et al.* demonstrated that exosomes can interact with recipient cells by fusion with the cells plasma membrane rather than endocytosis, thus releasing the exosomal material in terms of nucleotide into the cytosol of the recipient cell (Montecalvo et al. 2012). This was demonstrated by capturing luciferin inside the exosomes and co-culturing these with transgenic luciferase recipient cells. As luciferin is not able to cross lipid membranes, the measurement of luciferase activity in recipient cells, only 8 minutes after the cells received exosomes, indicates the delivery of the exosomal luminal cargo, including miRNA and mRNA (Montecalvo et al. 2012).

Messenger RNAs are not the only RNA species that transported via exosomes from donor to recipient cells. Moreover, several studies have demonstrated that microRNAs are not only transported by exosomes to recipient cells, but also they are potentially functional (Montecalvo et al. 2012; Pan et al. 2012; Pegtel et al. 2010). Pegtel *et al.* showed that Epstein-Barr virus (EBV)-transformed B cells release exosomes that contain mature EBV-miRNAs, which can be transferred to recipient cells (Pegtel et al. 2010). They also showed that when incubated with EVB-miRNA containing B cell-derived exosomes, there was an 80% reduction in luciferase activity in recipient cells transfected with a luciferase vector carrying an EBV-miRNA-regulated sequence. However, cells expressing disrupted EBV-miRNA binding sites were shown to have significantly less reduction in luciferase activity. This study suggested that exosomes

can regulate functional gene repression of specificmRNAs targets in recipient cells by the transfer of functional miRNAs (Pegtel et al. 2010). Montecalvo *et al.* also demonstrated the delivery of functional miRNA via exosomes, by transfecting recipient cells with a luciferase reporter gene containing copies of the complementary target sequence for a specific miRNA. When exosomes containing the specific miRNA were incubated with these cells, the result was inhibited luciferase activity in these cells (Montecalvo et al. 2012). The functionality of exosomal miRNA has also been demonstrated with the transfection of cells with lentiviral vector short hairpin RNA (LVsh). Exosomes released from cells transfected with LV-shCD81 resulted in the downregulation of CD81 surface expression in the recipient cells (Pan et al. 2012).

2.8.8 Exosomes in physiological processes

Exosomes can be secreted from different cell types in the extracellular environment where they can interact with other cells resulting in physiological changes (Thery et al. 2001). The entire mechanisms underlying how exosomes interact with the recipient cells have not been properly understood. While some studies showed that exosomes can fuse with the plasma membrane of the target cells leading to the release of the exosome content into the target cell (Parolini et al. 2009; Skog et al. 2008), other studies proposed a binding at the cell surface of the recipient cell *via* specific receptors (Segura et al. 2007) or the internalization by endocytosis or macropinocytosis following fusion with internal compartments (Fitzner et al. 2011; Morelli et al. 2004). Because exosomes are readily released by different cell types, they are likely involved in many physiological processes such as antigen presentation (Thery et al. 2001), tissue repair (Lai et al. 2010) or transfer of RNAs (Valadi et al. 2007). Later Lai *et al.* demonstrated that exosomes are the active components of conditioned medium from human embryonic stem cell-derived mesenchymal stem cells and the injection of these exosomes into swine and murine models of ischemia/reperfusion injury reduced the infarct size (Lai et al. 2010). Because most of the exosome studies were performed *in vitro* culture conditions or from different biological fluids, therefore, it is not perfectly assessed if the quantities of exosomes used to see effects *in vitro* are comparable to physiological amounts of exosomes secreted *in vivo*. Moreover, recently one study demonstrated that there are differences between exosomes derived from *in vitro* tumor

cells and tumor cells grown *in vivo* (Xiang et al. 2010). The remarkable finding of the presence of exosomes in biological fluids and their purification from these body fluids emphasizes their *in vivo* secretion and their physiological relevance. A study in human tonsils provides more compelling evidence for the *in vivo* existence of exosomes (Denzer et al. 2000). Using electron microscopy, exosomes bearing MHC class II antigen and tetraspan molecules were identified in tonsil germinal centers where they attach to the surface of follicular dendritic cells (Denzer et al. 2000). Thus, if exosomes have a physiological function *in vivo* remains to be answered.

2.8.9 Exosomes in pathological processes

Exosomes have been associated with the progression of disease conditions including neurodegenerative disease, cardiovascular diseases and cancer (Thery 2011). It was in 1983 when Tailor revealed for the first time the secretion of tumor-derived microvesicles in ovarian cancer patients (Taylor et al. 1983). Exosomes from cancer cells can transfer tumor antigens to dendritic cells and present tumor antigens, which induce immune responses (Thery 2011). In contrast, tumor exosomes can also suppress the function of immune cells by inducing apoptosis of activated cytotoxic T cells or promoting differentiation of regulatory T lymphocytes (Clayton et al. 2007). Other reports suggest that tumor exosomes can promote tumor growth by suppressing antitumor immune responses or by promoting neoangiogenesis or migration to other cells resulting in metastasis (Valadi et al. 2007). A recent study revealed that exosomes/microvesicles secreted from tumor-associated macrophages can transport miRNAs into breast cancer cells promoting the invasiveness of breast cancer cells (Yang et al. 2011). Further, exosomes were found in the serum of cancer patients displaying the characteristic surface molecules of tumors (Huber et al. 2005). Increased amount of exosomes correlates with advanced disease conditions (Bergmann et al. 2009). Consistently, studies in patients with ovarian cancer and lung cancer revealed that the plasma-exosome levels were increased in patients with advanced disease (2.85 mg/ml exosomes for lung cancer patients *vs.* 0.77 mg/ml exosomes for healthy patients) (Simpson et al. 2009). This suggests that tumor-derived exosomes can be used as diagnostic biomarkers of a disease. However, if this increase in exosomes is the cause of tumor size or the promotion of tumor growth is not clear.

Another report published by Stefanie Dimmler's lab revealed a new role of microvesicles/exosomes in cell–cell communication during atherosclerotic protection (Hergenreider et al. 2012). Smooth muscle cells (SMCs) are the key players of atherosclerosis, which is caused by the formation of a plaque within an artery leading to myocardial infarction. The study demonstrated that endothelial and smooth muscle cells communicate to each other *via* microvesicles/exosomes (Hergenreider et al. 2012). Endothelial cells release microvesicles enriched with miR-143/145, which are transferred to smooth muscle cells and regulate gene expression of miRNA target genes. The injection of miR-143/145 containing microvesicles in a mouse model of atherosclerosis resulted in a reduction of atherosclerotic lesion formation (Hergenreider et al. 2012). This further suggests a potential use of exosomes containing miRNAs as therapeutic tool to treat diverse pathological conditions.

3. Material and methods

3.1 Materials

3.1.1 List of chemicals and kits

During this experiment, various chemicals, kits and culture media purchased from different manufacturers were used. Besides, during data analysis multifarious software packages, tools and databases were utilized.

Chemicals	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
Coomassie Brilliant Blue	
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
Sequagel XR sequencing gel	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 recommended.

Name of the buffer/media	Constituents	Volume / amount
Physiological saline solution	Sodium chloride	9.0 g
(NaCl 9%)	added to water	1000.0 ml
Manipulation medium	TCM-199	1500.0 g
	Gentamycine sulfate	0.050 g
	Sodium pyruvate	0.022 g

	$NaHCO_3$	0.350 g
	BSA	1.00 g
	Water up to	1000.0 ml
70% Ethanol	Ethanol (100%)	700 ml
	Water	300 ml
10x CMF (Calcium manesium free PBS)	Sodium chloride (NaCl)	8 g
	Potasium chloride (KCl)	0.2 g
	Sodium hydrogen phosphate (Na_2HPO_4)	1.15
	Potasium hydrogen phosphate (KH_2PO_4)	0.2 g
	Water up to	1000 ml
10x PBS	NaCl	8.77 g
	Na_2HPO4	1.50 g
	NaH_2PO4	2.04 g
	Water up 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
	ddH_2O added to	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml

	ddH_2O added to	25 ml
BCB stock solution	BSA	0.2 g
	BCB	0.005 g
	PBS	Up to 50 ml
BCB working solution	BCB stock solution	1 ml
	3% BSA in PBS	14 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	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
3M Sodium acetate (pH 5.2)	Sodium Acetate	123.1 g
	Water added to	500.0 ml
4 % paraformaldehyde	Paraformaldehyde	4.0 g
	1X PBS	100.0 ml
Guanidin-ethanol solution (50 ml)	Guanidin	1.43 g
	95% Ethanol up to	50 ml
Proteinase K solution	Proteinase K	20 mg
	DEPC-treated H_2O	1 ml

Modified parker medium	HEPES	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Gentamicin	500 µl
	Medium 199	99 ml
	Hemi calcium lactate	0.06 g
	added to water	110 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
	dd water	1.65 ml
	APS (10%)	25 µl
	TEMED	5 µl
1x Blotting buffer (500 ml)	10x blotting buffer	50 ml
	Methanol	100 ml
	dd Water	350 ml
Fixation solution	Methanol	500 ml
	Glacial acetic acid	100 ml
	Water up to	1000 ml
Coomassie staining solution	Coomassie Blue R-250	2 ml
	Methanol	500 ml
	Glacial acetic acid	100 ml
	Water up to	1000 ml
Stacking solution	Rotephorese gel	0.725 ml

	Sammel gel buffer	0.5 ml
	dd water	2.825 ml
	APS (10%)	20 µl
	TEMED	5 µl
Stripping solution	Glycin	15 g
	SDS	1 g
	Tween 20	10 ml
	dd water up to	1000 ml
Urea-DTT solution	Urea	3 g
	Water	3 ml
	Stock DTT	250 µl

3.1.3 Equipments

Equipment	Manufacturer
ABI PRISM® 7000 SDS	Applied Bio Systems, CA, U.S.A.
ABI StepOnePlus™ PCR system	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

Programs (soft wares) and statistical packages	Source
BLAST program	http://www.ncbi.nlm.nih.gov/Blast/
DAVID Bioinformatic Resource 6.7	http://david.abcc.ncifcrf.gov/
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	Ingenuity® Systems, www.ingenuity.com

MiRBase v.16	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/pcr/arrayanalysis.php
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 layout

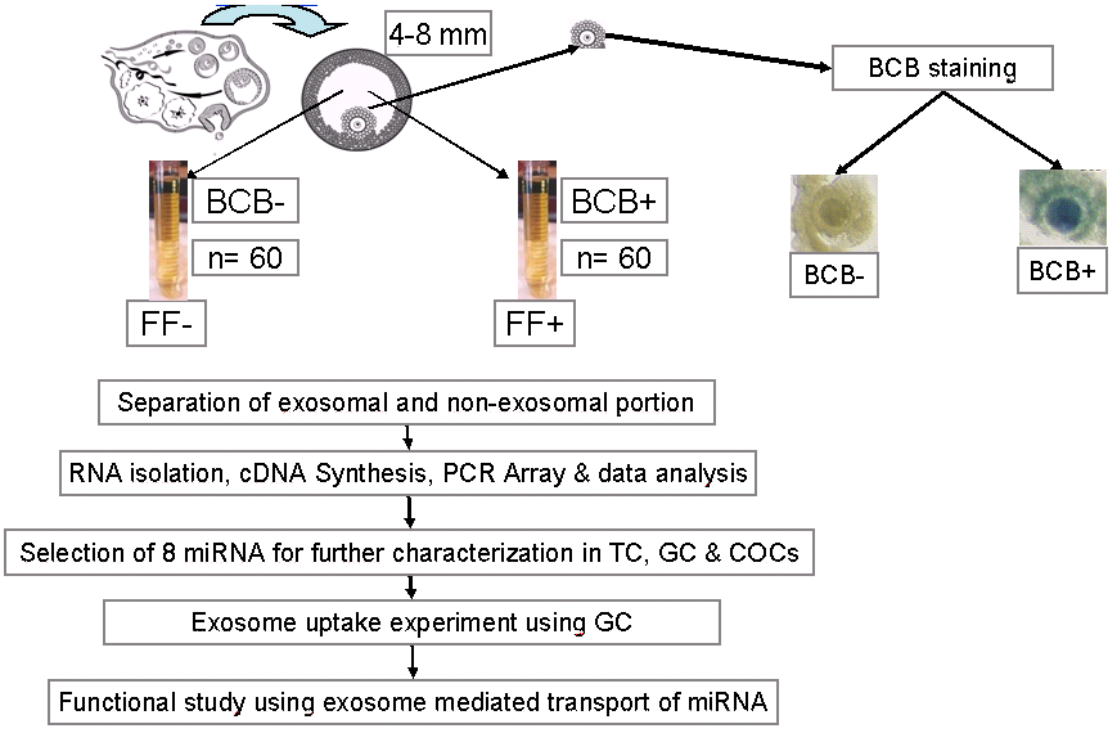


Figure 3.1 Brief overview of the present study

Brilliant cresyl blue (BCB), growing oocyte (BCB-), fully grown oocyte (BCB+), follicular fluid (FF), number of follicle (n), theca cells (TC), granulosa cells (GC) and cumulus oocyte complex (COCs).

3.2.2 Collection of follicular fluid and follicular cells

Ovaries were obtained from local slaughterhouse (Bernhard Frenken GmbH Vieh- und Fleischhandle Schlachthof Düren, Paradiesstrasse 19, 52349 Düren, Germany) after official permission by the slaughterhouse management to use ovaries for research purpose. Ovaries were transported in 0.9% saline solution at a temperature of 38°C in thermo flask within 2 hours after collection. Following washing with new saline solution, antral follicles of 4-8 mm in diameter were blunt-dissected from the ovaries

with scissors and forceps. A caliper was used to measure the diameter of the follicles. Follicular materials from 120 follicles were harvested individually by breaking onto the plastic sterile culture dish. The cumulus oocyte complex (COCs), retrieved from each follicle, were subjected to brilliant cresyl blue (BCB) to categorize as a fully grown oocyte (BCB+) or growing oocyte (BCB-) as described in next paragraph. The corresponding follicular fluid, granulosa cells and theca cells were collected and stored separately for further analysis. Follicular fluid, containing granulosa cells, was placed in a sterile microcentrifuge tube and centrifuge at 500×*g* for 3 minutes. While follicular fluid was collected as supernatant, the granulosa cells were collected as pellet from the bottom of the tube. Follicular fluid was snap frozen and stored at -80°C for further use. After two additional washes in DPBS (Gibco, Life Technologies, UK) containing 0.02% polyvinyl alcohol (PVA), granulosa cells were also stored at -80°C. The theca cell layer of the follicles was peeled carefully and scraped off with a plastic inoculation loop, washed several times for complete removal of granulosa cells, and stored at -20ºC in RNA later (Sigma). Following BCB staining, the COCs were stored at -80°C separately for further use.

3.2.3 Brilliant cresyl blue (BCB) staining of cumulus oocyte complexes

To investigate the expression of miRNAs follicular fluid containing oocytes of different competence, the COCs were retrieved from individual follicles and washed in mDPBS. Following washing COCs subjected to stain with 26 μM BCB (Sigma- Aldrich Chemie GmbH, Munich, Germany) diluted in mDPBS for 90 min at 38.5°C in humidified air atmosphere (Alm et al. 2005). After washing, the stained COCs were examined under stereo microscope and categorized into two groups according to oocyte cytoplasm coloration, which is dependent on the glucose 6 phosphate dehydrogenase (G6PDH) activity of the oocytes. Fully grown oocytes (competent oocyte) are known to have lower G6PDH activity, this decreased activity indicated that the oocyte achieved developmental competence, thereby the oocyte cytoplasm remain stained blue (BCB+), while growing oocytes (non competent oocyte) have a higher G6PDH activity which changes the oocyte cytoplasm to colorless (BCB-). Following this, the COCs were washed two times in PBS and were snap-frozen separately in cryo-tubes containing lysis buffer and stored at -80°C until further analysis.

3.2.4 Isolation of exosomes and non-exososmal fractions of follicular fluid

Exosomes were isolated from follicular fluid using two different methods: Exoquick precipitation and differential ultracentrifugation.

3.2.4.1 Isolation of exosomes using Exoquick™

Exosomes were isolated using Exoquick kit (SBI System Biosciences, Inc.) according to the manufacture's protocol with some modifications. 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 25,000×*g* for 30 minutes to remove further microparticles, and the microvesicles fraction remained in samples after filtration. All centrifugation steps were performed at 4°C. Cell free and microvesicle depleted follicular fluid samples were used for exosome precipitation with Exoquick. Exoquick reagent (120 µl) was added to 500 µl of follicular fluid, incubated 12 h at 4°C, and centrifuged at 1500 × *g* for 30 min to obtain pelleted exosomes. The supernatant (non-exosomal portion) of the samples were collected without disturbing the exosome pellets, and exosome pellets were resuspended in 200 µl of DPBS. Both the exosomes and exosome-depleted supernatant were further used for total RNA isolation and miRNA profiling.

3.2.4.2 Isolation of exosomes using ultracentrifugation

Exosome isolation from independent follicular fluid samples was also performed by ultracentrifugation using previously described methods (Arroyo et al. 2011; 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 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 uptake experiment.

3.2.5 Electron microscopy and visualization of exosomes

Morphological evaluation of exosomes isolated using Exoquick kit and ultracentrifugation was performed by visualizing them using electron microscope (Leo 922, Zeiss, Germany). For this, 15 µl drops of isolated exosomes in DPBS were placed on parafilm before the Formvar/carbon-coated grids were placed on top of exosome drops and allowed to stand for 5–10 min to absorb the exosomes. The grids with adherent exosomes were transferred to three 30 µl drops of DPBS for washing and subsequently fixed with 2% paraformaldehyde in DPBS for 7 min. Then the grids were incubated with 25 µl drops of 2% uranyl acetate followed by examination with electron microscope.

3.2.6 Protein isolation and western blot analysis

Exosomal and non exosomal proteins were isolated from organic phenol portion during total RNA isolation using Qiagen miRNeasy mini kit following optimized protocol and resuspended in 8M urea and used for protein detection and western blot analysis.

3.2.6.1 Isolation of protein

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 protein frection was transfered to a new cap lock tube and 1.5 ml of isopropanol was added and incubated for 10 min at room temperature. Samples were centrifuged at 12,000 × g for 10 min at 4°C, 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. After that, samples were centrifuged at 7500 × g for 5

min. This washing step with 100% ethanol was repeated twice. Following final centrifugation and removal of supernatant the pellets were air dried for 5-10 min. Then first 50 µl of urea-DTT solution was added to break up the pellet using a needle. After that 450 µl of urea-DTT solution was added and incubated at room temperature for 1 h. Following this, the samples were incubated at 95°C for 3 min. and then placed on ice. During the incubation period on ice samples were sonicated 10 times using short bursts to allow all proteins mixed properly in the solution. The solution containing protein was transferred into new tube and centrifuged at 10,000 × g for 10 min at room temperature. Then 500 µl of urea-DTT solution was added and incubate at 95°C for 3 min. The solution containing protein was transferred into 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 ND-1000 spectrophotometer.

3.2.6.2 Coomassie blue staining

Fifty µg of protein from each sample were loaded and resolved in 12% SDS-PAGE polyacrylamide gels (Bio-Rad, Corp., Hercules, CA, USA) at 120 V for 1 hr. Following this, the gel was incubated in fixation solution for over night with gentle agitation. After that the gel was stained with Comassie blue R-250 for 1 hr at room temperature. The gel was then washed with destaining solution several times until the background become clear and the stained proteins image were captured with a Chemidoc XRS (Bio-Rad) instrument.

3.2.6.3 Western blot analysis

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 were 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), EIF2C2 (0.4 µg/ml; Santa Cruz Biotechnology Inc, USA) and cytochrome C (CYCS; 0.4 µg/ml; Santa Cruz Biotechnology Inc, Texas, USA) overnight at 4°C. After subsequent washing, membranes preincubated with the respective primary antibody were further incubated with a horseradish peroxidase conjugated anti-goat,

anti-rabbit and anti-mouse secondary antibody (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.

3.2.7 Total RNA isolation

Total RNA (including miRNA) was isolated from exosome preparations, exosome-depleted portion of follicular fluid and surrounding follicular cells using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modification adopted for liquid samples.

3.2.7.1 Total RNA isolation from exosomes

Briefly, exosome pellets obtained from Exoquick^{TM} precipitation or ultracentrifugation were diluted in 200 µl of RNase free water. 4 × volumes (800 µl) of Qiazol lysis buffer was added to diluted exosomes (200 µl) and exosomes depleted portion (200 µl) of follicular fluid. To ensure complete digestion vortexed for 2-3 min and incubated for 6-7 min. After that, 200 µl of chloroform was added and shacked vigorously for 1 min and incubated for 5 min. The samples were then centrifuged at 12,000 × g for 15 min. This will allow the phase separation and after that clear aqueous phase containing total RNA was transferred to new 2 ml microcentrifuge tube. After retrieving the clear aqueous phase, 1.5 volume of 100% ethanol was added and mixed gently by pipetting. After mixing, 700 µl of sample were transferred to miRNeasy spin column and centrifuged at 10,000 × g for 15 sec. The supernatant was discarded. Since the sample volume was exceeding 700 µl, remaining samples were centrifuged in the same column. Following this, 700 µl of RWT buffer was added to the spin column and centrifuged at 10,000 × g for 15 sec at room temperature. After removing the flow-through, 500 µl of buffer RPE was added to each spin column and centrifuged at 10,000 × g for 15 sec at room temperature. This step was repeated twice with the same centrifugation speed for 2 min. The spin column was transferred to a new 2 ml collection tube (supplied by company) and centrifuged at full speed for 2 min to eliminate any possible carryover of buffer RPE and residual flow-through that remained on the spin column. Following this, the spin column was transferred to a new 1.5 µl collection tube (supplied by company) and

open the lid for 1 min which allow the evaporation of residual ethanol portion from the spin column. Total RNA was eluted by adding 30 µl of RNase free water to the membrane of the spin column and incubated for 1 min before centrifugation at 12,000 × g for 1 min at room temperature. The RNA was stored at 80°C.

3.2.7.2 Total RNA isolation from non-exosomal portion of follicular fluid

Total RNA was isolated from exosome depleted follicular fluid using miRNeasy mini kit (Qiagen, Hilden, Germany) according to the protocol adopted for serum or plasma with some modification. Briefly, 5 volume of Qiazol lysis buffer was added to the follicular fluid, vortexed for 3-4 min and incubated for 8 min at room temperature. Following incubation, 200 µl of chloroform was added to the sample, vigorously shacked for 1 min and incubated for 5 min. After that, same protocol was followed which was used for total RNA isolation from exosomes, total RNA was eluted by adding 30 µl of RNase free water and stored at -80°C for further analysis.

3.2.7.3 Total RNA isolation from surrounding follicular cells

Total RNA (including miRNA) was isolated from theca cells, granulosa cells and cumulus oocyte complexes using the miRNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Briefly, 700 µl of Qiazol lysis buffer was added to cells and vortex until complete digestion and incubate for 5 min. To ensure complete digestion vortexed for 2-3 min and incubated for 6-7 min. After that, 200 µl of chloroform was added and shacked vigorously for 1 min and incubated for 5 min. The samples were then centrifuged at 12,000 × g for 15 min. This will allow the phase separation and after that clear aqueous phase containing total RNA was transferred to new 2 ml microcentrifuge tube. After retrieving the clear aqueous phase, 1.5 volume of 100% ethanol was added and mixed gently by pipetting. After mixing, 700 µl of sample were transferred to miRNeasy spin column and centrifuged at 8,000 × g for 15 sec. The supernatant was discarded. Since the sample volume was exceeding 700 µl, remaining samples were centrifuged in the same column. Following this, 350 µl of RWT buffer was added to the spin column and centrifuged at 8,000 × g for 15 sec at room temperature. After that 80 µl (10 µl of DNase I + 70 µl of buffer RDD) of diluted DNase I solution was added to the RNeasy mini spin column membrane and incubated for 15

sec at room temperature on the benchtop. Following incubation 350 µl of buffer RWT was added to the spin column and centrifuged at 8,000 × g for 15 sec at room temperature. After removing the flow-through, 500 µl of buffer RPE was added to each spin column and centrifuged at 8,000 × g for 15 sec at room temperature. This step was repeated a second time. The spin column was then transferred to a new 2 ml collection tube (supplied by company) and centrifuged at full speed for 1 min to eliminate any possible carryover of buffer RPE and residual flow-through that remained on the spin column. Following this the spin column was transferred to a new 1.5 µl collection tube (supplied by company) and open the lid for 1 min that allowed the evaporation of residual ethanol portion from the spin column. Total RNA was eluted by adding 50 µl of RNase free water to the membrane of the spin column and incubating for 1 min before centrifugation at 12,000 × g for 1 min at room temperature. Total RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer.

3.2.8 Quality control of isolated RNA

Before committing RNA samples to profiling on microRNA qPCR panels, it is very important to ascertaining the purification yield and absence of PCR inhibitors. This was done by testing different RNA sample input volumes 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-103 and miR-191. The relative quantification of miRNAs expression was calculated using a comparative threshold cycle (Ct) method. Samples containing PCR inhibitors will show dilution curves without the expected linear relationship between sample input and signal. Subsequently samples with low RNA quality were excluded from further studies.

3.2.9 First strand cDNA synthesis

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 iindicated in the Table 3.1. RT master mix was dispended into nuclease free tube and then total RNA template was added to each tube and mixed by gentle vortexing or pipetting to ensure all reagents were thoroughly mixed. 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

3.2.10 miRNA profiling

The expression of miRNAs in exosomal and non-exosomal portion of follicular fluid derived from follicles with growing (BCB-) or fully grown (BCB+) oocyte was examined using Exiqon microRNA PCR Human Panels (I + II) and quantified using SYBR green based real time PCR technology. Subsequently, the resulting cDNA products were mixed with nuclease free water and ready to use SYBR-green mix. Briefly, 2x SYBR Green master mix (Exiqon, Vedbaek, Denmark) was placed on ice for 15-20 min for melting and during that time the vial contining SYBR Green was

protected from light by wrapping with aluminum foil. After that SYBR Green master mix was pipetted up and down for proper mixing. Following this, 4000 µl of SYBR Green master mix was pipetted and 3960 µl of water was added and mixed by gentle vortexing. At this point, 40 µl of cDNA was added to the mixed cocktail, mixed properly by gentle vortexing and centrifuged at 1000 × g for 1min. Before removing the plate seal, the plates were briefly centrifuged in a plate centrifuge machine. Then a multichannel laboratory automation workstation was employed to distribute mixed cocktail (Biomek® NXP, Beckman Coulter, Krefeld, Germany) on 384 well PCR plate containing miRNA specific primers. The real time PCR was run on a 7900HT thermocycler (ABI) using the following thermal-cycling parameters: 95°C for 10 min, 40 cycle of 95°C for 10 sec, 60°C for 1 min followed by a melting curve analysis.

3.2.11 miRNA expression analysis

Raw Ct values were calculated as recommended by Exiqon using the RQ manager software v1.2.1 (ABI) with manual settings for threshold and baseline, i.e. all miRCURY assays were analyzed using a ΔRn threshold of 60 and baseline subtraction using cycles 1-14. The PCR data were analyzed using web-based PCR array data analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php-). The raw miRNA data was first normalized using a global normalization method. To calculate the global normalization value, miRNAs with CT value range from 22-32 were taken under consideration. To minimize the potential noise introduced by measurements below detection threshold, miRNAs with Ct value less than 35 in all groups were considered as undetected. Based on their enrichment in exosomal and non-exosomal portion of follicular fluid from fully grown (BCB+) or growing (BCB-) oocytes, 8 candidate miRNAs were selected for further characterization of their expression in surrounding follicular cells. For this individual, miRNA specific LNA-TM primer assays were used to investigate their expression in triplicate cDNA samples obtained from the surrounding follicular cells from the contemporary oocyte competence group. Relative expression of each miRNA was analyzed using a comparative CT ($2^{-\Delta\Delta CT}$) method and global normalization strategy was employed to normalize the data.

3.2.12 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 Indivudual assay primer set for miR-654-5p, miR-640, miR-526b*, miR-373, miR-19b-1*, miR-29c, miR-381, miR-30e* and U6 was used as endogenous control. Complementary DNA was synthesized from total RNA of granulosa cells, theca cells and COCs 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 stability of the U6 expression was checked (Figure 3.2) before it was utilized for data normalization. The relative quantification of miRNAs expression was calculated using a comparative threshold cycle (Ct) method as described before in previous study (Tesfaye et al. 2009).

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

ΔCt = average Ct target miRNA - average Ct of normalizer

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

Calibrator was the one with the highest ΔCt value among the groups

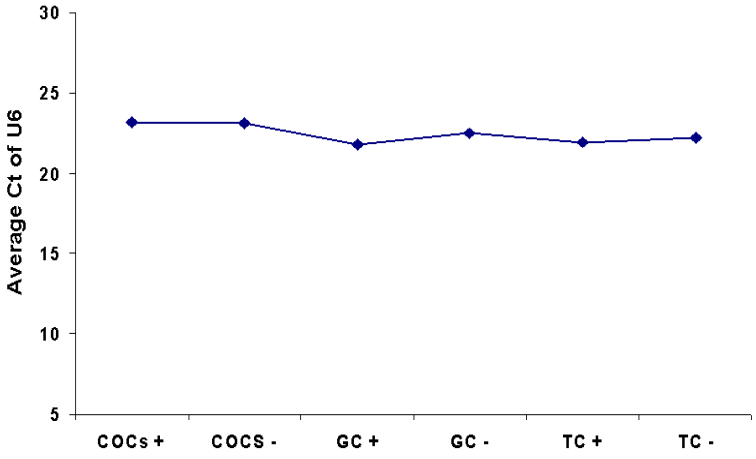


Figure 3.2: The average threshold cycle (Ct) value of endogenous control U6 RNA in different cell samples.

3.2.13 miRNA target prediction

To predict miRNA targets, we used miRecords at http://mirecords.biolead.org/, which contains animal miRNA targets according to combinations of the widely used target prediction programs DIANA MicroTest, Micro Inspector, mirTarget, miRDB, miRanda, TargetScanS, PicTar and experimentally supported targets from TarBase. For miRNAs annotated with *, as their target genes are not present in miRecords database, therefore we used miRDB to predict the target genes list for those miRNAs. To identify biological processes most involved in the biological phenomena under study we have performed a Gene Ontology (GO) analysis using the DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/). The predicted miRNA target genes were also analyzed by using the DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/) server for Annotation, Visualization, and Integrated Discovery to identify the pathway distribution. These pathways were presented according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/ kegg/).

3.2.14 Stability of exosomal miRNA under in vitro culture conditions

To check the stability of exosomes under culture conditions, follicular fluid samples were collected from random slaughterhouse ovaries and exosomes were isolated by ultracentrifugation according to previously mentioned protocol (section 3.2.4.2). After two additional washes in DPBS exosomes were resuspended in 800 µl of F-12 media containing 10% exosomes-free fetal bovine serum (FBS, Invitrogen, South America origin**)**. Exosomes were pre-cleared from the FBS via filtration using a 0.22 µm filter (Millipore) and ultracentrifugation at 120,000 *g* for 2 hours. The samples were incubated at 37ºC in humidified atmosphere of 5% CO2 for 0 hr, 6 hrs, 12 hrs and 24 hrs. After incubation total RNA was isolated using miRNeasy mini kit (Qiagen, Hilden, Germany) and individual miRNA expression was measured as described above and miRNAs used in this experiment were miR-654-5p, miR-640, miR-526b-1* & miR-373. Exosomes at 0 hr time point was used as control group.

3.2.15 Exosome labeling

Follicular fluid was collected from random slaughter house. Exosomes isolated from follicular fluid using ultracentrifugation were subjected to fluorescent labeling using PKH67 dye (Sigma-Aldrich), which is a green fluorescent dye that labels the lipid membranes, according to the manufacturer's instructions. Briefly, exosomes in DPBS were resuspended in 1 ml of Diluent C (Sigma-Aldrich, catalog number G8278) by gentle pipetting for complete dispersion to prepare "2x exosome suspension". "2x dye solution" was prepared by adding 4 µl of PKH67 ethanolic dye solution (Sigma-Aldrich, catalog number P7333) to 1 ml of Diluent C in a polypropylene centrifuge tube and mixed well by pipetting to disperse. To minimize the ethanol effects on exosomes, the volume of dye added in this step should not exceed 1-2% ethanol in the final mix. Following this, 1 ml of 2x exosome suspension was rapidly added to 1 ml of 2x Dye solution and immediately mixed by pipetting. This solution was incubated for 3-5 min with periodic mixing. Labeling was stopped by addition of an equal volume (2 ml) of exosome-free FBS and 1 min incubation, followed by the addition of F12 media with 10% exosome-free FBS to fill up the centrifuge tube and subsequently ultracentrifugation for 30 min at 120,000×*g* was performed. After two additional washes in F12 media (with 10% exosome-free FBS) using ultracentrifugation, the exosomes were resuspended in 100 µl of F12 media with 10% exosome-free FBS.

3.2.16 Co-incubation of bovine granulosa cells with labeled exosomes

Primary cultures of bovine granulosa cells were established as previously described (Spanel-Borowski et al. 1994) to undertake labeled exosomes uptake experiment. Briefly, ovaries were collected from slaughter house randomly and transported to the laboratory within 2-3 hrs of collection in a thermoflask containing physiological solution at 38°C. In the laboratory ovaries were then washed two times in 0.9% NaCl solution. Following washing steps granulosa cells were isolated from bovine follicles (4-8 mm) by aspirating with 5 ml syringe attached to 18 gauge needle. The aspirated follicular fluid was collected in a 50 ml sterilized tube and allowed to precipitate for 15 min. Following this, supernatant containing granulosa cells were transferred to new sterilized 50 ml tube and centrifuged at 500×*g* for 5 minutes. After that granulosa cell pellet were washed once in calcium-magnesium free PBS (CMF) followed by

centrifugation at 500×*g* for 5 minutes and resuspended in 2 ml of RBC lysis buffer for 3-5 minutes to remove erythrocytes. Osmality was restored by adding 8 ml of F-12 media containing 10% exosome free FBS. After two additional washes with media cell viability was determined by a trypan blue exclusion test. Granulosa cells (approximately 45000 viable cells) were seeded on the chamber slide (Lab-Tek^{TM}, Thermo Scientific, USA) and cultured for 24 hours in DMEM/F-12 media supplemented with 10% exosomes free FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO_2. On the next day, the cells were washed two times with DMEM/F-12 media to remove dead cells and DMEM/F-12 medium+10% exosome-free FBS was supplemented with labeled exosomes and co-cultured with the cells for 24 hrs. After 24 hours of co-culture with labeled exosomes, granulosa cells were washed three times with DPBS and fixed with 4% paraformaldehyde and mounted in mounting medium containing DAPI and observed under a laser scanning confocal microscope (LSM710-Carl Zeiss). These experiments were performed three times, and a parallel negative control was run each time in which cells were co-incubated with sterile PBS labeled with fluorescent dye.

3.2.17 Exosome mediated transportation of miRNAs in granulosa cells *in vitro*

Once the uptake of labeled exosomes by granulosa cells was confirmed, exosomes were isolated from independent follicular fluid from follicle containing BCB+ or BCB- oocytes according to the previously mentioned protocol. After confirming the candidate miRNA enrichment in each group of exosomal samples, a portion of exosomes from each group was co-incubated with granulosa cells. For this granulosa cells were cultured in 24 well culture plate and 1×10^5 viable cells were seeded to each well. After 24 hrs of co-culture with exosomes, the granulosa cells were washed three times in DPBS, collected using 200 µl of trypsin and snap frozen in lysis buffer before using for RNA isolation. The same volume of DPBS added to culture media was used as negative control.

3.2.18 Quantitative analysis of selected target genes after exosome transfection

To end up with limited number candidate genes for our analysis, we have refined the selection criteria based on the sequence compelementarity between 5′ “seed” region of

miRNAs and the 3'UTR of mRNAs of the potential target genes(Wang et al. 2009b). , As in animals, as little as 6 bp match with the target mRNA can be sufficient to suppress gene expression (Brennecke et al. 2005; Lewis et al. 2003), we searched for target genes with 3'UTR target sites for a 7-mers seed match (position 2–8) with the seed region of the respective miRNA. Based on this criteria and their potential involvement in critical pathways for follicular development, a total of seven target genes (ITGA3, MAP3K1, SOCS4, BRMS1L, ZNFX1, CD44 and VEGFA) were selected for quantitative real time PCR analysis after exosome mediated transfer of miRNAs in cultured granulosa cells. The respective primers were designed by using FASTA product of the GenBank mRNA sequences for *Bos taurus* using Primer3 program (Table 3.2). A portion of total RNA (used for miRNA abundance study) isolated from exosome transfected granulosa cells was used for cDNA synthesis performed as described elsewhere (Tesfaye et al. 2009). The corresponding cDNA samples were used to quantify all the target genes and GAPDH as endogenous control (Gad et al. 2011) in ABI PRISM® 7000 sequence detection system instrument (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was set up using a 2 µl first-strand cDNA template, 7.4 µl deionized H_2O, 0.3 µM of forward and reverse gene specific primers and 10 µl 1× Power SYBR Green I (Bio-Rad) master mix with ROX as a reference dye. The thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Relative expression of each mRNA was analyzed using a comparative CT ($2^{-\Delta\Delta CT}$) method.

Table 3.2: Details of primers used for quantitative real-time PCR analysis of selected target genes.

Gene	Accession number	Primer sequences (5' -> 3')	Annealing temperature (°C)	Product size (bp)
GAPDH	NM_001034034	F:CCAGGGCTGCTTTTAATTCT R:ATGGCCTTTCCATTGATGAC	60	247
CD44	NM_174013	F: CTGAAATGAGGGCCCAGTTA R: CCAACCCCACTTGAAAGAAA	57	236
BRMS1L	NM_001083428	F: GCCATCTCTCCAGTTCTGCT	59	211

		R: AAGTGGGACCCAACTCAGTG		
ZNFX1	NM_001205716	F: GTCAGCCAGGAGCGACTTAC R: GCTGAAGCTCAAACGCTTCT	58	189
ITGA3	XM_003587418	F: CAAGTCTGAGGGCCAGAAAC R: CTCCTTCACCACCAGGAATC	59	265
MAP3K1	NM_001205906	F: ACTGGCCAGCATTTCAGTAG R: TGTGTTTGAGGAGATGCAGA	56	217
SOCS4	NM_001076218	F: CATTCTTCAGGGCTTCCATC R: TGGTTATGACACAGGGCTGA	57	244

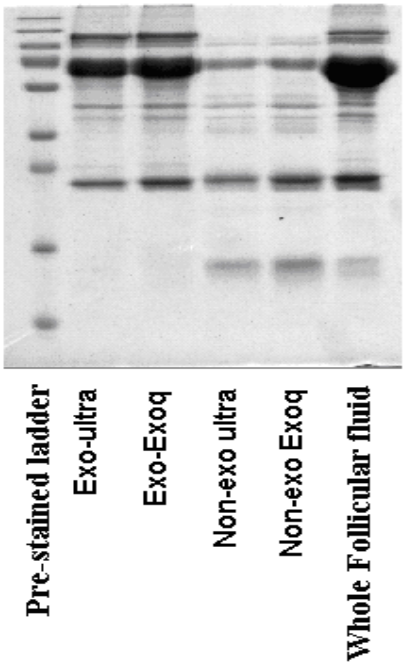
3.2.19 Statistical analysis

All graphs were made and statistical analyses performed using GraphPad Prism, or Microsoft Excel 2010. All experiments were performed with a minimum of three replicates. When two groups were compared (i.e., BCB- vs. BCB+) the Student's *t*-test was used to detect differences between treatment groups. A P value of ≤ 0.05 was considered to be significant. Data are expressed as mean ± SD of three biological replicates.

4 Results

4.1 Efficient recovery of exosomes from bovine follicular fluid

Exosomes were isolated from follicular fluid using two different methods, namely chemical precipitation (using Exoquick^{TM} Exosome precipitation kit) and mechanical separation (using differential ultracentrifugation). In order to determine the specificity of exosome isolation procedures, it was characterized at the protein level by detecting the presence of CD63, a membrane protein, which is abundantly present in exosomes derived from variety of cellular origin and absent in non-exosomal fraction of follicular fluid. Furthermore, exosomes derived from follicular fluid were morphologically characterized by analyzing the size and shape using electron microscope. However, for the non-exosomal fraction of follicular fluid the presence of Ago2 protein, abundantly present in non-exosomal fraction, was used to characterize the isolation specificity. On the other hand, in order to demonstrate that the samples were free of contamination of protein from cellular origin we checked the absence of cellular cytochrome C (CYCS), which is exclucively present in mitochondria.



Exo-ultra = Exosomes derived from ultracentrifugation

Exo-Exoq = Exosomes derived from Exoquick precipitation

Non-exo ultra = Non-exosomal from ultracentrifugation

Non-exo Exoq = Non-exosomal from Exoquick precipitation

Figure 4.1: Coommassie blue staining of proteins from different fraction of follicular fluid.

In order to check the specificity of exosome isolation, first we confirmed the abundance of protein in isolated protein samples from both exosomes and non-exosomal fraction of follicular fluid. Coomassie brilliant blue staining of SDS-PAGE gel revealed the presence of wide range of detectable proteins in both fractions of the follicular fluid (Figure 4.1).

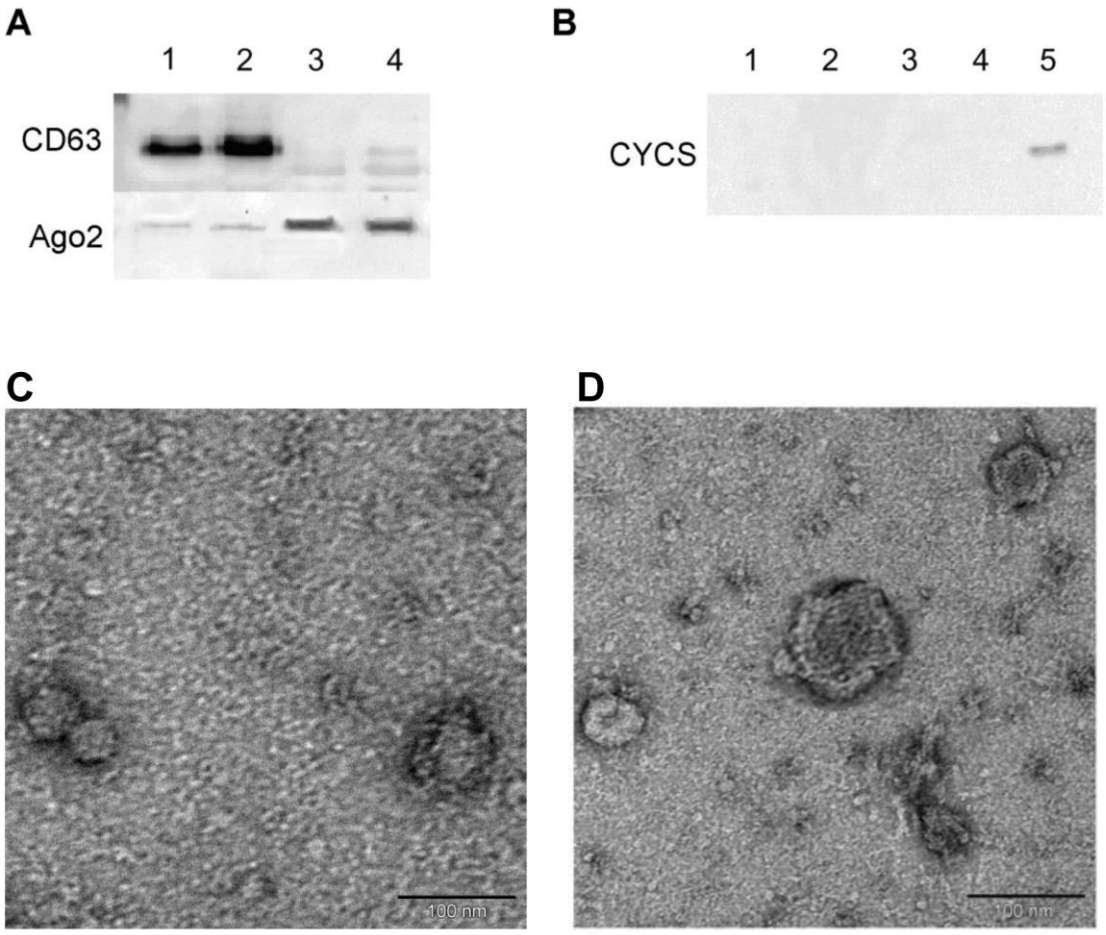


Figure 4.2: **(A)** Proteins (50 µg) from exosomal fraction (lane 1 & 2) and non exosomal fraction (lane 3 & 4) of follicular fluid isolated either by ultracentrifugation (lane 1 & 3) or Exoquick kit (lane 2 & 4) were resolved on a 12% SDS-PAGE gel to detect CD63 and Ago2 proteins. (**B)** Confirmation of absence of detection of CYCS (mitochondrial protein) in both exosomal and non exosomal proteins as isolated by ultracentrifugation (lane 1 & 3) and Exoquick kit (lane 2 & 4). A known cellular origin protein was used as a positive control (lane 5). Morphological characterization of exosomes isolated from bovine follicular fluid

using Exoquick kit (C) and ultracentrifugation (D) by transmission electron microscopy. The scale bar indicates 100 nm.

As shown in Figure 4.2 A, CD63 protein was detected in the exosomal fraction of follicular fluid derived from both isolation procedures, either by ultracentrifugation (lane 1) or Exoquick precipitation (lane 2). Efficient recovery of exosomes by both methods was also confirmed by the absence of Ago2 protein in the exosomal fraction of follicular fluid. Non-exosomal fraction of follicular fluid derived using the Exoquick isolations contained an ignorable amount of CD63 protein (Figure 4.2 A lane 4); however, both non-exosomal fractions (either from exoquick preparation or ultracentrifugation) contained Ago2 protein (Figure 4.2 A lanes 3 and 4). Furthermore, CYCS (mitochondrial protein) was not present in the exosomal or non-exosomal fraction of follicular fluid isolated by both methods, indicating that those protein samples are free of any protein of cellular origin (Figure 4.2B).

To investigate the morphological characteristics of exosomes in follicular fluid, exosomes were isolated using Exoquick Kit and differential ultracentrifugation. The exosomes were absorbed on formwar carbon coated grids, fixed with PFA, contrasted using uranyl acetate and observed under electron microscope. Observation by electron microscope revealed that both preparations contained vesicles (Figure 4.2C & D) which were similar in size (40-100 nm) to the previously described exosomes.

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 both exosome and non-exosomal fraction of follicular fluid (Figure 4.3). On the other hand, the expression of miR-191 in exosome samples showed a linear pattern, for non-exosomal fraction the deviation from linearity was acceptable (Figure 4.4).

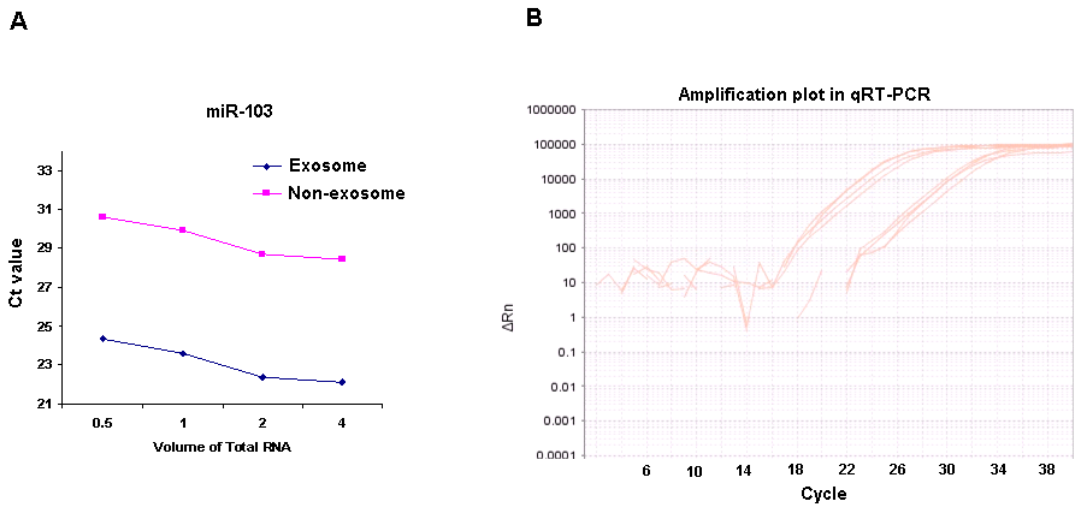


Figure 4.3: Expression of miR-103 in exosomes and non-exosomal fraction of follicular fluid across the input volume (A) and amplification graph in qRT-PCR (B). Where Ct is the threshold cycle value and Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. In Figure 4.3B amplification plot shows the variation of log (ΔRn) with PCR cycle number.

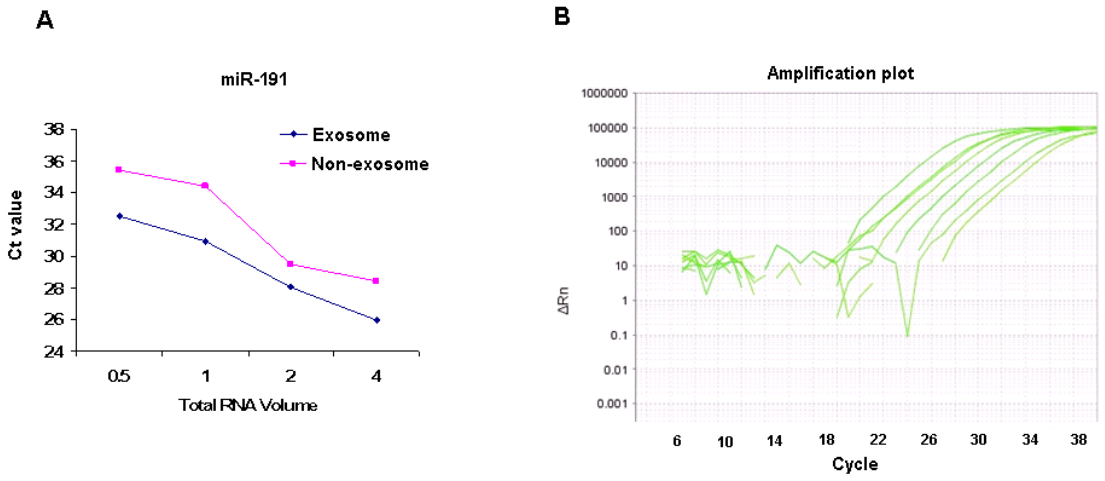


Figure 4.4: Expression of miR-191 in exosomes and non-exosomal fraction of follicular fluid across the input volume (A) with amplification graph in qRT-PCR (B). Where Ct is the threshold cycle value and Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. In Figure 4.3B amplification plot shows the variation of log (ΔRn) with PCR cycle number.

4.3 Detection of miRNAs in exosomal and non-exosomal fraction of bovine follicular fluid

The presence of circulatory miRNAs in exosomal and non-exosomal fractions of bovine follicular fluid was examined by quantitative real time RT-PCR. The expression of mature miRNAs were examined in exosomal and non-exosomal fractions isolated from follicular fluid of each catagory (BCB+ or BCB-) using microRNA ready-to-use Human PCR Panels (I + II) (Exiqon, Denmark). After comparative analysis of these mature human miRNA sequences to the recent bovine mature miRNA sequences available in miRBase version 19 (http://www.mirbase.org/), we found 241 miRNAs to be completely identical with bovine sequence, 126 miRNAs showed differences in size due to addition or deletion of up to 5 nucleotides, 58 miRNA sequences contained mismatches within the mature sequences, out of which 27 miRNAs showed only single mismatch. But no bovine homologous sequences were found for the 323 miRNAs in the present miRBase databank.The miRNAs with Ct value (number of cycles required to get a fluorescence signal above background) less than 35 were considered to be present

in bovine follicular fluid while those with Ct value of >35 were considered as undetected. This analysis revealed that both exosomal and non-exosomal fractions of follicular fluid contain a large array of miRNAs (detected miRNAs were listed in Table 4.1). Among the 748 miRNAs profiled, 509 and 356 miRNAs were detected in exosomal and non-exosomal fraction, respectively. From the detected miRNAs, a total of 331 miRNAs were commonly detected in both exosomal and non-exosomal part of follicular fluid. However, 178 and 25 miRNAs were detected only in exosomal and non-exosomal fractions, respectively (Figure 4.5).

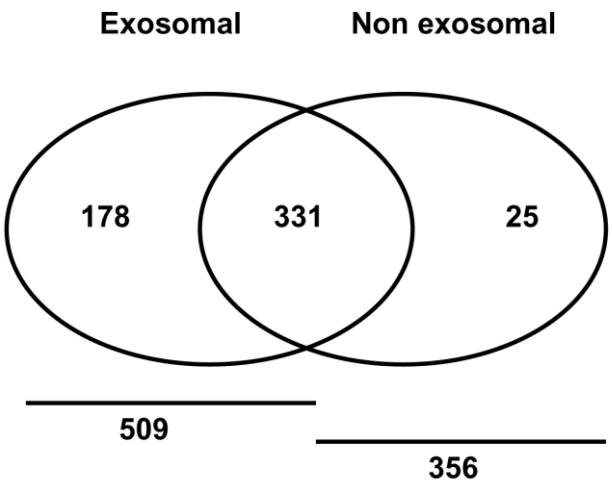


Figure 4.5 Venn diagram showing the number of detected miRNAs in exosomal and non exosomal fraction of follicular fluid. From a total of 748 miRNAs used in the PCR panel 509 and 356 miRNAs were detected (with threshold cycle value of ≤35 in real time PCR analysis) in exosomal fraction and non-exosomal fraction of bovine follicular fluid respectively.

Table 4.1: List of miRNAs detected in exosomal and non-exosomal portion of follicular fluid derived from follicles containing a growing and fully grown oocyte

Detected only in Exosomes	**Detected in both Exosomal and non exosomal**	**Detected only in non exosomal structures**
hsa-let-7c*, hsa-let-7f-2*, hsa-let-7g*, hsa-let-7i, miR-105, miR-10a, miR-1183, miR-1185, miR-122, miR-1227, miR-1247, miR-1249, miR-1256, miR-1272, miR-127-3p, miR-127-5p, miR-128, miR-129*, miR-129-5p, miR-130a*, miR-132*, miR-135a*, miR-135b, miR-136, miR-137, miR-143*, miR-146a, miR-147, miR-148b*, miR-153, miR-155*, miR-15a*, miR-17*, miR-181c, miR-181c*, miR-181d, miR-182*, miR-187, miR-188-3p, miR-190, miR-190b, miR-1911*, miR-192*, miR-193b*, miR-196a, miR-196b, miR-196b*, miR-198, miR-200a, miR-200c, miR-200c*, miR-205, miR-206, miR-208b, miR-20a*, miR-20b*, miR-21*, miR-2113, miR-214*, miR-216b, miR-22*, miR-223*, miR-23a*, miR-23b*, iR-24-1*, miR-24-2*, miR-26a-1*, miR-27b*, miR-28-3p, miR-296-3p, miR-296-5p, miR-297, miR-299-3p, miR-299-5p, **miR-29a***, miR-29c*, miR-301b, miR-31, miR-323-3p, miR-326, **miR-33a***, miR-335*, miR-338-3p, miR-338-5p, miR-33a*, miR-33b, miR-340, miR-342-5p, miR-345, miR-34a, miR-34a*, miR-34b, miR-361-3p, miR-362-3p, miR-365*, miR-370, miR-371-5p, miR-372, miR-377, miR-378, miR-379*, miR-383, miR-384, miR-409-5p, miR-410, miR-411, miR-411*, miR-429, miR-431*, miR-450b-5p, **miR-451**, miR-454, miR-487a, miR-487b, miR-490-5p, miR-492, miR-493*, miR-494, miR-502-3p, miR-504, miR-514, miR-517a, miR-518f, miR-520a-5p, miR-520d-3p, miR-520d-5p, miR-520h, miR-524-3p, miR-525-3p, miR-526a, miR-541, miR-544, miR-548c-3p, miR-551b*, miR-556-5p, miR-566,	hsa-let-7a, hsa-let-7a*, hsa-let-7b, hsa-let-7b*, **hsa-let-7c**, hsa-let-7d, hsa-let-7d, hsa-let-7d*, has, let-7e, hsa-let-7f, hsa-let-7f-1*, hsa-let-7g, **hsa-let-7i***, miR-1, miR-100, miR-101, **miR-103**, miR-103-2*, miR-103-as, miR-105*, miR-106a, miR-106b, miR-106b*, miR-107, miR-10a*, miR-10b, miR-10b*, miR-1179, miR-1181, miR-1207-5p, miR-1224-3p, miR-1237, miR-124, miR-1254, miR-125a-3p, miR-125a-5p, miR-125b, miR-125b-1*, miR-125b-2*, miR-126, miR-126*, miR-1260, miR-1266, miR-1272, miR-129-3p, miR-1296, miR-130a, miR-130b, miR-130b*, **miR-132**, **miR-133a**, miR-133b, miR-134, miR-135a, miR-138, miR-138-2*, miR-139-5p, miR-140-3p, miR-140-5p, miR-141, miR-141*, miR-142-3p, miR-142-5p, miR-143, miR-145, miR-1468, miR-146b-5p, miR-1471, miR-147b, miR-148a, miR-148a*, miR-148b, miR-149, miR-149*, miR-150, miR-151-3p, miR-151-5p, miR-152, miR-1538, miR-1539, **miR-155**, **miR-15a**, miR-15b, miR-15b*, miR-16. miR-17, miR-181a, **miR-181a***, miR-181a-2*, miR-181b, miR-183, **miR-184**, miR-185, **miR-186**, miR-187*, miR-188-5p, miR-18a, miR-18a*, miR-18b, miR-1909, miR-191, **miR-191***, miR-1913, miR-1914, miR-192, **miR-193a-3p**, miR-193a-5p, miR-193b, miR-194, miR-194*, miR-195, miR-195*, miR-197, miR-1972, miR-1979, miR-199a-3p, **miR-199a-5p**, miR-199b-5p, miR-19a, miR-19b, **miR-19b-1***, miR-200b, miR-200b*, miR-202, miR-202*, miR-203, miR-204, miR-20a, **miR-21**, mir-210,, miR-2110, miR-212, miR-214, miR-215, miR-218, miR-218-1*, miR-219-5p, miR-22, **miR-221**, miR-221*, **miR-222**, miR-224, miR-224*, miR-23a, miR-23b, miR-24, miR-25, miR-25*, miR-26a, miR-26a-2*, miR-26b, **miR-26b***, miR-27a, miR-27b, miR-28-5p, **miR-29a**, miR-29b, miR-29b-1*, **miR-29c**, miR-300, miR-301a, **miR-302c**, miR-302d*, miR-30a, miR-30a*, **miR-30b**, miR-30b*, miR-30c, miR-30c-1*, miR-30c-2*, miR-30d, miR-30d*, miR-30e, **miR-30e***, miR-31*, miR-320a, miR-320b, **miR-324-3p**, miR-324-5p, **miR-328**, miR-329, miR-330-3p, miR-330-5p, miR-331-3p, miR-331-5p, miR-335, miR-339-5p, miR-33a, miR-33b*, miR-342-3p, miR-346, miR-34c-3p, miR-34c-5p, miR-361-5p, miR-362-5p**, miR-363**, **miR-365**, miR-369-5p, **miR-373**, miR-373*, miR-374a, miR-374b, miR-375, miR-376a, miR-376a*, miR-376b, miR-376c, miR-378*, miR-379, **miR-381**, miR-382, miR-409-3p, miR-421, miR-422a, miR-423-3p, **miR-423-5p**, miR-423-5p, miR-424, **miR-424***, miR-425, **miR-425***, miR-432, miR-433, miR-449a, **miR-449b**, miR-450am, **miR-450b-3p**, miR-452, miR-454*, miR-455-3p, miR-455-5p, miR-483-3p, miR-484, miR-485-3p, miR-486-3p, miR-486-5p, miR-490-3p, miR-491-3p, **miR-491-5p**, miR-493,	**hsa-let-7a-2***, **miR-101***, miR-1244, miR-1267, **miR-145***, miR-185*, miR-216a, **miR-223**, miR-302a, miR-367, miR-431, miR-498, miR-501-5p, miR-518b, miR-518c*, miR-548j, miR-549, miR-572, miR-576-5p, miR-584, miR-622, miR-629, miR-646, **miR-654-3p**, miR-802, miR-887

miR-590-5p, miR-596, miR-600, miR-601, miR-605, **miR-608**, miR-615-5p, miR-616, miR-620, miR-627, miR-629*, miR-631, miR-649, miR-652, miR-660, miR-663b, miR-671-3p, miR-671-5p, miR-675*, miR-708, miR-7-1*, miR-767-5p, miR-769-5p, miR-876-5p, miR-877*, miR-885-5p, miR-886-3p, miR-888*, miR-891a, miR-9, miR-9*, miR-924, miR-92b*, miR-93*, miR-937, miR-941, miR-942, miR-95, miRPlus-C1076, miRPlus-C1089, miRPlus-D1061	miR-495, miR-496, miR-497, miR-499-5p, miR-500a, miR-502-5p, miR-503, **miR-505**, miR-505*, miR-509-3p, miR-510, miR-518c, miR-518f*, **miR-519d**, miR-521, miR-526b, **miR-526b***, miR-532-3p, miR-532-5p, miR-542-5p, miR-543, miR-545, miR-550a*, miR-551b, miR-552, miR-555, miR-558, miR-562, miR-564, miR-571, **miR-573**, miR-574-3p, miR-577, **miR-582-5p**, miR-589, miR-592, miR-593, miR-595, miR-598, miR-615-3p, **miR-617**, miR-624*, miR-628-3p, miR-628-5p, miR-638, miR-639, **miR-640**, miR-643, **miR-654-5p**, **miR-659**, miR-661, miR-662, miR-663, miR-664, miR-665, miR-668, miR-675b, miR-7, miR-708*, miR-720, miR-744, miR-744*, miR-758, miR-760, miR-765, miR-766, **miR-873**, miR-876-3p, miR-877, miR-885-3p, miR-886-5p, miR-888, miR-92a, miR-92a-1*, miR-92b, miR-93, miR-933, **miR-934**, miR-936, miR-940, miR-943, miR-98, miR-99a, **miR-99a***, miR-99b, miR-99b*, miRPlus-A1027, miRPlus-A1031, miRPlus-C1066	
miRNAs were considered as detected only when the threshold cycle (Ct) value is less than 35. Differentially expressed miRNAs (P<0.05 & 2 fold change) are bold and underlined		

4.4 Differential expression of miRNAs in exosomal and non-exosomal of follicular fluid based on oocyte competence

Next step is to test the hypothesis that miRNAs are present as circulatory molecules in follicular fluid and have different expression patterns depending on the stage of oocyte growth. The miRNAs were profiled in both exosomes and non-exosomal of follicular fluid of BCB+ & BCB- oocyte origin. Since global normalization showed better results within groups compared to spiked-in miRNA or mammalian U6 (Cui et al. 2011), we chose the same normalization procedure (correction with the sum of the expression levels of detected miRNAs) to normalize the expression in array data analysis. Comparisons were made for expression analysis of miRNA in follicular fluids of follicles containing growing oocyte (BCB-) versus a fully grown oocyte (BCB+) in exosomal and non-exosomal separately. The number of differentially expressed miRNAs (P<0.05) restricted by fold change (1.5- and 2-fold) for each comparison is summarized in Table 4.2. Accordingly, 25 and 30 miRNAs in the exosomal and non-exosomal, respectively, were differentially regulated (≥2 fold change, P< 0.05) between follicular fluids from BCB+ & BCB- groups (Table 4.3).

Table 4.2: Summary of the number of differentially expressed miRNAs obtained from comparisons (growing vs. fully grown) in exosomal and non exosomal of follicular fluid.

	Growing vs. fully grown		
	Exosomal	**Non exosomal**	**Overlap**
Number of DE miRNAs (P<0.05)	40 (↑23, ↓17)	41 (↑30, ↓11)	1
Number of DE miRNAs (P<0.05 & fold change > 1.5)	38 (↑22, ↓16)	35 (↑24, ↓11)	1
Number of DE miRNAs (P<0.05 & fold change > 2)	25 (↑16, ↓9)	30 (↑21, ↓9)	0

↑: Up-regulated in the growing vs. fully grown comparison.
↓: Down-regulated in the growing vs. fully grown comparison.
DE: differentially expressed.

Of the 25 exosomal miRNAs that were differentially expressed, 16 and 9 miRNAs were up- and down-regulated, respectively in the growing oocyte (BCB-) group. Among the up-regulated miRNAs, miR-654-5p & miR-640, and down-regulated miRNAs, miR-373 & miR-526b*, displayed the greatest fold change difference in the exosomal of follicular fluid. Among the 30 non-exosomal differentially expressed miRNAs, 21 and 9 miRNAs were found to be up- and down-regulated, respectively, in the growing oocyte group (BCB-). However, from the up-regulated miRNAs, miR-19b-1* and miR-29c, and from down-regulated miRNAs, miR-381 and miR-30e*, had the highest fold change in the non-exosomal of follicular fluid. Importantly, there was no overlap observed between the differentially expressed miRNAs identified from exosomal and non-exosomal, suggesting that each of follicular fluid transport a distinct subset of differentially regulated miRNAs.

Table 4.3. List of differentially expressed microRNAs in exosomal and non-exosomal fraction of bovine follicular fluid obtained from growing vs. fully grown oocyte comparison.

Exosomal portion (Growing vs. fully grown)			Non-exosomal portion (Growing vs. fully grown)		
MicroRNA name	Fold change (BCB-/VCB+)	P value	MicroRNA name	Fold change (BCB-/VCB+)	P value
miR-654-5p	49.64	0.0152	miR-19b-1*	9.54	0.0098
miR-640	8.09	0.0408	miR-29c	7.8	0.0138
miR-582-5p	5.02	0.0120	miR-659	7.72	0.0018
miR-449b	4.39	0.0120	miR-29a	7.51	0.0000
miR-155	3.36	0.0021	miR-424*	6.49	0.0470
miR-573	2.95	0.0005	miR-133a	6.07	0.0417
miR-451	2.78	0.0468	miR-193a-3p	5.64	0.0152
miR-221	2.76	0.0001	miR-617	5.62	0.0489
miR-363	2.73	0.0441	miR-145*	4.83	0.0313
miR-199a-5p	2.4	0.0491	miR-423-5p	4.19	0.0367
hsa-let-7c	2.35	0.0454	miR-365	3.7	0.0419
miR-491-5p	2.26	0.0353	miR-99a*	3.59	0.0266
miR-21	2.23	0.0282	miR-505	3.42	0.0048
miR-132	2.17	0.0349	miR-101*	3.12	0.0463
miR-873	2.16	0.0357	miR-15a	2.8	0.0309
miR-324-3p	2.01	0.0219	miR-222	2.72	0.0425
miR-450b-3p	-2	0.0002	miR-103	2.67	0.0108
miR-191*	-2.05	0.0015	miR-654-3p	2.64	0.0137
miR-26b*	-2.06	0.0013	miR-532-5p	2.45	0.0339
miR-1272	-2.27	0.0474	miR-145	2.18	0.0088
miR-29a*	-2.3	0.0080	miR-574-3p	2.03	0.0118
miR-30b	-2.44	0.0375	miR-184	-2.8	0.0119
miR-33a*	-2.59	0.0400	miR-425*	-2.99	0.0295
miR-526b*	-35.23	0.0399	miR-186	-3.43	0.0143
miR-373	-265.11	0.0210	miR-519d	-3.99	0.0211
			miR-302c	-5.02	0.0052
			miR-934	-7.75	0.0297
			miR-30e*	-9.28	0.0119
			miR-18a*	-14.35	0.0106
			miR-381	-17.41	0.0478

(P<0.05) with at least two-fold change.

4.5 Target prediction, biological functions and canonical pathways identified for differentially expressed miRNAs

To understand the biological relevance of the miRNA signature in follicular fluid (both exosomal and non-exosomal) we have performed *in silico* analysis in order to identify the potential target genes of the differentially expressed (≥2-fold change, P<0.05) up-

regulated miRNAs of growing oocyte (BCB-) group. As the number of experimentally validated miRNA targets is limited, we used miRecords at http://mirecords.biolead.org/, which contains animal miRNA potential targets according to combinations of the widely used target prediction programs DIANA, MicroTest, Micro Inspector, mirTarget, miRBD, miRanda, TargetScanS, and PicTar. We considered a gene as a potential target of a specific miRNA when it was predicted by at least 4 target prediction programs.

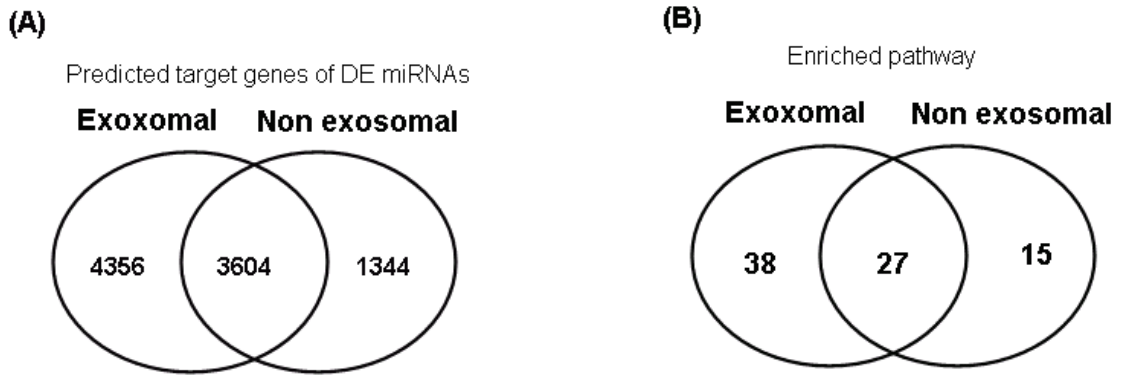


Figure 4.6: Venn diagram showing the overlap at gene and pathway levels targeted by differentially expressed miRNAs. (A)The targeted genes predicted (predicted by at least 4 target prediction tools) to be regulated by differentially expressed miRNAs. (B) The enriched pathways ($P<0.01$) in targeted genes predicted to be regulated by differentially expressed miRNAs.

A total of 7,960 and 4,948 potential targets were identified through this process for differentially expressed exosomal and non-exosomal miRNAs respectively (Figure 4.6 A). We then used DAVID (http://david.abcc.ncifcrf.gov/) server to identify the gene ontology (GO) and significantly enriched canonical pathways (P<0.01) in these conserved targets. As shown in Figure 4.6 B, although there are no overlaps at differentially regulated miRNA levels (Table 4.2), there is a much higher degree of convergence at pathway levels regulated by differentially expressed miRNAs and up-regulated miRNAs of growing oocyte (BCB-) group (Figure 4.6 B).

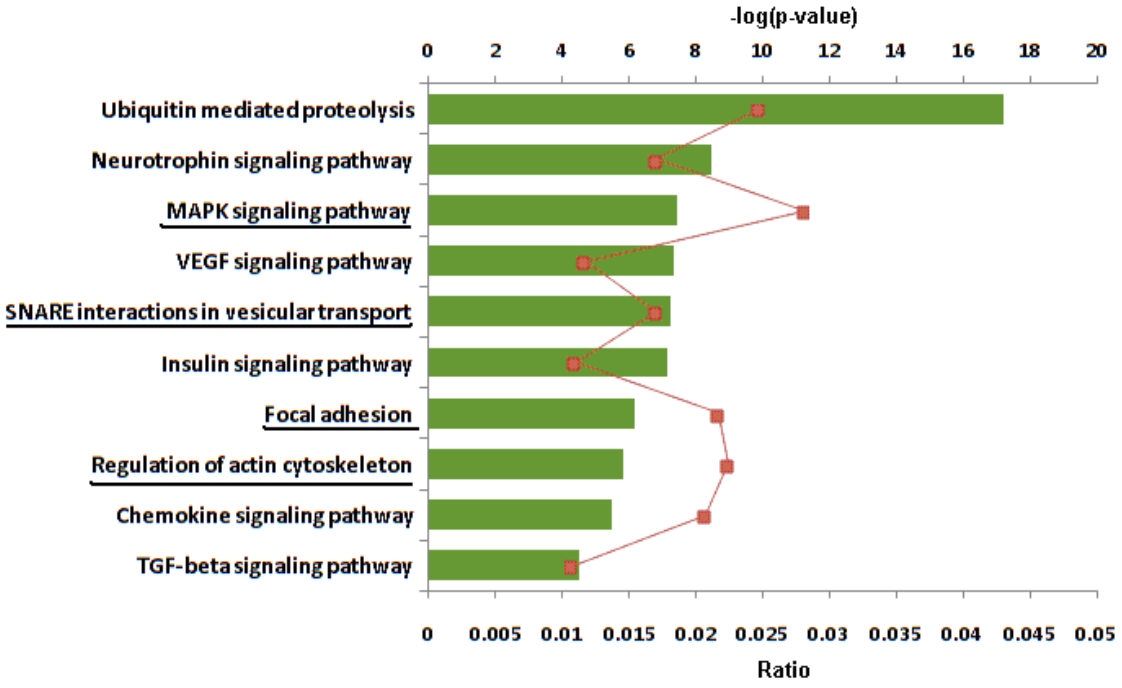


Figure 4.7: Pathways enriched by genes predicted to be targeted by miRNAs highly abundant in exosomal of follicular fluid derived from follicles with growing oocytes (BCB-) compared to fully grown oocytes (BCB+) group. The bars represent the –log(p-value) for each pathway. The red irregular line is a graph of the ratio (number of genes from the data set / total number of genes involved in respective pathways).

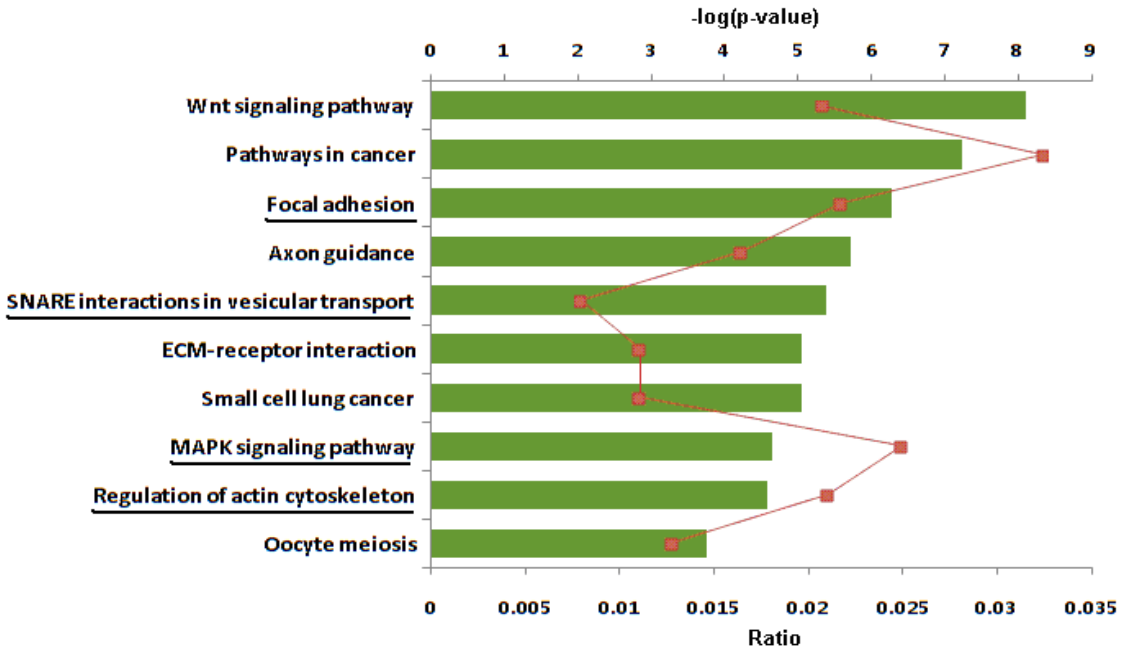


Figure 4.8: Pathways enriched by genes predicted to be targeted by miRNAs highly abundant in non exosomal of follicular fluid derived from follicles with growing oocytes (BCB-) compared to fully grown oocytes (BCB+) group. The bars represent the –log (p-value) for each pathway. The red irregular line is a graph of the ratio (number of genes from the data set / total number of genes involved in respective pathways). The common pathways affected by differentially expressed miRNAs in exosomal and non-exosomal fraction of follicular fluid are underlined.

Our analysis for GO revealed that the genes targeted by up-regulated miRNAs in both exosomal and non-exosomal fraction of follicular fluids of the growing oocyte group were associated with transcriptional categories (such as transcription, regulation of transcription, transcription factor activity), transport, secretion and gene expression (Table 4.4). As shown in Figure 4.7, ubiquitin-mediated proteolysis, neurotrophin signaling pathway, and MAPK signaling pathway are the most enriched pathways for the target genes of differentially expressed upregulated exosomal miRNAs. On the other hand, WNT signaling pathway, pathways in cancer and focal adhesion are the most enriched pathways for the genes potentially targeted by non-exosomal miRNAs (Figure

4.8). Furthermore, pathways involved in various signal transduction and cell-cell interactions such as VEGF signaling pathway, insulin signaling pathway, focal adhesion, pathways in cancer, and oocyte meiosis TGF-beta signaling pathways are also significantly enriched in both exosomal and non-exosomal fraction of follicular fluid from follicles containing growing oocytes.

Table 4.4: Gene ontology analysis of potential target genes of miRNAs differentially expressed in exosomal and non-exosomal fraction of follicular fluid derived from follicles containing growing vs. fully grown oocytes.

Exosomal portion		**Non exosomal portion**	
GO ID & terms	**Enrichment P value**	**GO ID & terms**	**Enrichment P value**
GO:0006350~transcription	9.69E-32	GO:0045449~regulation of transcription	4.16E-14
GO:0045449~regulation of transcription	3.18E-29	GO:0015031~protein transport	1.05E-11
GO:0051252~regulation of RNA metabolic process	1.10E-20	GO:0045184~establishment of protein localization	1.33E-11
GO:0006355~regulation of transcription, DNA-dependent	2.20E-20	GO:0051173~positive regulation of nitrogen compound metabolic process	1.61E-11
GO:0007242~intracellular signaling cascade	1.19E-18	GO:0010628~positive regulation of gene expression	5.45E-11
GO:0006814~sodium ion transport	1.50E-17	GO:0045941~positive regulation of transcription	1.74E-10
GO:0055085~transmembrane transport	3.42E-15	GO:0031328~positive regulation of cellular biosynthetic process	1.96E-10
GO:0007264~small GTPase mediated signal transduction	6.46E-14	GO:0009891~positive regulation of biosynthetic process	3.32E-10
GO:0015837~amine transport	3.32E-13	GO:0051252~regulation of RNA metabolic process	4.77E-10
GO:0015031~protein transport	3.37E-13	GO:0007167~enzyme linked receptor protein signaling pathway	6.00E-10
GO:0006865~amino acid transport	8.93E-13	GO:0006350~transcription	9.70E-10
GO:0046942~carboxylic acid transport	5.58E-12	GO:0051254~positive regulation of RNA metabolic process	1.21E-09
GO:0019941~modification-dependent protein catabolic process	6.45E-12	GO:0008104~protein localization	1.49E-09
GO:0015849~organic acid transport	7.81E-12	GO:0045893~positive regulation of transcription, DNA-dependent	2.80E-09
GO:0046034~ATP metabolic process	1.39E-11	GO:0046907~intracellular transport	4.31E-09

Note: only top 15 terms from each portion are listed here.

4.6 Expression analysis of candidate miRNAs in surrounding follicular cells

Based on their expression profile in both exosomal and non-exosomal fraction of the follicular fluid containing growing oocytes, we selected 8 candidate miRNAs to investigate in the surrounding follicular theca cells (TC), granulosa cells (GC) and cumulus oocyte complex (COCs). From the PCR array results we revealed that, in follicular fluid from follicles with growing oocytes (BCB-), miR-654-5p and miR-640 (in exosomal) and miR-19b-1* and miR-29c (in non exosomal) were highly abundant (Table 4.3). Similarly, miR-526b* and miR-373 (in exosomal) and miR-381 and miR-30e* (in non-exosomal) were more abundant in follicular fluid from follicles with a fully grown oocyte (BCB+) (Table 4.3).

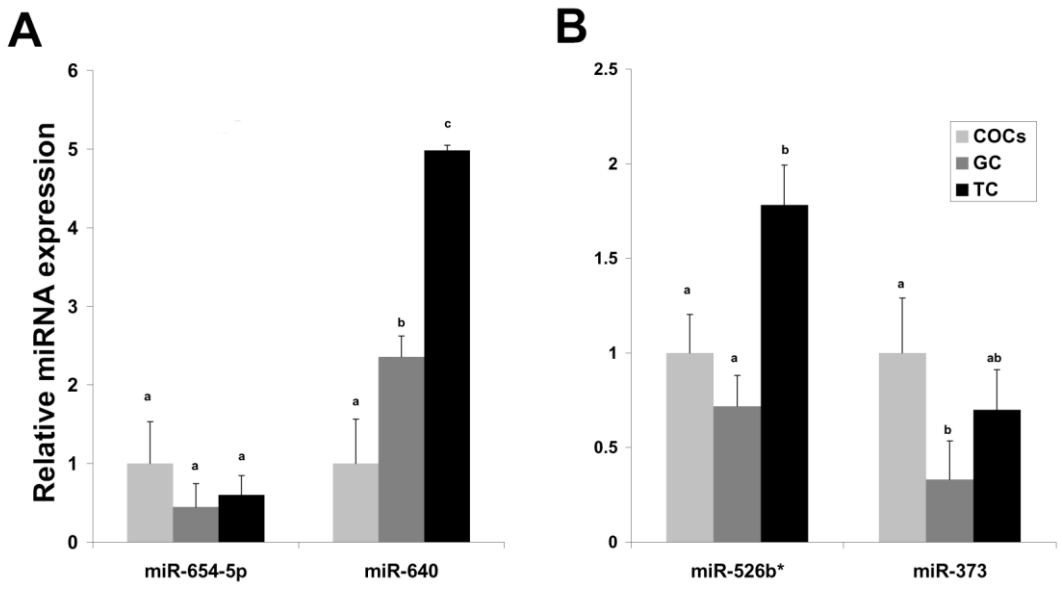


Figure 4.9: Analysis of exosomal miRNAs in follicular cells. Expression patterns of miR-654-5p and miR-640, which are up-regulated in follicular fluid of BCB- vs. BCB+ follicles (Figure 4.9 A), and miR-526b-1* and miR-373, which are down-regulated in BCB- vs. BCB+ follicles (Figure 4.9 B) were investigated in surrounding follicular cells namely cumulus oocyte complex (COCs), granulosa cells (GC) and theca cells (TC) from the same category of follicle which were used for PCR array analysis. The data is presented as relative abundance of

different miRNAs in different cell types compared to their expression in COCs as a control. Means with SD, three biological replicates and bars with different superscripts are different (P<0.05).

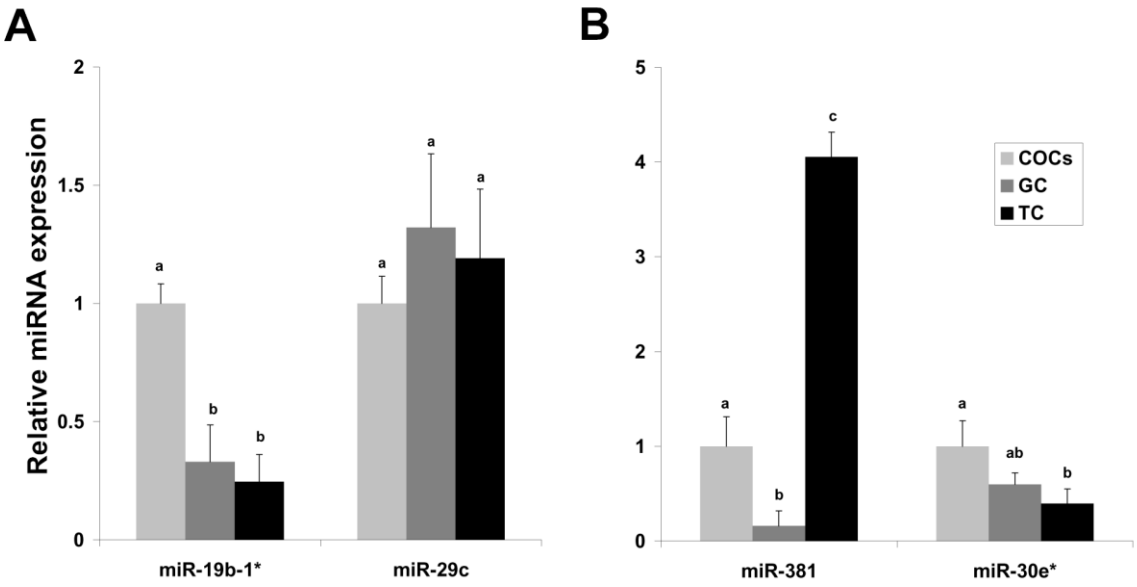


Figure 4.10: Analysis of non-exosomal miRNAs in follicular cells. Expression of two up-regulated namely miR-19b-1*& miR-29c (Figure 4.10 A) and two down-regulated namely miR-381 & miR-30e* (Figure 4.10 B) non-exosomal miRNAs were investigated in surrounding follicular cells namely cumulus oocyte complex (COCs), granulosa cells (GC) and theca cells (TC) from the same category of follicle which were used for PCR array analysis. The data is presented as relative abundance of different miRNAs in different cell types compared to their expression in COCs as a control. Means with SD, three biological replicates and bars with different superscripts are different (P<0.05).

Comparative expression analysis of 8 candidate miRNAs in follicular cells showed that they are present in all cell types with different expression level. While exosomal miRNAs namely, miR-640 and miR-526b* were more abundant in theca cells, miR-373 was detected at higher level in COCs. However, no significant difference was observed

in the expression level of miR-654-5p across different cell types (Figures 4.9 A & B). Similar analysis for non-exosomal miRNAs shows that miR-19b-1* and miR-30e* were highly abundant in cumulus oocyte complex (COCs) where as miR-381 was highly abundant in theca cells. However, no significant difference was observed for miR-29c across the cell types (Figures 4.10 A & B).

4.7 Stability of exosomal miRNAs under culture conditions

To demonstrate the stability of exosomes under culture condition (F-12 media + 10% exosomes free FBS, 37°C and 5% CO_2) we investigate the expression of four candidate exosomal miRNAs at three different time points (6 hr, 12 hr and 24 hr) and compared with freshly isolated exosomes (0 hr) samples derived from follicular fluid irrespective of oocyte competence. We choose miR-654-5p, miR-640, miR-526b-1* and miR-373 because these miRNAs were differentially expressed in exosomal fraction of follicular fluid and they were also substantially expressed in exosomes.

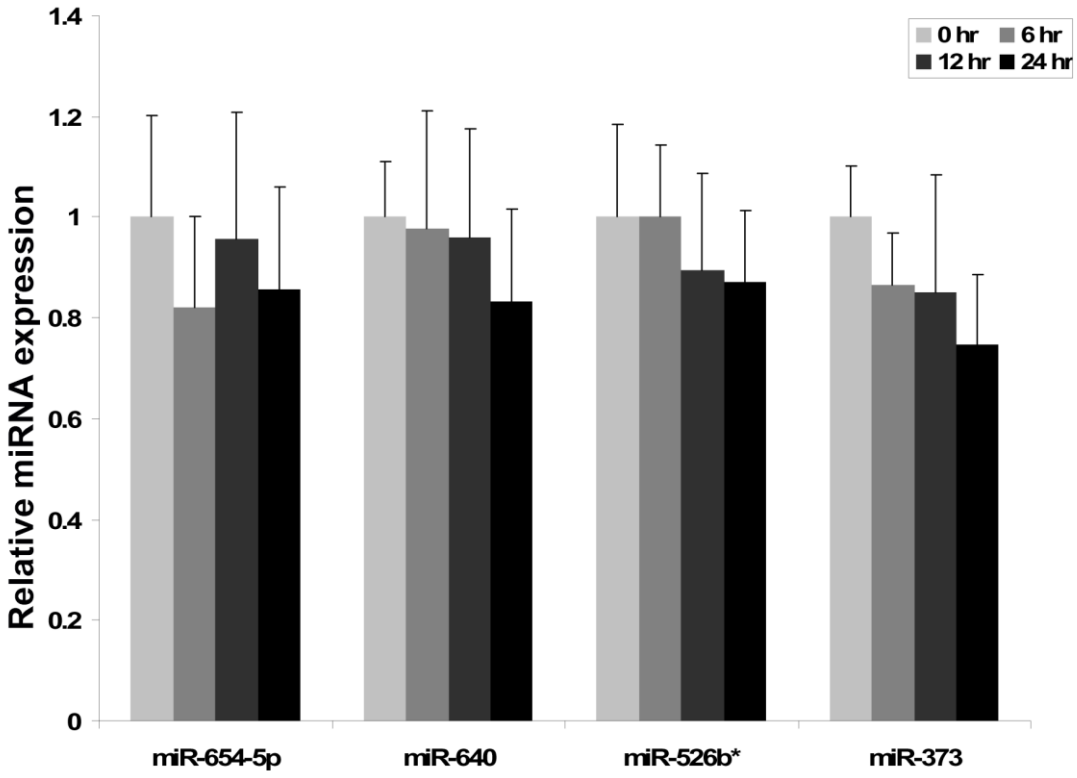


Figure 4.11: Stability of exosomal miRNAs under in vitro culture conditions. Exosomes from follicular fluid were incubated under *in vitro* cell culture conditions (37°C

& 5% CO_2) for 6 hr, 12 hr and 24 hr in a exosome-free culture medium in order to determine the stability of exosome miRNAs by quantitative real time PCR. Non-cultured exosomes (0 hr) were used as reference control to check the stability of exosomes coupled miRNAs in different time points. The data are presented as means with ± SD of three biological replicates.

The results indicate that the exosomal miRNAs of bovine follicular fluid shows more or less stable expression under culture conditions at different time points (Figure 4.11). As *in vitro* culture temperature is similar to body temperature of bovine, thus the results indicate that exosomal miRNAs may stable in follicular fluid long time (up to 48 hrs, data not presented) and may have potential role during follicular development.

4.8 Exosomes can be taken up by follicular cells and increase endogenous miRNA abundance

To evaluate the possibility of exosomes from follicular fluids can be taken up by recipient cells, granulosa cells were collected and 50,000 viable cells were seeded to each well of 8-well chamber slide. Purified bovine follicular fluid exosomes were labeled with PKH67 dye (a green fluorescent dye) and added to the culture media of granulosa cell. Following an incubation period of 24 hr, labeled exosomes treated granulosa cells were washed and fixed and examined by a confocal microscope. The PKH67 labeled exosomes were taken up by cultured bovine granulosa cells (Figures 4.12 A, B & C) and were located around the nucleus. More importantly, granulosa cells that cultured with PKH67 treated sterile PBS showed no signals for green fluorescence (Figure 4.12 D).

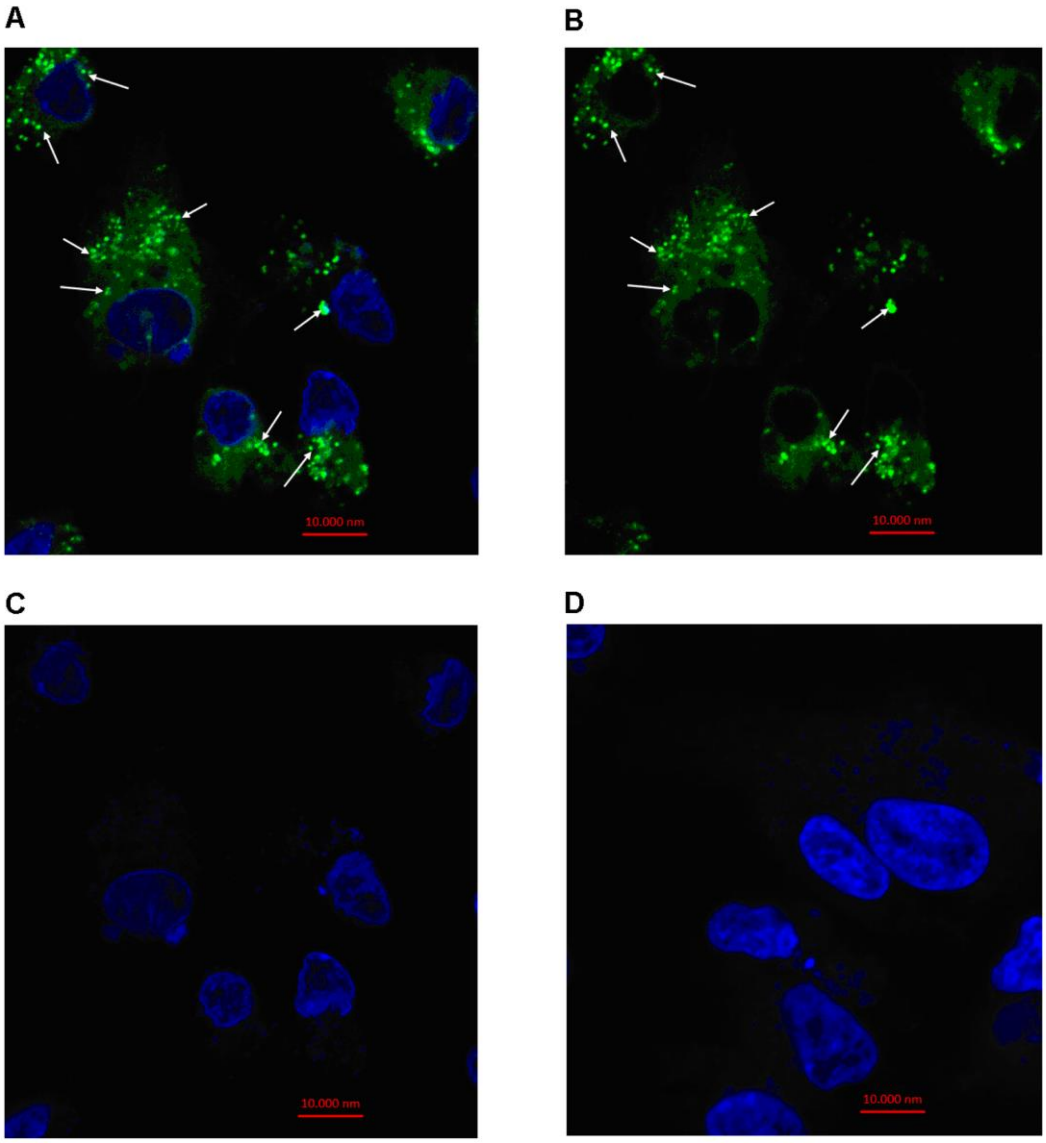


Figure 4.12: Uptake of PKH67-labeled exosomes by bovine granulosa cells *in vitro*. Exosomes, purified from follicular fluid, were labeled with PKH67 dye and added to primary culture of granulosa cells. Granulosa cells were co-cultured with labeled exosomes isolated from follicular fluid in exosome-free medium for 22 hours under optimum cell culture conditions (37°C & 5% CO_2). Nuclei are stained blue using DAPI (Figure 4.12 A, C & D) while PKH67-labeled exosomes are stained green (Figures 4.12 A, B). Arrows indicate exosomes that were taken up by granulosa cells. Granulosa cells cultured in exosomes free

medium containing PKH67-labeled sterile PBS served as a negative control (Figure 4.12 D). Scale bar: 10,000 nm.

4.9 Exosome mediated transport of miRNAs can increase the endogenous population of miRNAs at cellular level

To determine whether exosomes mediated transfection of miRNAs could increase the endogenous level of miRNA, we first purified exosomes from follicular fluid of BCB+ or BCB- oocyte source to check the abundance of candidate miRNAs in fresh exosomes. Importantly, we found that miR-654-5p, miR-640 and miR-526b*, and miR-373 were more abundant in exosomes from follicular fluid of follicles containing BCB- and BCB+ oocytes, respectively (Figure 4.13). Then a of exosomes from BCB+ and BCB- follicular fluid was co-cultured separately with primary granulosa cells in vitro for 24 hours, after which the expression level of those miRNAs was investigated. Granulosa cells co-cultured with same volume of sterile PBS were used as negative control. The results revealed that the level of endogenous miRNAs in exosome co-cultured granulosa cells was significantly increased compared to controls (Figures 14 A & B).

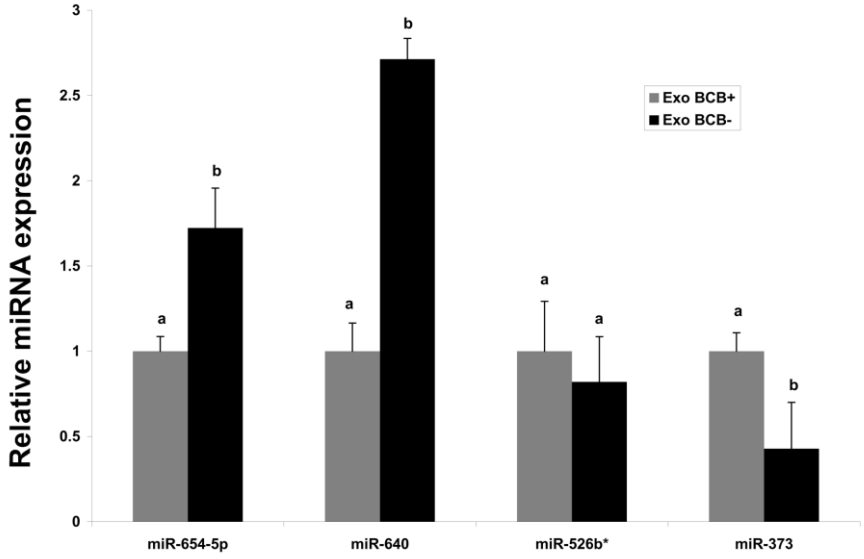


Figure 4.13: Validation of the enrichment of candidate miRNAs in exosomes taken up by granulosa cells. Purified exosomes from follicular fluid of follicles with

growing (BCB-) or fully grown (BCB+) oocytes were subjected to total RNA extraction. Then the expression level miR-654-5p and miR-640 (enriched in exosomes derived from BCB- follicular fluid) and miR-256b-1* and miR-373 (enriched in follicular fluid derived from follicles with BCB+ oocyte) were investigated by real time PCR. Different superscript letters (a,b) denote a significant difference between groups ($P<0.05$). The data is presented as means ± SD of three biological replicates.

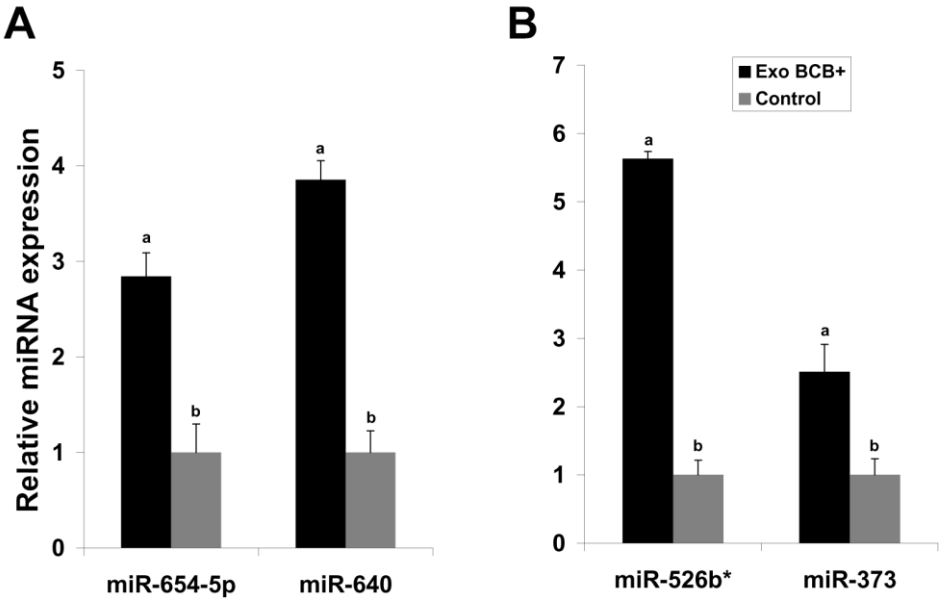


Figure 4.14: Exosome mediated delivery of miRNAs in bovine granulosa cells in vitro. Purified exosomes from follicular fluid of follicles from growing (Figure 4.14 A) or fully grown (Figure 4.14 B) oocytes were co-cultured with bovine granulosa cells. After 24 hrs of incubation at 37°C in a humidified incubator, cells were collected and subjected to total RNA extraction. The expression levels of selected miRNAs were investigated by real time PCR. In both cases the miRNAs the levels of endogenous miRNAs were significantly increased compared to untreated controls. Bars with different superscripts are different ($P<0.05$). The data is presented as means with SD of three biological replicates.

4.10 Changes in expression of target genes following treatment of exosomes in granulosa cells

To determine whether exosome-mediated delivery of miRNA can alter the mRNA abundance of selected target genes, we examined the expression of seven transcripts in granulosa cells collected after treatment with exosomes enriched by candidate miRNAs compared to untreated controls. Based on the criteria mentioned in material and methods part that we analyzed ITGA3 (as target of miR-654-5p), SOCS4 & MAP3K1 (as target of miR-640), BRMS1L & ZNFX1 (as target of miR-526b*) and CD44 & VEGFA (as target of miR-373). As shown in Figure 4.15, treatment of granulosa cells with exosomes enriched with candidate miRNAs down-regulated ITGA3, SOCS4, MAP3K1, BRMS1L, YNFX1 and CD44 genes by 20-65% compared to the untreated control. However, the relative abundance of VEGFA has increased in granulosa cells transfected with exosomes compared with untreated controls (Figure 4.15 B**)**

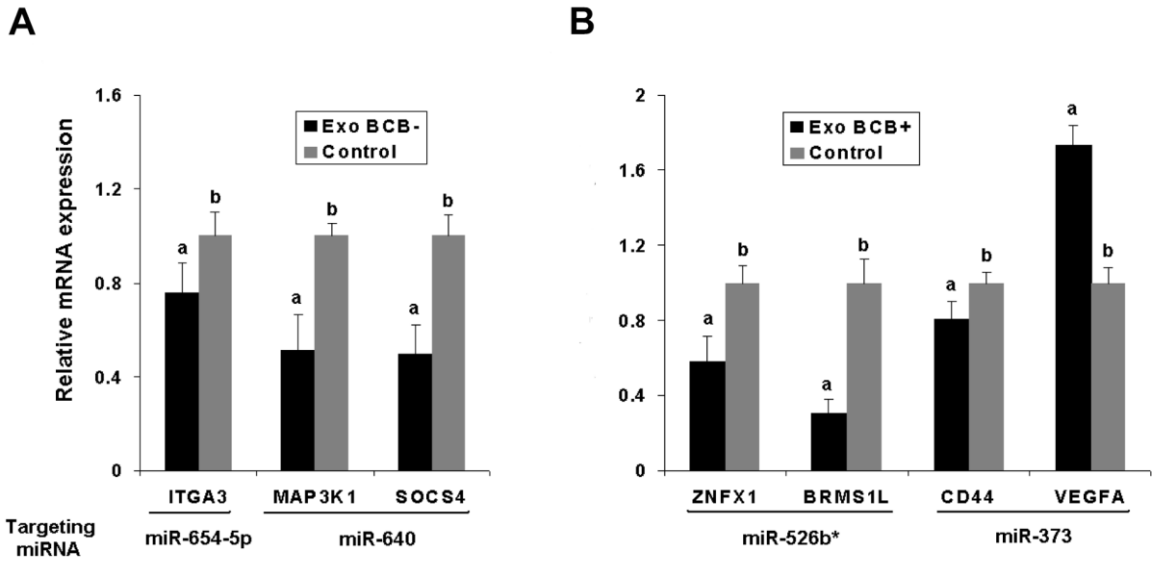


Figure 4.15: Changes in expression of target genes following exosomes transfection. Quantitative real-time PCR were carried out using gene-selective primers. miRNA target genes were selected using seed match and their involvement in important pathways. To elucidate the exosome mediated uptake of circulatory miRNAs can alter

the abundance of target mRNAs we used the same exosome co-cultured granulosa cells which was used for miRNA abundance study. The histogram (Figure 4.15 A & B) indicate that significantly lower expression of target mRNAs except VEGFA, suggesting possible involvement of transcriptional regulation of circulating miRNA. Different superscript letters (a, b) denote a significant difference between groups, such that groups not sharing a similar letter are significantly different form each other ($P<0.05$). The data is presented as means ± SD of three biological replicates.

5 Discussion

5.1 Extra-cellular miRNAs and follicular oocyte development

The follicular oocyte derived from slaughtered animals that is commonly used for *in vitro* maturation and subsequent embryo production is commonly heterogeneous in terms of developmental competences. This is because of the fact that the follicular population of the ovary may consist of oocytes that are either in the growing phase or already fully grown stage. It has been reported that a fully grown oocyte shows better competency than a growing oocyte in terms of *in vitro* maturation, fertilization and blastocyst rate (Mohammadi-Sangcheshmeh et al. 2012; Silva et al. 2011). In addition, there is a coordinated communication between follicular cells, which is done in terms of exchange of signaling molecules among them. To attain competence there is a higher degree of transcription and translation occurred during oocyte growth (Tatemoto and Horiuchi 1995). In this regard follicular fluid plays a significant role in mediating the transport of nutrients and signals from and to granulosa cells, theca cells and cumulus oocyte complex. This process demanded a high degree of miRNA management to support further development or attaining competence. Several studies have been conducted to correlate the dynamic process of oocyte development and gene regulatory network that include oocyte specific transcripts as well as regulators of transcripts like miRNA (Ghanem et al. 2007; Tesfaye et al. 2009; Torner et al. 2008). We hypothesized that mRNA regulatory molecules namely miRNAs as circulatory RNA in follicular fluid may play a significant role in this regard. The presence of certain circulatory miRNAs in follicular fluid may be associated with the oocyte growth phase. Therefore, we have profiled the expression of circulatory miRNAs in follicular fluid using a custom miRNA PCR array. However, our understanding of composition of the oocyte transcriptome and the regulatory role of key oocyte-expressed genes and miRNAs in folliculogenesis is far from complete. Recently miRNAs have been detected in extracellular environment mainly in different bio-fluids and their spectra could reflect altered physiological and pathological conditions (Gilad et al. 2008; Hunter et al. 2008; Schrauder et al. 2012; Valadi et al. 2007; Weber et al. 2010; Yamaura et al. 2012). To our knowledge, this study is the first to report on the presence of circulatory miRNAs in bovine follicular fluid that may be associated with oocyte growth. The results of this study clearly

support our hypothesis that miRNAs are also present in bovine follicular fluid in association with either exosomes or non-exosomal fractions and their signature may represent the altered physiological conditions.

5.2 Specificity of exosomes isolation procedure

Recent studies have demonstrated that circulating miRNAs are coupled with either exosomes (Taylor and Gercel-Taylor 2008; Thery 2011) or Ago2 protein complex (Arroyo et al. 2011) to bypass the high RNases activity in blood stream. By using a systemic approach, we have revealed that there are at least two population of circulating miRNAs in follicular fluid, namely exosomes and non exosome associated, in which the exosome-coupled represent the majority of miRNAs in follicular fluid. The specificity of exosome isolation was 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). It is crucial for any biomarker discovery project utilizing cell free microRNA from serum or plasma that the cellular content of the bio-fluid is minimized, that miRNA expression levels are interpreted in light of blood cell counts, or that miRNA biomarkers are sought among miRNAs that are not expressed in cells. Minimizing cellular content can be done by standardizing the sampling procedure as well as the handling of the material. After collection of follicular fluid we followed several spinning and filtration procedures which allow complete removal of cellular fraction from follicular fluid which might present during sampling. In order to demonstrate the purity of follicular fluid we further performed immunoblotting using CYCS which is exclusively expressed in cytosol/mitochondria. As expected, the result revealed that

both exosomal and non-exosomal protein preparation were negative for CYCS (da Silveira et al. 2011; Torri and Hajduk 1988).

5.3 Quality control of total RNA

Another source of possible variation in miRNA profiles from serum and plasma is the RNA isolation procedure and specifically 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 inhibitors which may be derived from the bio-fluid or introduced via reagent carry-over during sample preparation and suboptimal RNA isolations. 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. However, agarose gel analysis is time-consuming and also hazardous, as ethidium bromide staining is required to visualize RNA bands. RNA purity is generally determined 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, 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 we investigate using a small set of single qPCR assays where cDNA was synthesized with different total RNA input volume to make a dilution curve (e.g. 0.5 µl, 1.0 µl, 2.0 µl, and 4.0 µl in a 10 µl reaction). Before committing sample RNA to

profiling on miRNA qPCR panels, this pre-examination allowed us to discharge the samples which fail to generate a dilution curve with expected linear relationship between sample input and signals.

5.4 Differentially expressed miRNAs in exosomal and non-exosomal portion of follicular fluid

Recently RNAs, including miRNAs were detected in blood serum in association with microvesicles/exosomes (Hunter et al. 2008; Taylor and Gercel-Taylor 2008) and non-vesicle-associated (Ago2 complex associated) (Arroyo et al. 2011). Very recently, one report shows that miRNAs may be present in blood plasma in association of High Density Lipoprotein (HDL) and delivered to recipient cells with functional capabilities (Vickers et al. 2011). Indeed numerous studies have shown that secreted microRNAs can be implicated in pathogenic conditions such as various cancers, coronary heart disease and organ damage (Kosaka et al. 2010; Mitchell et al. 2008; Wang et al. 2010b). The precise role of circulating miRNAs is still largely unknown. Circulating or extracellular miRNAs have been shown to be stabilized and protected from RNAse degradation by inclusion in various protein complexes or membranous particles such as exosomes or microvesicles (Arroyo et al. 2011; Valadi et al. 2007). There seems to be a subset of cell-free microRNAs present in normal blood with possible functions within the circulatory and immune systems (Vasilatou et al. 2010). The miRNA profile in serum and plasma has been shown to reflect disease states such as cancer (Bryant et al. 2012) as well as organ damage and injury (Wang et al. 2010b). It has also been shown that miRNA containing subcellular vesicles can be taken up by cells and cause changes in cellular gene expression during pathological conditions (Kosaka et al. 2010; Vickers et al. 2011). These findings suggest a biological function for extra-cellular miRNA yet to be fully described. In the present study, in addition to the exosomal portion, miRNAs coupled with Ago2 protein were found in the non-exosomal portion (supernatant) of the follicular fluid. Subsequently, we have confirmed the presence of miRNAs in both exosomal and non-exosomal portion of the follicular fluid using a Human miRNome PCR array platform. Despite using a heterologous approach, the quantitative real time PCR analysis shows detection of the majority of miRNAs in both portion of follicular fluid indicating the cross species conservation feature of miRNAs between human and

bovine as it has been observed in a wide range of species (Bentwich et al. 2005). Of the total of 750 miRNAs in the PCR array panel, a total of 509 and 356 miRNAs were detected in the exosomal and non-exosomal portion of follicular fluid respectively (Figure 4.5). Among the detected miRNAs, 331 were commonly found in both portions, while 178 and 25 miRNAs were detected only in exosomal and non-exosomal portion of follicular fluid, respectively. This shows exosome mediated transport of miRNAs is the dominant pathway in bovine follicular fluid compared to the non exosomal way (Ago2 & HDL) as observed previously in blood plasma and saliva (Gallo et al. 2012). To explore the possible association of exosomal and non exosomal miRNA expression with oocyte growth in the follicle, we examined the relative miRNA expression level in exosomal and non exosomal portion of bovine follicular fluid collected from follicles containing growing (BCB-) and fully grown (BCB+) oocytes. Subsequently we found 25 and 32 miRNAs to be differentially regulated in exosomal and non-exosomal portion, respectively between two oocyte groups (BCB- vs. BCB+). The higher number of up-regulated miRNAs in both exosomal (16 miRNAs) and non-exosomal (21 miRNAs) portion of follicular fluid of growing oocyte group may indicate a higher degree of transcriptional activity during the growth phase of oocytes. In order to evaluate the potential role of these differentially expressed miRNAs, the target genes were predicted bioinformatically and their biological function and gene ontology was determined. The most dominant categories enriched by predicted genes are related to transcription and transport which may indicate that, growing oocytes have higher degree of transcription and translation resulting in efficient RNA management and storage in oocytes as maternal resource (Fair et al. 1995). Furthermore, the most significant pathways enriched by predicted targets for up-regulated exosomal miRNAs include ubiquitin-mediated pathway, neurotrophin signaling, MAPK signaling and insulin signaling pathways. All these pathways are known to be involved in ovarian follicular growth and many developmental processes. The ubiquitin mediated pathway modulates oocyte meiotic maturation (Huo et al. 2004), early mitotic division in developing embryos (Suzumori et al. 2003) and plays important role in many cellular processes (Ciechanover and Schwartz 1994). While Neurotrophin signaling pathway reported to be important in regulation of oogenesis and follicle formation (Dissen et al. 2009), the MAPK signaling has certain role in oocyte and cumulus cells (Zhang et al. 2009). Similarly, the overrepresented pathways in non-exosomal portion were WNT signaling

pathway and pathways in cancer and focal adhesion. WNT molecules are glycoproteins involved in fetal ovarian development and adult ovarian function including follicular growth, oocyte growth or maturation, steroidogenesis, ovulation and luteinization (Boyer et al. 2010; Harwood et al. 2008; Zheng et al. 2006). Moreover, the higher number of common pathways in both exosomal and non-exosomal portion may indicate that, miRNAs associated with either exosomes or Ago2/HDL have a complementary function in follicular microenvironment or the same miRNA species can be carried by both exosomes and Ago2/HDL components.

5.5 Expression of candidate miRNAs in surrounding follicular cells

To validate the hypothesis that circulating miRNAs in follicular fluid originate from different cell types within follicular microenvironment we investigated the expression of 8 selected candidate miRNAs in granulosa cells (GCs), theca cells (TCs) and cumulus oocyte complex (COCs). Results revealed that all these miRNAs are detected in all follicular cell types with varying expression level. MicroRNAs like miR-640, miR-526b* and miR-381 were abundant at higher levels in theca cells, while miR-373, miR-30e* and miR-19b-1* expressed more in COCs. However, no significant differences were observed between cell types in the expression of miR-654-5p and miR-29c. Although the origin of circulating miRNAs in body fluids remains elusive, several reports demonstrated that the origin of circulating miRNAs is closely related to the surrounding cells. For example, while a common set of miRNAs were found in equine follicular fluid and surrounding follicular cells (da Silveira et al. 2011), blood cells are reported to be the major contributor of circulating miRNAs in serum (Pritchard et al. 2011). Therefore, the presence of a significant level of candidate circulatory miRNAs in the surrounding follicular cells could enable us to postulate that the majority of circulatory miRNAs in follicular fluid originated from various cell types in the course of their communication during oocyte growth.

5.6 Functional delivery of miRNAs via exosomes

Cells release different types of vesicles into the extracellular space. Two types of vesicles can be distinguished depending on their cellular origin. Shedding vesicles are generated by budding from the plasma membrane into the extracellular space (Booth et

al. 2006; Cocucci et al. 2009), whereas exosomes have an endosomal origin in MVBs: MVBs either fuse with lysosomes for degradation or with the plasma membrane to release their intraluminal vesicles (ILVs) as exosomes. Mixed populations, containing shedding vesicles and exosomes, are referred as microvesicles. Microvesicles are increasingly recognized as important mediators of cell-to-cell communication. They can transfer receptors, proteins, mRNA and miRNA to target cells via interaction with specific receptors. Microvesicles derived from embryonic stem cells have been reported to reprogramme haematopoietic progenitors through the delivery of mRNA (Ratajczak et al. 2006a). The transfer of RNA species via microvesicles to endothelial cells induces angiogenesis (Deregibus et al. 2007), and progenitor mobilization (Zernecke et al. 2009). In the immune system, exosomal transfer of mRNA occurs between mast cells, and viral miRNAs secreted by EBV-infected cells can be taken up by uninfected recipient cells (Pegtel et al. 2010; Valadi et al. 2007). Synthetic miRNA mimetics and viral miRNAs have been reported to be transferred between leukocytes, although the direct involvement of exosomes/microvesicles in this case was not demonstrated (Rechavi et al. 2009). Exchange of proteins between immune cells has been extensively reported, but the mechanism of this transfer remains unclear (Davis 2007).

In order to elucidate the possible exchange of molecular signals in form of exosome coupled miRNAs between follicular cells, we have investigated the ability of granulosa cells to take up exosomes isolated from follicular fluid. Prior to uptake experiment, the stability of exosome coupled miRNA under *in vitro* culture environment was determined and result showed that exosome coupled miRNAs were stable not only up to 24 hours (Figure 7) but even until 48 (data not shown). Similar studies have also shown the stability of circulating serum miRNAs (Gilad et al. 2008) and breast milk exosomal miRNA stability in room temperature and multiple freeze-thaw cycle (Zhou et al. 2012). Following the confirmation of exosomal miRNA stability under culture conditions, we performed exosomes uptake experiment by primary granulosa cells. Fresh exosomes isolated from follicular fluid and labeled by PKH67 fluorescent dye were co-cultured with bovine primary granulosa cells in vitro for 24 hrs. The fluorescent microscopy observation of labeled exosomes co-cultured granulosa cells revealed the presence of green fluorescent exosomes in cultured primary granulosa cells. A similar observation was reported in equine granulosa cells (da Silveira et al. 2011), microglial cell line

(Fitzner et al. 2011), immune cells (Mittelbrunn et al. 2011) and other cell lines (Valadi et al. 2007). Fluorescent microscopy results also showed that majority of the exosomes taken up by granulosa cells were gathered around perinuclear region (Tian et al. 2010). Importantly, there were no fluorescent signals in plasma membrane indicating that exosomes were internalized in to granulosa cells via endocytosis. If fusion is the dominant pathway for exosomes uptake, plasma membrane would contain fluorescent signal after co-culture of cells and labeled exosomes. Similar results were observed in exosome internalization by resting PC12 cells (Tian et al. 2010). The consequence of transfection of exosome coupled miRNAs in endogenous miRNA abundance in granulosa cells was investigated by expression analysis of candidate miRNAs in exosomes derived from follicular fluid of either BCB+ or BCB- oocyte groups. Results have evidenced increased level of endogenous miRNA in exosome transfected granulosa cells compared to the control ones. This could enable us to conclude that exosome mediated exchange of miRNAs in follicular microenvironment is a route of communication between follicular cells.

In conclusion, to the best of my knowledge, this thesis reported for the first time the presence of circulatory miRNAs in bovine follicular fluid and also demonstrated the exosome and non-exosome mediated transport of miRNAs in follicular microenvironment. The stability of exosome coupled miRNA under in vitro culture condition will pave the way for functional analysis of cell to cell communication under *in vitro* environment. Finally, by the comparison of the miRNA expression profiles in follicular fluid of growing and fully grown oocyte source, we have identified several miRNAs which may be associated with growth status of oocyte. However, further functional investigation based on this data could help to find out key regulatory circulating miRNAs controlling oocyte developmental competence by facilitating cell to cell communication in follicular environment.

6 Summary

Growth and development of bovine ovarian follicles and oocytes is the result of series of complex and coordinated processes that involves extensive cell-to-cell communication in the follicle. This phenomenon involves enormous complex and heterogeneous biochemical substances existing in the oocytes and its surrounding cells and the follicular fluid. Thus, the dynamic chemical interactions signaled between these entities will determine the fate of follicular development, follicular cell proliferation and oocyte growth. It has been speculated that follicular fluid influence follicular development by facilitating the transportation and uptake of small membrane-enclosed vesicles called exosomes by surrounding follicular cells. In addition, reports also indicated that exosomes harbour a variety of miRNAs that are implicated in cell to cell communication. Indeed, investigating the presence and source of miRNAs in the follicular fluid will give insight into the role of miRNA in follicular cell development and function. Therefore, the present study was conducted to investigate the presence and expression pattern of circulating miRNA in exosomes and non-exosomal portion of bovine follicular fluid, in follicles containing fully grown and growing oocytes. In addition, this study also addressed the exosome mediated transfer of miRNAs in follicular microenvironment by using a granulosa cell culture model. For this purpose, follicles ranging from 4-8 mm in diameter were blunt-dissected and follicular fluid and cumulus oocyte complex (COCs) were collected from individual follicles, then the COCs were subjected to brilliant cresyl blue (BCB) staining and classified as BCB+ (fully grown oocytes) and BCB- (growing oocytes). The corresponding follicular fluid, granulosa cells and theca cells were also classified accordingly. A total of three replicate pools each containing follicular materials from 20 follicles from both BCB+ and BCB- oocyte category were used in this experiment. The follicular fluid was fractioned in to exosomal and non-exosomal portion using Exoquick™ (Exosome precipitation kit) and used for total RNA isolation using miRNeasy® mini kit. From each exosome and non-exosome 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) consisted of 748 miRNAs, was used for the expression profile of circulatory miRNA in both portion of follicular fluid. The qPCR

data was analyzed using a comparative threshold cycle (ΔCT) method after normalization by global normalization method. To minimize potential noise, miRNAs with Ct value of > 35 were filtered out.

The presence of miRNAs in cell-secreted exosomes and non-exosomal portion of follicular fluid was examined by real time qPCR. miRNAs were considered to be present when the Ct value of the corresponding miRNA was < 35. Thus, of 748 miRNA probes on the PCR array platform, 509 miRNAs and 356 miRNAs were detected in exosomes and non-exosomal portion respectively. While 331 miRNAs were commonly detected in both portion of the follicular fluid, 178 miRNAs were detected only in exosomes and 25 miRNAs were detected in the non-exosomal portion. These finding indicate that miRNAs are transported through exosomes and non-exosomal structures (i.e. Ago2 protein complexes & HDL) in bovine follicular fluid where exosomes mediated transportation of circulatory miRNAs is the dominant pathway.

The results of PCR array revealed that 25 and 30 miRNAs were differentially regulated (≥2 fold change, $P< 0.05$) in the exosomal and non-exosomal portion respectively between follicular fluids from BCB+ & BCB- oocyte origin. Of the 25 differentially expressed exosomal miRNAs, 16 miRNAs were elevated in the growing oocyte (BCB-) group and 9 miRNAs were elevated in fully grown oocyte (BCB+) group. In order to identify the potential target genes of 16 exosomal miRNAs elevated in BCB- group, we used in silico analysis based on target prediction software miRecord and subsequently we found 7960 genes were predicted to be targets of miRNAs enriched in the exosomal portion of BCB- group. The regulator pathway annotation analysis based on the Kyoto encyclopedia of genes and genome database revealed that the genes targeted by the miRNAs were mainly involved in ubiquitin-mediated proteolysis, neurotrophin signaling pathway, and MAPK signaling pathway, VEGF signaling pathway, focal adhesion and TGF-β signaling pathway. Furthermore, ontological classification revealed that the genes targeted by enriched miRNAs in exosomal portion of follicular fluids of the growing oocyte group were associated with transcriptional categories (such as transcription, regulation of transcription, transcription factor activity), transport, secretion and gene expression. On the other hand, among the 30 differentially expressed non-exosomal miRNAs, 21 miRNAs were highly abundant in growing oocyte (BCB-) category whereas 9 miRNAs were found to be highly expressed in fully grown oocyte

category (BCB+). *In silico* target prediction revealed that 4948 genes were potentially targeted by the non-exosomal portion of follicular fluid derived from growing oocyte (BCB-) origin and pathway analysis showed that those genes were found to be involved in WNT signaling pathway, pathways in cancer, focal adhesion, axon guidance, MAPK signaling pathway, regulation of actin cytoskeleton and oocyte meosis. In addition to pathway analysis gene ontological classification showed that potential target genes of enriched non-exosomal miRNAs of growing oocyte group were found to be involved in regulation of transcription, protein transport, establishment of protein localization, positive regulation of nitrogen compound metabolic process, positive regulation of gene expression and positive regulation of transcription. The differences in regulatory pathways and biological processes indicated the transcriptomic and molecular signaling alteration between exosomal and non-exosomal portion of follicular fluid containing growing oocyte. Based on expression intensity four exosomal miRNAs (miR-654-5p, miR-640, miR-526b* and miR-373) and four non-exosomal miRNAs (miR-19b-1*, miR-29c, miR-381 and miR-30e*) were selected for further characterization in surrounding follicular cells. Comparative expression analysis of eight candidate miRNAs in follicular cells using quantitative real time PCR showed that they are present in all cell types with different expression level. This finding may reflect the origin of circulating miRNAs in follicular fluid.

Following investigating the expression analysis of miRNas in follicular fluid (associated with exosomes & non-exosomal means) and surrounding follicular cells next we examined the function of follicular fluid exosomes. For this purpose, a granulosa cell culture model was used. First, exosomes from follicular fluid were isolated and checked for stability under culture condition by real time expression analysis of selected exosomal miRNAs. The result demonstrated that isolated follicular fluid exosomes were stable under culture conditions not only 24 h, but also up to 48 h. Once stability of exosomes was confirmed, next we checked the possibility of uptake of exosomes by recipient cells. For this, follicular fluid exosomes were labelled with PKH67 dye and co-cultured with granulosa cells for 24 hr. Confocal microscopy results revealed that the green fluorescent labelled exosomes were taken up by the granulosa cells. Furthermore, to determine the exosome mediated transport of miRNAs could increase the endogenous level of miRNAs in recipient cells, we isolated the exosomes from follicular fluid of growing oocytes (BCB-) and fully grown oocyte (BCB+) origin and co-cultured with

primary granulosa cells separately for 24 hr. Following this we investigated the expression of miR-654-5p & miR-640 in granulosa cells co-cultured with BCB- oocyte origin exosomes and miR-526b* & miR-373 in granulosa cells co-cultured with BCB+ oocyte origin exosomes using quantitative real time PCR. The results revealed that the level of endogenous miRNAs in exosome co-cultured granulosa cells was significantly increased compared to controls. Once the enrichment of endogenous miRNA levels in exosome co-cultured granulosa cells was uncovered, the mRNA expression pattern of some candidate target genes were assessed by quantitative real time PCR in the same pools of granulosa cell samples used for miRNA enrichment experiment. Based on 7-mer seed match with the 3´ untranslated region of target gene, we selected ITGA3 (as target of miR-654-5p), SOCS4 & MAP3K1 (as target of miR-640), BRMS1L & ZNFX1 (as target of miR-526b*) and CD44 & VEGFA (as target of miR-373). Subsequently the expression analysis revealed that treatment of granulosa cells with exosomes enriched with candidate miRNAs down-regulated ITGA3, SOCS4, MAP3K1, BRMS1L, YNFX1 and CD44 genes by 20-65% compared to the untreated control. However, the relative abundance of VEGFA has increased in granulosa cells transfected with exosomes compared with untreated controls.

In conclusion, the present study reported for the first time the presence of extra-cellular miRNAs in bovine follicular fluid and those could be coupled with exosomes or non-exosomal structures (i.e. Ago2, HDL). This data highlights a number of differentially expressed circulatory miRNAs in bovine follicular fluid in relation to oocyte development competence, which is a step forward in developing non invasive bio-markers for selecting competent oocytes. Moreover, this thesis also documented the exosome mediated transfer of miRNAs and alteration of endogenous levels of mRNAs and miRNAs in recipient cells which reflect the cell-cell communication during follicular development. Although this data open a new area of extra-cellular miRNA research regarding follicular microenvironment, in-depth functional studies will be required to understand the functional contribution of differentially expressed miRNAs to acquire oocyte developmental competence.

7 Zusammenfassung

Wachstum und Entwicklung boviner Follikel und Eizellen ist das Ergebnis einer Reihe von komplexen und koordinierten Prozessen die eine umfangreiche Zell-Zell-Kommunikation innerhalb der Follikel beinhaltet. Dieses Phänomen beruht auf komplexen und heterogenen biochemischen Vorgängen sowohl in den Eizellen, den umliegenden Zellen als auch in der Follikelflüssigkeit. Dementsprechend werden durch die dynamischen chemischen Wechselwirkungen zwischen diesen Einheiten die Follikelreifung, die follikuläre Zellproliferation und das Wachstum der Eizelle beeinflusst.

Das Ziel der vorliegenden Studie war es die Präsenz und Expression von zirkulierenden miRNA in exosomalen und nicht-exosomalen Teilen boviner Follikelflüssigkeit und in Follikeln mit ausgewachsenen und wachsenden Eizellen zu untersuchen. Zusätzlich sollte durch diese Studie mittels eines Granulosazellkulturmodells auch der durch Exosomen vermittelte miRNA Transfer in follikulärer Mikroumgebung untersucht werden.

Für diese Fragestellungen wurden Follikel mit einer Größe von 4-8 mm Durchmesser gewonnen und die Follikelflüssigkeit und der Cumulus-Oozyten-Komplex (COC) aus individuellen Follikeln gesammelt. Durch eine Brilliantkresylblau (BCB)-Färbung der COCs fand eine Unterteilung in BCB + (ausgewachsenen Eizellen) und BCB- (wachsenden Eizellen) statt. Die entsprechende Follikelflüssigkeit, Granulosazellen und Thekazellen wurden ebenfalls entsprechend klassifiziert. Insgesamt drei Pools mit jeweils follikulären Material aus 20 Follikeln sowohl aus der Kategorie BCB + und BCB- Eizellen wurden in diesem Experiment als Ausgangsmaterial verwendet. Die Fraktionierung der Follikelflüssigkeit in ihre exosomalen und nicht-exosomalen Bestandteile erfolgte mittels des ExoquickTM (Exosome Niederschlag kit) und die Total-RNA Isolierung mit dem miRNeasy ® Mini Kit. Nach der cDNA-Synthese wurde mittels des human miRCURY (Human Panel I+II, V2.M) Ready-to-Use PCR array (Exiqon), welcher 748 miRNAs enthält, miRNA Profile in beiden Bestandteilen der Follikelflüssigkeit erstellt werden. Die qPCR Daten wurden mit einer vergleichenden Schwellenwert-Zyklus (ΔCT)-Methode nach der Normalisierung mit dem globalen

Normalisierungsverfahren analysiert. Um mögliches Hintergrundrauschen zu minimieren, wurden miRNAs mit einem Ct-Wert > 35 herausgefiltert.

Die Ergebnisse zeigten, dass von 748 miRNA Sonden auf der PCR-Array-Plattform 509 miRNAs im exosomalen und 356 miRNAs im nicht-exosomalen Teil detektiert wurden. Davon konnten 331 miRNAs in beiden Bestandteilen nachgewiesen werden, während 178 miRNAs dem exosomalen und 25 miRNAs dem nicht-exosomalen Teil zugewiesen wurden. Unter den detektierten miRNAs waren 25 miRNAs unterschiedlich im exosomalen und 30 miRNAs unterschiedlich im nicht-exosomalen Bestandteil reguliert (≥ 2-fache Veränderung, P <0,05) im Vergleich zu BCB + und BCB- Eizellen. Von den 25 differentiell exprimierten miRNAs des exosomalen Teils waren 16 miRNAs in der Gruppe der wachsenden Eizelle (BCB-) und 9 miRNAs in der Gruppe der ausgewachsen Eizelle (BCB +) erhöht. Um potenzielle Zielgene dieser 16 exosomalen miRNAs zu identifizieren, welche in der BCB- Gruppe in ihrer Expression erhöht waren, verwendeten wir eine in silico Analyse. Diese basierte auf einer speziellen "target prediction software" und fanden somit 7960 Gene als mögliche Zielgene für die miRNAs in dieser Gruppe. Eine Signalwegsanalyse mit der Kyoto-Enzyklopädie von Genen und Genom-Datenbank ergab, dass diese 7960 Gene hauptsächlich an den Signalwegen in der Ubiquitin-vermittelten Proteolyse, Neurotrophin, MAPK, VEGF, fokale Adhäsion und TGF β beteiligt sind. Darüber hinaus ergab eine ontologische Klassifizierung, dass die Gene der miRNAs aus dem exosomal angereicherten Teil der Follikelflüssigkeiten der wachsenden Eizelle mit transkriptionalen Kategorien (z. B. Transkription, Regulation der Transkription, Transkriptionsfaktor-Aktivität), Transport, Sekretion und Genexpression assoziiert waren.

Auf der anderen Seite wurden unter den 30 unterschiedlich exprimierten nicht-exosomalen miRNAs eine übermäßig hoch Expression von 21 miRNAs in der Kategorie wachsender Eizellen (BCB-) gefunden, während 9 weitere miRNAs hoch in der Kategorie ausgewachsener Eizellen (BCB+) exprimiert waren. Die In silico Analysen offenbarten 4948 potenzielle Gene im nicht-exosomalem Teil der Follikelflüssigkeit aus der Gruppe der wachsenden Eizellen (BCB-). Analysen der Signalwege zeigten, dass diese Gene in WNT Signalweg, Krebssignalweg, fokale Adhäsion, Axonleitung, MAPK Signalweg und Eizellmeiose involviert sind. Zusätzlich zur Signalwegsanalyse erbrachten genontologische Klassifikationen der potentielle

Zielgene von nicht-exosomalen miRNAs wachsender Eizellengruppen das diese Gene nachweislich an der Regulation der Transkription, dem Proteintransport, der Feststellung der Proteinlokalisation sowie an einem positiven Einfluss auf Nitrogen zusammengesetzte metabolische Prozesse und eine positive Regelung der Genexpression und Transkription beteiligt sind. Der Unterschied zwischen regulatorischen Signalwegen und biologischen Prozessen ist gekennzeichnet durch die transkriptionalen und molekularen Signalveränderungen zwischen exosomalen und nicht-exosomalen Bestandteilen aus der Follikelflüssigkeit wachsender Eizellen. Eine vergleichende Expressionsanalysen dieser acht Kandidaten miRNAs in folikulären Zellen mittels quantitativer Real-Time PCR zeigte unterschiedliche Expressionsniveaus dieser in allen Zelltypen. Dieses Ergebnis gibt eine mögliche Erklärung über den Ursprung der zirkulierenden miRNAs in der Follikelflüssigkeit.

In einem zweiten Experiment wurde der miRNA Transfer mittels Exosomen in vitro untersucht. Als erstes wurden Exosomen aus der Follikelflüssigkeit isoliert. Das Ergebnis demonstrierte, dass die isolierten Exosomen aus der Follikelflüssigkeit bis zu 48 h unter Zellkulturbedingungen stabil waren. Nachdem die Exosomstabilität bestätigt war, wurde als nächstes die Möglichkeit der Aufnahme von Exosomen in die Empfängerzelle überprüft. Die Ergebnisse des Konfokalmikroskops ergaben, dass eine Aufnahme der PKG67 markierten Exosome von Granulosazellen statt gefunden hat. Darüber hinaus sollte der durch die Exosomen vermittelte Transport von miRNAs sowie der dadurch erhöht endogene Level der miRNAs in den Empfängerzellen bestimmt werden. Dafür isolierten wir Exosomen unterschiedlichen Ursprungs aus Follikelflüssigkeiten von wachsenden Eizellen (BCB-) sowie von ausgewachsen Eizellen (BCB+) und co-kultivierten diese seperat für 24 h mit primären Granulosazellen. Die Ergebnisse zeigten, dass das Niveau von endogenen miRNAs in Exosomen co-kultivierten Granulosazellen signifikant erhöht war im Vergleich zur Kontrolle. Nachdem die Anreicherung der endogenen miRNA Level in Exosomen co-kultivierten Granulosazellen aufgeklärt war, wurden in den selben Granulosazellproben die mRNA Expressionsmuster von einigen Kandidatengenen mittels quantitativer Real-Time PCR bestimmt. Die Expressionsanalyse zeigte, dass die Behandlung der Granulosazellen mit Exosomen im Vergleich zur unbehandelten Kontrolle eine Runterregulierung von 20-65% der miRNA Kandidatengene ITGA3, SOCS4,

MAP3K1, BRMS1L, YNFX1 und CD44 erbrachte. Allerdings kam es zu einem relativen Überschuss von VEGFA in Granulosazellen die mit Exosomen transfiziert waren im Vergleich zur unbehandelten Kontrolle.

Schlussfolgernd berichtet die vorliegende Studie das erste Mal über das Vorkommen von extra-zellulären miRNAs in bovinen Follikelflüssigkeiten und dieses könnte mit exosomalen und nicht-exosomalen Strukturen (z.B. Ago2, HDL) in Verbindung stehen. Diese Daten heben eine Anzahl von unterschiedlich exprimierten zirkulierenden miRNAs in boviner Follikelflüssigkeit bezüglich der Eizellkompetenzentwicklung hervor. Diese ist ein Schritt vorwärts in der Entwicklung von nicht invasiven Biomarkern für die Selektion kompetenter Eizellen. Darüber hinaus dokumentiert diese Doktorarbeit einen Exosomen vermittelten Transfer von miRNAs und eine Veränderung endogener Level von mRNAs und miRNAs in Empfängerzellen welches die Zell-Zell-Kommunikation während der follikulären Entwicklung reflektiert. Obwohl diese Daten ein neues Gebiet in der extra-zellulären miRNA Forschung im Bereich der follikulären Mikroumgebung eröffnen, wird eine in die Tiefe gehende Studie für das Verständnis über den funktionellen Beitrag der unterschiedlich exprimierten miRNAs beim Erlangen der Eizellentwicklungskompetens benötigt.

7 References

Adams GP. 1999. Comparative patterns of follicle development and selection in ruminants. J Reprod Fertil Suppl 54: 17-32.

Adams GP, Jaiswal R, Singh J, Malhi P. 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69(1): 72-80.

Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. 1992. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J Reprod Fertil 94(1): 177-188.

Admyre C, Johansson SM, Paulie S, Gabrielsson S. 2006. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. Eur J Immunol 36(7): 1772-1781.

Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A, Gabrielsson S. 2007. Exosomes with immune modulatory features are present in human breast milk. J Immunol 179(3): 1969-1978.

Aharon A, Katzenell S, Tamari T, Brenner B. 2009. Microparticles bearing tissue factor and tissue factor pathway inhibitor in gestational vascular complications. J Thromb Haemost 7(6): 1047-1050.

Ahn HW, Morin RD, Zhao H, Harris RA, Coarfa C, Chen ZJ, Milosavljevic A, Marra MA, Rajkovic A. 2010. MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. Mol Hum Reprod 16(7): 463-471.

Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y, Naoe T. 2011. Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages. Mol Ther 19(2): 395-399.

Alm H, Torner H, Lohrke B, Viergutz T, Ghoneim IM, Kanitz W. 2005. Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brillant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. Theriogenology 63(8): 2194-2205.

Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. 2011. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 29(4): 341-345.

Amsterdam A, Lindner HR, Groschel-Stewart U. 1977. Localization of actin and myosin in the rat oocyte and follicular wall by immunofluorescence. Anat Rec 187(3): 311-328.

Amsterdam A, Rotmensch S. 1987. Structure-function relationships during granulosa cell differentiation. Endocr Rev 8(3): 309-337.

Amzallag N, Passer BJ, Allanic D, Segura E, Thery C, Goud B, Amson R, Telerman A. 2004. TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway. J Biol Chem 279(44): 46104-46112.

Andersen MM, Kroll J, Byskov AG, Faber M. 1976. Protein composition in the fluid of individual bovine follicles. J Reprod Fertil 48(1): 109-118.

Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, Tursz T, Amigorena S, Raposo G, Angevin E, Zitvogel L. 2002. Malignant effusions and immunogenic tumour-derived exosomes. Lancet 360(9329): 295-305.

Araki M, Fukumatsu Y, Katabuchi H, Shultz LD, Takahashi K, Okamura H. 1996. Follicular development and ovulation in macrophage colony-stimulating factor-deficient mice homozygous for the osteopetrosis (op) mutation. Biol Reprod 54(2): 478-484.

Arita S, Baba E, Shibata Y, Niiro H, Shimoda S, Isobe T, Kusaba H, Nakano S, Harada M. 2008. B cell activation regulates exosomal HLA production. Eur J Immunol 38(5): 1423-1434.

Armstrong DG, Webb R. 1997. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod 2(3): 139-146.

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108(12): 5003-5008.

Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D, Witkin SS. 2008. Heat shock protein-containing exosomes in mid-trimester amniotic fluids. J Reprod Immunol 79(1): 12-17.

Babst M. 2011. MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. Curr Opin Cell Biol 23(4): 452-457.

Bagavandoss P, Midgley AR, Jr., Wicha M. 1983. Developmental changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. J Histochem Cytochem 31(5): 633-640.

Balaban B, Urman B. 2006. Effect of oocyte morphology on embryo development and implantation. Reprod Biomed Online 12(5): 608-615.

Baley J, Li J. 2012. MicroRNAs and ovarian function. J Ovarian Res 5: 8.

Barker EV, Cervigne NK, Reis PP, Goswami RS, Xu W, Weinreb I, Irish JC, Kamel-Reid S. 2009. microRNA evaluation of unknown primary lesions in the head and neck. Mol Cancer 8: 127.

Batista BS, Eng WS, Pilobello KT, Hendricks-Munoz KD, Mahal LK. 2011. Identification of a conserved glycan signature for microvesicles. J Proteome Res 10(10): 4624-4633.

Bellingham SA, Guo BB, Coleman BM, Hill AF. 2012. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? Front Physiol 3: 124.

Belting M, Wittrup A. 2008. Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease. J Cell Biol 183(7): 1187-1191.

Ben-Rafael Z, Meloni F, Strauss JF, 3rd, Blasco L, Mastroianni L, Jr., Flickinger GL. 1987. Relationships between polypronuclear fertilization and follicular fluid hormones in gonadotropin-treated women. Fertil Steril 47(2): 284-288.

Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. 2005. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet 37(7): 766-770.

Bergmann C, Strauss L, Wieckowski E, Czystowska M, Albers A, Wang Y, Zeidler R, Lang S, Whiteside TL. 2009. Tumor-derived microvesicles in sera of patients with head and neck cancer and their role in tumor progression. Head Neck 31(3): 371-380.

Beyer C, Pisetsky DS. 2009. The role of microparticles in the pathogenesis of rheumatic diseases. Nat Rev Rheumatol 6(1): 21-29.

Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS. 2007. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. Blood 110(9): 3234-3244.

Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, Hivroz C. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol 168(7): 3235-3241.

Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, Dahlsveen IK. 2013. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods 59(1): S1-6.

Bols PE. 2005. Puncture of immature ovarian follicles in bovine assisted reproduction. Verh K Acad Geneeskd Belg 67(3): 177-202.

Booth AM, Fang Y, Fallon JK, Yang JM, Hildreth JE, Gould SJ. 2006. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. J Cell Biol 172(6): 923-935.

Boyer A, Goff AK, Boerboom D. 2010. WNT signaling in ovarian follicle biology and tumorigenesis. Trends Endocrinol Metab 21(1): 25-32.

Braw-Tal R, Roth Z. 2005. Gene expression for LH receptor, 17 alpha-hydroxylase and StAR in the theca interna of preantral and early antral follicles in the bovine ovary. Reproduction 129(4): 453-461.

Bryant RJ, Pawlowski T, Catto JW, Marsden G, Vessella RL, Rhees B, Kuslich C, Visakorpi T, Hamdy FC. 2012. Changes in circulating microRNA levels associated with prostate cancer. Br J Cancer 106(4): 768-774.

Buccione R, Cecconi S, Tatone C, Mangia F, Colonna R. 1987. Follicle cell regulation of mammalian oocyte growth. J Exp Zool 242(3): 351-354.

Buccione R, Schroeder AC, Eppig JJ. 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod 43(4): 543-547.

Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. 2005. Exosomal-like vesicles are present in human blood plasma. Int Immunol 17(7): 879-887.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99(24): 15524-15529.

Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. 2010. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 78(9): 838-848.

Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. 2000. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. Dev Biol 226(2): 167-179.

Carayon K, Chaoui K, Ronzier E, Lazar I, Bertrand-Michel J, Roques V, Balor S, Terce F, Lopez A, Salome L, Joly E. 2011. Proteolipidic composition of exosomes changes during reticulocyte maturation. J Biol Chem 286(39): 34426-34439.

Carletti MZ, Christenson LK. 2009. MicroRNA in the ovary and female reproductive tract. J Anim Sci 87(14 Suppl): E29-38.

Carlsen AL, Schetter AJ, Nielsen CT, Lood C, Knudsen S, Voss A, Harris CC, Hellmark T, Segelmark M, Jacobsen S, Bengtsson AA, Heegaard NH. 2013. Circulating microRNA expression profiles associated with systemic lupus erythematosus. Arthritis Rheum 65(5): 1324-1334.

Castellana D, Kunzelmann C, Freyssinet JM. 2009. Pathophysiologic significance of procoagulant microvesicles in cancer disease and progression. Hamostaseologie 29(1): 51-57.

Cavender JL, Murdoch WJ. 1988. Morphological studies of the microcirculatory system of periovulatory ovine follicles. Biol Reprod 39(4): 989-997.

Cecconi S, Tatone C, Buccione R, Mangia F, Colonna R. 1991. Granulosa cell-oocyte interactions: the phosphorylation of specific proteins in mouse oocytes at the germinal vesicle stage is dependent upon the differentiative state of companion somatic cells. J Exp Zool 258(2): 249-254.

Chen C, Skog J, Hsu CH, Lessard RT, Balaj L, Wurdinger T, Carter BS, Breakefield XO, Toner M, Irimia D. 2010. Microfluidic isolation and transcriptome analysis of serum microvesicles. Lab Chip 10(4): 505-511.

Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38(2): 228-233.

Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. 2009. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. Nucleic Acids Res 38(1): 215-224.

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Zen K, Zhang CY. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18(10): 997-1006.

Cheng FP, Fazeli AR, Voorhout WF, Tremoleda JL, Bevers MM, Colenbrander B. 1998. Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances in vitro binding to the zona pellucida. Int J Androl 21(2): 57-66.

Cheng Y, Tan N, Yang J, Liu X, Cao X, He P, Dong X, Qin S, Zhang C. 2010. A translational study of circulating cell-free microRNA-1 in acute myocardial infarction. Clin Sci (Lond) 119(2): 87-95.

Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PS, Star RA. 2007. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. Am J Physiol Renal Physiol 292(5): F1657-1661.

Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. 2008. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 54(3): 482-490.

Chin LJ, Slack FJ. 2008. A truth serum for cancer--microRNAs have major potential as cancer biomarkers. Cell Res 18(10): 983-984.

Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet JM, Tedgui A. 2009. Endothelial microparticles in diseases. Cell Tissue Res 335(1): 143-151.

Choi YH, Takagi M, Kamishita H, Wijayagunawardane MP, Acosta TJ, Miyazawa K, Sato K. 1998. Effects of follicular fluid on fertilization and embryonic development of bovine oocytes in vitro. Theriogenology 49(6): 1103-1112.

Ciechanover A, Schwartz AL. 1994. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. FASEB J 8(2): 182-191.

Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. 2007. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Res 67(15): 7458-7466.

Clayton A, Turkes A, Dewitt S, Steadman R, Mason MD, Hallett MB. 2004. Adhesion and signaling by B cell-derived exosomes: the role of integrins. FASEB J 18(9): 977-979.

Cocucci E, Racchetti G, Meldolesi J. 2009. Shedding microvesicles: artefacts no more. Trends Cell Biol 19(2): 43-51.

Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, Elortza F, Lu SC, Mato JM, Falcon-Perez JM. 2008. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. J Proteome Res 7(12): 5157-5166.

Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, Wagner DR, Staessen JA, Heymans S, Schroen B. 2010. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. Circ Cardiovasc Genet 3(6): 499-506.

Cox NM. 1997. Control of follicular development and ovulation rate in pigs. J Reprod Fertil Suppl 52: 31-46.

Creemers EE, Tijsen AJ, Pinto YM. 2012. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res 110(3): 483-495.

Cui W, Ma J, Wang Y, Biswal S. 2011. Plasma miRNA as biomarkers for assessment of total-body radiation exposure dosimetry. PLoS One 6(8): e22988.

D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, Rubino M, Carena MC, Spazzafumo L, De Simone M, Micheli B, Biglioli P, Achilli F, Martelli F, Maggiolini S, Marenzi G, Pompilio G, Capogrossi MC. 2010. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. Eur Heart J 31(22): 2765-2773.

da Silveira JC, Veeramachaneni DN, Winger QA, Carnevale EM, Bouma GJ. 2011. cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. Biol Reprod 86(3): 71.

David GF, Anand Kumar TC, Baker TG. 1974. Uptake of tritiated thymidine by primordial germinal cells in the ovaries of the adult slender loris. J Reprod Fertil 41(2): 447-451

Davis DM. 2007. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. Nat Rev Immunol 7(3): 238-243.

Dbouk HA, Tawil A, Nasr F, Kandakarjian L, Abou-Merhi R. 2007. Significance of CEA and VEGF as diagnostic markers of colorectal cancer in lebanese patients. Open Clin Cancer J 1: 1-5.

de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. 2003. Lipid raft-associated protein sorting in exosomes. Blood 102(13): 4336-4344.

de Gassart A, Geminard C, Hoekstra D, Vidal M. 2004. Exosome secretion: the art of reutilizing nonrecycled proteins? Traffic 5(11): 896-903.

De La Fuente R, Eppig JJ. 2001. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev Biol 229(1): 224-236.

Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J Cell Sci 113 Pt 19: 3365-3374.

Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. 2007. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood 110(7): 2440-2448.

Dissen GA, Garcia-Rudaz C, Ojeda SR. 2009. Role of neurotrophic factors in early ovarian development. Semin Reprod Med 27(1): 24-31.

Donadeu FX, Schauer SN, Sontakke SD. 2012. Involvement of miRNAs in ovarian follicular and luteal development. J Endocrinol 215(3): 323-334.

Driancourt MA, Reynaud K, Cortvrindt R, Smitz J. 2000. Roles of KIT and KIT LIGAND in ovarian function. Rev Reprod 5(3): 143-152.

Duke KL. 1967. Ovogenetic activity of the fetal-type in the ovary of the adult slow loris, Nycticebus coucang. Folia Primatol (Basel) 7(2): 150-154.

Edwards RG. 1974. Follicular fluid. J Reprod Fertil 37(1): 189-219.

El-Hefnawy T, Raja S, Kelly L, Bigbee WL, Kirkwood JM, Luketich JD, Godfrey TE. 2004. Characterization of amplifiable, circulating RNA in plasma and its potential as a tool for cancer diagnostics. Clin Chem 50(3): 564-573.

Eppig JJ. 1979. A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro. J Exp Zool 209(2): 345-353.

Eppig JJ. 1991. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays 13(11): 569-574.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122(6): 829-838.

Eppig JJ, Peters AH, Telfer EE, Wigglesworth K. 1993. Production of cumulus expansion enabling factor by mouse oocytes grown in vitro: preliminary characterization of the factor. Mol Reprod Dev 34(4): 450-456.

Erickson GF, Magoffin DA, Dyer CA, Hofeditz C. 1985. The ovarian androgen producing cells: a review of structure/function relationships. Endocr Rev 6(3): 371-399.

Ericsson S, Boice M, Funahashi H, Day B. 1993. Assessment of porcine oocytes using brilliant cresyl blue. Theriogenology 39(1): 214.

Espey LL. 1980. Ovulation as an inflammatory reaction--a hypothesis. Biol Reprod 22(1): 73-106.

Espey LL. 1994. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. Biol Reprod 50(2): 233-238.

Etheridge A, Lee I, Hood L, Galas D, Wang K. 2011. Extracellular microRNA: a new source of biomarkers. Mutat Res 717(1-2): 85-90.

Evans AC, Duffy P, Hynes N, Boland MP. 2000. Waves of follicle development during the estrous cycle in sheep. Theriogenology 53(3): 699-715.

Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D, Croce CM. 2012. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A 109(31): E2110-2116.

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. 1997. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. Mol Reprod Dev 46(2): 208-215.

Fair T, Hyttel P, Greve T. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 42(4): 437-442.

Fevrier B, Raposo G. 2004. Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 16(4): 415-421.

Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM, Ethier JF. 2002. Recruitment and development of the follicle; the roles of the transforming growth factor-beta superfamily. Mol Cell Endocrinol 191(1): 35-43.

Fisch B, Goldberg I, Ovadia J, Tadir Y. 1990. Physicochemical properties of follicular fluid and their relation to in vitro fertilization (IVF) outcome. J In Vitro Fert Embryo Transf 7(2): 67-73.

Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. 2008. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 15(2): 272-284.

Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, Regen T, Hanisch UK, Simons M. 2011. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci 124(Pt 3): 447-458.

Fleischhacker M, Schmidt B. 2010. Free circulating nucleic acids in plasma and serum (CNAPS) -- Useful for the detection of lung cancer patients? Cancer Biomark 6(3-4): 211-219.

Fortune JE. 2003. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim Reprod Sci 78(3-4): 135-163.

Fraser HM. 2006. Regulation of the ovarian follicular vasculature. Reprod Biol Endocrinol 4: 18.

Furman A, Rotmensch S, Dor J, Venter A, Mashiach S, Vlodavsky I, Amsterdam A. 1986a. Culture of human granulosa cells from an in vitro fertilization program: effects of extracellular matrix on morphology and cyclic adenosine 3',5' monophosphate production. Fertil Steril 46(3): 514-517.

Furman A, Rotmensch S, Kohen F, Mashiach S, Amsterdam A. 1986b. Regulation of rat granulosa cell differentiation by extracellular matrix produced by bovine corneal endothelial cells. Endocrinology 118(5): 1878-1885.

Gad A, Besenfelder U, Rings F, Ghanem N, Salilew-Wondim D, Hossain MM, Tesfaye D, Lonergan P, Becker A, Cinar U, Schellander K, Havlicek V, Holker M. 2011. Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction. Hum Reprod 26(7): 1693-1707.

Gallo A, Tandon M, Alevizos I, Illei GG. 2012. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7(3): e30679.

Gatti JL, Metayer S, Belghazi M, Dacheux F, Dacheux JL. 2005. Identification, proteomic profiling, and origin of ram epididymal fluid exosome-like vesicles. Biol Reprod 72(6): 1452-1465.

Gerard N, Loiseau S, Duchamp G, Seguin F. 2002. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 124(2): 241-248.

Ghanem N, Holker M, Rings F, Jennen D, Tholen E, Sirard MA, Torner H, Kanitz W, Schellander K, Tesfaye D. 2007. Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave. BMC Dev Biol 7: 90.

Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. 2009. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol 11(9): 1143-1149.

Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. 2008. Serum microRNAs are promising novel biomarkers. PLoS One 3(9): e3148.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. 1996. Selection of the dominant follicle in cattle. Biol Reprod 55(6): 1187-1194.

Gosden RG, Hunter RH, Telfer E, Torrance C, Brown N. 1988. Physiological factors underlying the formation of ovarian follicular fluid. J Reprod Fertil 82(2): 813-825.

Gougeon A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17(2):121-155.

Gougeon A. 2010. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. Ann Endocrinol (Paris) 71(3): 132-143.

Gould GW, Lippincott-Schwartz J. 2009. New roles for endosomes: from vesicular carriers to multi-purpose platforms. Nat Rev Mol Cell Biol 10(4): 287-292.

Gould SJ, Booth AM, Hildreth JE. 2003. The Trojan exosome hypothesis. Proc Natl Acad Sci U S A 100(19): 10592-10597.

Greve T, Callesen H, Hyttel P. 1989. Follicular correlates with in-vitro fertilization in cattle. J Reprod Fertil Suppl 38: 117-126.

Grinsted J, Blendstrup K, Andreasen MP, Byskov AG. 1980. Temperature measurements of rabbit antral follicles. J Reprod Fertil 60(1): 149-155.

Hanson PI, Shim S, Merrill SA. 2009. Cell biology of the ESCRT machinery. Curr Opin Cell Biol 21(4): 568-574.

Harter BT. 1948. Glycogen and carbohydrate-protein complexes in the ovary of the white rat during the oestrous cycle. Anat Rec 102(3): 349-367.

Harwood BN, Cross SK, Radford EE, Haac BE, De Vries WN. 2008. Members of the WNT signaling pathways are widely expressed in mouse ovaries, oocytes, and cleavage stage embryos. Dev Dyn 237(4): 1099-1111.

Hasselmann DO, Rappl G, Tilgen W, Reinhold U. 2001. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin Chem 47(8): 1488-1489.

Hausler SF, Keller A, Chandran PA, Ziegler K, Zipp K, Heuer S, Krockenberger M, Engel JB, Honig A, Scheffler M, Dietl J, Wischhusen J. 2010. Whole blood-derived miRNA profiles as potential new tools for ovarian cancer screening. Br J Cancer 103(5): 693-700.

Hawkins SM, Matzuk MM. 2010. Oocyte-somatic cell communication and microRNA function in the ovary. Ann Endocrinol (Paris) 71(3): 144-148.

Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. 1999. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood 94(11): 3791-3799.

Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. 2010. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg 251(3): 499-505.

Henne WM, Buchkovich NJ, Emr SD. 2011. The ESCRT pathway. Dev Cell 21(1): 77-91.

Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T, Urbich C, Boon RA, Dimmeler S. 2012. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat Cell Biol 14(3): 249-256.

Herlands RL, Schultz RM. 1984. Regulation of mouse oocyte growth: probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. J Exp Zool 229(2): 317-325.

Hillier SG, van den Boogaard AM, Reichert LE, Jr., van Hall EV. 1980. Intraovarian sex steroid hormone interactions and the regulation of follicular maturation: aromatization of androgens by human granulosa cells in vitro. J Clin Endocrinol Metab 50(4): 640-647.

Hirshfield AN. 1989. Granulosa cell proliferation in very small follicles of cycling rats studied by long-term continuous tritiated-thymidine infusion. Biol Reprod 41(2): 309-316.

Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewith K, Le QT, Koong AC. 2010. Circulating miR-210 as a Novel Hypoxia Marker in Pancreatic Cancer. Transl Oncol 3(2): 109-113.

Hossain MM, Ghanem N, Hoelker M, Rings F, Phatsara C, Tholen E, Schellander K, Tesfaye D. 2009. Identification and characterization of miRNAs expressed in the bovine ovary. BMC Genomics 10: 443.

Hossain MM, Sohel MM, Schellander K, Tesfaye D. 2012. Characterization and importance of microRNAs in mammalian gonadal functions. Cell Tissue Res 349(3): 679-690.

Hristov M, Erl W, Linder S, Weber PC. 2004. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. Blood 104(9): 2761-2766.

Huang J, Ju Z, Li Q, Hou Q, Wang C, Li J, Li R, Wang L, Sun T, Hang S, Gao Y, Hou M, Zhong J. 2011. Solexa sequencing of novel and differentially expressed microRNAs in testicular and ovarian tissues in Holstein cattle. Int J Biol Sci 7(7): 1016-1026.

Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, Zaccheddu A, Colone M, Arancia G, Gentile M, Seregni E, Valenti R, Ballabio G, Belli F, Leo E, Parmiani G, Rivoltini L. 2005. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology 128(7): 1796-1804.

Hunter MG, Robinson RS, Mann GE, Webb R. 2004. Endocrine and paracrine control of follicular development and ovulation rate in farm species. Anim Reprod Sci 82-83: 461-477.

Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB. 2008. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 3(11): e3694.

Huo LJ, Fan HY, Zhong ZS, Chen DY, Schatten H, Sun QY. 2004. Ubiquitin-proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation. Mech Dev 121(10): 1275-1287.

Ireland JJ, Roche JF. 1983. Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: changes in concentration of hormones in follicular fluid and specific binding of gonadotropins to follicles. J Anim Sci 57(1): 157-167.

Irving-Rodgers HF, Rodgers RJ. 2000. Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa. J Reprod Fertil 118(2): 221-228.

Irving-Rodgers HF, Rodgers RJ. 2006. Extracellular matrix of the developing ovarian follicle. Semin Reprod Med 24(4): 195-203.

Irving-Rodgers HF, Morris S, Collett RA, Peura TT, Davy M, Thompson JG, Mason HD, Rodgers RJ. 2009. Phenotypes of the ovarian follicular basal lamina predict developmental competence of oocytes. Hum Reprod 24(4): 936-944.

Itahana K, Morikazu Y, Takeya T. 1996. Differential expression of four connexin genes, Cx-26, Cx-30.3, Cx-32, and Cx-43, in the porcine ovarian follicle. Endocrinology 137(11): 5036-5044.

Jaiswal R, Gong J, Sambasivam S, Combes V, Mathys JM, Davey R, Grau GE, Bebawy M. 2011. Microparticle-associated nucleic acids mediate trait dominance in cancer. FASEB J 26 (1): 420-429.

Johnson ML, Redmer DA, Reynolds LP, Grazul-Bilska AT. 1999. Expression of gap junctional proteins connexin 43, 32, and 26 throughout follicular development and atresia in cows. Endocrine 10(1): 43-51.

Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428(6979): 145-150.

Johnstone RM. 2006. Exosomes biological significance: A concise review. Blood Cells Mol Dis 36(2): 315-321.

Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 262(19): 9412-9420.

Juneja SC, Barr KJ, Enders GC, Kidder GM. 1999. Defects in the germ line and gonads of mice lacking connexin43. Biol Reprod 60(5): 1263-1270.

Kastelic JP, Ko JC, Ginther OJ. 1990. Suppression of dominant and subordinate ovarian follicles by a proteinaceous fraction of follicular fluid in heifers. Theriogenology 34(3): 499-509.

Katzmann DJ, Stefan CJ, Babst M, Emr SD. 2003. Vps27 recruits ESCRT machinery to endosomes during MVB sorting. J Cell Biol 162(3): 413-423.

Khamsi F, Roberge S. 2001. Granulosa cells of the cumulus oophorus are different from mural granulosa cells in their response to gonadotrophins and IGF-I. J Endocrinol 170(3): 565-573.

King HW, Michael MZ, Gleadle JM. 2012. Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer 12: 421.

Knight PG, Glister C. 2006. TGF-beta superfamily members and ovarian follicle development. Reproduction 132(2): 191-206.

Kogure T, Lin WL, Yan IK, Braconi C, Patel T. 2011. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology 54(4): 1237-1248.

Koh W, Sheng CT, Tan B, Lee QY, Kuznetsov V, Kiang LS, Tanavde V. 2010. Analysis of deep sequencing microRNA expression profile from human embryonic stem cells derived mesenchymal stem cells reveals possible role of let-7 microRNA family in downstream targeting of hepatic nuclear factor 4 alpha. BMC Genomics 11 (Suppl 1): S6.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. 2010. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 285(23): 17442-17452.

Krisher RL. 2004. The effect of oocyte quality on development. J Anim Sci 82 (E-Suppl): E14-23.

Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. 2010. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 4(3): 214-222.

Lakkaraju A, Rodriguez-Boulan E. 2008. Itinerant exosomes: emerging roles in cell and tissue polarity. Trends Cell Biol 18(5): 199-209.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129(7): 1401-1414.

Lange-Consiglio A, Arrighi S, Fiandanese N, Pocar P, Aralla M, Bosi G, Borromeo V, Berrini A, Meucci A, Dell'aquila ME, Cremonesi F. 2012. Follicular fluid leptin concentrations and expression of leptin and leptin receptor in the equine ovary and in vitro-matured oocyte with reference to pubertal development and breeds. Reprod Fertil Dev 25: 837-846.

Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, Tanaka WK, Johnson JM, Sina JF, Fare TL, Sistare FD, Glaab WE. 2009. Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem 55(11): 1977-1983.

Laulagnier K, Motta C, Hamdi S, Roy S, Fauvelle F, Pageaux JF, Kobayashi T, Salles JP, Perret B, Bonnerot C, Record M. 2004. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. Biochem J 380(Pt 1): 161-171.

Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, Hatton CS, Harris AL. 2008. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141(5): 672-675.

Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, Baumgart J, Trimbuch T, Eom G, Hinz M, Kaul D, Habbel P, Kalin R, Franzoni E, Rybak A, Nguyen D, Veh R, Ninnemann O, Peters O, Nitsch R, Heppner FL, Golenbock D, Schott E, Ploegh HL, Wulczyn FG, Lehnardt S. 2012. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 15(6): 827-835.

Lei L, Jin S, Gonzalez G, Behringer RR, Woodruff TK. 2010. The regulatory role of Dicer in folliculogenesis in mice. Mol Cell Endocrinol 315(1-2): 63-73.

Lenz RW, Ax RL, Grimek HJ, First NL. 1982. Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. Biochem Biophys Res Commun 106(4): 1092-1098.

Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. 1977. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 37(3): 646-650.

Lewis GD, Metcalf TG. 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl Environ Microbiol 54(8): 1983-1988.

Li M, Liu Y, Wang T, Guan J, Luo Z, Chen H, Wang X, Chen L, Ma J, Mu Z, Jiang AA, Zhu L, Lang Q, Zhou X, Wang J, Zeng W, Li N, Li K, Gao X, Li X. 2011. Repertoire of porcine microRNAs in adult ovary and testis by deep sequencing. Int J Biol Sci 7(7): 1045-1055.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433(7027): 769-773.

Lin F, Li R, Pan ZX, Zhou B, Yu de B, Wang XG, Ma XS, Han J, Shen M, Liu HL. 2012. miR-26b promotes granulosa cell apoptosis by targeting ATM during follicular atresia in porcine ovary. PLoS One 7(6): e38640.

Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, Perdicchio M, Marino ML, Federici C, Iessi E, Brambilla D, Venturi G, Lozupone F, Santinami M, Huber V, Maio M, Rivoltini L, Fais S. 2009. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One 4(4): e5219.

Luck MR, Griffiths S, Gregson K, Watson E, Nutley M, Cooper A. 2001. Follicular fluid responds endothermically to aqueous dilution. Hum Reprod 16(12): 2508-2514.

Luo S, Kleemann GA, Ashraf JM, Shaw WM, Murphy CT. 2010. TGF-beta and insulin signaling regulate reproductive aging via oocyte and germline quality maintenance. Cell 143(2): 299-312.

Luo SS, Ishibashi O, Ishikawa G, Ishikawa T, Katayama A, Mishima T, Takizawa T, Shigihara T, Goto T, Izumi A, Ohkuchi A, Matsubara S, Takeshita T. 2009. Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. Biol Reprod 81(4): 717-729.

Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, Plachy J, Stangassinger M, Erfle V, Schlondorff D. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med 6(7): 769-775.

Mahi-Brown CA, Yanagimachi R. 1983. Parameters influencing ovum pickup by oviductal fimbria in the golden hamster. Gamete Research 8(1): 1-10.

Mangia F, Epstein CJ. 1975. Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. Dev Biol 45(2): 211-220.

Mark AC, Michael PM. 2013. Regulation and function of gonadotropins throughout the bovine oestrous Cycle. Gonadotropin Chap 7: 143-154.

Marzesco AM, Janich P, Wilsch-Brauninger M, Dubreuil V, Langenfeld K, Corbeil D, Huttner WB. 2005. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. J Cell Sci 118(13): 2849-2858.

Mathivanan S, Simpson RJ. 2009. ExoCarta: A compendium of exosomal proteins and RNA. Proteomics 9(21): 4997-5000.

Mause SF, Weber C. 2010. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res 107(9): 1047-1057.

Maxfield FR, McGraw TE. 2004. Endocytic recycling. Nat Rev Mol Cell Biol 5(2): 121-132.

Mayor S, Riezman H. 2004. Sorting GPI-anchored proteins. Nat Rev Mol Cell Biol 5(2): 110-120.

McBride D, Carre W, Sontakke SD, Hogg CO, Law A, Donadeu FX, Clinton M. 2012. Identification of miRNAs associated with the follicular-luteal transition in the ruminant ovary. Reproduction 144(2): 221-233.

McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. 2011. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem 57(6): 833-840.

McNatty KP, Baird DT. 1978. Relationship between follicle-stimulating hormone, androstenedione and oestradiol in human follicular fluid. J Endocrinol 76(3): 527-531.

Menezo Y, Pluntz L, Chouteau J, Gurgan T, Demirol A, Dalleac A, Benkhalifa M. 2011. Zinc concentrations in serum and follicular fluid during ovarian stimulation and expression of Zn2+ transporters in human oocytes and cumulus cells. Reprod Biomed Online 22(6): 647-652.

Mishima T, Takizawa T, Luo SS, Ishibashi O, Kawahigashi Y, Mizuguchi Y, Ishikawa T, Mori M, Kanda T, Goto T. 2008. MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. Reproduction 136(6): 811-822.

Mitchell PA, Burghardt RC. 1986. The ontogeny of nexuses (gap junctions) in the ovary of the fetal mouse. Anat Rec 214(3): 283-288.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 105(30): 10513-10518.

Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, Bernad A, Sanchez-Madrid F. 2011. Unidirectional transfer of

microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun 2: 282.

Mohammadi-Sangcheshmeh A, Soleimani M, Deldar H, Salehi M, Soudi S, Hashemi SM, Schellander K, Hoelker M. 2012. Prediction of oocyte developmental competence in ovine using glucose-6-phosphate dehydrogenase (G6PDH) activity determined at retrieval time. J Assist Reprod Genet 29(2): 153-158.

Moley KH, Schreiber JR. 1995. Ovarian follicular growth, ovulation and atresia. Endocrine, paracrine and autocrine regulation. Adv Exp Med Biol 377: 103-119.

Monget P, Fabre S, Mulsant P, Lecerf F, Elsen JM, Mazerbourg S, Pisselet C, Monniaux D. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest Anim Endocrinol 23(1-2):139-154.

Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. 2012. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119(3): 756-766.

Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB, Papworth GD, Zahorchak AF, Logar AJ, Wang Z, Watkins SC, Falo LD, Jr., Thomson AW. 2004. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood 104(10): 3257-3266.

Mortimer D, Camenzind AR. 1989. The role of follicular fluid in inducing the acrosome reaction of human spermatozoa incubated in vitro. Hum Reprod 4(2): 169-174.

Muntasell A, Berger AC, Roche PA. 2007. T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. EMBO J 26(19): 4263-4272.

Naslund TI, Gehrmann U, Qazi KR, Karlsson MC, Gabrielsson S. 2013. Dendritic cell-derived exosomes need to activate both T and B cells to induce antitumor immunity. J Immunol 190(6): 2712-2719.

Nazarenko I, Rana S, Baumann A, McAlear J, Hellwig A, Trendelenburg M, Lochnit G, Preissner KT, Zoller M. 2010. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. Cancer Res 70(4): 1668-1678.

Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ. 2009. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 58(10): 1375-1381.

Nguyen DG, Booth A, Gould SJ, Hildreth JE. 2003. Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. J Biol Chem 278(52): 52347-52354.

Nussey S, Whitehead S. 2001. Endocrinology: An Integrated Approach. St. George's Hospital Medical School, Oxford, London.

Nuttinck F, Peynot N, Humblot P, Massip A, Dessy F, Flechon JE. 2000. Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary. Mol Reprod Dev 57(1): 60-66.

O'Shea JD. 1981. Structure-function relationships in the wall of the ovarian follicle. Aust J Biol Sci 34(4): 379-394.

Pakarainen T, Zhang FP, Nurmi L, Poutanen M, Huhtaniemi I. 2005. Knockout of luteinizing hormone receptor abolishes the effects of follicle-stimulating hormone on preovulatory maturation and ovulation of mouse graafian follicles. Mol Endocrinol 19(10): 2591-2602.

Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT. 2010. Nanostructural and transcriptomic analyses of human saliva derived exosomes. PLoS One 5(1): e8577.

Palma GA, Arganaraz ME, Barrera AD, Rodler D, Mutto AA, Sinowatz F. 2012. Biology and biotechnology of follicle development. ScientificWorldJournal 2012: 938138.

Pan BT, Teng K, Wu C, Adam M, Johnstone RM. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol 101(3): 942-948.

Pan Q, Ramakrishnaiah V, Henry S, Fouraschen S, de Ruiter PE, Kwekkeboom J, Tilanus HW, Janssen HL, van der Laan LJ. 2012. Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi). Gut 61(9): 1330-1339.

Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT. 2009. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. Clin Cancer Res 15(17): 5473-5477.

Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C, Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S. 2009. Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem 284(49): 34211-34222.

Parrott JA, Skinner MK. 1998a. Developmental and hormonal regulation of hepatocyte growth factor expression and action in the bovine ovarian follicle. Biol Reprod 59(3): 553-560.

Parrott JA, Skinner MK. 1998b. Developmental and hormonal regulation of keratinocyte growth factor expression and action in the ovarian follicle. Endocrinology 139(1): 228-235.

Parrott JA, Skinner MK. 1998c. Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and Kit ligand during ovarian follicular development. Endocrinology 139(5): 2240-2245.

Parrott JA, Vigne JL, Chu BZ, Skinner MK. 1994. Mesenchymal-epithelial interactions in the ovarian follicle involve keratinocyte and hepatocyte growth factor production by thecal cells and their action on granulosa cells. Endocrinology 135(2): 569-575.

Patsoula E, Loutradis D, Drakakis P, Kallianidis K, Bletsa R, Michalas S. 2001. Expression of mRNA for the LH and FSH receptors in mouse oocytes and preimplantation embryos. Reproduction 121(3): 455-461.

Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Wurdinger T, Middeldorp JM. 2010. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 107(14): 6328-6333.

Pelchen-Matthews A, Kramer B, Marsh M. 2003. Infectious HIV-1 assembles in late endosomes in primary macrophages. J Cell Biol 162(3): 443-455.

Pierson RA, Ginther OJ. 1984. Ultrasonography of the bovine ovary. Theriogenology 21(3): 495-504.

Pierson RA, Ginther OJ. 1986. Ovarian follicular populations during early pregnancy in heifers. Theriogenology 26(5): 649-659.

Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM. 2010. Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS One 5(10): e13515.

Pincus G, Enzmann EV. 1935. The comparative behavior of mammalian eggs in vivo and in vitro : I. The activation of ovarian eggs. J Exp Med 62(5): 665-675.

Pisitkun T, Shen RF, Knepper MA. 2004. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A 101(36): 13368-13373.

Pohler KG, Geary TW, Atkins JA, Perry GA, Jinks EM, Smith MF. 2012. Follicular determinants of pregnancy establishment and maintenance. Cell Tissue Res 349(3): 649-664.

Prado N, Marazuela EG, Segura E, Fernandez-Garcia H, Villalba M, Thery C, Rodriguez R, Batanero E. 2008. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. J Immunol 181(2): 1519-1525.

Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M. 2011. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila) 5(3): 492-497.

Pujol M, Lopez-Bejar M, Paramio MT. 2004. Developmental competence of heifer oocytes selected using the brilliant cresyl blue (BCB) test. Theriogenology 61(4): 735-744.

Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. 2009. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer 10(1): 42-46.

Rajakoski E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, end left-right variations. Acta Endocrinol Suppl (Copenh) 34(Suppl 52): 1-68.

Ranta T, Knecht M, Baukal AJ, Korhonen M, Catt KJ. 1984. GnRH agonist-induced inhibitory and stimulatory effects during ovarian follicular maturation. Mol Cell Endocrinol 35(1): 55-63.

Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. 1996. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 183(3): 1161-1172.

Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. 1997. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol Biol Cell 8(12): 2631-2645.

Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. 2006a. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20(5): 847-856.

Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. 2006b. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia 20(9): 1487-1495.

Rathi R, Colenbrander B, Stout TA, Bevers MM, Gadella BM. 2003. Progesterone induces acrosome reaction in stallion spermatozoa via a protein tyrosine kinase dependent pathway. Mol Reprod Dev 64(1): 120-128.

Rechavi O, Erlich Y, Amram H, Flomenblit L, Karginov FV, Goldstein I, Hannon GJ, Kloog Y. 2009. Cell contact-dependent acquisition of cellular and viral nonautonomously encoded small RNAs. Genes Dev 23(16): 1971-1979.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 7: 40.

Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M, Norman RJ. 2009. Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. J Clin Endocrinol Metab 94(5): 1533-1540.

Rodgers RJ, Irving-Rodgers HF. 2010a. Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod 82(6): 1021-1029.

Rodgers RJ, Irving-Rodgers HF. 2010b. Morphological classification of bovine ovarian follicles. Reproduction 139(2): 309-318.

Rodriguez-Gonzalez E, Lopez-Bejar M, Velilla E, Paramio MT. 2002. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 57(5): 1397-1409.

Romero-Arredondo A, Seidel GE, Jr. 1996. Effects of follicular fluid during in vitro maturation of bovine oocytes on in vitro fertilization and early embryonic development. Biol Reprod 55(5): 1012-1016.

Salustri A, Yanagishita M, Hascall VC. 1990. Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. Dev Biol 138(1): 26-32.

Saunderson SC, Schuberth PC, Dunn AC, Miller L, Hock BD, MacKay PA, Koch N, Jack RW, McLellan AD. 2008. Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor. J Immunol 180(12): 8146-8152.

Savina A, Fader CM, Damiani MT, Colombo MI. 2005. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. Traffic 6(2): 131-143.

Savina A, Furlan M, Vidal M, Colombo MI. 2003. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. J Biol Chem 278(22): 20083-20090.

Savio JD, Keenan L, Boland MP, Roche JF. 1988. Pattern of growth of dominant follicles during the oestrous cycle of heifers. J Reprod Fertil 83(2): 663-671.

Schrauder MG, Strick R, Schulz-Wendtland R, Strissel PL, Kahmann L, Loehberg CR, Lux MP, Jud SM, Hartmann A, Hein A, Bayer CM, Bani MR, Richter S, Adamietz BR, Wenkel E, Rauh C, Beckmann MW, Fasching PA. 2012. Circulating micro-RNAs as potential blood-based markers for early stage breast cancer detection. PLoS One 7(1): e29770.

Schwarz T, Kopyra M, Nowicki J. 2008. Physiological mechanisms of ovarian follicular growth in pigs--a review. Acta Vet Hung 56(3): 369-378.

Seekallu SV, Toosi BM, Grazul-Bilska AT, Rawlings NC. 2010. Markers of ovarian antral follicular development in sheep: comparison of follicles destined to ovulate from the final or penultimate follicular wave of the estrous cycle. Reproduction 140(4): 559-568.

Segura E, Guerin C, Hogg N, Amigorena S, Thery C. 2007. CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. J Immunol 179(3): 1489-1496.

Shalgi R, Kraicer PF, Soferman N. 1972. Gases and electrolytes of human follicular fluid. J Reprod Fertil 28(3): 335-340.

Shantsila E, Kamphuisen PW, Lip GY. 2010. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. J Thromb Haemost 8(11): 2358-2368.

Shefler I, Salamon P, Reshef T, Mor A, Mekori YA. 2010. T cell-induced mast cell activation: a role for microparticles released from activated T cells. J Immunol 185(7): 4206-4212.

Shemesh M. 1979. Inhibitory action of follicular fluid on progesterone and prostaglandin synthesis in bovine follicles. J Endocrinol 82(1): 27-31.

Shen J, Todd NW, Zhang H, Yu L, Lingxiao X, Mei Y, Guarnera M, Liao J, Chou A, Lu CL, Jiang Z, Fang H, Katz RL, Jiang F. 2010. Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. Lab Invest 91(4): 579-587.

Shet AS. 2008. Characterizing blood microparticles: technical aspects and challenges. Vasc Health Risk Manag 4(4): 769-774.

Shigoka M, Tsuchida A, Matsudo T, Nagakawa Y, Saito H, Suzuki Y, Aoki T, Murakami Y, Toyoda H, Kumada T, Bartenschlager R, Kato N, Ikeda M, Takashina T, Tanaka M, Suzuki R, Oikawa K, Takanashi M, Kuroda M. 2010. Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. Pathol Int 60(5): 351-357.

Silberstein T, Saphier O, Paz-Tal O, Gonzalez L, Keefe DL, Trimarchi JR. 2009. Trace element concentrations in follicular fluid of small follicles differ from those in blood serum, and may represent long-term exposure. Fertil Steril 91(5): 1771-1774.

Silva DS, Rodriguez P, Galuppo A, Arruda NS, Rodrigues JL. 2011. Selection of bovine oocytes by brilliant cresyl blue staining: effect on meiosis progression, organelle distribution and embryo development. Zygote 21 (3): 1-6.

Simon AM, Goodenough DA, Li E, Paul DL. 1997. Female infertility in mice lacking connexin 37. Nature 385(6616): 525-529.

Simons M, Raposo G. 2009. Exosomes-vesicular carriers for intercellular communication. Curr Opin Cell Biol 21(4): 575-581.

Simpson RJ, Lim JW, Moritz RL, Mathivanan S. 2009. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics 6(3): 267-283.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. Theriogenology 65(1): 126-136.

Sirois J, Fortune JE. 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 39(2): 308-317.

Sisco KL. 2001. Is RNA in serum bound to nucleoprotein complexes? Clin Chem 47(9): 1744-1745.

Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Jr., Carter BS, Krichevsky AM, Breakefield XO. 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 10(12): 1470-1476.

Spicer LJ, Convey EM, Leung K, Short RE, Tucker HA. 1986. Anovulation in postpartum suckled beef cows. II. Associations among binding of 125I-labeled gonadotropins to granulosa and thecal cells, and concentrations of steroids in serum and various sized ovarian follicles. J Anim Sci 62(3): 742-750.

Srivastava A, Suy S, Collins SP, Kumar D. 2011. Circulating MicroRNA as Biomarkers: An Update in Prostate Cancer. Mol Cell Pharmacol 3(3): 115-124.

Stoorvogel W. 2012. Functional transfer of microRNA by exosomes. Blood 119(3): 646-648.

Su YQ, Wu X, O'Brien MJ, Pendola FL, Denegre JN, Matzuk MM, Eppig JJ. 2004. Synergistic roles of BMP15 and GDF9 in the development and function of the

oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. Dev Biol 276(1): 64-73.

Subra C, Grand D, Laulagnier K, Stella A, Lambeau G, Paillasse M, De Medina P, Monsarrat B, Perret B, Silvente-Poirot S, Poirot M, Record M. 2010. Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins. J Lipid Res 51(8): 2105-2120.

Subra C, Laulagnier K, Perret B, Record M. 2007. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie 89(2): 205-212.

Suzumori N, Burns KH, Yan W, Matzuk MM. 2003. RFPL4 interacts with oocyte proteins of the ubiquitin-proteasome degradation pathway. Proc Natl Acad Sci U S A 100(2): 550-555.

Swain JE, Pool TB. 2008. ART failure: oocyte contributions to unsuccessful fertilization. Hum Reprod Update 14(5): 431-446.

Tanaka M, Oikawa K, Takanashi M, Kudo M, Ohyashiki J, Ohyashiki K, Kuroda M. 2009. Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PLoS One 4(5):e5532.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev 61(3): 414-424.

Tatemoto H, Horiuchi T. 1995. Requirement for protein synthesis during the onset of meiosis in bovine oocytes and its involvement in the autocatalytic amplification of maturation-promoting factor. Mol Reprod Dev 41(1): 47-53.

Taylor DD, Akyol S, Gercel-Taylor C. 2006. Pregnancy-associated exosomes and their modulation of T cell signaling. J Immunol 176(3): 1534-1542.

Taylor DD, Gercel-Taylor C. 2008. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110(1): 13-21.

Taylor DD, Homesley HD, Doellgast GJ. 1983. "Membrane-associated" immunoglobulins in cyst and ascites fluids of ovarian cancer patients. Am J Reprod Immunol 3(1): 7-11.

Telfer EE. 2004. Germline stem cells in the postnatal mammalian ovary: a phenomenon of prosimian primates and mice? Reprod Biol Endocrinol 2: 24.

Teo H, Veprintsev DB, Williams RL. 2004. Structural insights into endosomal sorting complex required for transport (ESCRT-I) recognition of ubiquitinated proteins. J Biol Chem 279(27): 28689-28696.

Tesfaye D, Worku D, Rings F, Phatsara C, Tholen E, Schellander K, Hoelker M. 2009. Identification and expression profiling of microRNAs during bovine oocyte maturation using heterologous approach. Mol Reprod Dev 76(7): 665-677.

Thery C. 2011. Exosomes: secreted vesicles and intercellular communications. F1000 Biol Rep 3: 15.

Thery C, Amigorena S, Raposo G, Clayton A. 2006. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 3(3): 22.

Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol 166(12): 7309-7318.

Thery C, Zitvogel L, Amigorena S. 2002. Exosomes: composition, biogenesis and function. Nat Rev Immunol 2(8): 569-579.

Tian T, Wang Y, Wang H, Zhu Z, Xiao Z. 2010. Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. J Cell Biochem 111(2): 488-496.

Tijsen AJ, Pinto YM, Creemers EE. 2012. Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases. Am J Physiol Heart Circ Physiol 303(9): H1085-1095.

Tilly JL, Rueda BR. 2008. Minireview: stem cell contribution to ovarian development, function, and disease. Endocrinology 149(9): 4307-4311.

Tomari Y, Zamore PD. 2005. Perspective: machines for RNAi. Genes Dev 19(5): 517-529.

Tomasoni S, Longaretti L, Rota C, Morigi M, Conti S, Gotti E, Capelli C, Introna M, Remuzzi G, Benigni A. 2012. Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. Stem Cells Dev 22(5): 772-780.

Tomic D, Miller KP, Kenny HA, Woodruff TK, Hoyer P, Flaws JA. 2004. Ovarian follicle development requires Smad3. Mol Endocrinol 18(9): 2224-2240.

Torner H, Ghanem N, Ambros C, Holker M, Tomek W, Phatsara C, Alm H, Sirard MA, Kanitz W, Schellander K, Tesfaye D. 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. Reproduction 135(2): 197-212.

Torri AF, Hajduk SL. 1988. Posttranscriptional regulation of cytochrome c expression during the developmental cycle of Trypanosoma brucei. Mol Cell Biol 8(11): 4625-4633.

Tripurani SK, Xiao C, Salem M, Yao J. 2010. Cloning and analysis of fetal ovary microRNAs in cattle. Anim Reprod Sci 120(1-4): 16-22.

Tsai SJ, Wiltbank MC. 2001. Differential effects of prostaglandin F2alpha on in vitro luteinized bovine granulosa cells. Reproduction 122(2): 245-253.

Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, Konishi H, Morimura R, Deguchi K, Fujiwara H, Okamoto K, Otsuji E. 2010. Circulating

microRNAs in plasma of patients with gastric cancers. Br J Cancer 102(7): 1174-1179.

Turchinovich A, Weiz L, Langheinz A, Burwinkel B. 2011. Characterization of extracellular circulating microRNA. Nucleic Acids Res 39(16): 7223-7233.

Urbe S, Sachse M, Row PE, Preisinger C, Barr FA, Strous G, Klumperman J, Clague MJ. 2003. The UIM domain of Hrs couples receptor sorting to vesicle formation. J Cell Sci 116(Pt 20): 4169-4179.

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9(6): 654-659.

Vanderhyden BC, Caron PJ, Buccione R, Eppig JJ. 1990. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. Dev Biol 140(2): 307-317.

van Dessel HJ, Schipper I, Pache TD, van Geldorp H, de Jong FH, Fauser BC. 1996. Normal human follicle development: an evaluation of correlations with oestradiol, androstenedione and progesterone levels in individual follicles. Clin Endocrinol (Oxf) 44(2): 191-198.

van Niel G, Porto-Carreiro I, Simoes S, Raposo G. 2006. Exosomes: a common pathway for a specialized function. J Biochem 140(1): 13-21.

van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ, Jr., Olson EN. 2009. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell 17(5): 662-673.

van Wezel IL, Rodgers RJ. 1996. Morphological characterization of bovine primordial follicles and their environment in vivo. Biol Reprod 55(5): 1003-1011.

Vasilatou D, Papageorgiou S, Pappa V, Papageorgiou E, Dervenoulas J. 2010. The role of microRNAs in normal and malignant hematopoiesis. Eur J Haematol 84(1): 1-16.

Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 13(4): 423-433.

Vlassov AV, Magdaleno S, Setterquist R, Conrad R. 2012. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim Biophys Acta 1820(7): 940-948.

Vozzi C, Formenton A, Chanson A, Senn A, Sahli R, Shaw P, Nicod P, Germond M, Haefliger JA. 2001. Involvement of connexin 43 in meiotic maturation of bovine oocytes. Reproduction 122(4): 619-628.

Wakim LM, Bevan MJ. 2011. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. Nature 471(7340): 629-632.

Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q. 2010b. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J 31(6): 659-666.

Wang J, Chen J, Chang P, LeBlanc A, Li D, Abbruzzesse JL, Frazier ML, Killary AM, Sen S. 2009a. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. Cancer Prev Res (Phila) 2(9): 807-813.

Wang K, Zhang S, Weber J, Baxter D, Galas DJ. 2010c. Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res 38(20): 7248-7259.

Wang L, Lin J, Huang J, Wang J, Zhao Y, Chen T. 2012. Selection of ovine oocytes by brilliant cresyl blue staining. J Biomed Biotechnol 2012: 161372.

Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. 2008. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 15(2): 261-271.

Webb R, Gong JG, Law AS, Rusbridge SM. 1992. Control of ovarian function in cattle. J Reprod Fertil Suppl 45: 141-156.

Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. 2010. The microRNA spectrum in 12 body fluids. Clin Chem 56(11): 1733-1741.

Williams RL, Urbe S. 2007. The emerging shape of the ESCRT machinery. Nat Rev Mol Cell Biol 8(5): 355-368.

Wise T. 1987. Biochemical analysis of bovine follicular fluid: albumin, total protein, lysosomal enzymes, ions, steroids and ascorbic acid content in relation to follicular size, rank, atresia classification and day of estrous cycle. J Anim Sci 64(4): 1153-1169.

Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. 2008. Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue. Clin Cancer Res 14(9): 2588-2592.

Wright CS, Becker DL, Lin JS, Warner AE, Hardy K. 2001. Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation. Reproduction 121(1): 77-88.

Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem 278(13): 10963-10972.

Xiang X, Liu Y, Zhuang X, Zhang S, Michalek S, Taylor DD, Grizzle W, Zhang HG. 2010. TLR2-mediated expansion of MDSCs is dependent on the source of tumor exosomes. Am J Pathol 177(4): 1606-1610.

Xu S, Linher-Melville K, Yang BB, Wu D, Li J. 2011. Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatase. Endocrinology 152(10): 3941-3951.

Yamamoto Y, Kosaka N, Tanaka M, Koizumi F, Kanai Y, Mizutani T, Murakami Y, Kuroda M, Miyajima A, Kato T, Ochiya T. 2009. MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. Biomarkers 14(7): 529-538.

Yamaura Y, Nakajima M, Takagi S, Fukami T, Tsuneyama K, Yokoi T. 2012. Plasma microRNA profiles in rat models of hepatocellular injury, cholestasis, and steatosis. PLoS One 7(2): e30250.

Yang M, Chen J, Su F, Yu B, Lin L, Liu Y, Huang JD, Song E. 2011. Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells. Mol Cancer 10: 117.

Yang X, Zhou Y, Peng S, Wu L, Lin HY, Wang S, Wang H. 2012. Differentially expressed plasma microRNAs in premature ovarian failure patients and the potential regulatory function of mir-23a in granulosa cell apoptosis. Reproduction 144(2): 235-244.

Yao N, Lu CL, Zhao JJ, Xia HF, Sun DG, Shi XQ, Wang C, Li D, Cui Y, Ma X. 2009. A network of miRNAs expressed in the ovary are regulated by FSH. Front Biosci 14: 3239-3245.

Yao G, Yin M, Lian J, Tian H, Liu L, Li X, Sun F. 2010. MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. Mol Endocrinol 24(3): 540-551.

Yin M, Lu M, Yao G, Tian H, Lian J, Liu L, Liang M, Wang Y, Sun F. 2012. Transactivation of microRNA-383 by steroidogenic factor-1 promotes estradiol release from mouse ovarian granulosa cells by targeting RBMS1. Mol Endocrinol 26(7): 1129-1143.

Yuan A, Farber EL, Rapoport AL, Tejada D, Deniskin R, Akhmedov NB, Farber DB. 2009. Transfer of microRNAs by embryonic stem cell microvesicles. PLoS One 4(3): e4722.

Yurchenco PD, Schittny JC. 1990. Molecular architecture of basement membranes. FASEB J 4(6): 1577-1590.

Yu X, Harris SL, Levine AJ. 2006. The regulation of exosome secretion: a novel function of the p53 protein. Cancer Res 66(9): 4795-4801.

Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C. 2009. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2(100): ra81.

Zhang M, Ouyang H, Xia G. 2009. The signal pathway of gonadotrophins-induced mammalian oocyte meiotic resumption. Mol Hum Reprod 15(7): 399-409.

Zhang Y, Jia Y, Zheng R, Guo Y, Wang Y, Guo H, Fei M, Sun S. 2010. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. Clin Chem 56(12): 1830-1838.

Zheng P, Vassena R, Latham K. 2006. Expression and downregulation of WNT signaling pathway genes in rhesus monkey oocytes and embryos. Mol Reprod Dev 73(6): 667-677.

Zhou H, Ohno N, Terada N, Saitoh S, Fujii Y, Ohno S. 2007. Involvement of follicular basement membrane and vascular endothelium in blood follicle barrier formation of mice revealed by 'in vivo cryotechnique'. Reproduction 134(2): 307-317.

Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, Zhou X, Gao X, Li X. 2012. Immune-related microRNAs are abundant in breast milk exosomes. Int J Biol Sci 8(1): 118-123.

Zhu W, Qin W, Atasoy U, Sauter ER. 2009. Circulating microRNAs in breast cancer and healthy subjects. BMC Res Notes 2: 89.

Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med 4(5): 594-600.

Zomer A, Vendrig T, Hopmans ES, van Eijndhoven M, Middeldorp JM, Pegtel DM. 2010. Exosomes: Fit to deliver small RNA. Commun Integr Biol 3(5): 447-450.

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