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Expression analysis of regulatory MicroRNA in bovine cumulus oocyte complex and preimplantation embryos

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Expressionsanalyse von regulierenden MircoRNA im bovinen Kumulus-Oocyten Komplex und präimplantierten Embryonen

MicroRNAs (miRNAs) sind kleine nicht-kodierende endogene Moleküle, die an der post-trankriptionellen Genregulierung beteiligt sind. Diese kleinen RNAs sind an einer Reihe von biologischen Prozessen, wie Entwicklung, Zelldifferenzierung, Zellproliferation und Apoptose in verschiedenen Organismen, involviert. Jedoch ist bisher nur wenig über ihre Rolle in bovinen zellen und umgebenden Kumuluszellen während der Eizellenentwicklung bekannt. Um dieses aufzuklären, untersuchten wir die relativen Expressionsmuster zwischen bovinen Eizellen und den umgebenden Kumuluszellen während der *in vitro* Maturation. Die Expressionsmuster eines Sets von 88 miRNAs wurden mittels eines miRNA PCR Arrays für humane Zellentwicklung und-differenzierung erfasst. Die Ergebnisse zeigten, dass insgesamt 47 bzw. 51 miRNAs unterschiedlich zwischen immaturen (GV) bzw. reifen Eizellen (MII) und ihren umgebenden Kumuluszellen exprimiert waren ($p \leq 0.05$, fold change ≥ 2). Dabei wurden 8 bzw. 6 miRNAs stärker in den Kumuluszellen exprimiert beim Vergleich von Eizellen im reifen bzw. unreifen Stadium. Weitere Expressionsanalysen wurden von sechs ausgewählten miRNAs (miR-205, -150, -122, -96, -146a und 146b-5p) in bovinen Embryonen des Präimplantationsstadiums durchgeführt, da diese in Eizellen angereichert auftraten. Die Ergebnisse zeigten, dass die Expression dieser miRNAs stark erhöht in frühen Stadien der Embryonalentwicklung und reduziert nach dem 8-Zellen-Stadium. Zu beobachten waren bis zum Blastozysten Stadium folgten die Embryonen den typischen maternalen Transkriptionsmustern. Vergleichbare Ergebnisse wurden bei der *in situ* Lokalisierung von miR-205 in Embryonen des Präimplantationsstadiums beobachtet. Leider zeigten die ausgewählten miRNAs (miR-210 und miR-452), die in Kumuluszellen vermehrt exprimiert waren, kein definiertes Profil. Die gleichen acht miRNAs wurden in einer hohen Menge in voll ausgewachsenen Eizellen (BCB+) im Vergleich zu noch wachsenden Eizellen (BCB-) detektiert. Interessanterweise konnte beobachtet werden, dass die Präsenz oder Abwesenheit von Eizelle oder Kumuluszellen während der Maturation einen Einfluss auf die Expression der ausgewählten miRNAs in den entsprechenden Zelltypen hatte. Des Weiteren wurden miR-205 und miR-210 *in situ* in Follikeln lokalisiert und zeigten eine raum-zeitliche Expression während der Follikelentwicklung. Unsere Ergebnisse zeigen die Präsenz einer definierten Gruppe von miRNAs in Eizellen und Kumuluszellen, welch möglicherweise eine Rolle in der Regulation der bidirektionellen Kommunikation zwischen diesen zwei Zelltypen spielen.

Expression analysis of regulatory MicroRNA in bovine cumulus oocyte complex and preimplantation embryos

MicroRNAs (miRNAs) are small non coding endogenous molecules which participate in gene regulation post transcriptionally. These small RNAs are shown to be involved in a wide range of biological processes including development, cell differentiation, cell proliferation, and apoptosis in diverse organisms. However, little is known about their abundance in bovine oocytes and their surrounding cumulus cells during oocyte development. To elucidate this, we investigated the relative expression pattern of a set of 88 miRNAs between bovine oocytes and the surrounding cumulus cells during *in vitro* maturation, using miRNA PCR array related to human cell development and differentiation. Results revealed a total of 47 and 51 miRNAs to be differentially expressed ($p \leq 0.05$ and fold change ≥ 2) between immature (GV) and matured (MII) oocytes, respectively compared to their surrounding cumulus cells. However, 8 and 6 miRNAs were found to be highly abundant in cumulus cells compared to oocytes at immature and matured stages, respectively. The expression analysis of six oocytes enriched miRNAs (miR-205, -150, -122, -96, -146a and -146b-5p) in bovine preimplantation embryo stages was performed. Results showed that expression of these miRNAs were highly abundant in early stages of embryo development and reduced after 8-cell until the blastocyst stage following a typical maternal transcript profile. Comparable results were obtained by *in situ* localization of miR-205 in preimplantation embryo stages. However, the two selected miRNAs enriched in cumulus cells (miR-210 and miR-452) showed no defined profile. The same eight miRNAs were detected at high level in fully grown oocyte (BCB+) compared to growing one (BCB-). Interestingly, the presence or absence of oocytes or cumulus cells during maturation was found to affect the expression of selected miRNAs in each of the two cell types. Furthermore, miR-205 and miR-210 were *in situ* localized in ovarian follicle and revealed a spatio-temporal expression during follicular development. Hence, our results evidenced the presence of distinct set of miRNAs in oocytes and cumulus cells which may have potential role in regulation of bidirectional communication between the two cell types. Moreover, maternal miRNAs were found to persist until the major genome activation in bovine.

Dedicated to my family, my husband Mohamed and my lovely son Eyad

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List of abbreviations

A	: Adenine
ActRIIA	: Activin receptor IIA
ADM2	: Adrenomedullin 2
Ago	: Argonaute protein
ALDOA	: Aldolase A, fructose-bisphosphate
ALK	: Activin receptor-like kinase
AREs	: AU-rich elements
PABP	: Poly(A)-binding protein
Bax	: BCL2-associated X protein
BCB	: Brilliant cresyl blue stain
BCL2L11	: BCL2-like 11
BMP	: Bone morphogenetic protein
BMPRII	: Bone morphogenetic protein receptor, type II
Bta	: *Bos taurus*
BTC	: Betacellulin
CALML4	: Calmodulin-like 4
cAMP	: Cyclic adenosine monophosphate
CAT	: Catalase
CC	: Cumulus cell
CC-OO	: Cumulus cells matured without oocyte (Oocytectomy)
CCND2	: Cyclin D2
CCNG1	: Cyclin G
cDNA	: Complementray DNA

COC : Cumulus oocyte complex

COX-2 : Cytochrome c oxidase subunit II

CSDC2 : Cold shock domain containing protein

CTBP1 : C terminal binding protein-1

CTNND1 : Catenin delta 1

Cx37 : Connexin 37

Cx43 : Connexin 43

CXCR4 : Chemokines receptor 4

CYP19A1 : Cytochrome P450, family 19, subfamily A, polypeptide 1

Daf-12 : Abnormal DAuer Formation

DCP1 : Decapping protein 1

ddH_2O : Deionised and demineralised millipore water

DDX5 : DEAD box RNA helicases p68

DDX17 : DEAD (Asp-Glu-Ala-Asp) box polypeptide 17

DEPC : Diethylpyrocarbonate

Dgcr8 : DiGeorge syndrome critical region gene 8

DHCR7 : 7-dehydrocholesterol reductase

DIG : Digoxigenin

DNA : Deoxynuclic acid

DO : Denouded oocyte

dsRNA : Double-stranded RNA

dsRBDs : Double-strand RNA binding domains

DTCS : Dye terminator cycle sequencing

DTT : Dithiothretiol

DVL3 : Dishevelled dsh homolog 3

EDC3 : Enhancer of mRNA decapping 3 homolog

eIF4A : Eukaryotic initiation factor 4A

eIF6 : Eukaryotic initiation factor 6

eIF4E : Eukaryotic translation initiation factor 4E

eIF4G : Eukaryotic translation initiation factor 4G

EIF4G3 : Eukaryotic translation initiation factor 4 gamma, 3

ENO1 : Enolase 1, (alpha)

FGF : Fibroblast growth factor

ER : Endoplasmic reticulum

FF-MAS : Follicular fluid meiosis-activating sterol

FSH : Follicle stimulating hormone

FXR1 : Fragile X mental retardation–related protein 1

G6PDH : Glucose 6 phosphate dehydrogenase

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

GC : Granulosa cell

GDF-9 : Growth differentiation factor 9

GEMIN : Gem (nuclear organelle) associated protein

GPI : Glycosylphosphatidylinositol

GPX3 : Glutathione peroxidase 3

GSH : Gluthatione

GV : Germinal vesicle

GVBD : Germinal vesicle break down

HAS2 : Hyaluronan synthase 2

HCV : Hepatitis C virus

HMC : Handmade cloning

hnRNPs : Heterogeneous nuclear ribonucleoproteins

hr: : Hour

HSPB1 : Heatshock 27 kDa protein 1

IL24 : Interleukin 24

IL32 : Interleukin 32

imc : Immature cumulus cells

IMD : Intermedin

imo : Immature oocyte

INHBA : Inhibin beta A

ISH : In situ hybridization

IPA : Ingenuity pathway analysis

IP3R1 : 1,4,5-inositol triphosphate receptor type 1

IPTG : Isopropyl -D- thiogalactopyranoside

ITI : Inter-α trypsin inhibitor

IRAK1 : Interleukin-1 receptor-associated kinase 1

ITPR1 : 1,4,5-triphosphate receptor 1

IVF : In vitro fertilization

IVM : In vitro maturation

IVP : In vitro production

kDa : Kilo Dalton

Ldh1 : L-lactate dehydrogenase

LH : Luteinizing hormone

LH/hCGR : Luteinizing hormone/human chorionic gonadotrophins

LHR : Luteinizing hormone receptor

LNA : Locked nucleic acid

Loqs : Loquacious

m7G : 7-methyl guanine-cap

MAPK : Mitogen-activated protein kinase

mc : Matured cumulus cells

MeOH : Methanol

MET : Maternal-to-embryonic transition

mg : Milligrams

MGC : Mural granulosa cells

MII : Metaphase II

min : Minute

miRNA : MicroRNA

miRNPs : MiRNA-containing ribonucleoprotein complex

MPF : Maturation-promoting factor

MPM : Modified Parker Medium

mo : Matured oocyte

mRNA : Messenger ribonucleic acid

mRNP : Messenger ribonucleoprotein

ncRNAs : Non coding ribonucleic acids

NFIB : Nuclear factor I/B

NOBOX : NOBOX oogenesis homeobox

nt : Nuclotied

OCS : Ostrous cow serum

OO+CC : Oocyte matured with cumulus cells (Cumulus oocyte complex)

OO-CC : Oocyte matured without cumulus cells (Denuded oocyte)

OOX : Oocytectomy

OSFs : Oocytes secreted factors

PABPC1 : Poly(A) binding protein, cytoplasmic 1

PFA : Paraformaldehyde

RANGAP1 : Ran GTPase activating protein 1

PANX3 : Pannexin 3

Pbp1 : Penicillin- binding protein

PBS : Phosphate buffered saline

PCK1 : Phosphoenlpyruvate carboxykinas 1

PCNA : Proliferating cell nuclear antigen

PCR : Polymerase chain reaction

PFKP : Phosphofructokinase, platelet

PKM2 : Pyruvate kinase, muscle

PLCB1 : Phospholipase C beta 1

Pre-miRNA : Precursor miRNA

Pri-miRNA : Primary miRNA

PTGS2 : Prostaglandin synthase 2

PTX3 : Pentraxin 3

Ran : Ras-related nuclear protein

RANGAP1 : Ran GTPase activating protein 1

RNASEN : Ribonuclease type III, nuclear

RISC : RNA-induced silencing complex

RNA : Ribonucleic acid

rpm : Revoulution per minute

RT : Real time

SCNT : Somatic-cell nuclear transfer

SD : Stander deviation

sec : Second

SF1 : Steroidogenic factor-1

Slc38a3 : Solute carrier family 38, member 3

SNPs : Single nucleotide polymorphisms

ssRNA : Single-stranded RNA

StAR : Steroidogenic acute regulatory protein

TRBP : Tar-binding protein

TCM : Tissue culture media

TdT : Deoxynucleotidyl transferase terminal

TGF-β : Transforming growth factor beta

TKDP : Trophoblast kunitz domain protein

TNFAIP6 : Tumor necrosis factor-induced protein 6

TNFα : Tumor necrosis factor alpha

TPI : Triose phosphate isomerase

TRAF6 : TNF-receptor-associated factor 6

TRIM28 : Tripartite motif-containing 28

Tu : Transcription unit

uPA : Plasminogen activator, urokinase

UTR : Untranslated region

Xrn1p : Single-stranded RNA 5'->3' exonuclease

α : Alpha

β : Beta

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1 Introduction

Bidirectional communication between oocytes and cumulus cells is essential for proper maturation of oocytes, fertilization and further embryonic development (Buccione et al. 1990; Eppig 1991; Matzuk et al. 2002). During oocyte maturation, the cumulus cells provide a network of gap junction transmembrane channels that allows the transport of nutrient, regulatory molecules and paracrine factors between oocytes and cumulus cells to promote the nuclear and cytoplasmic maturation of oocyte and acquisition of developmental competence (Eppig 1991; Gilchrist et al. 2004; Grazul-Bilska et al. 1997; Tanghe et al. 2002). This has been evidenced from removal of cumulus cells before maturation whereby the oocyte maturation was suppressed (Assidi et al. 2008; Fatehi et al. 2002; Vozzi et al. 2001; Wongsrikeao et al. 2005). On the other hand, the oocyte, through its secreted paracrine growth factors, play an important role in regulating the proliferation and differentiation of granulosa cells into two phenotypically and functionally distinct subtypes namely, cumulus granulosa cells and mural granulosa cells (Eppig et al. 1997; Li et al. 2000). Furthermore, oocytes secreted factors (OSFs) are believed to regulate key cumulus cell functions which in turn enhance positive regulatory factors that are required during subsequent development of the oocytes (Sugiura et al. 2005). Therefore, the bidirectional communication between oocyte and their surrounding cumulus cells is essential for oocyte and cumulus cells development during folliculogenesis.

Oocytes are known to have high transcriptional activity in the germinal vesicle (GV) stage leading to the metaphase II (MII). The stored mRNAs and proteins in the oocyte support the early embryogenesis following fertilization (Dalbies-Tran and Mermillod 2003; Fulka et al. 1998; Vallee et al. 2005). However, as development proceeds and maternally inherited informational molecules decay, early embryogenesis becomes dependent on the expression of genetic information from the embryonic genome (Telford et al. 1990). The dynamic change in transcript abundance before and after *in vitro* maturation of bovine oocytes has been documented underlying the tight temporal control of gene expression required for oocyte maturation, fertilization and early embryo development (Dalbies-Tran and Mermillod 2003; Fair et al. 2007). Recently, it

has been postulated that the mRNA storage and translations are post transcriptionally under the control of non coding regulatory small RNAs mainly microRNAs.

MicroRNAs (miRNAs), the key regulator of many biological function, are small noncoding RNAs (~18-22 nt) that originate from endogenous hairpin transcripts called primary miRNA (pri-miRNA) which processed by the RNase III Drosha in the nucleus before being exported as ~70-nucleotide precursor miRNA (pre-miRNA) to the cytoplasme, where further processed by another RNase, Dicer, into mature double-stranded miRNA (van den Berg et al. 2008). One strand of this matured miRNA incorporated into the RNA-induced silencing complex (RISC), where it acts as a guide to mediate their targets messages either by translational repression or/and mRNA decay via a deadenylation and decapping mechanism (Behm-Ansmant and Izaurralde 2006; Giraldez et al. 2006; Wu et al. 2006). Although under certain conditions, miRNAs have been reported to induce gene expression (Vasudevan et al. 2007). Similar to mRNA, miRNAs have been reported to show a dynamic change during oocyte maturation in bovine (Tesfaye et al. 2009), mouse and human (Tang et al. 2007; Xu et al. 2011a). Furthermore, the presence of regulatory miRNAs has been reported in oocytes and numerous reproductive tissues (Carletti and Christenson 2009; Creighton et al. 2010; Hong et al. 2008; Murchison et al. 2007; Nagaraja et al. 2008). In addition, disruption of Dicer (a polymerase II enzyme responsible for miRNA processing) in mouse oocytes was found to be result in defects in meiotic spindle organization and chromosome congression, reduced maturation rate and reduced the transcripts of Dicer miRNAs (Liu et al. 2010; Murchison et al. 2007).

Apart from oocytes, miRNAs have been identified in embryo of mice, cow and human (Coutinho et al. 2007; Cui et al. 2009; McCallie et al. 2010). The role of miRNAs in early embryo development and maternal-to-zygotic transition has been reported, where loss of maternal miRNAs by specific deletion of Dicer results in an arrest of zygotic development in mice (Tang et al. 2007) and a specific miRNA such as miR-430 and miR-427, has been shown to be responsible of the degradation of many maternal mRNAs in zebrafish and xenopus laevis embryos, respectively (Giraldez et al. 2006; Lund et al. 2009). Recently, the expression pattern of miR-196a in bovine

preimplantation embryo stages indicated the involvement of this miRNA in maternal transcript degradation during maternal-to-zygotic transition (Tripurani et al. 2011).

Although, the accumulating evidences support the notion of potential role of these non coding miRNAs in post transcriptional regulation of genes involved in various aspects of mammalian reproduction, so far little is known about the distinct abundance of miRNAs in bovine oocyte and its surrounding cumulus cells before and after oocyte maturation. Therefore, the objectives of the present study were: 1) To identify differentially expressed miRNAs in bovine oocytes and surrounding cumulus cells at immature or germinal vesicle (GV) and matured or metaphase II (MII) stages, 2) To characterize the expression pattern of selected oocyte and cumulus cells enriched miRNAs and their predicted target genes in bovine preimplantation embryo stages, 3) To investigate the association of the expression of selected miRNAs with oocytes developmental potential, 4) To reveal the effect of presence or absence of oocyte or cumulus cells during maturation on the expression of selected miRNAs in either of the two cell types and 5) To identify spatio-temporal expression of two selected miRNAs in ovarian sections, COCs and bovine preimplantation embryo stages.

2 Literature review

2. 1 Oogenesis and folliculogenesis

Oogenesis is the process of oocyte formation involves several steps including primordial germ cell formation, migration to the gonads and differentiation into oogonia, followed by the initiation of meiosis and subsequent arrest at the diplotene stage during prophase I (van den Hurk and Zhao 2005). When the bovine embryo is about 15 mm in length, the primordial germ cells (precursors of the oocyte) migrated by amoeboid movement from the epithelium of the yolk sac via the dorsal mesentery of the hindgut to the genital ridges located at the ventral sides of the mesonephroi (Gordon 2003a; Smitz and Cortvrindt 2002). The primordial germ cells in female fetal gonads expand the population of germ cells through a high frequency of mitotic division and eventually undergo one last round of DNA replication, before entering meiosis and becoming oocytes (Gosden and Bownes 1995). The onset of oocyte first meiotic division occurs between days 75 and 80 of pregnancy in cattle (Erickson 1966). The oocyte passes through leptotene, zygotene, pachytene and arrested at diplotene stage. During this time the transcription level become nearly undetectable by early pachytene while its higher at the diplotene stage (Picton et al. 1998).

Folliculogenesis is the developmental process in which an activated primordial follicle develops to a preovulatory follicle following the growth and differentiation of the oocyte and its surrounding granulosa cells (Gougeon 1996; Knight and Glister 2001; Senger 1997). During folliculogenesis, a follicle may be classified as primordial, primary, secondary or tertiary (Figure 2.1) (Pedersen and Peters 1968). This classification is based on the size of oocyte, the morphology of granulosa cells, and the number of granulosa cell layers surrounding the oocyte (Braw-Tal and Yossefi 1997; Lussier et al. 1987). It's accepted that folliculogenesis in bovine occur during fetal development. Once oocytes reaching to diplotene stage of prophase I, it's surrounded by a single layer of squamous pre-granulosa cells resting on a basement membrane and forming the non-growing or primordial follicle (Baker and Hunter 1978; Picton et al. 1998). The large population of non-growing primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life, when

granulosa cells surrounding the oocytes become cuboidal and the oocyte begin its extensive growth phase, the primordial follicles are transformed into primary follicles (Eppig 2001; van Wezel and Rodgers 1996). Progression to the secondary follicle stage also known as preantral follicle or growing follicle is characterized by the appearance of a second layer of granulosa cells, increased oocyte diameter (Driancourt 1991; Hyttel et al. 1997), zona pellucida and cortical granule formation, development of gap junction between oocyte and granulosa cell which may serve both to maintain meiotic arrest and oocyte growth (Braw-Tal and Yossefi 1997; Fair et al. 1997a), first detectable signs of oocyte RNA synthesis (Fair et al. 1997b) and gonadotrophin responsiveness where the follicular stimulating hormone (FSH) receptor mRNA expression has been detected in follicles with one to two layers of granulosa cells in cattle (Bao and Garverick 1998; Fair 2003; Xu et al. 1995). Development beyond the early antral follicle stage is clearly dependent upon gonadotrophins, antral follicles are sometimes called tertiary follicles and well developed antral follicles are often referred to as Graafian follicle (Eppig 2001). The transition to antral follicle characterized by formation of antrum which is fluid filled cavity surrounded by multiple layer of granulosa cells, this fluid contain important regulatory substance to be delivered to oocyte (Braw-Tal and Yossefi 1997; Eppig 2001; Lussier et al. 1987). After antrum formation, granulosa cells are divided into two distinct subtypes, the cumulus granulosa surrounding and in intimate metabolic contact with the oocyte forming a structure called cumulus oocyte complex (COC) and the mural granulosa lining the follicle wall and adjacent to the basal lamina, the mural granulosa cells nearest the antrum are called periantral granulosa cells (Eppig 2001). Finally, in response to preovulatory gonadotrophins surge, two marked events occur in cumulus oocyte complex (Figure 2.1) : 1- The fully grown oocyte (GV) resume meiosis to complete first meiotic division via germinal vesicle break down (GVBD), chromatin re-condensation, the pairs of homologous chromosomes are separated and half of them are expelled forming the first polar body and the mature oocyte arrested again in metaphase of second meiotic division and 2- The cumulus cell surrounding the oocyte undergo expansion or mucification through secretion of hyaluronic acid, a non-sulphated glycosaminoglycan bound to the cumulus cells by linker proteins (Buccione et al. 1990; Chen et al. 1996; Eppig 1979; Salustri et al. 1989), and when the hyaluronic acid becomes hydrated, the spaces between cumulus cells become enlarged and the cells

become embedded in a sticky mucified matrix. If cumulus expansion is suppressed, ovulation rate is greatly reduced (Chen et al. 1993).

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

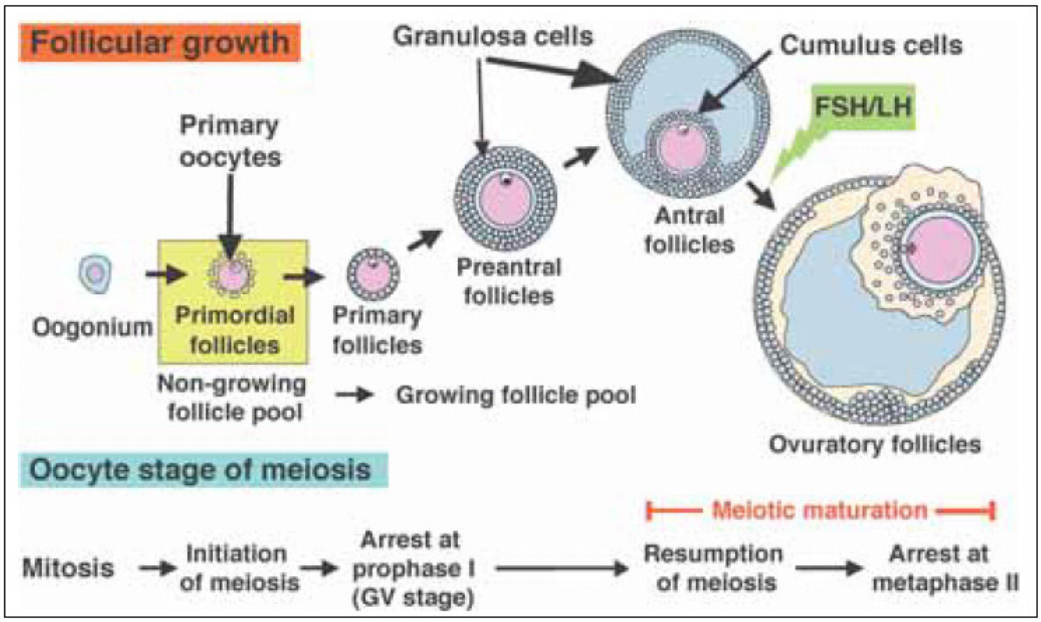


Figure 2.1: Follicular growth and oocyte meiotic maturation (Kimura et al. 2006).

Growth of the mammalian oocyte is coordinated with the follicle that enclosed it (Figure 2.2), as follicle size increased there is a corresponding increase in oocyte diameter until follicle has grown to 3 mm and oocyte reach maximum size (Fair et al. 1995). The transcriptional activity in bovine oocyte, where the messenger RNA and ribosomal RNA are synthesized, is activated in the secondary follicle and is maintained up to an oocyte diameter of about 110 mm in the tertiary follicle (Crozet 1989; Fair et al. 1995; Fair et al. 1996). At a diameter of 110 mm, the oocyte achieves competence to undergo meiotic maturation and sustain embryonic development (Fair et al. 1995).

The accumulation of messenger RNAs (mRNAs), ribosomes and polypeptides by the oocyte during the growth phase is important to later development (Moor et al. 1998).

The maternal mRNAs are stored in an inactive form as message-ribonucleoprotein (mRNP) complexes and they can be released from the mRNP compartment and recruited for translation in a stage-specific manner during oocyte maturation or early embryo development (Mtango et al. 2008).

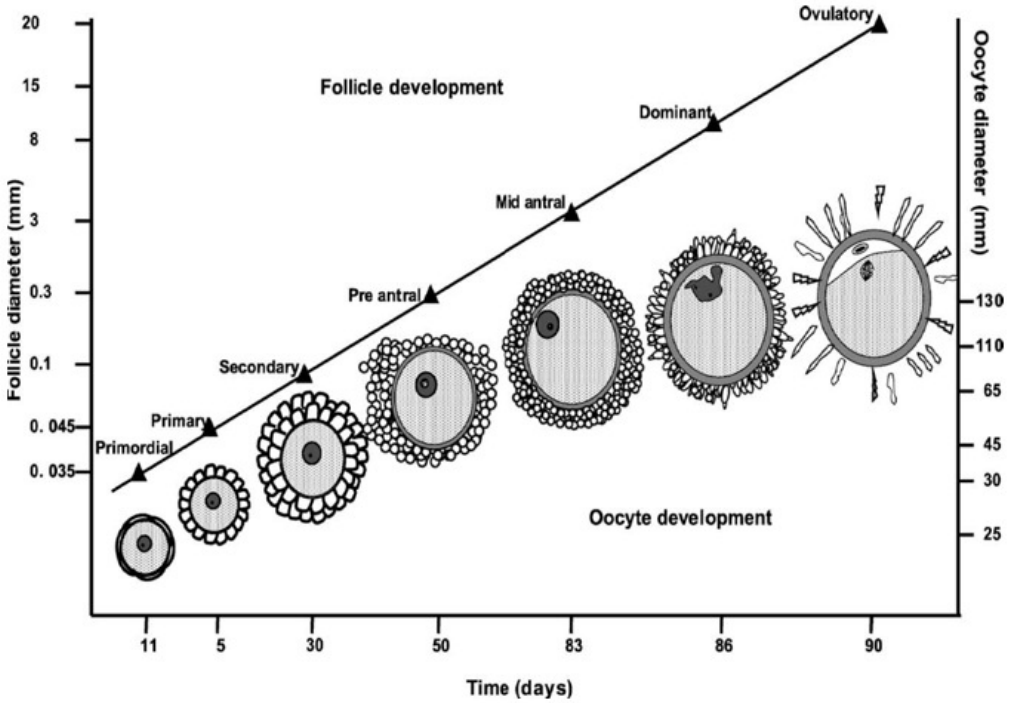


Figure 2.2: The relationship between follicle development and oocyte development in cattle. The growth of oocytes (surrounded by granulosa cells) is shown schematically in relation to follicle diameter, oocyte diameter and estimated duration of stage of folliculogenesis from the primordial to preovulatory stage (Fair 2003, based on data from Hulshof et al. (1992) and Lussier et al. (1987)).

2.2 Oocyte maturation

Oocyte maturation is progression of meiosis from the GV stage up to the formation of the metaphase plate of the second meiotic division (MII) and involves nuclear and cytoplasmic maturation. Nuclear maturation mainly involves chromosomal segregation while cytoplasmic maturation involves organelle reorganization and storage of mRNAs, proteins and transcription factors that act in the overall maturation process, fertilization and early embryogenesis (Ferreira et al. 2009). Luteinizing hormone (LH) surge or removal of the oocyte from its follicular environment leads to induced nuclear

maturation. In bovine oocyte nuclear maturation starts with germinal vesicle breakdown which is characterized by gradual chromatin condensation, the disappearance of a compact nucleolus and nuclear membrane disintegration, arrangement of condensed homologues chromosome on equatorial plate of the meiotic spindle. After that, the oocyte extrude the first polar body into the perivitelline space and now the oocyte called a secondary oocyte, begins the second meiotic division and proceeds until it reaches MII (Gordon 2003b), at this stage the oocyte contains the haploid number of chromosomes, however, the second meiotic division is not completed unless sperm penetration.

The essential enzyme complex in oocyte maturation is maturation-promoting factor (MPF), a protein complex composed of cyclin B1 and P32cdc2, which is synthesized and/or activated depending on the species (Sirard and Blondin 2006). The MPF activity appears shortly before GVBD reaches a maximal level in MI oocytes, decreases markedly during the transition from MI to MII and regains its peak level in MII oocytes. This changeable pattern of MPF activity means that the transition from MI to MII is correlated with a decrease in MPF activity (Gordon 2003b). In addition, Gordo et al. (2001) mentioned that, mitogen-activated protein kinase (MAPK) activity is also necessary for metaphase II arrest, the maintenance of MPF activity and spindle organization.

During cytoplasmic maturation, many events that have relevance on developmental competence occur as: redistribution of mitochondria, lipid droplet, cortical granules (Hosoe and Shioya 1997; Hyttel et al. 1997; Kruip et al. 1983), reorganization of endoplasmic reticulum (ER) and calcium store, increased number of 1,4,5-inositol triphosphate receptor type 1 (IP3R1) and their biochemical prosperities (Ajduk et al. 2008; Shiraishi et al. 1995; Stricker 2006) and modified organization of the oocyte cytoskeleton that forms a network in which the organelles encased by a membrane move and occupy defined positions (Albertini 1992). Morphological changes are accompanied by molecular processes which correspond to the phases of oocyte growth and maturation and it involves the transcription, storage and processing of the mRNAs expressed by the chromosomes, which will be further translated into proteins by the ribosomes (Ferreira et al. 2009). The proteins derived from these mRNAs are being

stored until the appropriate time for their utilization (Fulka et al. 1998). These proteins involved both in maturation and in subsequent cellular events such as fertilization, pronucleus formation and early embryogenesis. The molecular changes in the oocyte are under the influence a signal from follicular cells which produced in response to precisely timed changes in circulating level of gonadotrophins before ovulation (Moor et al. 1998).

2.3 Oocyte-somatic follicular cell interaction

The cumulus oocyte complex is consisting of secondary oocyte which arrested at metaphase I and surrounding cumulus cells which contact to each other via gap junction and the innermost layer of cumulus cells surrounding the oocyte extend cytoplasmic processes through the zona pellucida, forming gap junctions with the oocyte plasma membrane (Albertini et al. 2001; Hyttel 1987; Kruip et al. 1983). These interactions are essential for the development and function of both cell compartments (Eppig 2001). The gap junction channels allowing intercellular communication and transfer of low molecular weight substrates such as ions, nucleotides, amino acids and metabolites that are necessary for oocyte development, as well as, regulatory molecules such as cAMP that control nuclear oocyte maturation (Buccione et al. 1990; Downs et al. 1986; Kumar and Gilula 1996), while the large molecules are transported by receptor mediated endocytosis. The essential role of gap junction in folliculogenesis has came from the genetic deletion of oocyte-specific gap junction subunit, connexin 37 (Cx37) which its deficiency display arrested folliculogenesis and oocyte growth and oocyte development arrested before meiotic competence is achieved (Carabatsos et al. 2000; Simon et al. 1997). Moreover, mice deficient in connexin (Cx43) fail to properly develop granulosa cell layers in antral follicle (Ackert et al. 2001). In experiments designed to decrease Cx43 *in vitro*, the primary gap junctional protein in COCs, a recombinant adenovirus expressing the antisense code for Cx43 resulted a two-fold decrease in coupling between cumulus cells and the oocyte and an overall fifty percent decrease in oocyte maturation. Thus, this experiment demonstrated that gap junctional communication between oocytes and the surrounding cumulus cells is vital for oocyte maturation (Vozzi et al. 2001).

Granulosa cells are supporting oocyte growth and development by providing essential nutrients to oocyte, participate in maintenance of oocyte meiotic arrest, global suppression of oocyte transcriptional activity, and the induction of oocyte meiotic and cytoplasm maturation (Buccione et al. 1990; Eppig et al. 2004). In turn, oocyte itself is not passive structure within the follicle, it plays a central role in regulating folliculogenesis through paracrine secretion of peptide growth factor called oocyte secreting factors (OSFs). Oocyte secreting factors regulate many functions within the follicle including: proliferation of granulosa cells/cumulus cells, inhibition of apoptosis of cumulus cells, stimulates prostaglandin and progesterone synthesis and/or signaling pathways in preovulatory cumulus granulosa cells, suppresses the luteinization of cumulus granulosa cells by inhibiting LH receptor expression, regulation of cumulus cell metabolism and promotion of cumulus cells expension (Elvin et al. 2000; Gilchrist et al. 2008; Sugiura et al. 2005). However, not all of these OSFs are known. The much focused OSFs are transforming growth factor beta (TGF-β) superfamily members, particularly growth differentiation factor 9 (GDF-9), GDF-9B also known as bone morphogenetic protein 15 (BMP-15) and to a lesser bone morphogenetic protein 6 (BMP6) and activin (Dube et al. 1998; Gilchrist et al. 2004; Laitinen et al. 1998; McGrath et al. 1995). This bidirectional communication mode in the ovary is essential for development and fertility (Simon et al. 1997) and though to play a key role in disseminating local and endocrine signal to the oocyte via the cumulus cells (Figure 2.3) (Gilchrist et al. 2004).

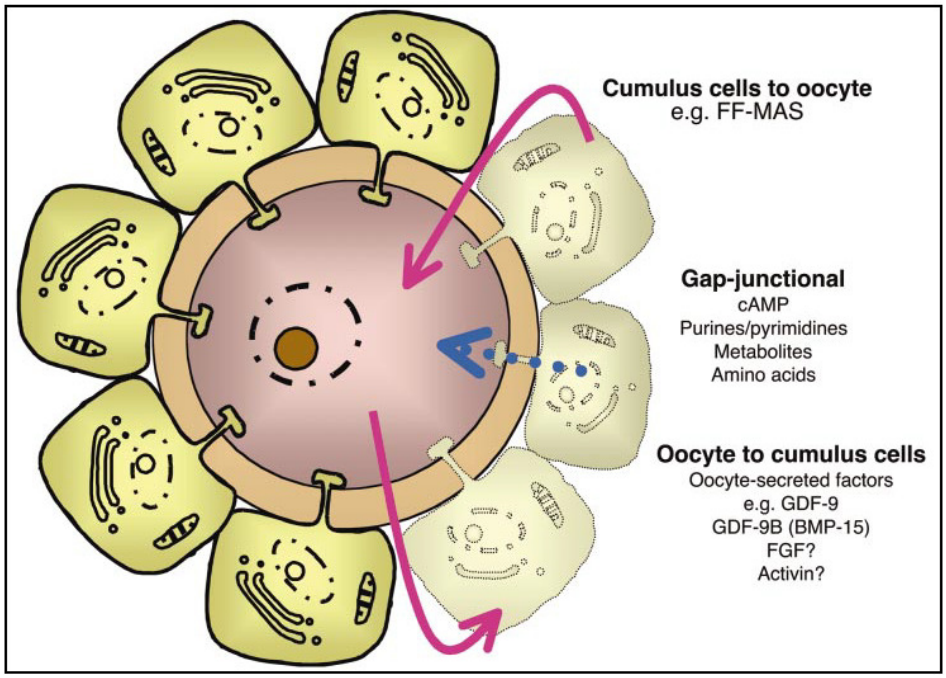


Figure 2.3: Oocyte cumulus cell communication. Both paracrine (bold arrow) and gap-junctional (dashed arrow) communication between the oocyte and cumulus cells are required for normal oocyte and follicle development. Both communications pathways are bidirectional. Factors transmitted via these pathways include follicular fluid meiosis-activating sterol (FF-MAS), cAMP, purines and pyrimidines, metabolites, amino acids, growth differentiation factor-9 (GDF-9) and GDF-9B or bone morphogenic protein (BMP-15), fibroblast growth factor (FGF) and activin (Sutton et al. 2003b).

2.3.1 Role of oocyte secreting factors

The role of oocyte not only in transmission of genetic information and raw material to early embryo but the oocyte is a central regulator of follicular cell function and play a critical role in regulation of oogenesis, ovulation rate and fecundity (Eppig 2001; Gilchrist et al. 2004; Gilchrist and Thompson 2007; McNatty et al. 2004). The oocyte do this by secreting paracrine growth factors (OSFs) which act on surrounding follicular cells to regulate several aspect of its function and thereby the oocytes create and control their microenvironment to regulate their own development.

Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are two members of transforming growth factor β (TGF-β) superfamily, which are translated as preproproteins, consisting of a signal peptide, prodomain, and mature domain (Chang et al. 2002; Shimasaki et al. 2004). Expression of ovarian GDF-9 and BMP-15 are restricted exclusively to oocyte in many species (Juengel and McNatty 2005). They are considered probably major players of the oocyte-granulosa cell regulatory loop and participate in many functions of oocytes (Eppig 2001; Erickson and Shimasaki 2001; Matzuk et al. 2002; McNatty et al. 2004). Mutation in these genes causes major effect on ovarian function and fertility in mice and sheep (Bodin et al. 2007; Dong et al. 1996; Galloway et al. 2000; Hanrahan et al. 2004). More sever fertility defect was observed in double mutant BMP-15$^{-/-}$ GDF-9$^{+/-}$ than in BMP-15$^{-/-}$ mice females which appear to be due to abnormalities in ovarian folliculogenesis, cumulus cell physiology and fertilization (Yan et al. 2001).

Addition of recombinant GDF-9 or BMP-15 to granulosa cells *in vitro* mimic several regulatory actions of oocyte on granulosa cells activities, for example increased follicle growth and inhibin α production (Hayashi et al. 1999), reduced the levels of FSH receptor mRNA (Otsuka et al. 2001), stimulated DNA synthesis and steriodogenesis in rat granulosa cells (Vitt et al. 2000) and induced cytochrome c oxidase subunit II (COX-2), hyaluronan synthase 2 (HAS2), steroidogenic acute regulatory protein (StAR) mRNA synthesis, but suppressed plasminogen activator, urokinase (uPA) and LHR mRNA synthesis in mouse granulosa cells (Elvin et al. 1999a).

The GDF-9 and BMP-15 signal depend on the TGF-β superfamily receptor which in turn activate the SMAD intracellular cascade (Juengel and McNatty 2005; Kaivo-oja et al. 2006; Shimasaki et al. 2004). The TGF-β superfamily growth factors are binded to type–I receptor also known as activin receptor-like kinase (ALK) or type–II receptor leads to ALK phosphorylation then intracellular receptor-regulated signal transducers (SMADs) phosphorylation (Massague 2000; Shimasaki et al. 2004). Formation of heterodimeric complex (between receptor-regulated SMADs and receptor independent co-SMADs, such as SMAD4) occurred to mediate ligand-induced gene transcription. The TGF-β superfamily growth factors intracellular signaling can be broadly divided into two distinct groups: those utilizing the TGF-β/activin signaling pathway leading to

activation of the SMAD2 and SMAD3 proteins and those using the BMP pathway leading to activation of SMAD1, SMAD5 and/or SMAD8 molecules (Massague 2000). In granulosa cells and cumulus cells (Figure 2.4), BMP-15 and BMP-6 use the classic BMP signaling pathway through binding the BMP type-II and ALK6, and activating the SMAD1/5/8 intracellular pathway and GDF-9 used TGF-β superfamily signaling systems, GDF-9 binding BMP type-II receptor and ALK5 leads to activation of ALK5 which in turn phosphorylates SMAD2 and SMAD3 signal transducers which associates with the common SMAD4 and then this complex translocates to the nucleus to interact with specific DNA motifs and transcriptional regulators, leading to expression of target genes (Gilchrist et al. 2008). Furthermore, GDF-9 and BMP-15 may also activate alternate pathways, particularly when acting synergistically or with non superfamily members such as fibroblast growth factors (FGFs) (Sugiura et al. 2007).

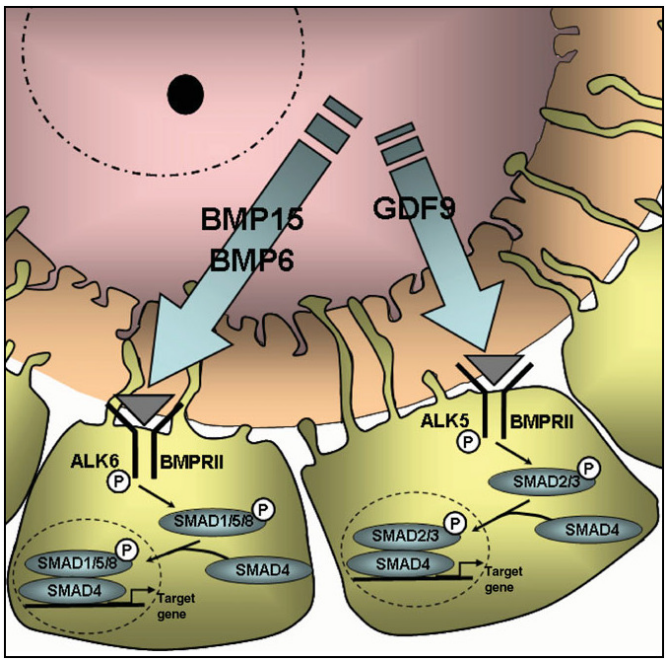


Figure 2.4: Molecular basis of oocyte–CC paracrine signaling, details of the molecules mediating oocyte paracrine signaling to CCs are still emerging; however, TGF-β superfamily signaling is central to this communication axis. Key OSFs include GDF-9, BMP-15 and possibly BMP-6. GCs and CCs express a large compliment of TGF-β superfamily receptors, co-receptors and intracellular signal transducer molecules (SMAD). BMPRII is the critical type-II receptor utilized by all three OSF ligands, although BMP6 also binds ActRIIA (Gilchrist et al. 2008).

The oocyte secreted factor promotes the expansion of cumulus through enabling the cumulus cells to respond to the gonadotrophic/EGF signal to induce expression of several transcripts (HAS2, TNFAIP6, PTX3, and PTGS2) required for formation and stability of the hyaluronan rich matrix (Diaz et al. 2006; Dragovic et al. 2005; Dragovic et al. 2007; Elvin et al. 1999b; Varani et al. 2002; Yoshino et al. 2006). Hyaluronan synthase 2 (HAS2), is the key enzyme required for the production of hyaluronic acid, a non-sulfated glycosaminoglycan secreted by cumulus cells that is a main component of the cumulus matrix (Camaioni et al. 1993; Chen et al. 1993). Prostaglandin-endoperoxide synthase 2 (PTGS2) is produced by cumulus cells as well as mural granulosa cells and is required for maximum cumulus expansion and ovulation (Lim et al., 1997). Tumor necrosis factor-induced protein 6 (TNFAIP6) is a secreted protein that binds both hyaluronic acid and inter-α trypsin inhibitor (ITI), a serum derived protein important for matrix formation (Fülöp et al. 2003; Mukhopadhyay et al. 2004). Pentraxin 3 (PTX3) is also secreted by cumulus cells and acts to stabilize TNFAIP6 protein to maintain the expanded matrix (Salustri et al. 2004; Varani et al. 2002). The tumor necrosis factor alpha-induced protein 6 (TNFAIP6) was not only a hyaluronan binding protein involved in cumulus cells expansion, but it has been also reported to be associated with the expression of a common receptor of luteinizing hormone/human chorionic gonadotrophin (LH/hCGR) in cumulus cells (Fülöp et al. 2003; Haouzi et al. 2009). On the contrary, cow, rat and pig COC expansion can be induced by FSH in absence of the oocyte (Ralph et al. 1995; Singh et al. 1993; Vanderhyden 1993). Although, some factor produced by bovine oocytes enabled expansion of murine oocytectomised complexes (OOX) when cultured in media conditioned by bovine oocytes (Ralph et al. 1995).

In bovine oocyte cumulus cell communication, Hussein and his colleagues (2005) demonstrated that oocyte removal led to increase in cumulus cell apoptosis compared with COC and the level of apoptosis in cumulus cells without oocyte (OOX) was reversed in dose dependant manner when co-culture with oocyte. They also revealed that oocyte secreted factors, particularly BMP-15 and BMP-6 are responsible for the low incidence of apoptosis within cumulus cells, through the establishment of a paracrine network of BMP growth factors and their binding proteins (Figure 2.5).

Furthermore, the incidence of apoptosis was lowest close to the oocyte and apoptosis increased with increasing distance from the oocyte (Hussein et al. 2005).

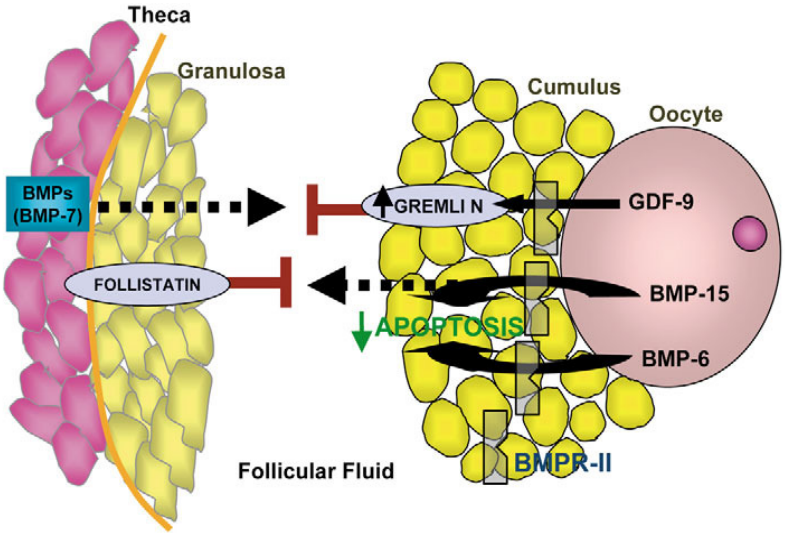


Figure 2.5: Proposed model by which the paracrine network of BMP/GDF-9 growth factors and their binding proteins interact to regulate apoptosis in the COC microenvironment. Oocyte-secreted BMP-15 and BMP-6 signal through the cumulus cell receptor BMPR-II to actively prevent cumulus cell death. Oocyte-secreted GDF-9, also acting through BMPR-II but using a different type-I receptor to the BMPs, does not prevent cumulus cell apoptosis but induces cumulus cell gremlin expression. GDF-9 stimulated gremlin expression may in turn block theca and granulosa cell-derived BMPs from competing with BMP-15, BMP-6 and GDF-9 for BMPR-II binding. In addition, follistatin produced by mural granulosa cells and present in follicular fluid, may limit the anti-apoptotic effects of oocyte BMP-15 specifically to the COC microenvironment (Hussein et al. 2005).

Recently, in addition to oocyte secreted factors, there is another oocyte derived ligand called intermedin (IMD) also known as adrenomedullin 2 (ADM2) which according to studies based on *in vitro*-cultured COCs and animal models *in vivo*. The IMD/ADM2 plays a direct role in enhancement cumulus cell survival because its exhibiting an anti-apoptosis effect and regulating cell-cell contacts in COCs, moreover, intrabursal injections of IMD antagonist led to oocyte atresia and reduction of follicular cyclin D2 (activator of cell cycle progression) expression (Chang et al. 2011). Thus, oocyte considers the main regulator of follicular cell functions through its secreting substances.

Exposure of bovine cumulus oocyte complexes (COCs) during oocyte maturation to oocyte-secreted factors (OSFs), whether in their native form as an uncharacterized mix of growth factors secreted by the oocyte or as recombinant exogenous BMP-15 or GDF-9, substantially improved subsequent oocyte developmental competence, where the blastocyst rate increased from ~40% to ~60% (Hussein et al. 2006).

2.3.2 The role of cumulus cell during oocyte development

During ovarian folliculogenesis in mammals, the transition from a secondary follicle to an antral follicle is signaled by the formation of a fluid-filled antrum, differentiation of granulosa cells and development of a cumulus oocyte complex. Cumulus cells have a characteristic function different from mural granulosa cells such as: cumulus cells have high proliferation rate, low steriodogenic capacity, very low LH receptor expression and ability to produced and secret hyalouronic acid and undergo expansion (Armstrong et al. 1996; Eppig et al. 1997; Li et al. 2000).

Bovine COC have very high capacity to incorporate tritiated thymidine (used as marker of DNA synthesis) and lower capacity produced progesterone in comparison to mural granulosa cells (MGC). In the same study when oocytes were microsurgically removed from oocyte cumulus cell complexes (oocytectomy), OOX displayed *in vitro* more characteristic like MGC (reduced DNA synthesis and increased secretion of progesterone) however, this MGC character of OOX complex was completely restored to COC level by co-culture with denuded oocyte (DO) (Li et al. 2000). Likewise, another study showed dramatic elevation of the expression of LHR mRNA in the OOX complex and this expression was suppressed below detectable level in OOX after co-culture of OOX complex with oocyte (Eppig et al. 1997). Both previous studies support the hypothesis that the oocyte actively promotes the development of the cumulus cell phenotype.

Cumulus cells are known to play a critical role during oocyte maturation, in keeping the mammalian oocyte under meiotic arrest, in participating in the induction of meiotic resumption and in supporting cytoplasmic maturation (Tanghe et al. 2002). Also in some species, it is evident that cumulus cells and their inter-cellular matrix participate

the capture of COC by oviduct infundibulum through its adhesion with ciliated oviductal epithelium (Kölle et al. 2009; Lam et al. 2000) and involved in the induction of sperm capacitation and acrosome reaction (Chian et al. 1995; Cox et al. 1993; Fukui 1990). Recently, the evidence of crosstalk between sperms and cumulus mass and cumulus induced acrosome reaction has emerged via a sperm membrane protein, NYD-SP8 which is glycosylphosphatidylinositol (GPI) anchored membrane protein cleaved from sperm head upon its interaction with the cumulus and binds to the cumulus cells, resulted in calcium mobilization and progesterone production in cumulus cells. The secreted progesterone subsequently initiates sperm acrosome reaction within the cumulus layer (Sun et al. 2011; Yin et al. 2009). Moreover, removal of CCs at the beginning of oocyte *in vitro* maturation (IVM) or shortly before *in vitro* fertilization or the blockade of the gap junction is detrimental to oocyte maturation, fertilization and subsequent development (Fatehi et al. 2002; Fukui and Sakuma 1980; Vanderhyden and Armstrong 1989; Vozzi et al. 2001; Zhang et al. 1995). In recent study, removal of companion CCs at GV stage appeared to affect the gene expression of MII oocytes as a number of genes are over-expressed in oocytes cultured with CCs relative to those cultured without (Regassa et al. 2011).

During oocyte maturation, the metabolic requirement of COC increased, such as the consumption of glucose, oxygen and pyruvate increased by two fold over the 24 hr period of bovine COC culture (Sutton et al. 2003a). The COC used glucose for energy production and other numerous essential metabolic processes such as nucleic acid and purine synthesis, mucification and cellular homeostasis (Sutton et al. 2003b). However, the oocyte itself has a poor capacity to utilize glucose (Biggers et al. 1967) and relies on cumulus cells to convert glucose mainly via glycolysis to substrate it can readily utilize such as pyruvate (Donahue and Stern 1968; Sutton-McDowall et al. 2010; Sutton et al. 2003b), then oocyte metabolized pyruvate through oxidative phosphorylation to produce energy for growth and maturation (Biggers et al. 1967; Brinster 1971; Eppig 1976). In mice, oocytes regulate the co-operativity between oocytes and cumulus cells for energy metabolism by paracrine secreted factors which regulating expression levels of genes encoding glycolytic enzymes (such as ENO1, PKM2, TPI, ALDOA, Ldh1, and PFKP) in granulosa/cumulus cells (Sugiura et al. 2005). While, the oocyte secreted factors didn't appear to affect the glucose consumption rate in bovine as COCs, OOX

and OOX co-culture with denuded oocytes all showed similar rate of glucose consumption over a 24 hr culture period (Sutton et al. 2003a). This indicated that, might be there is species difference in regulatory role of oocyte to cumulus cell glycolysis (Sutton-McDowall et al. 2010). In addition to nutritional support of the developing oocyte, cumulus cells have another metabolic and protective role in oocyte cytoplasmic maturation, for instance, cumulus cells have the ability to reduce cystine to cysteine and promote its uptake by bovine oocyte during maturation (de Matos et al. 1997), as consequence, bovine cumulus enclosed oocyte have higher contain of gluthatione (GSH) than denuded oocyte (Geshi et al. 2000). Gluthatione participate in sperm decondansation in parallel with oocyte activation and transformation of penetrated sperm head into male pronucleus during fertilization (Perreault et al. 1988; Sutovsky and Schatten 1997; Yoshida 1993). Moreover, GSH is well known to play an important role in protecting cells against the destructive effect of reactive oxygen intermediates and free radicals (Lim et al. 1996; Meister 1983). Cumulus cells play a critical role in protecting the oocyte against oxidative stress which induced apoptosis through the enhancement of the GSH content in pig oocyte (Tatemoto et al. 2000). Another example of metabolic cooperation between oocyte and cumulus cells, involved in amino acid uptake. Some amino acids such as L-alanine can not uptake by the oocyte, however, the cumulus cells take them up and then transfer to the oocytes through gap junctions and this supported by the high expression of Slc38a3 which known as a transcript encoding sodium-coupled neutral amino acid transporter in cumulus cell but not in oocytes (Colonna and Mangia 1983; Eppig et al. 2005).

During *in vitro* maturation, cumulus cell benefits bovine oocyte development either by secreting soluble factors, which induce developmental competence, or by removing inhibitory or toxic components from the maturation medium (Hashimoto et al. 1998). Additionally, cumulus cells might have unknown promoting effects on subsequent oocyte development which might be attributable to intracellular changes such as pH or calcium ions (Mori et al. 2000). The gap junction communication between oocyte and surrounding cumulus cells are required during IVM of bovine oocyte for cytoplasmic maturation and subsequent early development, where the absence of cumulus cells during maturation did not affect the development of the 2–8 cell stages 72 hr after insemination but development beyond the blastocyst stage was significantly lower in

cumulus free than in cumulus-intact oocytes (Atef et al. 2005) while, sheep oocytes matured without cumulus cells failed to undergo cleavage after fertilization (Crosby et al. 1981).

The cumulus and its communication with the oocyte have role in oocyte competence acquisition process, as we mentioned before, the presence of cumulus cells and intact gap junctions were reported to support oocyte competence *in vitro* and conversely, the inhibition of the functional coupling between the oocyte and its cumulus using gap junction inhibitors significantly reduced the oocyte competence expressed in terms of blastocyst rate (Atef et al. 2005; Hashimoto et al. 1998).

The oocyte developmental competence is the intrinsic ability of oocytes to undergo meiotic maturation, fertilization and embryonic development. Several studies used microarray technology and reverse transcription polymerase chain reaction to identify and validate gene expressed in cumulus cell and used as biomarkers related to oocyte competence and associated with embryo quality and pregnancy outcome (Assidi et al. 2008; Assou et al. 2008; Caixeta et al. 2009; McKenzie et al. 2004; van Montfoort et al. 2008). The prostaglandin-endoperoxide synthase-2 (PTGS2), hyaluronan synthase 2 (HAS2), inhibin beta A (INHBA), betacellulin (BTC) which is a member of the EGF family of growth factors, CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), follicle stimulating hormone receptor (FSHR), growth hormone receptor (GHR) and epidermal growth factor receptor (EGFR) have been identified as biomarkers in bovine cumulus cell that predict oocyte competence and select higher embryo quality (Assidi et al. 2008; Caixeta et al. 2009). Similarly, expression of PTGS2, HAS2 and GREM1 were increased in human cumulus cell isolated from oocyte which produced high quality embryos (cleavage stage) than those detected in cumulus cell from poor quality embryos, hence theses genes may be markers of human oocyte developmental competence (McKenzie et al. 2004). The same results were obtained for HAS2 and GREM1 by Cillo et al. (2007), which indicate that the measurement of transcriptional levels of these genes in cumulus cells would reliably complement the morphological evaluation providing a useful tool for selecting oocytes with greater chances to be fertilized and develop *in vitro*. Other genes expressed in cumulus cells and associated with embryo quality based on early cleavage rates during embryonic development:

glutathione peroxidase 3 (GPX3), chemokines receptor 4 (CXCR4), cyclin D2 (CCND2), catenin delta 1 (CTNND1), 7-dehydrocholesterol reductase (DHCR7), dishevelled dsh homolog 3 (DVL3), heatshock 27 kDa protein 1 (HSPB1) and tripartite motif-containing 28 (TRIM28). These genes reflect hypoxic conditions or delayed oocyte maturation in non early cleavage embryo, hence these also provide a set of potential markers could be used for oocyte selection (van Montfoort et al. 2008). Assou et al. (2008) have proposed that three of genes expressed in cumulus cells issued from oocytes that developed into embryos with a good morphology, BCL2L11 (involved in apoptosis), PCK1 (phosphoenlpyruvate carboxykinas 1 involved in gluconeogenesis) and NFIB (a transcription factor) could be used as biomarkers for embryo potential and pregnancy outcome.

2.4 Short history for microRNAs

MicroRNAs (miRNAs) represent an exciting discovery in the field of biology in recent years. Originally described in 1993, lin-4 was the first miRNA to be discovered in the *Caenorhabditis elegans*, by the joint efforts of Ambros's and Ruvkun's laboratories (Bartel 2004; Lee et al. 1993), lin-4 discovered by the isolation of a null mutation that caused a failure in temporal development (Chalfie et al. 1981; Horvitz and Sulston 1980). The activity of lin-4 is required for the transition from the L1 to L2 stage of larval development (Chalfie et al. 1981; Lee et al. 2004a). Ferguson et al. (1987) found that a suppressor mutation in the gene lin-14 was able to revert the null-lin-4 mutation phenotype. So this opposite phenotype between defects in lin-4 and lin-14 genes indicated that lin-4 could negatively regulate lin-14 (Lee et al. 2004a). In June 1992, Ambros and Ruvnkun independently concluded that lin-4 transcripts were complementary to a repeated sequence in the 3'-UTR of the lin-14 gene. Morover, in December 1993, Ambros and Ruvkun independently reported that the small and non-protein-coding transcript lin-4 regulates lin-14 through its 3'-UTR (Lee et al. 2004a; Lee et al. 1993; Wightman et al. 1993). Likewise lin-4, let-7 is a heterochronic gene of *C.elegans* and was the second miRNA to be discovered, in 2000, seven years after the finding of the first miRNA. Reinhart et al. (2000) at Ruvkun's laboratory reported that let-7 was a 21-nt RNA controlling the L4-to-adult transition of larval development and loss of let-7 activity caused reappearance of larval cell fates during the adult stage of

development, while increased let-7 activity caused precocious expression of adult fates. Counter to lin-4, the let-7 sequence is conserved across species from flies to humans (Pasquinelli et al. 2000) and this conserved across species triggered a revolution in the research of a new class of small ncRNAs, called miRNAs. Presently, thousands of miRNAs have been identified in humans and other species, and miRNA sequences are available online in database, such as miRbase (Griffiths-Jones 2004; Griffiths-Jones et al. 2006; Griffiths-Jones et al. 2008).

2.5 Genomic location of microRNAs

Nowadays, 1424, 720 and 662 miRNAs were identified in human, mice and bovine, respectively (miRBase, release 17, http://www.mirbase.org/). Approximately 50% of miRNAs are in miRNA clusters that are found to be encoded as a polycistronic transcript that is subsequently cleaved into multiple miRNAs (Lee et al. 2002). The clustered miRNAs may be functionally related in terms of targeting the same gene or different genes in the same biochemical pathway (Singh et al. 2008). In most cases, polycistronic miRNAs share the same expression pattern. Initially, it was thought that most miRNA are encoded in intergenic region and transcript from their promoter as independent genes (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Although, a number of miRNA genes have been also reside within the transcription unit (Tu) of other genes, both intronic and exonic (Lagos-Quintana et al. 2003; Lau et al. 2001; Lee et al. 2002). Rodriguez et al. (2004) showed that about 40% of mammalian miRNAs are located in the intronic region of either protein-coding or non-coding transcription unit (e.g miR-208 and miR-100 respectively), whereas 10% are encoded within the exonic region. With few exceptions, miRNAs that are embedded in, or overlap with, exons of known transcripts are always in the same orientation, and most of these known transcripts are in the non-coding 5' or 3'-UTRs (e.g. miR-198 in follistatin-like 1) and their maturation often excludes host gene function (Cullen 2004; Rodriguez et al. 2004). A new class of intronic miRNAs (called ‘mirtrons’) has been derived from short hairpin introns via a non-classical miRNA pathway and constitute 5-10% of miRNA genes in invertebrates and vertebrates (Berezikov et al. 2007; Glazov et al. 2008; Okamura et al. 2007; Ruby et al. 2007) So, concerning the genomic location of miRNA genes, it can be categorized into three groups (Figure 2.6):

a) Intergenic miRNA which located in between protein coding genes and have its own promoter

b) Intronic miRNA which located within intron of their host protein coding genes

c) Exonic miRNA which located within exon of host protein coding genes or overlap an exon and an intron of non-coding gene

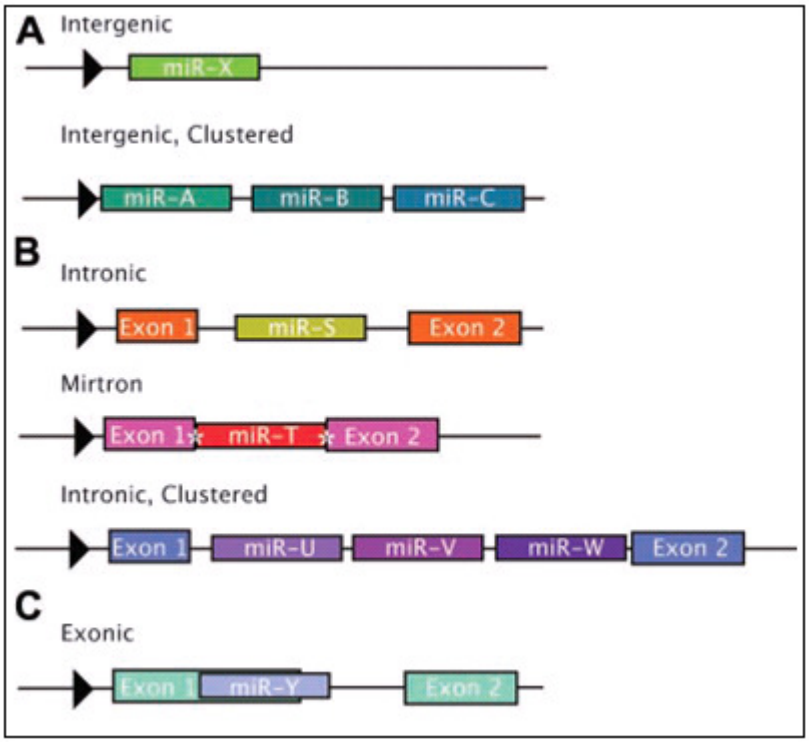


Figure 2.6: Genomic location of microRNAs (Olena and Patton 2010).

2.6 MicroRNA biogenesis

MicroRNA biogenesis is a multistep process; the first two steps are localized in the nucleus followed by maturation in the cytoplasm (Figure 2.7). The majority of miRNAs are transcribed by RNA polymerase II as long primary miRNAs (pri-miRNAs) that contain a hairpin stem carry a 7-methyl guanylate cap at the 5' end and poly (A) tail at the 3' end (Cai et al. 2004; Kim 2005; Lee et al. 2004b). RNA polymerase III has also been demonstrated to generate the transcripts of a subset of miRNAs (Borchert et al. 2006; Ozsolak et al. 2008).

Following transcription, the nuclear microprocessor complex, which contains the RNase-III type endonuclease Drosha and its dsRNA-binding partner, Dgcr8/Pasha cleaved the pri-miRNA transcripts and release a short stem–loop precursor miRNA (pre-miRNA) (Denli et al. 2004; Han et al. 2004; Lee et al. 2003). DiGeorge syndrome critical region gene 8 (Dgcr8) functions at least in part by binding to single-stranded RNA–double-stranded RNA (ssRNA-dsRNA) junction of the pri-miRNA stem and directing Drosha to cleave ~11 bp away from this junction produce a pre-miRNA which containing ~60-70 nt stem-loop with a 5' phosphate and a 3' 2-nt hydroxyl overhang that act as recognition site for the next processing step (Han et al. 2006). Also, microprocessor complex contains other accessory proteins, such as the DEAD box RNA helicases p68 (DDX5), p72 (DDX17) and heterogeneous nuclear ribonucleoproteins (hnRNPs) (Fukuda et al. 2007; Gregory et al. 2004; Guil and Caceres 2007; Liu et al. 2008). These accessory proteins may function to promote the fidelity, specificity, and/ or activity of Drosha cleavage. Moreover, these binding protien act as regulator for miRNA processing (Slezk-Prochazka et al. 2010).

After nuclear processing, the short 3' overhang structure in the pre-miRNA is recognized by Exportin-5, a nuclear transport receptor, in complex with Ran-GTP and export the pre-miRNA into the cytoplasm after hydrolysis of Ran-GTP to Ran-GDP (Lund et al. 2004; Okada et al. 2009; Yi et al. 2003). Exportin-5 is also important for stabilizing pre-miRNAs in the nucleus (Zeng and Cullen 2004).

In the cytoplasm, the pre-miRNA is processed by Dicer (an RNase III enzyme), together with its dsRBD protein partner, Loquacious (Loqs) in Drosophila or the trans-activator RNA (tar)-binding protein (TRBP) in mammals, cleaves the pre-miRNA into a ~19- 24bp double-stranded miRNA termed miRNA duplexes by cutting near the hairpin loop (Chendrimada et al. 2005; Grishok et al. 2001; Ketting et al. 2001; Saito et al. 2005). The miRNA duplex are then unwound and one of the strands (mature miRNA) are associates with an Argonaute protein (Ago) within RNA-induced silencing complex (RISC) or miRNA-containing ribonucleoprotein complexs (miRNPs), where it acts as a guide to repress target messages (Hutvagner and Simard 2008; Hutvagner and Zamore 2002; Mourelatos et al. 2002; Murchison and Hannon 2004). An Argonaute protein is the core component of RISC, its highly conserved family of proteins involved in the

RNA interference and miRNA pathway. The Ago have PIWI domain flanked by N-terminal (N) and middle (Mid) domains, PAZ domain which binding to 3' end of small RNA while Mid domain providing a binding pocket for the phosphorylated 5' end and the PIWI domain shows an RNase H-like structure (Carthew and Sontheimer 2009; Lingel et al. 2004; Liu et al. 2004; Ma et al. 2004).

The strand selection is identified by inherent features of the miRNA duplex, including thermodynamic asymmetry/stability (Khvorova et al. 2003; Schwarz et al. 2003), where the RNA strand which thermodynamically least stable at 5' end will be more frequently selected as the functional guide strand and the other strand (the passenger strand or miRNA*) is assumed to be excluded from miRISC assembly and subsequently degraded (Liu et al. 2008). However, deep-sequencing efforts have revealed that a large number of miRNA* strands can be appreciably detected in Ago complexes, and are in large part functional (Czech et al. 2009; Okamura et al. 2008; Seitz et al. 2008). Additionally, in recent study they demonstrated that miR-223 and miR-223* targeted the insulin-like growth factor 1 receptor/phosphatidylinositol 3-kinase axis and the high miR-223* levels were associated with increased overall survival in patients with acute myeloid leukemia, thus indicate the active role for both arms of the miR-223 duplex (Kuchenbauer et al. 2011).

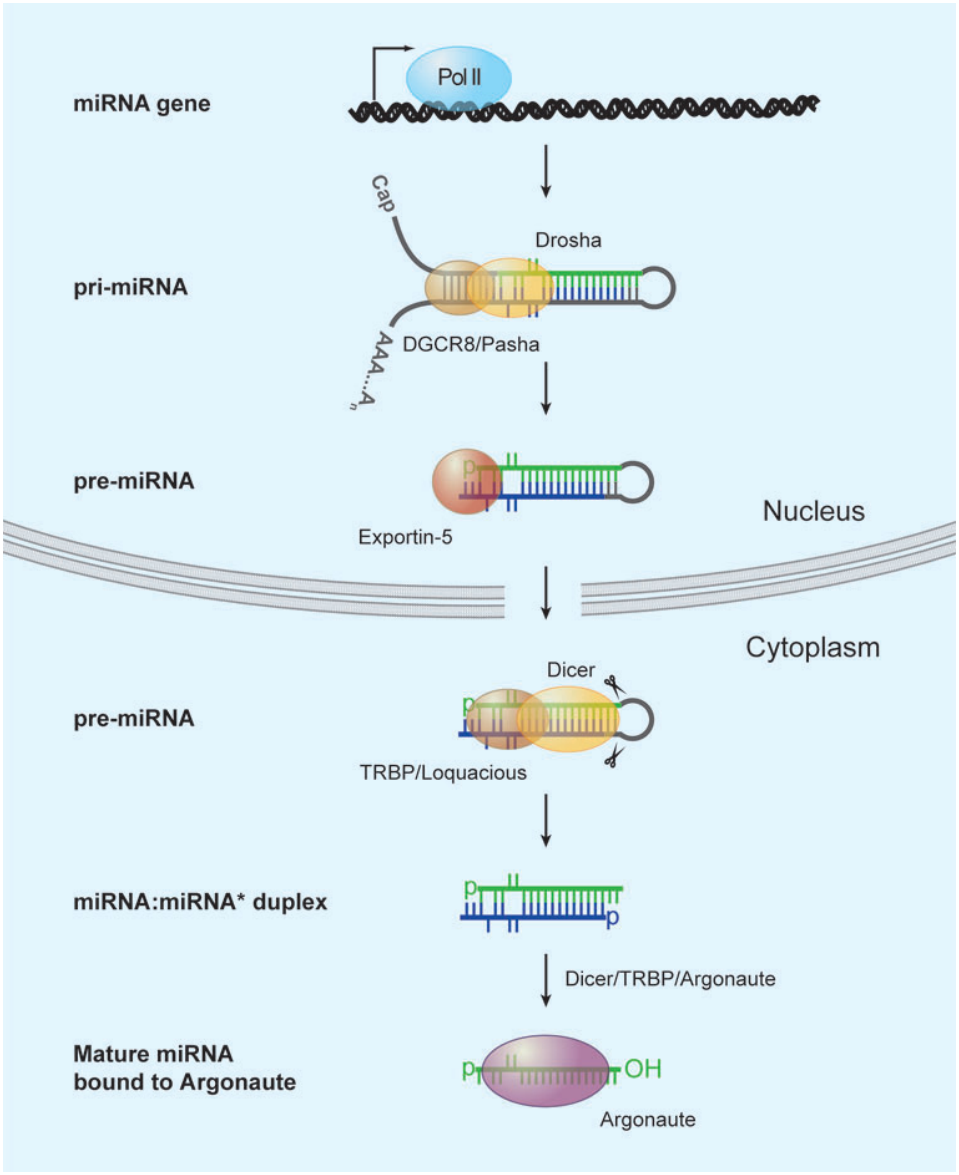


Figure 2.7: MicroRNA biogenesis (Bushati and Cohen 2007).

2.7 Principal of miRNA-mRNA complementary

MicroRNAs control in expression of target mRNA by Watson-Crick base pairing between to sequence motifs in the 3'-UTR of mRNAs and the miRNA at position 2–7 in the 5' end of the mature miRNA with perfect or near perfect complementarities (Bartel 2009; Lai 2002; Lewis et al. 2003; Xie et al. 2005). Also, in the 3'-UTR there is a certain AU-rich elements (AREs) which interact with miRNAs and act both directly and indirectly as potent post-transcriptional regulatory signals (Vasudevan and Steitz

2007). The analysis of miRNA target sites indicated that genes with longer 3'-UTRs usually have higher density of miRNA binding sites and are mainly involved in developmental modulations, while genes with shorter 3'-UTRs usually have lower density of miRNA-binding sites and tend to be involved in basic cellular processes (Cheng et al. 2009; Osada and Takahashi 2007; Sandberg et al. 2008). So these facts give indication about the importance of 3'-UTR interaction with miRNA, although, some miRNA from plants and animals repression regulation by specifically targeting the 5'-UTR or coding region of some mRNAs (Easow et al. 2007; Fang and Rajewsky 2011; Jopling et al. 2005; Selbach et al. 2008; Sunkar and Zhu 2004). However, sites located in coding regions appear to be less robust than those in the 3'-UTR (Easow et al. 2007; Gu et al. 2009). There are five types of seed-matched sites are known (Figure 2.8 A): two 6mer, two 7mer and one 8mer. The 6mer which have perfect 6 nt match to the miRNA seed (miRNA nucleotides 2–7) (Lewis et al. 2005) and the 6mer contains six matches to nucleotides 3–8, referred to as offset 6mer sit, but they are thought to have a limited functionality (Friedman et al. 2009; Witkos et al. 2011). The two 7mer site, one called 7mer-m8 site which the seed match augmented by a match to miRNA nucleotide 8 (Brennecke et al. 2003; Brennecke et al. 2005; Krek et al. 2005; Lewis et al. 2005; Lewis et al. 2003), while the other one is the 7mer-A1 site, which contains the seed match augmented by an adenine (A) in target position 1 at the 5' end of miRNA (Lewis et al. 2005). The 8mer site includes the seed match flanked by both the match at position 8 and the A at position 1 (Lewis et al. 2005). The site which have complete pairing within the seed region (7mer-m8, 7mer-A1 and 8mer site) known as canonical sites which account for the majority of validated conserved targets, 7mer sites are most abundant for highly conserved miRNAs (Friedman et al. 2009). Moreover, the adenine opposite position 1 of miRNA improves the degree of gene silencing (Nielsen et al. 2007).

All the pervious sites can have additional paring within the 3' region of the miRNA, at least 3-4 nucleotides consecutively paired in positions 13-16 of miRNA are usually required to enhance the effectiveness of miRNA-mRNA interaction which facilitates target prediction and such sites are called “3' supplementary sites” (Figure 2.8B) (Friedman et al. 2009; Grimson et al. 2007). However, this pairing to the 3' region of the

miRNA not only supplementary, it can be also compensate for a single-nucleotide bulge or mismatch in the seed region (Figure 2.8C) (Vella et al. 2004; Yekta et al. 2004).

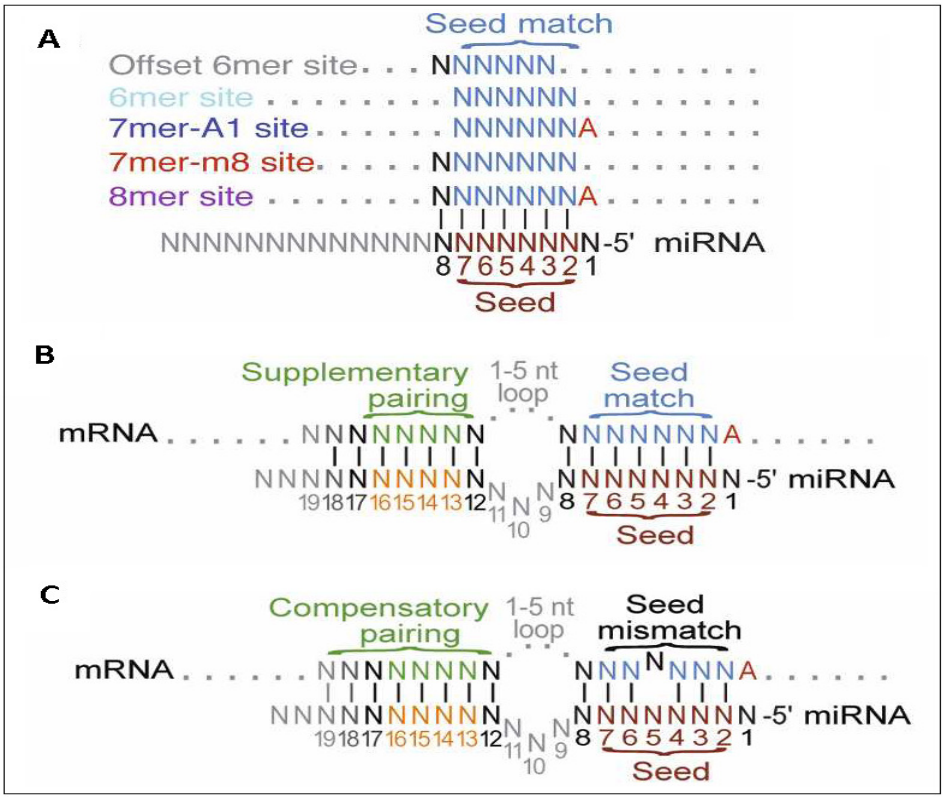


Figure 2.8: Sites matching the miRNA seed region (A), share six contiguous Watson–Crick matches to the miRNA seed (nucleotides 2–7); the offset 6mer contains six contiguous matches to nucleotides 3–8. Conserved pairing to the 3' ends of miRNAs (B, C) preferential occurrence of pairing to the 3' region of the miRNA as supplementary pairing or compensatory pairing, respectively, modified from Friedman et al. (2009).

Many different algorithms have been developed for the prediction of miRNA targets such as PicTar, TargetScan, miRanda, DIANA-microT, rna22 and PITA (John et al. 2004; Kertesz et al. 2007; Krek et al. 2005; Kuhn et al. 2008; Lewis et al. 2003; Rajewsky 2006). These algorithms based on various important features of miRNA-target nucleotide sequence interaction to search for mRNA target. At least some of the following features used by prediction programs: base pairing pattern of the seed region at the 5' end of miRNA and 3′-UTR of target mRNA and this criteria are the most common ones, evolutionary conservation of putative miRNA-binding site, secondary

structure of target transcript and nucleotide composition of target sequences which often added to increased accuracy (Grimson et al. 2007; Grün et al. 2005; Kertesz et al. 2007; Lewis et al. 2003; Long et al. 2007). The miRanda, TargetScan, and PicTar are most popular softwares. A proper integration of miRNA prediction algorithms can significantly improve the prediction accuracy (Zhang and Verbeek 2010).

2.8 Mechanisms of microRNA-mediated gene regulation

It is believed that the degree of miRNA-mRNA complementarity determines the mechanism of miRNA-mediated regulation. Perfect or almost perfect complementarity, like most of miRNAs in plants, allows Ago catalyzed direct cleavage of their target mRNAs as siRNA functions (Llave et al. 2002; Rhoades et al. 2002). However, perfect complementarity between miRNAs and their targets is rare in animals. A very few extensive microRNA target interactions can lead to mRNA cleavage in mammals, for example miR-196 and miR-434-5p which directs the cleavage of the Homeobox protein (HOXB8) and Rtl1/Peg11 transcript, respectively (Davis et al. 2005; Yekta et al. 2004). Most animal miRNAs mediate regulation of their targets through translational repression or/and mRNA decay via a deadenylation and decapping mechanism (Behm-Ansmant and Izaurralde 2006; Giraldez et al. 2006; Wu et al. 2006).

The precise mechanism by which miRNAs regulate gene expression still elusive, there are numbers of models has been proposed over the last years and have been the subject of an intense discussion in various reviews (Carthew and Sontheimer 2009; Chekulaeva and Filipowicz 2009; Eulalio et al. 2008a; Filipowicz et al. 2008; Jackson and Standart 2007; Nilsen 2007; Pillai et al. 2007; Richter 2008; Standart and Jackson 2007; Wu and Belasco 2008; Zhao and Liu 2009). Moreover, some studies suggest that, miRNAs can activate rather than repress their targets under specific conditions (Henke et al. 2008; Orom et al. 2008; Vasudevan et al. 2007).

2.8.1 Translation repression

Many experiments investigating miRNA function point to initiation of translation as a target of repression, however, evidence also exists that miRNA inhibition occurs at

various post-initiation steps (Eulalio et al. 2008a; Filipowicz et al. 2008). Briefly, in eukaryotic mRNA translation can be divided into three steps: initiation, elongation and termination. Initiation starts with the recognition of the mRNA 5'-terminal cap structure m7GpppN by the eIF4E subunit of the eukaryotic translation initiation factor eIF4F complex, which also contains eIF4A (an RNA helicase) and eIF4G (an important scaffold for the assembly of the ribosome initiation complex) (Edery et al. 1983; Grifo et al. 1983; Sonenberg et al. 1979). The eIF4G interact with another initiation factor, eIF3, and contributes to the small ribosomal subunit (40S) assembly at the 5' end of the mRNA to enable the preinitiation complex (Kapp and Lorsch 2004; Merrick 2004). The 40S preinitiation complex joins with the large ribosomal subunit (60S) at the AUG codon to begin elongation phase which can be regulated by phosphorylation of the elongation factor eEF2 (Herbert and Proud 2007; Taylor et al. 2007). The eIF4G also interacts with the polyadenylate binding protein 1 (PABP1) that decorates the 3' end of the message (Gingras et al. 1999; Imataka et al. 1998). The interaction of eIF4G with eIF4E and PABP1 lead to circularization of mRNA, this circularization stimulates translation initiation by increasing the affinity of eIF4E for m7GpppN, and might facilitate ribosome recycling (Derry et al. 2006; Wells et al. 1998). The termination process mediate by translation release factors when the ribosome reaches the stop codon (UAA, UGA and UAG) (Ehrenberg et al. 2007). Some viral mRNAs initiate translation without the involvement of the m7G cap and eIF4F. In such cases, the 40S ribosomes are bind to mRNA through the internal ribosome entry site (IRES) directly (Pestova et al. 2001; Pestova et al. 1998).

2.8.1.1 Repression at the initiation step

There is different models proposed translation repression at initiation step, early study shown that miRNAs and their targets are not associated with the polysomal fraction in sucrose gradients but rather with the free mRNP pool in mammalian cells, indicating that translation inhibition occurs at the initiation step (Pillai et al. 2005). Additionally in other study, miRNA can affect translation initiation and implicate the cap-binding protein eukaryotic initiation factor 4E as a molecular target. In this study, replacing the cap structure with an internal ribosome entry site from either the cricket paralysis virus or the encephalomyocarditis virus impairs miRNA-mediated repression (Humphreys et

al. 2005). In agreement with a role for miRNAs in repression translation initiation, Kiriakidou et al. (2007) reported that the MID domain of Ago2 protein, the core of the miRNA effector complex (RISC), contains limited sequence homology to m7G cape binding region of eIF4E, this indicated that Ago2 can compete with eIF4E for m7G binding and represses translation initiation process (Figure 2.9C) but this has been controversial in Drosophila cells (Eulalio et al. 2008b). Also, there is evidence suggesting that miRNAs inhibit an early translation step through inhibition ribosomal subunit joining. The Eukaryotic initiation factor 6 (eIF6) is known to interacts with the 60S ribosomal subunit and prevents its premature association with the 40S ribosomal subunit to generate transnational competent 80S ribosome particles (Ceci et al. 2003). Chendrimada and co-workers identify eIF6 as a binding partner of miRISC and depleting eIF6 in either human cells or *C.elegans* effectively abolishes miRNA-mediated translational repression (Chendrimada et al. 2007). Other data from Drosophila S2 cells suggest that eIF6 may not be widely required for miRNA function (Eulalio et al. 2008b). In addition, the experiments in reticulocyte lysate showed that miRNA-targeted mRNAs are enriched for 40S, but not 60S, ribosome components, thus author postulated that miRNA may repress initiation by inhibiting 60S subunit joining (Figure 2.9D) (Wang et al. 2008). Furthermore, miRNA can affect translation initiation by inhibit mRNA circularization (Figure 2.9E). The reported interaction of the GW182 protein, one of the component of miRISC, with the poly (A)-binding protein (PABP) (Fabian et al. 2009; Zekri et al. 2009) may interfere with the closed-loop formation mediated by the eIF4G-PABP interaction and thus contribute to the repression of translation initiation (Fabian et al. 2010). The interaction of GW182 with PABPC1 is also required for the degradation of miRNA targets, most likely because the interaction facilitates the recruitment of the CCR4-NOT deadenylase complex (Zekri et al. 2009).

For instance some natural or model miRNA repress translation at the initation step such as miR-122 in human hepatoma cells targeted CAT mRNA (Bhattacharyya et al. 2006), let-7 in *C. elegans* targeted Daf-12 and lin-41 (Ding and Grosshans 2009) and let-7 in mammalian cell (Humphreys et al. 2005; Pillai et al. 2005).

2.8.1.2 Repression at post-initiation step

The observation that miRNA and their repressed target in both *C. elegans* and mammalian cells are associated with polyribosome (no change in polyribosome profiles) compared with non repressed mRNAs, suggesting that most miRNA targets are regulated after translational initiation (Maroney et al. 2006; Nottrott et al. 2006; Olsen and Ambros 1999; Petersen et al. 2006; Seggerson et al. 2002). Furthermore, the cap independent translation initiated by internal ribosome entry site (IRES) was also repressed by miRNA (Lytle et al. 2007; Petersen et al. 2006). The post-initiation repression by miRNA could result from degradation of nascent polypeptide chain derived from mRNA under miRNA control or miRNA leading to increase rate of ribosome drop off and increased immature termination during elongation, resulting in incomplete protein product, (Figure 2.9A,B) (Nottrott et al. 2006; Petersen et al. 2006).

2.8.2 mRNA decay mediated by microRNA

It was originally thought that miRNAs affect primarily translation of mRNAs, it is now clear that the repression of many miRNA targets is frequently associated with their destabilization as demonstrated by microarray analysis of transcript level in cells and organ in which the miRNA pathway was inhibited (Bagga et al. 2005; Behm-Ansmant and Izaurralde 2006; Eulalio et al. 2007; Giraldez et al. 2006; Rehwinkel et al. 2005; Schmitter et al. 2006) or in which miRNA level were experimentally changed (Esau et al. 2006; Krutzfeldt et al. 2005; Lau et al. 2008; Lim et al. 2005; Linsley et al. 2007; Webster et al. 2009).

In animal cells, the majority of miRNA target degradation is not because of endonucleolytic cleavage by Ago but rather miRNA direct their targets to the cellular 5' -to-3' mRNA decay pathway, where mRNAs are first deadenylated by the CAF1–CCR4–NOT deadenylase complex, and then decapped by the decapping enzyme DCP2 (Figure 2.9F), which require several decapping activators including DCP1, Ge-1, EDC3, and RCK/p54, followed by the exonucleolytic decay (Behm-Ansmant and Izaurralde 2006; Eulalio et al. 2008b; Eulalio et al. 2009; Eulalio et al. 2007; Giraldez et al. 2006; Rehwinkel et al. 2005; Valencia-Sanchez et al. 2006; Wu et al. 2006). The

exonucleolytic decay can occur for deadenylated mRNA from the 3' end by the exosome and cofactors and also can be subsequent for decapping by the 5'-to-3' exoribonuclease, Xrn1p (Parker and Song 2004; Valencia-Sanchez et al. 2006). The exact process of target selection to be degraded or not was not clear, but it has been proposed that the number, type, and position of mismatches in the miRNA/mRNA duplex play an important role in triggering degradation or translation arrest (Aleman et al. 2007).

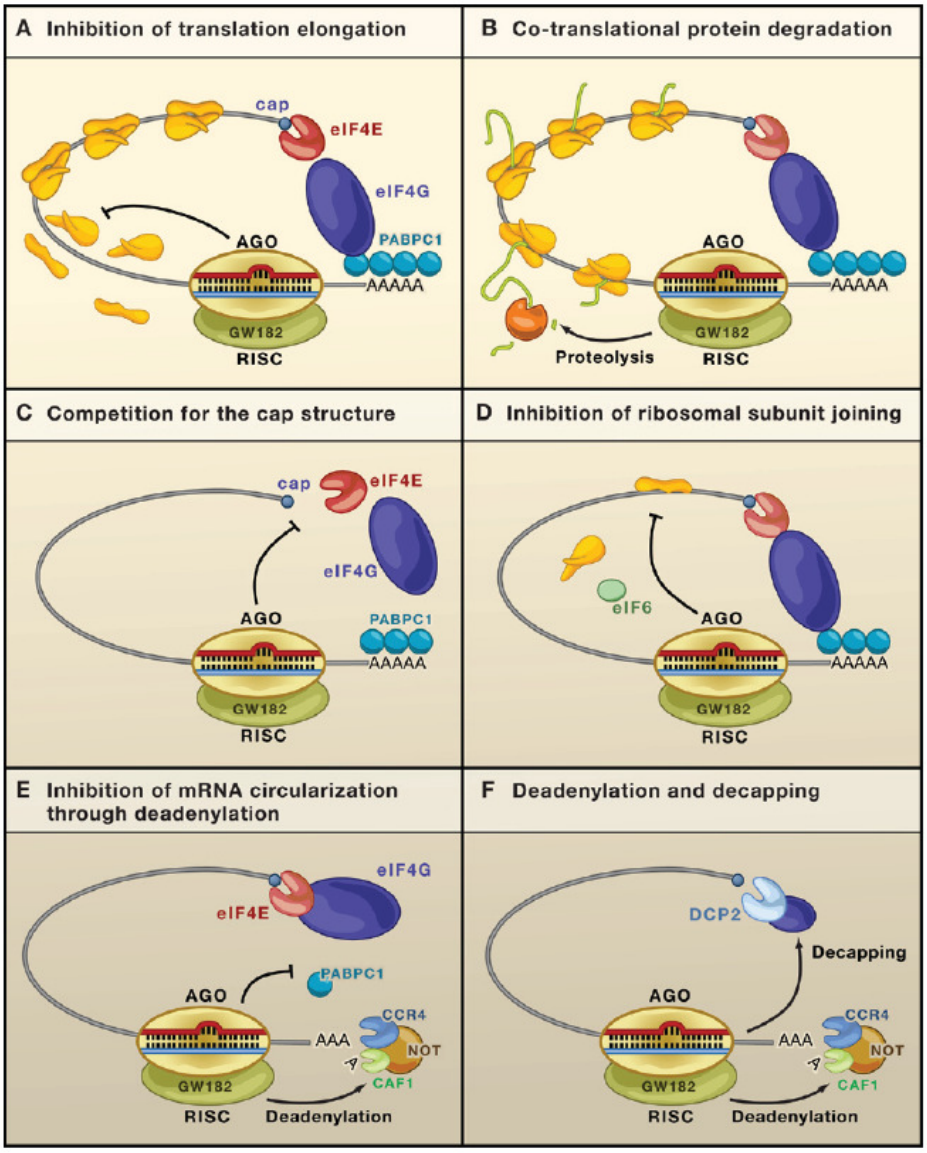


Figure 2.9 : Mechanisms of miRNA-mediated gene silencing (Eulalio et al. 2008a).

2.8.3 Another mode of action (Activation by microRNA)

Several studies give evidences that miRNA may also function to induce gene expression through different mechanism under certain conditions, Vasudevan and collaborators demonstrated that miRNA act as translation repression in proliferating cells and as activator in cells arrested in G0/G1. Their result showed that miR-369-3 directs association of Ago and fragile X mental retardation–related protein 1 (FXR1) with AREs in tumor necrosis factor alpha (TNFα) to activate translation under serum starvation by direct base pairing between miRNA seed sequence and complementary AREs in TNFα (Vasudevan et al. 2007). In another studies, miR-10a bind to 5'-UTR of mRNA encoding ribosomal protein and enhanced their translation during amino acid starvation, although this miRNA caused translation repression of Ras-related nuclear protein (Ran) and penicillin- binding protein (Pbp1) when binding to its 3'-UTR (Orom et al. 2008).

The effect of miRNA on gene expression showed to be position dependant, for instance, liver specific miRNA (miR-122) was shown to up-regulate the level of hepatitis C virus (HCV) RNA by stimulating RNA replication following its interaction with the 5'-UTR of HCV RNA genome, while binding of miR-122 to 3'-UTR of reporter mRNA lead to down regulation of mRNA expression (Henke et al. 2008; Jopling et al. 2008), miR-122 may protect HVC RNA from nucleolytic degradation through binding to 5' terminus of HVC RNA with 3' overhanging nucleotide and masking the 5' terminal sequence of the virus genome (Machlin et al. 2011).

Some miRNA induced gene expression through targeted promoter sequence of this gene as miR-373 which have target sit within promoter sequence of E-cadherin and cold shock domain containing protein (CSDC2) and induced expression of their protein, moreover, enrichment of RNA polymerase II was detected at both promoter after miR-373 transfection (Place et al. 2008). Similarly, miR-205 induced expression of tumor suppressor genes IL24 and IL32 at both the messenger RNA and protein levels by targeting specific sites in their promoters (Majid et al. 2010).

The ability of miRNAs to concurrently down-regulate transcripts by post-transcriptional gene silencing mechanisms and potentially up-regulate target genes reveal a more complex nature and fundamental importance of miRNAs in regulating gene expression (Place et al. 2008).

2.9 Regulation of microRNA

MicroRNAs have been shown to be important regulators of many biological process including: temporal development events, cell differentiation, proliferation, cell cycle regulation, oocyte maturation, spermatogenesis, embryonic development, apoptosis, metabolism, placenta formation, immunity and tumorigenesis (Asangani et al. 2008; Carleton et al. 2007; Cloonan et al. 2008; Cui et al. 2009; Esquela-Kerscher and Slack 2006; Houbaviy et al. 2003; Maatouk et al. 2008; Silber et al. 2008; Taganov et al. 2007; Tesfaye et al. 2009; Wilfred et al. 2007; Zhao and Srivastava 2007). However, miRNA itself also has developmental and tissue specific regulation mechanisms although a considerable amount of known miRNAs are expressed universally in various tissues and species (Biemar et al. 2005). Regulation and control of miRNAs biogenesis, expression and function affected by different factors and regulatory mechanisms such as sequence specific RNA binding proteins with double-strand RNA binding domains (dsRBDs), single nucleotide polymorphisms (SNPs) which created by changes in DNA sequences of miRNA-coding genes or in an miRNA-binding site in mRNAs, RNA editing which displays a micro-regulatory role in controlling the miRNA processing machinery, methylation and circadian clock modulated mechanisms (Han et al. 2007; Kawahara et al. 2007; Kedde et al. 2007; Lehmann et al. 2008; Slezak-Prochazka et al. 2010; Sun et al. 2009; Yang et al. 2006).

2.10 MicroRNA in oocyte and ovarian somatic cells

The target deletion of Dicer, the RNase III enzyme that processes precursor microRNA into mature single stranded miRNA, within female reproductive tissues indicated to the importance of miRNA in a variety of developmental and functional processes. First report of miRNA processing role in reproductive function was reported for the female germline during meiosis in mouse (Murchison et al. 2007). In this study, Murchison and

his colleagues (2007) showed that knockouts of Dicer in mouse oocyte lead to specific arrest in meiosis I and exhibit both chromosome segregation defects and multiple disorganized spindles, moreover, the authors posit that loss of miRNAs could deregulate the levels of gene products needed for successful meiosis and in support of this, they indicated that analysis of the transcripts found in developing oocytes lacking Dicer are enriched for genes involved in microtubule related processes including twelve genes with predicted miRNA targets, among them being the GTPase Ran whose activity is known to be directly regulated by the condensation state of chromosomes. Similar result obtained from mouse oocyte injected with Dicer siRNA showed significant reduction in maturation rate, increased spindle disorganization and chromosome misalignment rates and reduced transcript of Dicer, miRNA, spindle formation proteins and spindle check point genes, thus, indicated that Dicer and miRNA appear to have important role during oogenesis and meiosis completion (Liu et al. 2010).

Reduced expression of dicer in females results in infertility (Carletti and Christenson 2009; Otsuka et al. 2008). In addition, females without Dicer exhibited disrupted oviduct and uterine development, as well as reduced ovarian function, as evidenced by decreased ovulation rate and presence of oocyte being trapped within the luteinized follicles (Hong et al. 2008).

The expression of microRNA genes is tightly regulated spatially among tissues and temporally during development within tissues in all species studied, indicating the importance of determining miRNA expression profiles to fully understand livestock biology (Aboobaker et al. 2005; Coutinho et al. 2007; Wienholds and Plasterk 2005). Several studies have identify and analyzed the expression profiles of miRNAs in female reproductive tissue of different species, such as, Ro et al. (2007) identified 122 (15 are novel) miRNAs in adult mouse ovary, Tang et al. (2007) showed dynamic changes in miRNA expression during oogenesis by a real-time PCR-based miRNA expression profiling method in single mouse oocyte, Mishima et al. (2008) obtained 154 known miRNAs in the adult ovary, Hossain et al. (2009) identified 74 miRNAs in the bovine ovary, 36 were found to be reported in miRBase 12.0 for different species including bovine, 14 are registered only for other species and 24 were completely new, Tesfaye et al. (2009) revealed the differential expression of 59 miRNAs, of which 31 and 28

miRNAs were found to be differentially expressed in immature and matured oocytes, respectively, Yao et al. (2010b) identified 260 mature miRNA transcripts in rat granulose cells via microarray analysis, Tripurani et al. (2010a) identified 58 miRNAs in the bovine fetal ovary, 42 are known bovine miRNAs present in the miRBase database and the remaining 16 miRNAs include 15 new bovine miRNAs that are homologous to miRNAs identified in other species, and one novel miRNA and the expression analysis of these miRNAs in fetal ovaries in comparison to somatic tissue pools identified 8 miRNAs predominantly expressed in fetal ovaries. Moreover, further analysis of the eight miRNAs in germinal vesicle (GV) stage oocytes identified two miRNAs that are highly abundant in both GV oocytes and Metaphase II stage oocytes. Ahn et al. (2010) identified 398 known miRNA species and 118 are novel miRNAs in new born mouse ovary using the Illumina Genome Analyzer and Creighton et al. (2010) reported the identification of 132 novel miRNAs through deep sequencing of small RNA libraries from 103 tissues or cell lines derived from human female reproductive organs in both normal and diseased states.

The ovary contains multiple somatic cell types such as granulosa cells, cumulus cells and theca cells in addition to oocytes. Bidirectional communication between the oocyte and companion somatic cells plays a very important role in follicle development (Matzuk et al. 2002). Deletion of Dicer1 in follicular granulosa cells led to impaired functional gene expression in granulosa cells, degenerate oocytes with obvious morphological defects and down-regulation of oocyte-specific genes (Lei et al. 2010). Although, ovulation could occur in presence of the deletion of Dicer1 in follicle granulosa cells, but impaired egg development is one of the major reasons for the infertility of dicer conditional knockout mice (Gonzalez and Behringer 2009; Nagaraja et al. 2008).

Many miRNA have been identified in ovarian granulosa cell (Fiedler et al. 2008; Yao et al. 2010b) and the putative function of some identified miRNA being revealed. Specific microRNAs (miR-21, miR-132 and miR-212) appear to be up-regulated after the LH surge and inhibition of miR-132 using complementary locked nucleic acid (LNA) oligonucleotides decrease C terminal binding protein-1 (CTBP1) protein levels, which in turn may directly affect granulose cell steroid production as was shown to regulate

adrenal steroidogenesis by serving as a corepressor of steroidogenic factor-1 (SF1) activity (Dammer and Sewer 2008; Fiedler et al. 2008). Furthermore, the ectopic expression of miR-224 can enhance TGF-β1 induced GC proliferation through targeting SMAD4 and partial inhibition of these miRNA lead to suppression of GC proliferation induced by TGF-β1. In addition, both miR-224 and TGF-β1 can promote estradiol release from GC, at least in part, through increasing CYP19A1 mRNA levels. Such miRNA-mediated effects could be potentially used for regulation of reproductive processes or for treatment of reproductive disorders (Yao et al. 2010a). Conversely, miR-378 down-regulates porcine ovarian garnulosa cell aromatase protein levels and estradiol production via binding within the 3'-UTR of the aromatase transcript (Xu et al. 2011b). In human primary granulose cells, multiple microRNAs can control reproductive functions resulting in enhancement or inhibition the release of ovarian progesterone, androgen and estrogen. So, such miRNA-mediated effects could be potentially used for regulation of reproductive processes, including fertility, and for treatment of reproductive and other steroid-dependent disorders (Sirotkin et al. 2010). On the other hand, expression of 31 miRNAs altered during the process of FSH induced progesterone production of cultured rat granulosa cell by using microarray analysis and this indicated that FSH may have role in regulation of miRNA expression (Yao et al. 2010b).

MicroRNAs are arising as important mediators of proliferation, differentiation, and apoptotic events (Ademokun and Turner 2008; Bueno et al. 2008; Chivukula and Mendell 2008; Gangaraju and Lin 2009; He et al. 2007; Jovanovic and Hengartner 2006). Recent study, demonstrated the involvement of miRNAs in controlling both proliferation and apoptosis by ovarian granulose cells, 11 and 53 from 80 tested miRNAs constructs resulted in stimulation and inhibition of PCNA (proliferation marker) expression, respectively and 11 of the 80 tested miRNAs promoted accumulation of Bax (apoptosis marker), while 46 miRNAs caused a reduction in Bax in human ovarian cells. Furthermore, the opposite effects of tested miR-15a antagonist and precursor on PCNA, as well as the similarity of effect of some selected miRNAs on the expression of two markers of cell proliferation (PCNA and cyclin B1) and of three markers of apoptosis (Bax, TdT and caspase 3) confirm the specificity of miRNAs effect on both proliferation and apoptosis of human ovarian cells (Sirotkin et al. 2010).

MiR-21 acts as an anti-apoptotic factor in *in vivo* and *in vitro* cultured murine granulosa cells, also loss of miR-21 *in vivo* leads to reduced ovulation rates (Carletti et al. 2010).

All studies mentioned above provide direct evidence for the significant role of miRNA in regulation of gene function in the ovary. However, other contradictory reports demonstrated that miRNA function is limited in mouse oocyte and early embryo based on Dgcr8-deficient oocytes, although miRNA levels of mouse oocytes with Dgcr8 deletion were reduced to the similar levels as Dicer-deficient mice, meiosis was not affected as in the oocytes from Dicer knockout mice but the Dgcr8 knockout mice suffered from unexpected low fecundity (Ma et al. 2010; Suh et al. 2010). In addition the limite miRNA change in human oocytes during meiosis (Xu et al. 2011a) supports the notion that miRNAs may play specific, rather than global, regulatory functions in gene expression during oocyte maturation. Furthermore, species difference may interpret the distinct temporal miRNA expression patterns during oocytes development as Mtango et al. (2009) showed that the expression of genes involved in miRNA processing in oocyte (DICER, GEMIN, TNRC6B, RANGAP1 and RNASEN) appeared to be regulated differently in mouse and rhesus monkey. Up to now, the exact role of miRNAs in the oocytes remains unclear.

2.11 MicroRNA in embryonic development

Embryonic development in mammals including a various critical events: transition from oocyte to embryo, the first cell cleavage, the establishment of cellular contacts and the first cell lineage. During all previous steps there are several molecular events that the preimplantation embryo passes it including maternal mRNA degradation, transcriptional zygotic activation, compaction and inner cell mass/trophoectoderm differentiation. Early embryo development depends on maternal transcripts accumulated during oocyte growth. The maternal control period on embryonic development vary among species according to the beginning of embryonic genome activation and the degradation of maternal gene products (DeRenzo and Seydoux 2004). The embryonic genome activation begins in mice at two-cell stage, in humans, rats and pigs at four-cell stage and in cattle and sheep at eight-cell to 16-cell stage (Barnes and First 1991; Telford et al. 1990).

Degradation of maternal mRNA is critical for early embryo development, There are multiple negative regulatory mechanisms which have important role in post-transcriptional regulation of maternal transcripts, for instance, transcript deadenylation and interaction with RNA-binding proteins in a nonspecific or sequence-specific fashion (Bettegowda and Smith 2007). The first direct evidence of miRNA action in early embryonic development became clear after the establishment of null embryos for Dicer in mice (Bernstein et al. 2003). Also in mouse embryos, 390 miRNAs were detected using deep sequencing and some of these miRNAs showed temporal expression profiles during prenatal development (Mineno et al. 2006).

The role of microRNAs as key regulatory molecules targeting maternal mRNA for degradation during the maternal-to-embryonic transition (MET) have been established in zebrafish, where the miR-430 level increased at the onset of embryonic genome activation and targeting several hundred maternal mRNAs by binding to their 3'-UTR and promotes their deadenylation (Giraldez et al. 2006). Furthermore, maternal deposited miRNAs are needed for zygotic function in mouse as the zygotes result from occyte lacking dicer failed to complete the first cell division (Tang et al. 2007). Another specific miRNA has been shown to be responsible of the degradation of many maternal mRNAs such as miR-290 in mouse and miR-427 in Xenopus (Lund et al. 2009; Tang et al. 2007). Additionally, zygotic specific miRNA (miR-135a) modulates the first cell cleavage through regulating expression of E3 ubiquitin ligase, Seven in absentia homolog 1A (Siah1a), which in turns affect destabilization of chemokinesin DNA binding protein (Kid), which mediates chromosome compaction and is degraded by the proteasome pathway during mitosis (Germani et al. 2000; Ohsugi et al. 2008; Pang et al. 2011). In addition, embryonic tissues of Drosophila and mouse expressed more miRNA than that of adults (Yu et al. 2007). So, these previous data could implicate the active role of miRNA in embryonic development.

In bovine, little is known about the expression of miRNA in embryonic tissue. The first profile of miRNA expression in day 30 bovine embryos was providing data for at least 17 new miRNAs not previously known in cattle and not matching published human or mouse data (Coutinho et al. 2007). Moreover, expression profiling of miRNA during late elongation of bovine *in vitro* produced and somatic cell nuclear transfer (SCNT)

embryos identified 39 miRNAs expressed in elongated Day 17 cloned bovine embryos produced by handmade cloning (HMC), 32 miRNAs expressed in time-matched IVP embryos, and 22 miRNAs in the somatic cells used for cloning. The subset of miRNAs expressed in the IVP embryos could be considered embryonic miRNAs, most were also present in cloned bovine embryos (Castro et al. 2010), as well as, the most highly expressed miRNAs in IVP bovine embryo (miR-30d and miR-26a) interact with Trophoblast kunitz domain protein (TKDP) which involved in maternal recognition of pregnancy in cattle (Castro et al. 2010; Lagos-Quintana et al. 2001). Tesfaye et al. (2009) showed highly variable trends in expression patterns of six miRNAs (miR-125a, miR-25, miR-127, miR-208, miR-145 and miR-496) during bovine preimplantation development.

From eight miRNAs predominantly expressed in bovine fetal ovary, two miRNA bta-miR-424 and bta-miR-10b being highly abundant in GV and MII stage oocytes as well as in early stage embryos (until 16-cell) with a tendency of decreasing in morula and blastocyst stage embryos, such expression pattern of these two miRNAs indicated that these miRNAs are maternally inherited and may potentially be involved in the maternal transcript turnover during zygotic gene activation as miR-430 in Zebrafish (Giraldez et al. 2006; Tripurani et al. 2010a).

Recently, Tripurani et al. indicated that miR-196a involvement in maternal transcript degradation during the maternal-to-zygotic transition through its expression which increased near the eight-cell stage and declines at morula and blastocyst stages of embryogenesis, furthermore, they demonstrated that miR-196a is negative regulator of NOBOX, which is maternal transcription factor and have functional role in bovine early embryonic development (Tripurani et al. 2010b; Tripurani et al. 2011).

3 Materials and methods

3.1 Materials for laboratory analysis

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

3.1.1 Chemicals, kits, biological and other materials

Chemicals, kits, biological and other materials	Supplier
Acetic acid	Roth, Karlsruhe, Germany
Acetic anhydride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ampicillin	Roth, Karlsruhe, Germany
Anti-Digoxigenin-AP Roche Diagnostics	Roche Diagnostics GmbH, Mannheim, Germany
BME EAA 50X (essential amino acids)	Invitrogen Life Technologies, Karlsruhe, Germany
Brilliant cresyl blue stain (BCB)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Bromophenol blue	Roth, Karlsruhe, Germany
Calcium chloride dihydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Chloroform	Roth, Karlsruhe, Germany
Citric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe, Germany
Dithiothreitol (DTT)	Invitrogen Life Technologies, Karlsruhe, Germany
dNTPs	Roth, Karlsruhe, Germany
Dye Terminator Cycle Sequencing (DTCS)	Beckman Coulter, Krefeld, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Fast Red Substrate System	Dako Deutschland GmbH, Hamburg, Germany
Fetal Calf Serum (FCS)	Gibco, Karlsruhe, Germany

Follicular stimulating hormon (FSH)	Folltropin, Vetrepharm, Canada
Formamide 65%	Roth, Karlsruhe, Germany
GenElute TM plasmid Miniprep kit	Sigma-Aldrich Chemie GmbH, Munich, Germany
Gentamycin	Invitrogen Life Technologies, Karlsruhe, Germany
Gentamycin sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
Heparin	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 (HCL)	Roth, Karlsruhe, Germany
Igepal	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isopropyl -D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
iTaq™ SYBR® Green Supermix with ROX	Bio-Rad, Hercules, CA
L-Glutamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
LNA-modified oligonucleotide ISH probes (1pM) for miR-205, miR-210, U6 RNA and scrambled RNA sequence	Exiqon, Vedbaek, Denmark
Magnesium chloride hexahydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Medium 199	Sigma-Aldrich Chemie GmbH, Munich, Germany
MEM NEAA 100X (non-essential amino acids)	Invitrogen Life Technologies, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
MiRNA PCR array	SABiosciences, Frederick, MD
MiRNeasy® mini kit	QIAGEN, Hilden, Germany
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
Oligo (dT) $_{23}$	Promega, WI, USA
pGEM®-T vector	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
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Proteinase K	Roth, Karlsruhe, Germany
QIAquick PCR Purification Kit	QIAGEN, Hilden, Germany
Random hexamer	Invitrogen, CA, USA
Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA
RT^2 miRNA first Strand kit	SABiosciences, Frederick, MD
RT^2 miRNA PCR Array	SABiosciences, Frederick, MD
RT^2 qPCR-Grade miRNA isolation kit	SABiosciences, Frederick, MD
RT^2 miRNA qPCR assay primer set and U6 (endogenous controls)	SABiosciences, Frederick, MD
RT^2 Real-Time^{TM} PCR master mix (RT^2 SYBR Green PCR master mix)	SABiosciences, Frederick, MD
Sample loading solution (SLS)	Beckman Coulter, Krefeld, Germany
Sequagel XR Sequencing Gel	Biozym Diagnostic, Epicentre Technologies, Hessisch-Oldendorf
Sodium acetate (NaOAc)	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium citrate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Sodium hydrogen carbonate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium monohydrogen phosphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium dihydrogenphosphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium pyruvate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium lactate solution 60%	Sigma-Aldrich Chemie GmbH, Munich, Germany
Streptomycin sulfate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sucrose	Roth, Karlsruhe, Germany
Superscript II reverse transcriptase	Invitrogen, CA, USA
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
TCM-199	Sigma-Aldrich Chemie GmbH, Munich, Germany

T4 DNA ligase	Promega, WI, USA
Tissue-Tek OCT reagent	Sakura Finetek, Zoeterwoude, NL
Triethanolamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Tris	Roth, Karlsruhe, Germany
TURBO DNA-free^{TM} kit	Ambion, Foster City, CA
Tween-20	Roth, Karlsruhe, Germany
X-Gal (5-bromo-4-chloro-3- indolylbeta -D-galactopyranoside)	Roth, Karlsruhe, Germany
Vectashield containing DAPI	Vector laboratories, Burlingame, CA
Yeast extract	Roth, Karlsruhe, Germany
Yeast tRNA	Invitrogen, CA, USA
10X PCR buffer	Promega, WI, USA
5 α DH *Escherichia coli* competent Cells	Stratagene, Amsterdam
5X First strand buffer	Invitrogen Life Technologies, Karlsruhe, Germany
2X rapid ligation buffer	Promega, WI, USA
3'-Digoxigenin (DIG) labeled	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 (ddH_2O) and when necessary, the pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl). In addition, the solutions or buffers were subsequently filtered through 0.2 µ filter and autoclaved at 120°C for 20 minutes when it is necessary.

Name of the medium/buffer	Constituents	Amount/ Volume
Physiological saline solution	: Sodium chloride	9.0 g
(NaCl 9%)	added to water	1000.0 ml
Maturation medium	: HEPES	0.149 g
(Modified Parker Medium)	Sodium pyruvate	0.025 g
	L-Glutamine	0.010 g
	Gentamycin	500.0 µl
	Medium 199	99.0 ml

	Hemi Calcium lactate	0.06 g
	Add to water	110.0 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
Fertilization medium (Fert-TALP medium)	: Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin	0.0032 g
	Magnesium chloride hexahydrate	0.0050 g
	Calcium chloride dihydrate	0.0150 g
	Sodium lactate solution (60%)	93.0 µl
	Phenol red solution (5% in D-PBS)	100.0 µl
	Water up to	50.0 ml
Capacitation medium (50 ml)	: Sodium chloride	0.2900 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogene phosphate	0.0017 g
	Hepes	0.1190 g
	Magnesium chloride dihydrate	0.0115 g
	Calcium chloride dihydrate	0.0145 g
	Sodium lactate solution (60%)	184.0µl
	Phenol red solution (5% in D-PBS)	100.0 µl
	ddH_2O up to	50.0 ml
Penicillamine hypotaurine adrenaline medium	: Physiological saline (0.9%)	16.0 ml
	Hypotaurin solution	10.0 ml
	Epinephrin solution	4.0 ml
CR1aa culture media (50 ml)	: Hemi-calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g

	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100.0 µl
	Sodium hydrogen carbonate	0.080 g
Lysis buffer (100 µl)	: Igepal (0.8%)	0.8 µl
	40U RNasin	5.0 µl
	DTT	5.0 µl
	ddH_2O added to	100.0 µl
SOC media (400 ml)	: Peptone	4.0 g
	Yeast extract	4.0 g
	NaCl 1M	2.0 ml
	KCl 1M	500.0 ml
	ddH_2O added to	400.0 ml
	Mg^{+2} 2M	2.0 ml
	Glucose 2M	2.0 ml
LB-agar (800 ml)	: Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
dNTP solution	: dATP (100 Mm)	10.0 ml
	dCTP (100 mM)	10.0 ml
	dGTP (100 mM)	10.0 ml
	dTTP (100 mM)	10.0 ml
	ddH_2O added to	400.0 ml
Agarose loading buffer	: Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25.0 ml

IPTG solution	: IPTG	1.2 g
	ddH_2O added to	10.0 ml
X-gal	: X-gal	50.0 mg
	ddH_2O added to	1000.0 ml
DEPC-treated water (1000 ml)	: DEPC	1.0 ml
	ddH_2O added to	1000.0 ml
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
	ddH_2O added to	500.0 ml
Stop solution	: 3M Sodium Acetate (NaOAc) pH = 5.2	2.0 µl
	100 mM EDTA	2.0 µl
	Glycogen (20 mg/ml)	1.0 µl
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	100.0 ml
	DEPC added to	900.0 ml
4% paraformaldehyde (pH 7.3)	: Paraformaldehyde	4.0 g
	1X PBS	100.0 ml
Proteinase K solution	: Proteinase K	20 mg
	DEPC-treated H_2O	1 ml
Yeast tRNA (10 mg/ml)	: Yeast tRNA	25.0 mg
	DEPC-treated H_2O	2.50 ml
20X SSC	: NaCl	87.65 g
	Sodium citrate	44.1 g
	Water upto	500.0 ml

5X SSC	: 20X SSC	250.0 ml
	DEPC water	750.0 ml
2X SSC	: 20X SSC	100.0 ml
	DEPC water	900.0 ml
0.2X SSC	: 20X SSC	10.0 ml
	DEPC water	990.0 ml
1X PBST	: 1X PBS	999.0 ml
	Tween-20	1.0 ml
0.5 M Sucrose/PBS	: Sucrose	85.57 g
(30% sucrose)	1X PBS upto	500.0 ml
Acetylation solution	: Triethanolamine	2.33 ml
	Acetic anhydride	500.0 µl
	DEPC water added to	200.0 ml
B1 solution	: 1 M Tris (pH 7.5)	100.0 ml
	5 M NaCL	30.0 ml
	ddH_2O added to	1000.0 ml
Blocking Solution	: FCS	2.0 ml
	B1 solution	18.0 ml
Hybridization solution	: Formamide 65%	32.25 ml
	20X SSC	12.5 ml
	Tween-20, 0.1%	50.0 µl
	1M citric acid	460.0 µl
	Heparin 50 µg/ml	2.5 mg
	tRNA 500µg/ml	2.5 ml
	DEPC water upto	50.0 ml
Hybridization washing buffer	: Formamide 65%	65.0 ml
	20X SSC	25.0 ml
	Tween -20, 0.1%	100.0 ml
	1M citric acid	1.2 µl
	DEPC water upto	100.0 ml
1X PBS-Tween (PBST)	: 1X PBS	999.0 ml

	Tween-20	0.50 ml
50X TAE buffer, pH 8.0	: Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH_2O added to	1000.0 ml
2% Agarose gel	: Agarose	2.0 g
	1X TAE buffer	100.0 ml

3.1.3 Equipment used in the study

Equipments	Manufacturer
ABI PRISM® 7000 SDS	Applied Biosystems, Foster City, CA
Agilent 2100 bioanalyzer	Agilent Technologies, CA, USA
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Centrifuge (small, medium, larg)	Hermle, Wehingen, Germany
CEQ ™ 8000 genetic analysis system	Beckman Coulter, Krefeld, Germany
Confocal laser scanning microscope-510	Carl Zeiss, Germany
Cryo-tube	Nunc, Roskilde, Germany
Electrophoresis chamber (for agarose gels)	Bio-Rad, Munich, Germany
Four well dishes	Thermo Fisher Scientific, Nunc, Denmark
Hybridization chamber	GFL, Dülmen, Germany
IKA vortex mixer	Agilent technologies, Germany
Incubator (BB16)	Heraeus, Hanau, Germany
Inverted microscope	Leica DM-IRB, Germany
Laminar flow chamber	Heraeus, Germany
MAXQ6000 shacking incubator	Thermo scientific, Germany
Millipore apparatus	Millipore corporation, USA
Mini centrifuge	Labnet, UK
Micromanipulator	Leitz, Germany
Microplates for real-time PCR	STARLAB GmbH, Ahrensburg
Microwave	Micro maxx®, Germany
Molecular imager chemidoc XRS System	Bio-Rad, Munich, Germany
MyCycler thermal cycler	Bio-Rad, Munich, Germany
NanoDrop 8000 spectrophotometer	Thermo scientific,Germany
PH meter	Kohermann, Germany
PTC-100 thermal cyclers	MJ Research, USA
Rapid sectioning cryostat	Leica microsystem Nussloch GmbH, Heidelberger, Germany

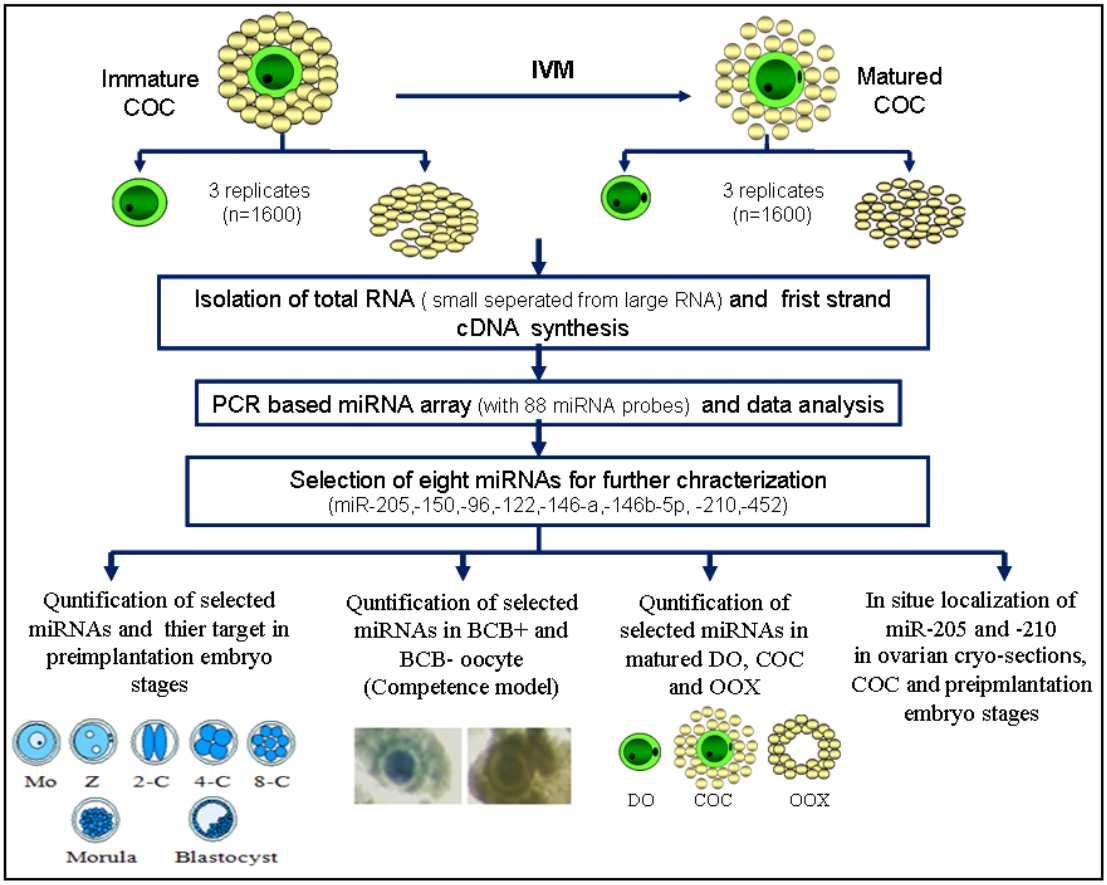
Refrigerated universal centrifuge Z233MK	Hermle Labortechnik, Wehingen, Germany
Savant speed vac	GMI, Inc. Minnesota, USA
Slide SuperFrost® Plus	Braunschweig, Germany
Stereo microscope SMZ 645	Nikon, Japan
Thermalshake Gerhardt	John Morris scientific, Melbourne
Tuttnauer autoclave	Connections unlimited, Wettenberg
Ultra low freezer (-80°C)	Labotect GmbH, Gottingen
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.4 Programs (softwares) and statistical packages used

Software and packages	Source
BLAST program	http://www.ncbi.nlm.nih.gov/Blast/
ENSEMBL genome browser	http://www.ensembl.org/
Entrez Gene	http://www.ncbi.nlm.nih.gov/
EndNote X1	Thomoson
GraphPad prism v.5	GraphPad software, Inc.
Ingenuity Pathways Analysis software (Ingenuity IPA 8.5)	Ingenuity® Systems, www.ingenuity.com
MiRBase v.12	http://www.mibase.org/
miRDB	http://www.mirdb.org/
MicroRNA.org-Target and Expression	http://www.microrna.org/
PicTar	http://www.pictar.org/
Primer Express® software v.2.0	Applied Biosystems, Foster City, CA, USA
SAbiosciences's PCR array data analysis	http://www.sabiosciences.com/pcrarraydataanalysis.php
TargetScan 5.1	http://www.targetscan.org/
Weight to molar quantity (for nucleic acids) converter	http://www.molbiol.ru/eng/scripts/01_07.html
ZEN 2008 Light Edition	Carl Zeiss, Germany

3.2 Methods

3.2.1 Experimental design



Cumulus oocyte complex (COC), in vitro maturation (IVM), brilliant cresyl blue (BCB), matured oocyte (Mo), zygote (Z), denuded oocytes (DO) and oocyteoctimized (OOX)

Figure 3.1: Overview of the present study

3.2.2 Cumulus oocyte complexes collection and *in vitro* maturation

Bovine ovaries were collected from local abattoirs and transported to the laboratory within 2-3 hrs in a thermo flask containing 0.9% physiological saline solution (NaCl) at 39ºC. Before aspiration of COCs, the ovaries were washed twice in 70% ethanol. Cumulus oocyte complexes were aspirated from antral follicles having 2-8 mm diameter using 5 ml syringe attached to 18 gauge needle. The aspirated follicular fluid was collected in 50 ml sterilized tube and allowed to precipitate for 15 min. Only morphologically uniform and good quality COCs (oocytes with multiple layers of

cumulus cells and has a translucent and homogeneous ooplasm) were picked using glass-pipette under stereo microscope (Nikon, Japan) and washed three times in maturation medium (Modified Parker Medium (MPM)) supplemented with 15% ostrous cow serum (OCS), 0.5 mM l-glutamine, 0.2 mM sodium pyruvate, 50 mg/ml gentamycin sulphate and 10 µl/ml follicular stimulating hormone (FSH) (Folltropin, Vetrepharm, Canada) before set into culture to obtain matured oocytes or directly freezed as immature oocytes. The COCs were cultured in a group of 50 in 400 µl of maturation medium under mineral oil in four-well dishes (Thermo Fisher Scientific, Nunc, Denmark). Maturation was performed for 24 hr at 39°C under a humidified atmosphere of 5% CO_2 in air. Both immature and matured COCs were collected and subjected to mechanical separation of the oocytes and the surrounding cumulus cells using gentle vortexing. Denuded oocytes in a group of 200 and the corresponding cumulus cells were snap-frozen in cryo-tubes containing lysis buffer [0.8% Igepal (Sigma-Aldrich Chemie GmbH, Munich, Germany), 40U RNasin (Promega, WI, USA) and 5 mM DTT (Invitrogen Life Technologies, Karlsruhe, Germany)] and stored at -80°C until further use. A total of three replicate pools each containing 1600 oocytes and their corresponding cumulus cells from both immature and matured COCs were utilized for analysis of miRNA using a PCR based miRNA array.

3.2.3 In vitro embryo production

In vitro matured oocytes were collected after 24 hr. A total of six independent *in vitro* maturation runs were conducted to collect three independent pools of 450 matured oocytes. For generating different stages of preimplantation embryo, proven bull spermatozoa were used at the concentration of $1x10^6$ cells/ml of fertilization medium (Fert-TALP). The frozen sperm straws were thawed in a water bath at 39°C for 8 sec and the best motile sperm separated by means of the ˝swim up˝ technique (Parrish et al. 1988). In this technique, the thawed sperm cells were incubated in a tube containing 5 ml capacitating medium supplemented with heparin at 39°C under a humidified atmosphere of 5% CO_2 in air for 50 min. During this time the good quality motile sperm will go to the upper layer of the media. This layer which contains the motile sperm was transferred to new sterilized tube and centrifuged at 10000 rpm for 10 min. The sperm pellet was washed two times then resuspended in 3.5 ml capacitating medium for IVF.

In vitro matured COCs were washed twice in the fertilization medium and transferred into a four-well dish (50 matured COC/well) containing 400 µl of fertilization medium (Fert-TALP) supplemented with 2 µg/ml heparin and 0.2 mM sodium pyruvate (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 25 µl/ml penicillamine hypotaurine adrenaline medium was added to each well to initiate sperm motility than covered with mineral oil. After 20 hr of co-incubation with spermatozoa at standard incubation conditions with 39ºC and humidified atmosphere containing 5% CO_2 in air, the presumptive zygotes were gently vortexes to separate them from the surrounding cumulus cells and dead spermatozoa. Cumulus-free zygotes were washed three times and cultured in CR-1aa culture medium (Rosenkrans and First 1994) supplemented with 10% OCS, 10 µl/ml BME (essential amino acids) and 10 µl/ml MEM (non-essential amino acids) (Tesfaye et al. 2010) in the same incubator and under the same conditions as used for maturation and fertilization. The oocytes and embryos were collected from the culture at specific time points after maturation or fertilization. Matured oocytes were collected at 24 hr after maturation, while zygote, 2-cell, 4-cell, 8-cell, morula, and blastocyst stage embryos were collected from the culture at 20, 30, 42, 70, 120 and 168 hr after fertilization, respectively. From six independent IVF runs, three independent pools each consisting of 150 zygotes, 100 two-cell, 80 four-cell, 60 eight-cell, 50 morula and 30 blastocyst stage embryos were collected and snap-frozen in cryo-tubes containing lysis buffer until RNA isolation. All frozen oocytes and embryos were stored at -80°C. These embryos and matured oocytes were utilized for the expression analysis of selected miRNAs and their predicted target mRNAs.

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

To investigate the expression of selected miRNAs in the oocytes of different competence, the immature COCs were 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; Bhojwani et al. 2007). After washing, the stained COCs were examined under stereo microscope and categorized into two groups according to oocyte cytoplasm colouration, 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 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 colourless (BCB-). From each group triplicate pools of COCs (n=500 COCs per pool) were used for analysis after separation of the oocytes from their surrounding cumulus cells mechanically. Denuded oocytes and the corresponding cumulus cells of each group were washed two times in PBS and were snap-frozen separately in cryo-tubes containing lysis buffer and stored in -80°C until further analysis.

3.2.5 Preparation and culture of oocytectomised cumulus cells and denuded oocytes

To reveal the effect of presence or absence of cumulus cells on expression of miRNA in oocytes and vice versa, oocytes or cumulus cells were *in vitro* matured in the presence or absence of the other. For this, mechanical removal of oocyte cytoplasm from COCs was done using a micromanipulator (Leitz, Germany) fitted to an inverted microscope (Leica DM-IRB, Germany). Each time a group of 50 COCs were placed into manipulation medium under mineral oil for removal of all contents of the oocyte cytoplasm including the nucleus. This was done by holding the COCs with a holding pipette and sucking out the cytoplasm contents using an enucleating pipette attached to the micromanipulator. In order to get denuded oocytes for maturation, immature COCs were subjected to gentle vortexing in PBS for mechanical removal of the cumulus cells. After being checked for complete removal of the cumulus cells, denuded oocytes and oocyteoctimized cumulus cells were washed twice in drops of fresh maturation medium and placed in culture for *in vitro* maturation. Each time morphologically good quality COCs were categorized into three groups. Group-1 includes COCs *in vitro* matured as intact, group-2 includes those oocytes matured without the surrounding cumulus cells (denuded oocytes) and group-3 includes cumulus cells matured without the oocyte cytoplasm (oocytectomised). The experiment was conducted four times with the same handling and culturing conditions. Each time 250 COCs from each of the three mentioned groups were snap-frozen in cryo-tubes containing lysis buffer. Collected samples were stored in -80°C until expression analysis of selected miRNAs.

3.2.6 RNA isolation and first strand cDNA synthesis

3.2.6.1 Total RNA isolation

The total RNA enriched with miRNAs was isolated from the experimental samples using miRNeasy® Mini kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Briefly, three pools of samples from each group, namely: immature oocytes and their corresponding cumulus cells, matured oocytes and their corresponding cumulus cells, matured oocyte and preimplantation embryo stags, BCB+ and BCB- oocyte, cumulus cells derived from BCB+ and BCB-, oocyte matured in presence of cumulus cell and vice versa, denuded oocyte and oocyteoctimized cumulus cells. The cells were disrupted by adding 700 µl QIAzol Lysis Reagent and homogenized by vortexing for 1 min then incubated at room temperature for 5 min. One hundred fourty µl chloroform was added to the homogenate, the tube shacked vigorously for 15 sec and centrifuged for 15 min at 12000 xg at 4°C in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany) after incubation at room temperature for 3 min. The upper aqueous phase was transferred carefully to a new 2 ml collection tube and 525 µl of 100% ethanol was added. After mixing, 700 µl of the sample was pipetted onto RNeasy Mini spin column and centrifuged for 15 sec at room temperature. The flow-through was discarded and the previous step was repeated with the remaining samples. Following this, the spin column was washed by 700 µl RWT buffer and centrifuged for 15 sec at 10000 rpm. After discarding the flow-through, 500 µl RPE buffer was added to the spin column followed by 15 sec centrifugation 10000 rpm. This step was repeated for the second time and centrifuged at the same speed for 2 min. The spin column was then transferred to a new 2 ml collection tube and centrifuged at 14000 rpm for 1 min to eliminate any possible carryover of RPE buffer and residual flow-through remained on the spin column. To elute total RNA, the RNeasy spin column was transferred into new 1.5 ml collection tube to which 30 µl of RNase-free water was added to the centre of the RNeasy spin column membrane and centrifuged at speed of 10000 rpm for 1 min. Isolated total RNA was stored in -80°C until the further step.

3.2.6.2 Small and large RNA isolation

The small RNAs were separated from the large RNA using RT^2 qPCR-Grade miRNA isolation kit (SABiosciences, Frederick, MD) following manufacturer's instructions. Briefly, lysis and binding buffer G6 was added to bring each total RNA sample to a final volume of 400 µl and then 215 µl 100% ethanol was added directly to each sample and mixed by vortexing gently. The mixture were added to first spin column and centrifuged at 11000 xg for 30 sec. The first spin column contains large RNA (stored at 4°C until used) and the eluate contains small RNA. Then, 750 µl 100% ethanol was added to the eluate. After mixing, 700 µl from ethanol mixture transferred to second spin column and centrifuged at 11000 xg for 30 sec. The flow-through was discarded and the previous step was repeated with the remaining of the ethanol mixture. Following this, 200 µl of washing Buffer working solution was added to both first and secand spin column and centrifuged at 11000 xg for 30 sec. After discarding flow-through, 250 µl of 70% ethanol was added to each first and second spin column and centrifuged at 11000 xg for 3 min. Afterwards, the first and second spin column was transferred to a new elution tubes and 40 µl RNase-free H_2O was added directly to the spin column membrane. The spin column membrane was stand for 1 min at room temperature, then centrifuged for 1 min at 11000 xg to elute the large and small RNA. For all the experiments, the resulting small RNAs and fractionated large RNAs were checked for their concentration and integrity (based on 260/280 nm measurement) using nanodrop 8000 spectrophotometer (Thermo scientific, Germany) and subsequent analysis by using agilent 2100 bioanalyzer (Agilent Technologies, CA, USA).

3.2.6.3 First strand cDNA synthesis

Small RNAs from each treatment groups were used for first strand cDNA synthesis using RT^2 miRNA first strand kit (SABiosciences, Frederick, MD) following manufacturer's instructions. Briefly, 50 ng of small RNA samples were incubated with a mix containing 2 µl of 5X miRNA RT buffer, 1 µl miRNA RT primer, 1 µl DTT (100 mM) and 1 µl miRNA RT enzyme mix in a 10 µl reaction volume. Reaction incubation was performed at 37°C for 2 hr followed by heating at 95°C for 5 min to inactivate the reverse transcriptase. Depending on the type of experiment and the amount of small

RNA used for RT-PCR, the resulting cDNA samples were diluted before use as template for miRNA qPCR assay. The large RNA fractions from the same samples of preimplantation embryo stags were recovered and utilized for RT-PCR after removal of contaminating DNA using TURBO DNA-free kit (Ambion, Foster City, CA) according to manufacturer's instructions. All DNA-free RNA samples were reverse transcribed using Superscript II reverse transcriptase kit (Invitrogen, Karlsruhe, Germany). Briefly, maximum volume of 10 µl RNA (with 100 ng of input RNA) from each treatment group were co-incubated with 1µl of random hexamer (Invitrogen, CA, USA) and 1 µl of oligo $(dT)_{23}$ (Promega, WI, USA) at 70°C for 3 min followed by incubation on ice for 2 min. Reverse transcription was performed in a 20 µl reaction volume containing 4 µl 5X first strand buffer, 2 µl DTT, 1 µl dNTP, 0.3 µl RNasin and 0.7 µl RT enzyme. The reaction was incubated for 90 min at 42 ºC, followed by heat inactivation for 15 min at 75ºC. The synthesized cDNA was stored at -20ºC for further use.

3.2.7 RT profiler PCR array and data analysis

The RT profiler PCR array were performed using sequence specific miRNA qPCR primer sets and RT^2 Real-Time™ PCR master mix (SABiosciences, Frederick, MD) in ABI Prism® 7000 SDS (Applied Biosystems, Foster City, CA). Expression profiling of miRNAs between oocytes and cumulus cells before and after *in vitro* maturation was performed using RT^2 miRNA PCR array related to human cell development and differentiation (SABiosciences, Frederick, MD) containing 88 well known human homologue miRNAs, four housekeeping small RNAs (SNORD44, SNORD47, SNORD48 and U6) and RT negative controls in a 96-well format. Prior to real time PCR profiling, 90 µl DNase/RNase-free water was added to each of the 10 µl first strand cDNA product from each biological replicates of immature and matured oocytes and their corresponding cumulus cells. The PCR master mix was prepared using the 100 µl diluted cDNA template, 1275 µl 2X RT^2 SYBR Green PCR master mix (SABiosciences, Frederick, MD) and 1175 µl DNase/RNase-free water. Twenty five µl of this mix was distributed to each well of the 96-well plate containing sequence specific miRNA primer sets and the respective controls. Following brief centrifugation, the plate was loaded onto ABI prism 7000 real time PCR apparatus and run with a thermal programme of initial heating at 95°C for 10 min followed by 40 cycles of 95°C

for 15 sec and 60°C for 1 min. The specificity of amplification was controlled using a melting curve generated at the end of the PCR protocol.

The data was analyzed by comparative threshold cycle (Ct) method and normalization was done using geometric mean of four endogenous controls (SNORD44, SNORD47, SNORD48 and U6) through SAbiosciences's PCR array data analysis online web portal provided with t-test (http://www.sabiosciences.com/pcrarraydataanalysis.php). The threshold level of ≥ 2 fold changes as a cut off value and significant level of $p \leq 0.05$ was taken to screen differentially expressed miRNAs. From this, we selected miRNAs for further characterization based on the differential expression intensity between oocytes and their cumulus cells at both before and after maturation.

3.2.8 Quantitative real time PCR analysis of selected microRNAs

The expression profiling of selected miRNAs in matured oocyte and preimplantation embryo stages has been examined using RT^2 miRNA qPCR assay primer set for miR-205, miR-150, miR-96, miR-122, miR-146a, miR-146b-5p, miR-210, miR-452 and U6 as endogenous controls with RT^2 Real time™ PCR master mix (SABiosciences, Frederick, MD). The PCR master mix was prepared using the 1µl cDNA template, 1µl RT^2 miRNA qPCR assay primer set, 12.5 µl RT^2 Real time™ PCR master mix and 10.5 µl ddH_2O. The reaction was done by using ABI prism 7000 real time PCR apparatus with a thermal programme of initial heating at 95°C for 10 min followed by 40 cycles of 95°C for 15 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 desciprd 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 (U6)

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

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

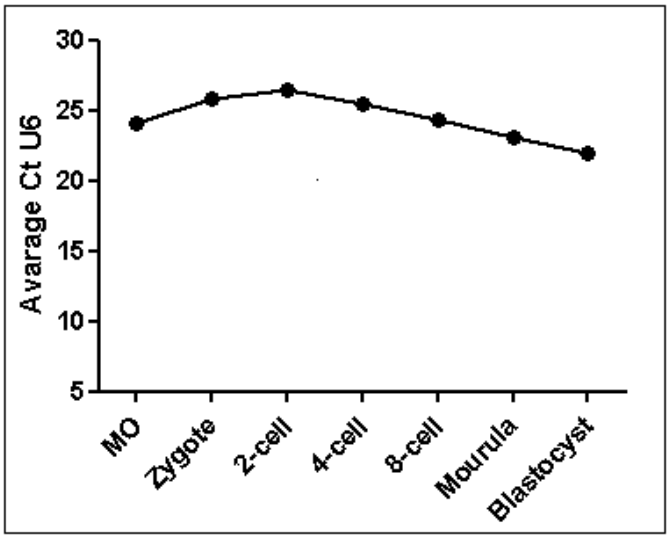


Figure 3.2: The average threshold cycle (Ct) of the endogenous control U6 RNA throughout matured oocyte and the preimplantation embryo stages.

3.2.9 MicroRNA target prediction and *in silico* functional analysis

The target mRNAs of the differentially expressed selected miRNAs were predicted using four different search algorithms: TargetScan 5.1 (http://www.targetscan.org/), PicTar (http://www.pictar.org/), miRDB (http://www.mirdb.org/) and MicroRNA.org-Target and Expression (http://www.microrna.org/). The targets with high score and predicted at least in two or more target search tools were accepted as a target gene for the miRNA under consideration. The biological functions of these predicted targets and the molecular pathways in which they could be involved were analysed through the use of IPA v.8.5 (Ingenuity® System, www.Ingenuity.com).

3.2.10 Quantification of selected microRNAs target genes

In this study, quantitative RT-PCR was performed for one target gene per miRNA using an ABI Prism® 7000 SDS (Applied Biosystems, Foster City, CA). Predicted target transcripts quantified in matured oocyte and preimplantation embryo stages include phospholipase C beta 1 (PLCB1) for miR-205, calmodulin-like 4 (CALML4) for miR-150, cyclin G (CCNG1) for miR-122, 1,4,5-triphosphate receptor 1 (ITPR1) for miR-96, interleukin-1 receptor-associated kinase 1 (IRAK1) for miR-146a, TNF-receptor-

associated factor 6 (TRAF6) for miR-146b-5p, eukaryotic translation initiation factor 4 gamma 3 (EIF4G3) for miR-452 and pannexin 3 (PANX3) for miR-210. Quantification of all targets was done in comparison to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control and standard curve were generated using serial dilution of plasmid DNA (10^1-10^9 molecules).

3.2.10.1 Primer design, target gene amplification and purification

The primer of selected miRNA target genes were designed for PCR amplification according to bovine cDNA sequence using Primer Express software v 2.2 (Applied Biosystems, Foster City, CA). Names of the primers, their accession numbers, sequences and product sizes are shown in table (1).

Table 1: List of specific primers used for quantitative PCR

Gene	ACC. No	Primer sequence (5'–3')	BP
PLCB1	NM_174817	F:GAAATGAAGGACCTGGTTAAGAGA	111
		R:AGCTGCTCTCCTTCTCAAGTAGTC	
CALML4	BC102423	F: CCCAGGACCAAATTAATGAGT	153
		R: TGTGAGTCTGTAGGTGCCG	
ITPR1	NM_001099952	F: CGCCCTTCTGCACAATAAT	152
		R: CCGGAATGGATTTGTTCAT	
CCNG1	NM_001013364	F: CCAACTTAAGGCATGCTACTG	153
		R: CCACTGATCTTGGAATGTTTC	
TRAF6	NM_001034661	F: TGGCAAATGTCATCTGTGAA	153
		R: CGTGCCAAGTGATTCCTCT	
IRAK1	NM_001040555	F: CCCCCTCTTCTTCATTCTG	155
		R: CCTCCTTGAGCCTCTTCAC	
PANX3	NC_007330	F: ATCACATGCAGGCTGACCT	151
		R: GGAGCATCTCGTAGATGGAG	
EIF4G3	NM_001078002	F: TCAGGCCAATCAGCACATCA	209
		R: AAGGCGTTCCGTAAGCACTG	
GAPDH	NM_001034034.1	F: AATGGAGCCATCACCATC	240
		R: GTGGTTCACGCCCATCACA	

ACC. No = Gene bank accession number, BP= number of base pairs amplified

Amplification of target genes were preformed in 20 µl PCR reaction volume containing 2 µl of 10X PCR buffer (Promega, WI, USA), 0.5 µl of each primer (10 pmole), 0.5 µl of dNTP (50 µM), 0.2 µl of Taq DNA polymerase (Sigma-Aldrich Inc, MO, USA) and 14.3 ddH_2O which finally added to 2 µl of cDNA templates or 2 µl genomic DNA (50 ng/µl) as positive control and 2 µl of ddH_2O as negative control. The reactions were carried out in a mycycler thermal cycler (Bio-Rad, Munich, Germany) and thermal cycling program denaturizing at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, annealing at the corresponding temperatures of each primer for 30 sec and extended at 72°C for 1 min, final extension step at 72°C for 10 min. After that, 5 µl of the PCR products were loaded with 2 µl of loading buffer onto ethidium bromide staining agarose gel 2% run in 1X TAE buffer to check for amplification of the desired product. Electrophoresis of the amplified PCR products was done for 20 min at 120 voltages and DNA bands were observed under molecular imager chemidoc XRS System (Bio-Rad, Munich, Germany). To confirm the specify of amplified PCR product, sequencing PCR reaction was performed using 5 µl purified PCR product, 2 µl reverse primer or forward specific primer, 4 µl DTCS (Beckman Coulter, Krefeld, Germany), 9 µl ddH_2O and the reaction carried out for 30 cycles at 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min, followed by holding at 4°C. Then, the sequencing PCR product was transferred to a 1.5 ml sterile tube and mixed with 5 µl stop solution (2.0 µl of 3M Sodium Acetate (NaOAc) pH = 5.2, 2.0 µl of 100 mM EDTA (pH =2.0) and 1.0 µl of glycogen (20 mg/ml). A volume of 60 µl cold ethanol (98%) was added and mixed by vortex and then centrifuged at 14000 rmp for 15 min at 4°C in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany). The supernatant was removed and the pellet washed 2 times with 200 µl cold ethanol (70%) and centrifuged for 5 min at 4°C. The pellet was dried by the savant speed vac (GMI, Inc. Minnesota, USA) at 35°C and resuspended in 40 µl sample loading solution (SLS). The sample was loaded in sequencing plate, covered with mineral oil and immediately sequenced using the CEQ ™ 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany). The final sequence of each DNA fragment was blast into GenBank database (http://www.ncbi.nlm.nih.gov/blast/). The specific PCR product was purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, buffer PB (Five volumes) was added to one volume of the amplified PCR product and mixed. The 700 µl of the mix was pipetted to

QIAquick spin column and centrifuged for 30 sec at 12000 rpm. The resulting flow-through was discarded and the column was placed back into the same tube and centrifuged for 30 sec at 12000 rpm after adding 750 µl buffer PE (washing buffer). The flow-through was discarded again and the column was centrifuged for additional 1 min at 14000 rpm. Finally, the column was transferred to new 1.5 ml micro centrifuge tube and the DNA was eluted by adding 30 µl ddH_2O to the centre of the QIAquick membrane and centrifuged for 1 min at 12000 rpm. The quality of purified DNA was analyzed on 2% agarose gel and stored at -20°C.

3.2.10.2 Ligation, transformation, colony screening and sequencing.

The purified DNA fragments were ligated into the pGEM®-T vector (Promega, WI, USA). The ligation reaction was performed in 6 µl total reaction volume containing 2.5 µl 2X rapid ligation buffer, 0.5 µl of pGEM®-T vector (50 ng/µl), 0.5 µl T4 DNA ligase (3 U/µl) and 2 µl of template purified DNA. The reaction was incubated at 4°C overnight. In the next day, transformation was done by combining 3 µl of ligation reaction with 80 µl of *Escherichia coli* competent cells (Stratagene, Amsterdam) in a 15 ml sterilized tube and incubating on ice for 20 min. The mixture was incubated in water bath at exactly 42°C for 90 sec and immediately transferred to ice for 2 min. Afterwards, 750 µl SOC media was added to the previous mixture and all together was incubated at 37°C for 90 min on a MAXQ6000 shaking incubator (Thermo scientific, Germany) at 130 rpm. After 70 min, 20 µl of IPTG and 20 µl of X-gal were uniformly distributed over the surface of the LB agar/ampicillin plate with a glass spreader. At the end of incubation period, the transformation culture was spread completely on two of previously prepared LB-agar plates and incubated at 37°C overnight till the colonies become visible. Successful cloning of DNA insert in the pGEM®-T vector was checked based on the activity of β-galactosidase. The β-galactosidase is an enzyme produced by lacZ gene in pGEM®-T vector which interacts with IPTG to produce a blue colony. On the other hand, when an insert was successfully cloned, the lacZ gene is disrupted and β-galactosidase is not produced any more which resulted in white colonies. Four independent white colonies in addition to two blue colonies were picked up and suspended in 30 µl 1X PCR buffer for M13 reaction for further confirmation of transformation and sequencing. The same colonies were also cultured in 600 µl

ampicillin/SOC media in a MAXQ6000 shaking incubator 130 rpm at 37°C for 90 min until the result of M13 product was known. Bacteria that were suspended in 30 µl 1X PCR were lysis by heating at 95°C for 15 minutes. Then, colonies were screened for the insert by performing a PCR with primers designed in M13 promoter region of the vector. For this, 20 µl of reaction volume containing 10 µl of lysate, 0.5 µl dNTPs (10 mM), 0.5 µl of each of primer (forward: 5´-TTGAAAACGACGGCCAGT-3 and reverse: 5´ CAGGAAACAGCTATGACC-3), 1µl 10X PCR buffer, 7.4 µl ddH_2O and 0.1 µl of Taq DNA polymerase were amplified in myCycler thermal cycler (Bio-Rad, Munich, Germany) and the thermal cycling program denaturizing at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, annealing at 60°C for 30 sec and extended at 72°C for 1 min, final extension step at 72°C for 10 min. At the end of the reaction, 5 µl of the M13 product mixed with 2 µl loading buffer was loaded to 2% agarose gel stained with ethidium bromide. The colonies that contain PCR fragments (white colonies) were identified depending on the distance travelled by DNA fragment in 2% agarose gel electrophoresis. The blue colonies were at lower position compared to the white colonies. Following this, the colonies confirmed for the presence of PCR fragment were transferred to 15 ml sterile tube and additional 5 ml of SOC media with ampicillin were added and incubation continued overnight in a MAXQ6000 shaking incubator 130 rpm at 37°C for further plasmid isolation. The identity of inserts was confirmed by sequencing, the M13 PCR products from white colonies containing inserts were used as a template for subsequent sequencing. A volume of 5 µl of M13 products was purified by adding 1 µl of ExoSAP-IT (USB, Ohio, USA) then incubated at 37°C for 45 min followed by enzyme inactivation step at 80°C for 15 min. The purified DNA product (6 µl) was subsequently used as template for the sequencing PCR which contains 8 µl of ddH_2O, 2 µl (1.6 pmole) M13 forward or reverse primer, 4 µl of DTCS. The PCR sequencing reaction was performed for 30 cycles at 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min, followed by holding at 4°C. The rest of the procedures were done as mention before in the previous section.

3.2.10.3 Plasmid isolation and serial dilution preparation

Plasmid DNA was isolated by using GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich Chemie GmbH, Munich, Germany) according to manufacturer's instructions. Briefly, 5

ml overnight cultured competent cells were pelleted by centrifuging at 14000 rpm for 1 min and the medium was discarded. These pellets were resuspended and vortexes in 200 µl of resuspension solution. Then, 200 µl of lysis solution was adding and the mixture was subsequently mixed by inversion of tubes until it became clear and viscous. After incubating at room temperature for 4 min, cell precipitation was done by adding 350 µl of neutralization buffer, mixed gently and centrifuged at 14000 rpm for 10 min. Following this, the GenElute Miniprep binding column was inserted into a provided microcentrifuge tube and 500 µl of the column preparation solution was added followed by centrifugation at 14000 rpm for 30 sec and discarding the flow-through. After that, the cleared lysate was transferred to the column and centrifuge at 14000 rpm for 30 sec. The flow-through was discarded and the column was washed by adding 750 µl of wash solution followed by centrifugation at 14000 rpm for 1 min. Again, the flow-through was discarded and the spin column centrifuge at 14000 rpm speed for 2 min without any additional wash solution. To elute DNA, the spin column was transferred into a new collection tube to which 30 µl of ddH_2O was added to the centre of the spin column membrane and centrifuged at speed of 14000 rpm for 1 min.

For determination of plasmid size and quality, 5 µl of the plasmid DNA was analyzed in ethidium bromide stained 2% agarose gel electrophoresis running in 1X TAE buffer. In addition, the concentration of the plasmid was determined using nanodrop 8000 spectrophotometer, the rest was stored at -20°C to be used as template for setting up the standard curve in real-time PCR. The serial dilution consisting of 10^1 to 10^9 copy number of molecules was prepared from plasmid DNA using the online software, weight to molar quantity converter (http://molbiol.edu.ru/eng/scripts/h01_07.html) which converts known DNA concentration to copy number of molecules. Briefly, to determine the number of molecules, the plasmid concentration (ng/µl) and the nucleic acid size (The size of the pGEM®-T vector + PCR fragment of each gene) were submitted to the software. The dilution that contains 10^9 molecules was then determined in 50 µl volume based on the number of molecules obtained in 1 µl plasmid DNA. Following this, 10^8 dilution was determined using 5 µl of 10^9 dilution and 45 µl ddH_2O. The remaining 10^7-10^1 dilutions were determined in the similar way. The PCR was performed to test whether the serial dilution could be a suitable standard curve achieved

for real-time PCR. Afterwards, the plasmid DNA serial dilutions were used as template to generate the standard curve during real time PCR analysis.

3.2.10.4 Real time PCR quantification

The real time PCR quantification was performed for target genes using iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, Hercules, CA). The quantification based on the changes in fluorescence proportional to the increase of product. SYBR Green, which emits a fluorescent signal upon binding to double stranded DNA, was used as a detector. Fluorescence values were recorded during every cycle representing the amount of product amplified to a point known as threshold cycle (Ct). The higher initial transcript amount, the sooner accumulated product was detected in the PCR. Prior to quantification, the optimum primer concentration was obtained by trying different combinations from 200 nM to 600 nM. Results from these primer combinations were compared and the one with lowest threshold cycle and minimizing non-specific amplification was selected for subsequent reaction. After selection of primer concentration, a final assay consisted of 2 µl cDNA as template, forward and reverse primers and 10 µl iTaq™ SYBR® Green Supermix with ROX in a total volume of 20 µl. Thermal parameters used to amplify the template started with an initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec denaturation and 60°C for 1 min annealing and extension. A dissociation curve was generated at the end of the last cycle by collecting the fluorescence data at 60°C and taking measurements every 7 sec until the temperature reached 95°C. The quantification analysis was done by amplifying serial dilutions of target plasmid DNA. Finally, quantitative analysis was done using the relative standard curve method (user bulletin #2 ABI PRISM 7700 SDS, http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf) and results were reported as fold change as compared to the calibrator after normalization of the transcript amount to GAPDH gene as endogenous control.

3.2.11 In situ hybridization of microRNAs in ovarian cryo-sections, whole mount cumulus oocyte complexes and preimplantation embryo stages

For *in situ* hybridization of miRNAs, as described previously in (Hossain et al. 2009; Obernosterer et al. 2007). Bovine ovaries at 21 days of estrous cycle were fixed in 4% paraformaldehyde (PFA) overnight at 4°C followed by overnight incubation in PBS with 30% sucrose at 4°C. In the next day, remove the specimen from the PBS/sucrose solution and tissue-Tek OCT (Sakura Finetek, Zoeterwoude, NL) was added to cover the tissue. Cryo-sections of frozen ovarian tissue (10 µm thickness) were prepared at -20ºC using rapid sectioning cryostat (Leica microsystem Nussloch GmbH, Heidelberger, Germany). The sections were mounted on slides and directly fixed in 4% PFA for 10 min. The fixed sections were washed three times for 5 min in 1x PBS then incubated in different concentration of Ethyl alcohol 50%, 70%, 90% and 100% (Freshly prepared in DEPC-treated H_2O) for 5 min for each concentration, followed by another different concentration 90%, 70% and 50% Ethyl alcohol for 5 min for each concentration, respectively. The sections were washed in 1X PBS for 5 min. Afterwards, the fixed sections were immersed in acetylation solution for 10 min, then washed three times in 1X PBS for 5 min per each times. Proteinase K treatment was done with proteinase K solution for 5 min, then washed three times for 5 min in 1X PBS. Two hours of pre-hybridization were performed at 61°C in hybridization solution. Ovarian sections were incubated overnight at 61°C with 3'-Digoxigenin (DIG) labeled, LNA-modified oligonucleotide ISH probes (1pM) for miR-205, miR-210, U6 RNA and scrambled RNA sequence (Exiqon, Vedbaek, Denmark) in hybridization solution under humidified condition. Hybridized sections were washed two times, one time with pre-warmed 5X SSC for 10 min and the next time with 0.2X SSC for 1 hr at 61°C. Ovarian sections were treated with 0.1 M HCL for 10 min, blocked in B1 solution for 10 min at room temperature. Afterward, the ovarian sections were incubated in humidified condition at room temperature with 500 µl blocking solution for each slide for 1 hr. The anti-DIG-AP antibody was diluted at 1:1500 in blocking solution. Then incubations of ovarian sections overnight at 4°C with diluted anti-DIG-AP antibody. The sections were washed three times in B1 solution for 5 min, and then incubated with 700 µl Tris solution (1 M, pH 8.2) for 10 min. The colour reaction was performed by incubation the slides for 3 hr with fast red solution (Dako Deutschland GmbH, Hamburg, Germany) in

dark place. After three hours, the colour reaction stopped by slides washing thee time in 1X PBST for 10 min. Finally, the slides were mounted with Vectashield containing DAPI (Vector laboratories, Burlingame, CA) and analyzed by confocal laser scanning microscope -510 (Carl Zeiss, Germany).

For whole mount *in situ* hybridization, cumulus oocyte complexes were aspirated from more than 8 mm of ovarian follicles. The COCs (n=5) and embryos (n =7 embryos per stage from zygote to blastocyste) were handled and managed with handmade glass pipettes (opening 250 µm) under stereo microscope. The pre-fixation, acetylation, digestion with Proteinase K, pre-hybridization, hybridization, post-hybridization washing was performed in 4-well embryo culture dishes according to the high-resolution whole mount *in situ* hybridization protocol from Exiqon with some modification. The COCs and embryos were collected and washed two times in PBS then in 4% (PFA) overnight at 4°C. In the next day, the COC and embryos transferred into 100% Methanol (MeOH) and washed for 5 min. Afterward, rehydration was performed by series incubations in 75% MeOH /25% 1X PBS, 50% MeOH /50% 1X PBS, 25% MeOH / 75% 1X PBS for 5 min in each time and then washed two times in 1X PBS for 3 min. After this repeated wash, the COCs and embryos were incubated for 10 min at room temperature in freshly prepared acetylation solution then washed in 1x PBS for 5 min. Proteinase K treatment (10 µg/ml) was preformed for 5 min at room temperature followed by re-fixation in 4% PFA-1X PBS for 5 min and washed in 1X PBS for 3 min. The specimen was pre-hybridized in 800 µl of hybridization solution for 2 hr at 61°C then incubated overnight with 3'-Digoxigenin (DIG) labeled LNA-modified oligonucleotide probes (1pM) for miR-205, miR-210, U6 RNA and scrambled RNA sequence (Exiqon, Vedbaek, Denmark) in hybridization solution in humidified chamber at 61°C. After overnight incubation, embryos were washed briefly in hybridization washing buffer then series wash in 75% hybridization washing buffer /25% 2X SSC, 50% hybridization washing buffer/50% 2X SSC, and 25% hybridization washing buffer /75% 2X SSC all at hybridization temperature for 10 min. Then the specimens were washed in 2X SSC for 10 min at 61°C then two time in 0.2X SSC for 30 min at 61°C. The rest of the procedures were similar to cryo-section hybridization protocol. Images for the different layers or position of COCs and embryos were taken by confocal laser scanning microscope-510 (Carl Zeiss, Germany) using Z-Stack with 1

μm interval. Three dimensional images from all the layers or Z positions of single embryo were constructed using the software ZEN 2008 Light Edition (Carl Zeiss, Germany).

4 Results

4.1 Expression profiling of miRNAs in immature oocytes vs. their surrounding cumulus cells

Expression profiling of 88 miRNAs between immature oocytes and their surrounding cumulus cells revealed that a total of 47 differentially expressed miRNAs ($p \leq 0.05$ and fold change ≥ 2) (Figure 4.1A) between the two cell types.

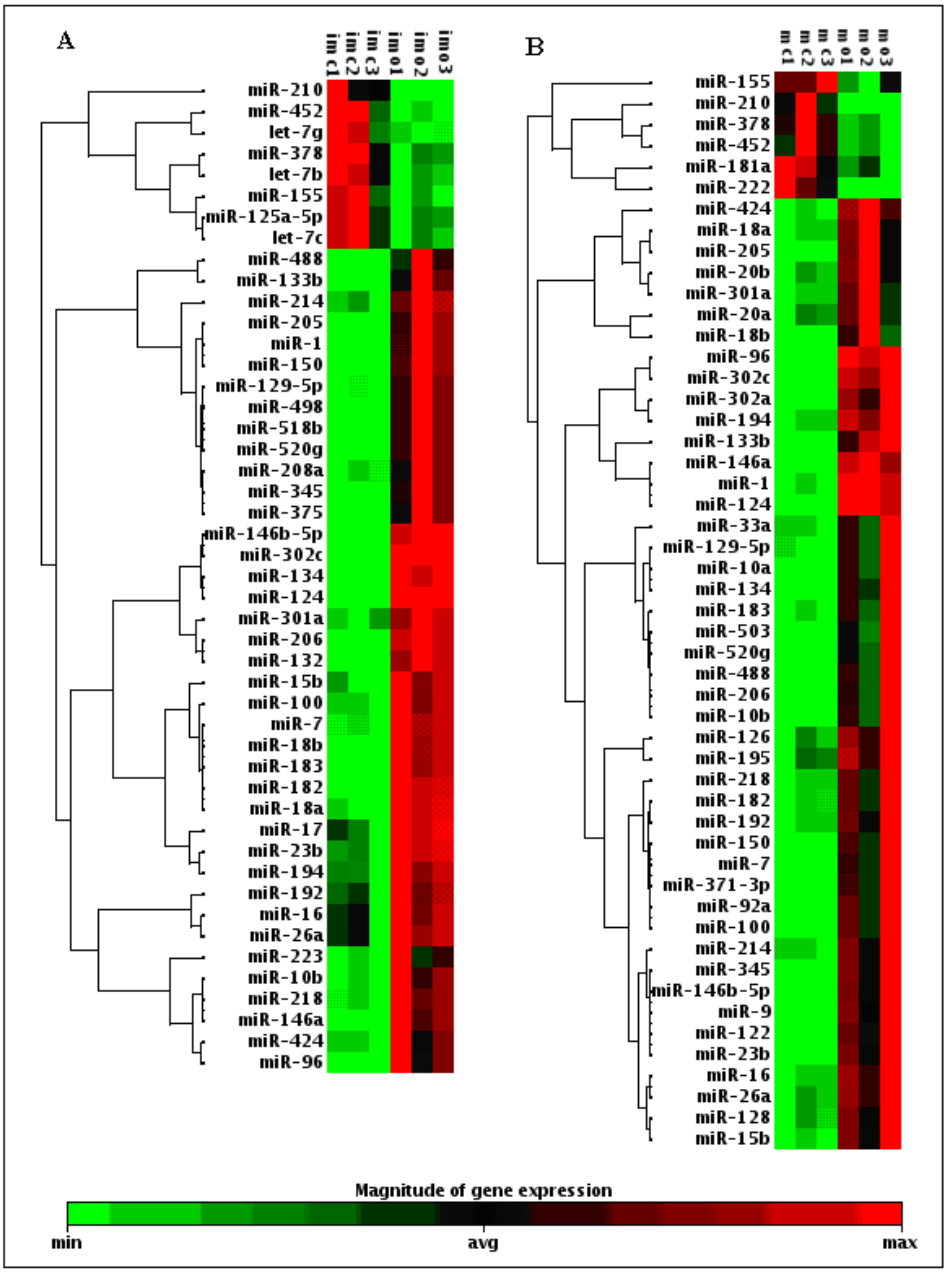


Figure 4.1: Hierarchical clustering of differentially expressed miRNAs between immature oocytes (imo) vs. cumulus cells (imc) (A) and matured oocytes (mo) vs.

cumulus cells (mc) (B). Columns represent the similarities or differences in the expression pattern within or between biological replicates of oocyte or cumulus cells and the horizontal dendrogram represent the clustering of miRNAs according to their expression similarities. Red and green colours indicate the higher and lower expression level, respectively

Among these, the expression level of eight miRNAs including miR-125a-5p, miR-155, miR-210, miR-378 and miR-452 were enriched in cumulus cells. On the other hand, the expression level of 39 differentially expressed miRNAs was enriched in immature oocytes compared to their surrounding cumulus cells. Among these, miR-146a, miR-146b-5p, miR-183, miR-18b, miR-96, miR-150 and miR-205 exhibited between 56 to 2109 fold change increases in the oocytes. The list of all differentially expressed miRNAs between immature oocytes and their surrounding cumulus cells is described in table (2).

Table 2**:** List of differentially expressed miRNAs between immature oocytes and their surrounding cumulus cells

MiRNA	Fold change (Mean ± SD)	p-value
miR-488	49.39 ± 0.95	0.026091
miR-133b	54.18 ± 0.65	0.011529
miR-214	2.43 ± 0.60	0.001691
miR-205	2109.88 ± 0.35	0.002081
miR-1	31.41 ± 0.49	0.001502
miR-150	715.57 ± 0.20	0.001268
miR-129-5p	19.24 ± 0.08	0.003916
miR-498	49.39 ± 0.45	0.00321
miR-518b	58.33 ± 0.40	0.003193
miR-520g	74.18 ± 0.29	0.003134
miR-208a	14.89 ± 1.03	0.005478
miR-345	12.58 ± 1.03	0.004175
miR-375	20.72 ± 0.66	0.004603
miR-146b-5p	92.17 ± 0.56	0.000002
miR-302c	37.09 ± 0.98	< 0.00001
miR-134	32.44 ± 0.74	0.000001
miR-124	20.39 ± 0.53	< 0.00001
miR-301a	5.43 ± 1.46	0.000413
miR-206	28.11 ± 0.60	0.000027

miR-132	20.06 ± 0.54	0.000125
miR-15b	2.54 ± 1.04	0.000976
miR-100	13.20 ± 0.74	0.000327
miR-7	20.48 ± 0.77	0.000055
miR-18b	197.58 ± 0.82	0.000043
miR-183	127.38 ± 0.60	0.000052
miR-182	87.00 ± 0.54	0.000007
miR-18a	8.36 ± 0.87	0.000014
miR-17	2.40 ± 0.79	0.002948
miR-23b	3.51 ± 0.57	0.000781
miR-194	4.25 ± 0.83	0.001866
miR-192	2.50 ± 0.75	0.012918
miR-16	2.27 ± 0.77	0.030143
miR-26a	2.09 ± 0.64	0.024601
miR-223	4.79 ± 1.07	0.02624
miR-10b	19.78 ± 0.96	0.002339
miR-218	18.72 ± 0.89	0.001003
miR-146a	56.48 ± 0.91	0.001352
miR-424	5.76 ± 1.00	0.009166
miR-96	323.95 ± 0.90	0.004585
miR-210	-16.96 ± 1.09	0.0193
miR-452	-3.14 ± 0.54	0.027883
let-7g	-2.62 ± 0.78	0.041633
miR-378	-20.49 ± 3.82	0.015314
let-7b	-3.12 ± 0.59	0.009613
miR-155	-17.55 ± 2.19	0.045894
miR-125a-5p	-47.63 ± 6.36	0.043974
let-7c	-2.14 ± 0.47	0.037073

Positive fold change means up-regulated in oocytes and negative fold change means down regulated in oocytes compared to surrounding cumulus cells**.**

4.2 Expression profiling of miRNAs in matured oocytes vs. their surrounding cumulus cells

The expression analysis of miRNAs between matured oocytes vs. their surrounding cumulus cells was preformed by using the same miRNA PCR array that consists of 88 miRNAs. The result revealed that 51 miRNAs were differentially expressed ($p \leq 0.05$ and fold change ≥ 2) between these two groups of cells. From those, six miRNAs (miR-155, miR-210, miR-378, miR-452, miR-181a and miR-222) were enriched in cumulus cells and 45 miRNAs were enriched in matured oocytes (Figure 4.1B). Several miRNAs including miR-317-3p, miR146a, miR-18b, miR-146b-5p, miR-424, miR-9, miR-150, miR-205 and miR-122 were among the top miRNAs (109 to 2433 fold changes)

enriched in matured oocytes compared to their surrounding cumulus cells. The detail list of all differentially expressed miRNAs is described in table (3)

Table 3**:** List of differentially expressed miRNAs between matured oocytes and their surrounding cumulus cells

MiRNA	Fold change (Mean ± SD)	p-value
miR-424	237.06 ± 7.95	0.001934
miR-18a	6.51 ±1.47	0.010765
miR-205	1773.55 ± 1.51	0.008389
miR-20b	3.52 ±1.41	0.010345
miR-301a	4.14 ± 1.50	0.018357
miR-20a	2.12 ± 1.43	0.047371
miR-18b	133.04 ± 1.84	0.032683
miR-96	15.09 ± 0.90	0.000021
miR-302c	37.86 ± 1.26	0.000106
miR-302a	46.28 ± 0.84	0.001537
miR-194	7.24 ± 0.82	0.000568
miR-133b	30.96 ± 1.65	0.00331
miR-146a	116.63 ± 1.21	0.000083
miR-1	39.19 ± 1.89	0.000008
miR-124	14.75 ± 1.09	0.000003
miR-33a	3.46 ± 0.98	0.044276
miR-129-5p	4.96 ± 0.84	0.029574
miR-10a	68.55 ± 0.51	0.029162
miR-134	16.94 ± 0.28	0.026959
miR-183	6.30 ± 0.35	0.046364
miR-503	85.57 ± 0.55	0.047496
miR-520g	55.04 ± 0.10	0.045680
miR-488	53.66 ± 0.19	0.035662
miR-206	30.25 ± 0.30	0.042302
miR-10b	62.07 ± 0.26	0.039812
miR-126	13.54 ± 2.15	0.007329
miR-195	2.81 ± 0.77	0.010008
miR-218	6.13 ± 0.48	0.019668
miR-182	5.00 ± 0.52	0.02036
miR-192	7.83 ± 0.54	0.013054
miR-150	1666.29 ± 0.98	0.017642
miR-7	54.91 ± 0.43	0.020461
miR-371-3p	109.83 ± 0.91	0.019111
miR-92a	5.51 ± 0.77	0.014021
miR-100	37.94 ± 0.48	0.015319
miR-214	3.50 ± 1.01	0.008226
miR-345	19.50 ± 0.65	0.004917

miR-146b-5p	139.34 ± 0.46	0.008366
miR-9	269.80 ± 0.52	0.007456
miR-122	2433.96 ± 0.64	0.010694
miR-23b	13.51 ± 0.62	0.009962
miR-16	4.92 ± 0.69	0.004289
miR-26a	4.35 ± 0.69	0.007508
miR-128	5.63 ± 0.80	0.010281
miR-15b	6.86 ± 0.80	0.010547
miR-155	-2.37 ± 0.41	0.046329
miR-210	-2.67 ± 1.22	0.036641
miR-378	-3.10 ± 1.45	0.012184
miR-452	-2.68 ± 1.47	0.044323
miR-181a	-2.92 ± 2.08	0.034214
miR-222	-4.93 ± 1.49	0.012613

Positive fold change means up-regulated in oocytes and negative fold change means down regulated in oocytes compared to the surrounding cumulus cells**.**

4.3 Commonly differentially expressed miRNAs in immature or matured oocyte vs. their surrounding cumulus cells

To identify the miRNAs that are constantly expressed in oocytes or cumulus during oocytes maturation, we merged the two data sets described above. From this analysis, we have identified 33 miRNAs commonly differentially expressed between oocytes and surrounding cells before and after maturation. From those miR-155, miR-210, miR-378, miR-452 were enriched in cumulus cells compared to the oocytes before and after maturation (Table 4). On the other hand, 29 miRNAs including miR-205, miR-150, miR-96, miR-122, miR-146a and miR-146b-5p were enriched in immature and matured oocytes compared to their surrounding cumulus cells (Table 4).

Table 4: List of commonly differentially expressed miRNAs between oocytes and their surrounding cumulus cells after and before maturation

MiRNA	Immature oocyte vs. its surrounding cumulus cells		Matured oocyte vs. its surrounding cumulus cells	
	Fold change (mean ± SD)	p-value	Fold change (mean ± SD)	p-value
MiR-182	87.00 ± 0.54	0.000007	5.00 ± 0.52	0.02036
MiR-1	31.41 ± 0.49	0.001502	39.19± 1.89	0.000008
MiR-146b-5p	92.17 ± 0.56	0.000002	139.34 ± 0.46	0.008366
MiR-301a	5.43 ± 1.46	0.000413	4.14± 1.50	0.018357
MiR-488	49.39 ± 0.95	0.026091	53.66 ± 0.19	0.035662

MiR-18a	8.36 ± 0.87	0.000014	6.51 ± 1.47	0.010765
MiR-100	13.20 ± 1.03	0.000327	37.94 ± 0.48	0.015319
MiR-134	32.44 ± 0.74	0.000001	16.94 ± 0.28	0.026959
MiR-206	28.11 ± 0.60	0.000027	30.25 ± 0.30	0.042302
MiR-23b	3.51 ± 0.57	0.000781	13.51 ± 0.62	0.009962
MiR-214	2.43 ± 0.60	0.001691	3.49 ± 1.01	0.008226
MiR-18b	197.58 ± 0.82	0.000043	133.04 ± 1.84	0.032683
MiR-345	12.58 ± 1.03	0.004175	19.50 ± 0.65	0.004917
MiR-129-5p	19.24 ± 0.08	0.003916	4.95 ± 0.84	0.029574
MiR-96	323.95 ± 0.90	0.004585	15.09 ± 0.90	0.000021
MiR-183	127.38 ± 0.60	0.000052	6.30 ± 0.35	0.046364
MiR-194	4.25 ± 0.83	0.001866	7.24 ± 0.82	0.000568
MiR-218	18.71 ± 0.89	0.001003	6.13 ± 0.48	0.019668
MiR-205	2109.88 ± 0.35	0.002081	1773.55 ± 1.51	0.008389
MiR-10b	19.78 ± 0.96	0.002339	62.07 ± 0.26	0.039812
MiR-133b	54.17 ± 0.65	0.011529	30.96 ± 1.65	0.00331
MiR-520g	74.18 ± 0.29	0.003134	55.04± 0.10	0.04568
MiR-124	20.39 ± 0.53	< 0.00001	14.75 ± 1.09	0.000003
MiR-150	715.57 ± 0.20	0.001268	1666.29 ± 0.98	0.017642
MiR-15b	2.54 ± 1.04	0.000976	6.86 ± 0.80	0.010547
MiR-302c	37.09 ± 0.98	< 0.00001	37.86 ± 1.26	0.000106
MiR-7	20.48 ± 0.77	0.000055	54.91 ± 0.43	0.02046
MiR-146a	56.48 ± 0.91	0.001352	116.63 ± 1.21	0.000083
MiR-122	66.09 ± 4.89	0.367784	2433.95 ± 0.64	0.010669
MiR-155	-17.55 ± 2.19	0.045894	-2.37 ± 0.41	0.046329
MiR-210	-16.96 ± 1.09	0.0193	-2.67 ± 1.22	0.036641
MiR-378	-20.49 ± 3.82	0.015314	-3,09 ± 1.45	0.012184
MiR-452	-3.14 ± 0.54	0.027883	-2.68 ± 1.47	0.044323

Positive fold change means up-regulated in oocytes and negative fold change means down regulated in oocytes compared to the surrounding cumulus cells

4.4 Expression pattern of miRNAs during bovine oocyte maturation

The differential expression of miRNA identified during oocyte maturation in both oocyte and cumulus cells by the comparison between immature and matured oocytes which showed that 18 miRNAs to be differentially expressed and all were highly abundant in matured oocytes (Figure 4.2A and Table 5). Similarly, the comparative analysis of miRNAs between cumulus cells surrounded the oocyte before and after maturation showed that 16 miRNAs to be differentially expressed, of which 13 and 3 miRNAs were highly abundant in cumulus cells derived from matured and immature COCs, respectively (Figure 4.2B and Table 5).

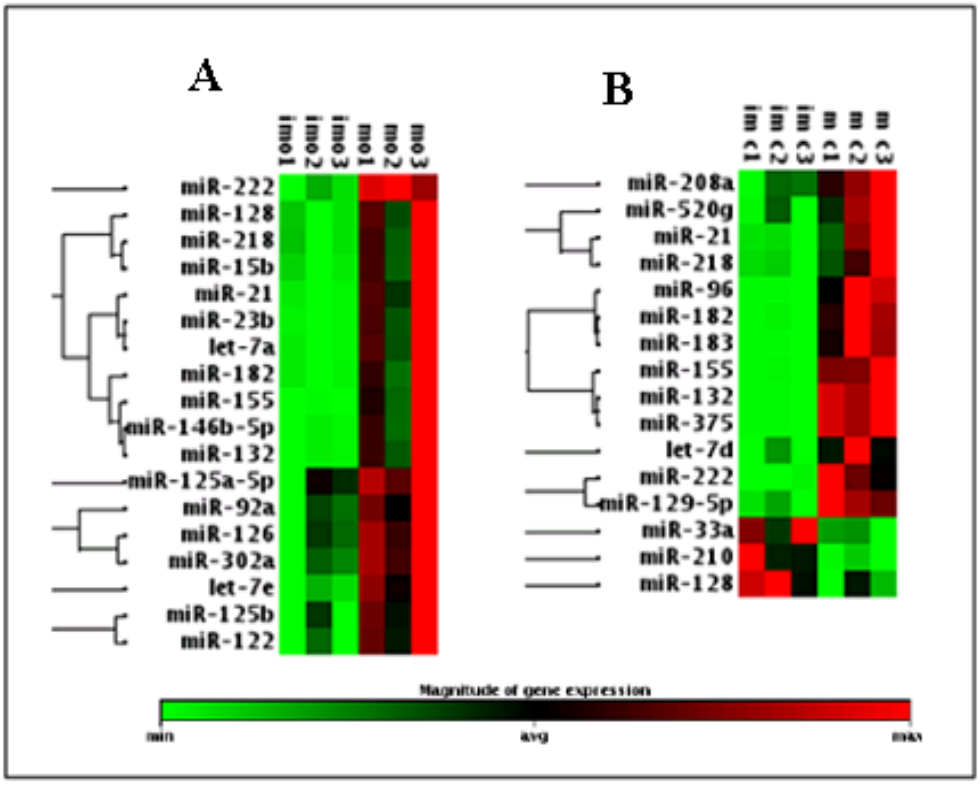


Figure 4.2: Hierarchical clustering of miRNAs differentially expressed between matured oocytes (mo) vs. immature oocyte (imo) (A) and cumulus cells derived from matured COCs (mc) vs. immature COCs (imc) (B). Columns represent the expression from a single PCR array with miRNA represented by raws, which are clustered according to their pattern of expression in three biological replicates from both stages of oocytes and cumulus cells. Shades of colours indicate the level of expression of miRNAs in matured and immature oocytes (A) or matured and immature cumulus cells (B) with a level increasing from green to red

Table 5: List of differentially expressed miRNAs between matured vs. immature oocytes and matured vs. immature cumulus cells

Matured oocytes vs. immature oocyte			Matured cumulus cells vs. immature cumulus cells		
MiRNA	Fold change (mean ± SD)	p-value	MiRNA	Fold change (mean ± SD)	p-value
let-7e	2.75 ± 0.67	0.007809	miR-155	75.79 ± 1.09	0.000643
miR-125a-5p	25.64 ± 6.38	0.048675	miR-182	43.93 ± 1.14	0.003009

miR-125b	164.30 ± 5.97	0.042853	miR-222	33.99 ± 0.82	0.006578
miR-122	125.97 ± 4.97	0.029304	miR-132	89.31 ± 0.61	0.000053
miR-155	560.36 ± 2.12	0.039682	miR-21	13.09 ± 1.60	0.033336
miR-126	12.47 ± 3.88	0.021730	miR-129-5p	6.03 ± 0.87	0.001556
miR-92a	48.62 ± 7.39	0.046121	miR-96	40.81 ± 0.73	0.005935
miR-182	2.53 ± 0.63	0.043930	miR-183	68.63 ± 0.94	0.004014
miR-302a	7.80 ± 2.25	0.010798	miR-218	8.25 ± 1.37	0.036779
miR-146b-5p	2.48 ± 0.55	0.040028	miR-520g	3.87 ± 1.16	0.035594
miR-222	4.89 ± 1.30	0.000319	Let-7d	2.04 ± 0.72	0.039373
miR-132	4.81 ± 0.50	0.031369	miR-208a	3.08 ± 0.99	0.01712
miR-23b	2.88 ± 0.63	0.029389	miR-375	18.77 ± 0.32	0.000063
miR-21	11.58 ± 0.91	0.018912	miR-210	-4.79 ± 0.75	0.034768
let-7a	2.75 ± 0.67	0.007809	miR-33a	-2.51 ± 0.59	0.036418
miR-218	2.70 ± 0.77	0.042421	miR-128	-2.57 ± 0.59	0.048758
miR-128	2.21 ± 0.77	0.042421			
miR-15b	2.19 ± 0.71	0.039268			

Positive fold change means up-regulation and negative fold change means down regulation

4.5 Target prediction of selected miRNAs and their functional annotation

Following identification of commonly differentially expressed miRNA in immature or matured oocyte vs. surrounding cumulus cells, six miRNAs enriched in oocyte (miR-205, miR-150, miR-96, miR-122, miR-146a and miR-146b-5p) and two miRNAs enriched in cumulus cells were selected based on their expression pattern. The target genes of selected miRNAs were identified using four online tools, TargetScan, PicTar, miRDB and MicroRNA.org-Target and Expression, based on their score and their prediction at least in two or more target search tools. The result revealed that, accordingly, 854 and 234 genes were found to be targeted by miRNAs enriched in oocytes and cumulus cells, respectively. To obtain an overview on the functional role of differentially expressed selected miRNAs, the predicted targets genes were then uploaded into the Ingenuity pathway analysis system (IPA) to identify the molecular networks (biological functions) and pathways based on the information in the IPA data base. The analysis identified 25 and 13 molecular networks involved by the target genes of miRNAs enriched in oocyte and cumulus cells, respectively (Table 6 and 7). Among these, cellular assembly and organization, cell cycle, carbohydrate metabolism, molecular transport, cellular development, cell signaling, cell death, cellular growth and proliferation, reproductive system development and function, embryonic development, amino acid metabolism, small molecule biochemistry, gene expression, cell to cell

signaling and interaction, and lipid metabolism were the most representative molecular networks identified by this analysis.

Table 6: Molecular networks involved by target genes of selected miRNAs enriched in oocyte

ID	Molecule in network	Score	Focus molecules	Top function
1	AFF1, AIFM2, ANLN, ARIH2, BAI3, C13ORF34, CACYBP, CCAR1, CCNG1, CENPF, CEP55, ENSA, FBXL3, FBXL5, FBXO4, HS3ST1, ING4, JMJD1C, KDM2B, LIN9, MSL2, P4HA1, PERP, PROSC, SCN3B, SIAH1, SIAH2, SKP1, TBL1X, THAP8	47	33	Cell Cycle, Cellular Movement, Cell Morphology
2	ACSL1, ADIPOR2, ATXN1, C10ORF118, C2ORF3, CMTM6, CXORF21, CYP4A11, DOCK1, E2F3, ERO1L, FADS1, FOXO1, ICMT, MYB (includes EG:4602), NLK, NOVA1, PRMT3, PSMA4, RAB23, RAPGEF4, RDH11, RIMS1, RXRG, SGMS1, ZHX1, ZHX2	36	27	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
3	ABR, AOC3, BZW1, CALCR, CCDC80, CLDN11, CREB3L2, CYBB, ESM1, EXT1, FOXF1, FOXF2, KLF7, LTBP2, MAGI1, MMD, NFAT5, PC, PGM2L1, PTP4A1, PTPRJ, RAB32, SLA, SLC1A2, SLC7A1, SQLE, TNF, TNFAIP8, TNRC6A, ZFP36L1	35	30	Developmental Disorder, Skeletal and Muscular Disorders, Gastrointestinal Disease
4	ADAM19, C1q, CBFA2T3, CCK, CDKN1B, CFH, COPS8, DCN, DLG2, ERBB4, EREG, ESRRG, GYPA, HBP1, INADL, LRP1, LRP2, LRP10, NLGN2, PDE7A, PDGF BB, PLEKHA1, PSMF1, QKI, , RBM11, RGS2, RHOXF2, SLC1A1	35	26	Cell Cycle, Cellular Growth and Proliferation, Immunological Disease
5	BRPF3, CAMK2G, CASP7, CREBL2, EGR2, FOXK2, FYN, GLP1R, HAS2, HERC3, HNRNPU, LRRC7, MAMLD1, NBEA (includes EG:26960), OVOL1, PSMD3, PTGER3, RPS6KA3, SFRP4, SMAD4, SMARCD1, SRP72, TCR, TOR1A, TOR1AIP2, UCK2, ZNF331	35	26	Cellular Development, Reproductive System Development and Function, Cell-mediated Immune Response
6	ADAMTS9, ALDH1B1, AMPH, CACNB4, GABRA4, GABRG2, GLRB, GLRX3, GPHN, HOXA2, ITCH, MTSS1, NECAP1 NUMB, OCRL,	30	24	Connective Tissue Development and Function, Embryonic Development, Skeletal and Muscular

	PPP3R1, PRDX4, SOX6, SOX11, SOX5 (includes EG:6660), SYNJ2, SYNJ2BP, TNFSF4, ZFAND5			System Development and Function
7	COX11, CPEB1, DPYD, EIF4B, EIF4E, EIF4G2, ENTPD1, FBXW2, HNRNPD, HTR2B, KIAA1199, KL, MED1, MPZL1, NTN4, PDCD4, PLXNA2, PRKAR1A, SEMA3A, SHC4, SLC9A1, SMC3, UBR5	29	23	Gene Expression, Protein Synthesis, RNA Post-Transcriptional Modification
8	ADCY6, Arp2/3, ARPC5, DTNA, ENC1, EPB41, EZR, FN1, GNE, GPLD1, GRPR, HSP90B1, MLPH, MORF4L1, MSN, MYO5A, MYO5B, MYRIP, OCLN, PFN2PRKCA, RAB8B, SAR1A	27	22	Cellular Assembly and Organization, Hair and Skin Development and Function, Genetic Disorder
9	C5ORF22, CACNA1G, CASP2, CDA, CLIC4, CUX1, DHFR, DUOX2, E2F5, FOXD3, HOXA5, Importin alpha, Importin beta, IPO5, IPO8, KAT2B, KPNA1, KPNB1, MLL, PAPPA, RARB, SMC2, STAR, SUZ12	26	22	Cancer, Endocrine System Development and Function, Organ Development
10	ABI2, C3ORF10, CAST, CDH11, CTH, DIO2, IFRD1, ITGAX, MARCH6, PPBP, RANBP9, RASGRP1, SHPRH, SNAP23, TLL1, UBE2G1, UBE2J1, UBE2N, VAMP2, VAPA	23	20	Cell Signaling, Cellular Assembly and Organization, Cellular Function and Maintenance
11	AICDA, ATF5, CALCRL, CD226, CD40LG, HSP90AA1, IL7, ITGB3, KIF11, LDLR, LIPG, LRRK2, LYN, METAP1, NR2F2, NR3C2, NRCAM, PTCH1, STIL, STK24, ZC3HAV1	23	21	Cell-To-Cell Signaling and Interaction, Skeletal and Muscular System Development and Function, Cellular Movement
12	ADRBK2, AKT3, CASR, DLAT, GOLGA7, MTCH2, MUC4, NRAS, NTRK2,, PDK1, PDK4, PI3K, PIK3AP1, PIP4K2A, PKM2, PLCB1, PLCB4, RFXAP, STYK1, TP53BP2,	22	19	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
13	AKAP9, AMPK, ASAH1, CADM1, CAMK2N1, CLIC5, CPD, ERRFI1, GYS1, ITPR1, KCNJ16, PDE3B, PFKFB2, PHKB, PPP1R15B, PPP1R3A, SLC8A1, STK3, TACC1	20	18	Carbohydrate Metabolism, Nervous System Development and Function, Behavior
14	ALK, CBL, CD80, DIXDC1, DLGAP2, DUSP4, FRK, FRS2, GPC3, LCOR, MECOM, PNN, RALGPS1, SATB2, SORBS1, YES1, ZDHHC17, ZEB1	20	18	Cellular Growth and Proliferation, Tumor Morphology, Tissue Development
15	BCL2A1, CFL2, DYRK1A, HSPA2, IRAK1, IRAK2, MAP2K3, MAP3K2, MAP3K3, MAP3K12, MS4A1, MYO1B, NEK2, PMAIP1, SCIN, STK39, UBA52	18	17	Cell Signaling, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function

16	BTBD3, CAMSAP1L1, DULLARD, GALNT2, GALNT3, GALNT12, GGNBP2, KIAA2022, LRCH2, MAMDC2, MBD6, MEGF9, RC3H1, VEZF1, ZC3H12B, ZFAND3	17	16	Post-Translational Modification, Genetic Disorder, Skeletal and Muscular Disorders
17	BAI2, C15ORF29, C3ORF34, C8ORF41, CACNA2D2, DNAL1, DPH5, HOOK3, KLHL20, NBPF3, NSL1, PAPOLA, PJA2, SLC25A1,TUBE1, ZNF589	16	16	Cellular Development, Cellular Growth and Proliferation, Reproductive System Development and Function
18	AK3, C3ORF58, COMMD10, DDAH2, ELOVL3, ETNK1, GAA, KIAA1949, MGRN1,MYPN, NEU1, TMSB4Y, TMX1,TRAPPC2,YKT6	15	15	Cellular Assembly and Organization, Cellular Compromise, Hair and Skin Development and Function
19	ANKIB1, ANKRD27, C19ORF12, DEPDC1, FAM35A (includes EG:54537), FMO2, GNPDA2, LIMCH1, LRRC58, PLP2, RBM47, SLC16A9, SLC22A5, SLC30A9, UST	15	15	Gene Expression, Drug Metabolism, Genetic Disorder
20	AGMAT, BCORL1, CLASP2, CMTM4, ELOVL7, GPR124, INSIG2, KANK4, MSL2, PKP4, PRTFDC1, RBPMS2, SLC10A3, TDRD6, VAT1	15	15	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Lipid Metabolism
21	ABAT, ANKRD57, AVIL, C9ORF86, CHN1 (includes EG:1123), CHST1, FEZ2, GAN, IGSF5, RAB35, RAB11FIP4, SNX7, STRBP, TBRG1	14	14	Cell Morphology, Cellular Development, Cancer
22	ADAMDEC1, C9ORF72, DNAJB7, FAM49B, IFIT5, KBTBD8, METTL9 (includes EG:51108), MTAP (includes EG:4507), PHF3, RIMBP2, RORB, SHOX2, SLITRK2, UPP2	14	14	Drug Metabolism, Lipid Metabolism, Small Molecule Biochemistry
23	ACSL4, ARF6, CS, ERLIN1, ETF1, GXYLT1, NPEPPS, PLSCR4, PPARGC1A, SFRS6, SRPRB, TRAF6, TRDMT1, WWC2	14	14	RNA Post-Transcriptional Modification, Carbohydrate Metabolism, Nucleic Acid Metabolism
24	ADK, C1ORF25, IMPACT, IPO9, LFNG, LMNB2, MOBKL1A, MYO9A, PRKRA, RASSF8, SIRPB1,SLC25A19,TNRC6B, ZFP30	13	14	Protein Synthesis, Energy Production, Molecular Transport
25	C6ORF97, CASC4, N6AMT1,SEC23IP, SLC19A3, SLC35A3, SLC38A4, TDRKH, TMEM216, TRMT12, ZNF253, ZNF577, ZNF614	12	13	Gene Expression, Endocrine System Disorders, Genetic Disorder

Table 7: Molecular networks involved by target genes of selected miRNAs enriched in cumulus cells

ID	Molecule in network	Score	Focus molecules	Top function
1	BECN1, CAPN9, CASD1, CNP, CORO2B, DR1, FUBP1, GABPA, GDE1, IGF2, LAMC1, LIFR, LIMS1, MLL2, PNN, PPP1CB, PPP1R2, PPP1R10, PPP1R3D, PTPN21, RAB31, RASGRP1, RELN,TLX1, ZEB1	46	25	Cancer, Reproductive System Disease, Skeletal and Muscular System Development and Function
2	ACVR1B, ANGPT2, INPP5D, IRS2, LCP2, LIG4, MYBL1, NKIRAS1, PGM2L1, PKN2, PSAP, PTGER2, PTPRJ, RPS6KA3, RUNX3, SIPA1L1, SMAD2, SNAI2, SYNGAP1, VEGFA	29	20	Tissue Morphology, Cell Signaling, Cardiovascular System Development and Function
3	AKAP5, CACNA1C, DYRK1A, ELK4, ELOVL5, PRKAR2B, PRKD3, RAB3B, RAD18, RGS13, SERTAD2, SF3B1, SHPRH, SLC6A1, SPRED2, VBP1	24	16	Gene Expression, Immunological Disease, Connective Tissue Development and Function
4	C18ORF45, COL23A1, EXOSC3, FAM76B, GGNBP2, HIATL1, KCMF1, NR1D2, PAN3, RUNX1T1, SLC8A3, TMEM165, YIPF4, YTHDC1, ZNF692	23	15	Cell Death, Cellular Development, Reproductive System Disease
5	ALG2, CASC1, CHN1 (includes EG:1123), DSG1, DSG3, EFNA3, FAM73B, GOLPH3, IRF8, NPHP1, POU2AF1, PPA2, RPA1, SERPINB9, VAMP7	23	15	Genetic Disorder, Ophthalmic Disease, Cellular Development
6	AGL, AIFM3, ARHGEF10, CNTN3, , ENOX2, IPO5, NECAB1, PAPD5, PHF6, RAB27B, RNF111, SEMA5B, SLC17A8, SLC7A11, SOX7	23	15	Amino Acid Metabolism, Small Molecule Biochemistry, Molecular Transport
7	AKAP2, ARMC10, ATIC, DACT1, DNAH5, ELOVL6, ERC2, KLF12, NPEPPS, PLCB4, RRP15, SLC4A11, SNRK, USH2A	21	14	Cell Death, Lymphoid Tissue Structure and Development, Cardiovascular System Development and Function
8	ALDH5A1, ANAPC4, ECM2, ELAVL2, FCHSD2, GGPS1, HNMT, NOC3L, PGM2L1, TTC13, XYLT1, ZDHHC21, ZNF532	19	13	Cellular Development, Cellular Growth and Proliferation, Connective Tissue Development and Function
9	BICD2, C1ORF212, ISCU, LUC7L2, NAP1L5, NDUFA4, NFIC, RYK, SDF2, SERP1, TNPO3, ZFR, ZNF292	19	13	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular

				Function and Maintenance
10	APBA3, CIAPIN1, CYGB, DLX1, E2F3, FGFRL1, IMMP2L, MED20, MYOM1, PANX3, RCAN2, WDR35 (includes EG:57539)	17	12	Gene Expression, Cell Death, Cancer
11	DIMT1L, GPRIN3, LXN, METTL13, MKLN1, RPL22, SLC6A15, SSX2, ZNF462, ZNF526, ZNF644	15	11	Cell-To-Cell Signaling and Interaction, Cancer, Cellular Development
12	ADD3, CCKBR, HMGCS1, KCNH5, LAPTM5, MFN1, REPS2, SH3BGRL, TMEM55A, TOPORS, ZNF140	15	11	Cellular Assembly and Organization, Cell Morphology, Lipid Metabolism
13	ANKRD17, BAZ2B, C12ORF5, GEMIN8, H2AFY, NUFIP1, PER3, SCARA3, SNX18, TMED7	13	10	Cell Cycle, Cancer, Cellular Compromise

Moreover, genes targeted by miRNAs enriched in oocytes were found to be involved in eleven significant pathways including the GnRH, cell cycle, G-protein coupled receptor signaling pathway, apoptosis signaling pathway (Figure 4.3A). Similarly, the genes targeted by miRNA enriched in cumulus cells were found to be involved in seven different key molecular pathways including CDK5 signaling pathway, insulin receptor signaling and protein kinase A signaling pathway (Figure 4.3B).

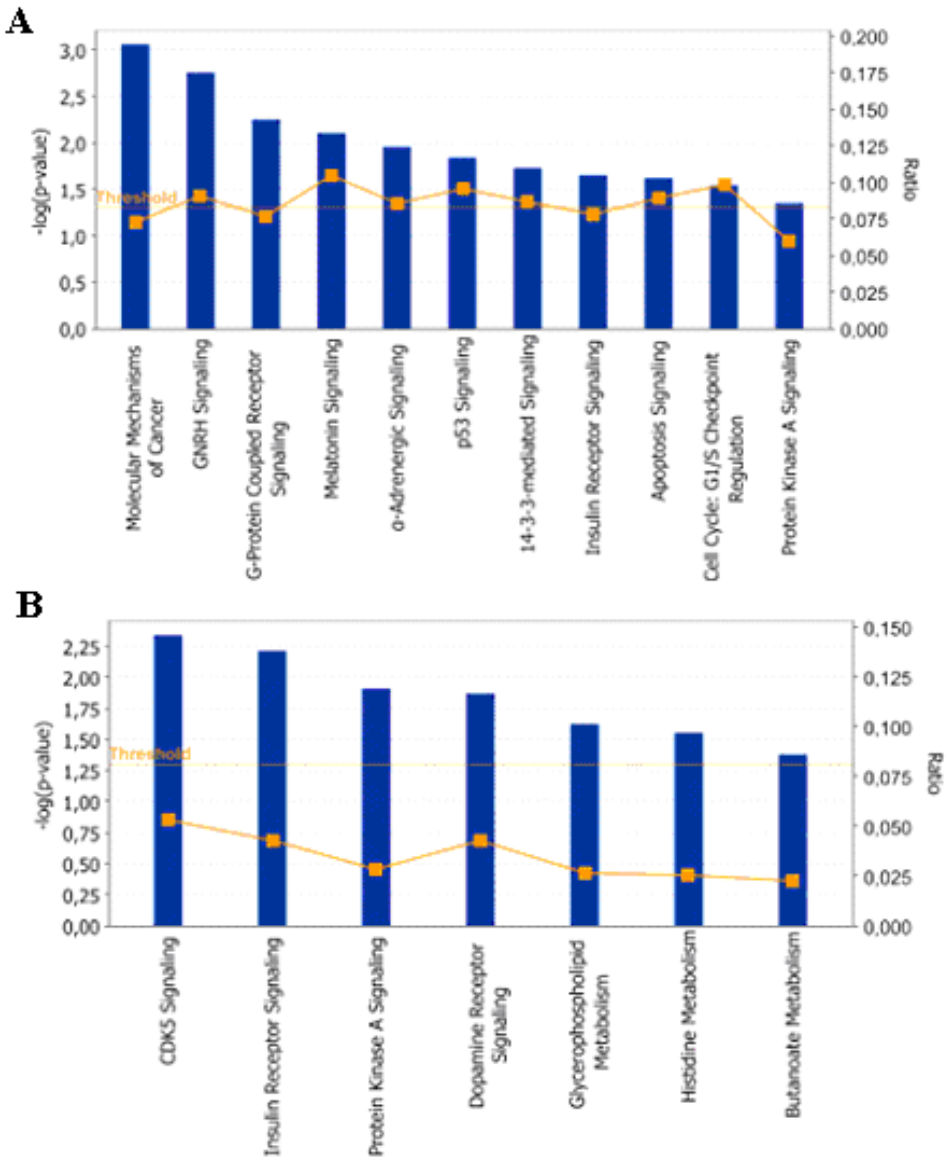


Figure 4.3: The most significant canonical pathway involved by genes targeted of miRNAs enriched in oocytes (A) and miRNAs enriched in cumulus cells (B). The bars represent the p-value for each pathway. The orange irregular line is a graph of the ratio of genes from the data set to the total number of genes involved in the pathway for the different pathways

Due to large number of target genes, we systematically selected one gene for each selected miRNA and the sequence complementarities between seed region of selected miRNAs and the 3'-UTR of target genes is indicated in figure 4.4. The further characterization of target genes was done only for these genes in the following sections.

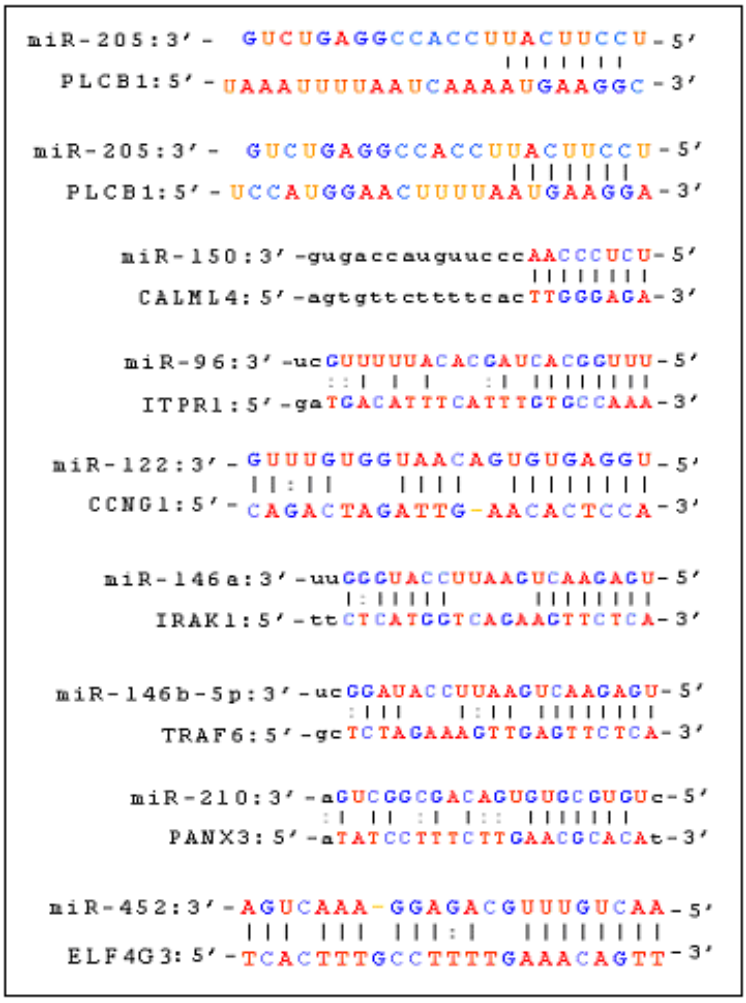


Figure 4.4: Sequence complementarities between selected miRNAs and their target mRNA 3'-UTR

4.6 Co-expression study of selected miRNAs and target genes in early developmental stages

To evaluate whether the expression of the selected miRNAs will be retained or degraded during the early embryo formation, the expression patterns of these miRNAs were then analyzed in matured oocyte and different stages of preimplantation embryo

(zygotes, 2-cell, 4-cell, 8-cell, morula and blastocyst). The results have shown that the expression of all oocyte enriched miRNAs were highly abundant in early stages of development up to 4-cell embryo, followed by dramatic reduction in expression from 8-cells until the blastocyst stage. Similarly, two miRNAs (miR-452 and miR-210) enriched in cumulus were selected based on their expression pattern and their expression profile was analyzed in different stages of preimplantation embryo development. Unlike those enriched in oocytes, these miRNAs showed no defined pattern of expression throughout the developmental stages and could be detected at relatively lower level in all stages of development (Figure 4.5).

After selecting the targets genes using four different target search tools based on their score and their prediction at least in two or more target search tools, the expression profiling of one target gene for each selected miRNA, based on sequence complementarities, were analyzed in matured oocyte and different stages of preimplantation embryo. The results have shown that three genes, namely PLCB1, ITPR1 and IRAK1 showed a reversed expression profile compared to the profile observed by their targeting miRNAs (miR-205, miR-96 and miR-146a), respectively (Figure 4.5). However, CALML4 and TRAF6 transcripts showed a similar expression profile to their targeting miRNAs, namely miR-150 and miR-146b-5p (Figure 4.5). On the other hand, the relative abundance of CCNG1 which is the target of miR-122 was detectable until later stages of development irrespective of the expression of the targeting miRNA. As it is the case for targeting miRNAs (miR-210 and miR-452), the target genes PANX3 and EIF4G3, were showed no clear trend in expression profile throughout the preimplantation period (Figure 4.5).

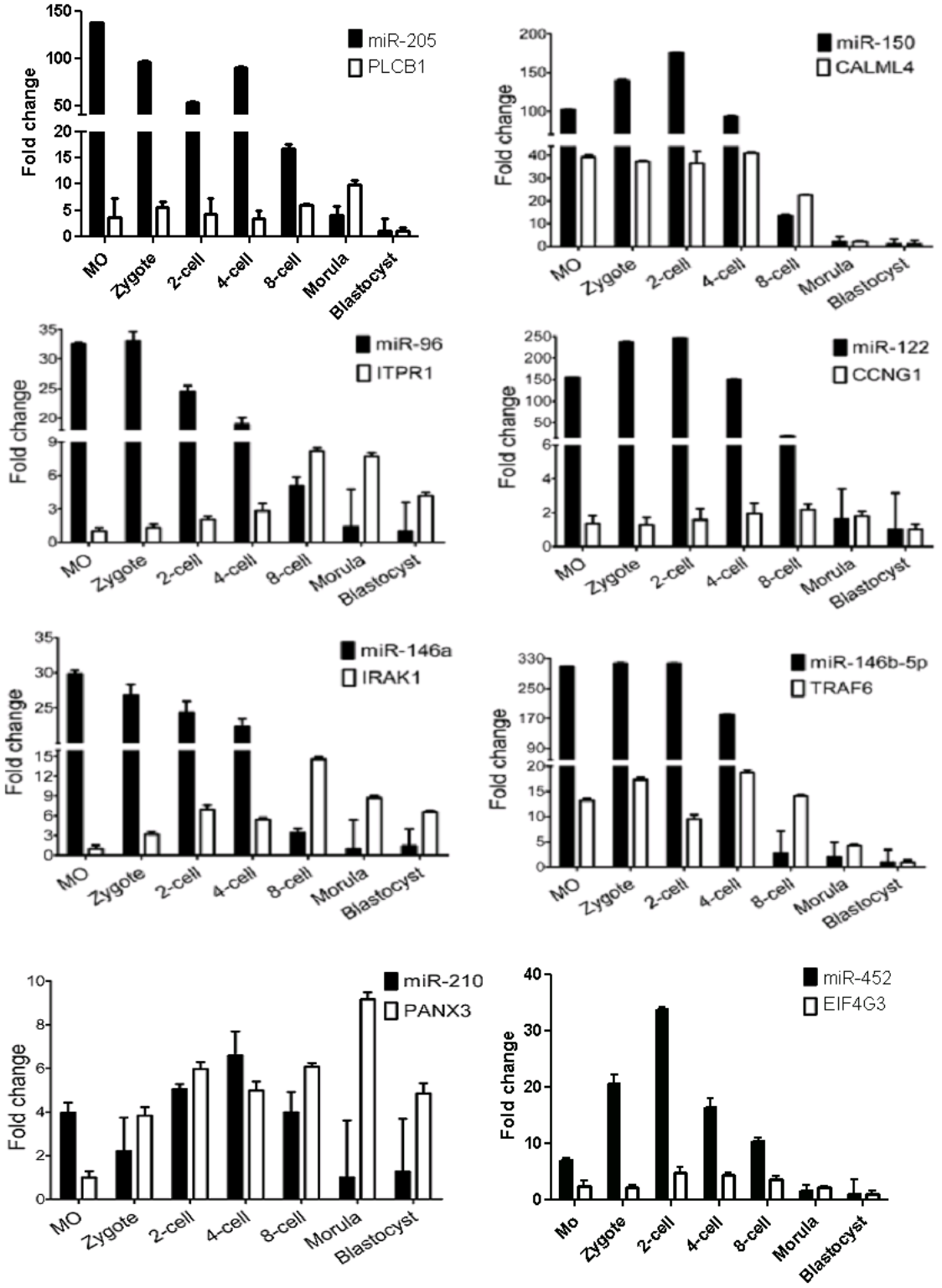


Figure 4.5: Relative abundance of miRNAs enriched in oocyte (miR-205, 150, 96, 122, 146a and 146b-5p), miRNAs enriched in cumulus cells (miR-210 and miR-452) and their target genes in bovine matured oocyte (mo) and preimplantation embryo stages

4.7 Expression of selected miRNAs in cumulus oocyte complexes of varying competence

The brilliant cresyl blue (BCB) staining was used to differentiate fully grown (BCB+) and growing (BCB-) oocytes based on the level of G6PDH activity. All studied miRNAs are found to be abundant at higher level ($p \leq 0.05$) in fully grown (BCB+) oocytes compared to their growing (BCB-) counterparts (Figure 4.6A). Specially, miR-150, miR-122 and miR-146b-5p have shown a remarkable difference in their relative abundance between BCB+ and BCB- oocytes. However, except miR-96, miR-122, miR-146a and miR-452 none of the selected miRNAs showed significant difference in their expression between cumulus cells derived from BCB+ and BCB- COCs (Figure 4.6B).

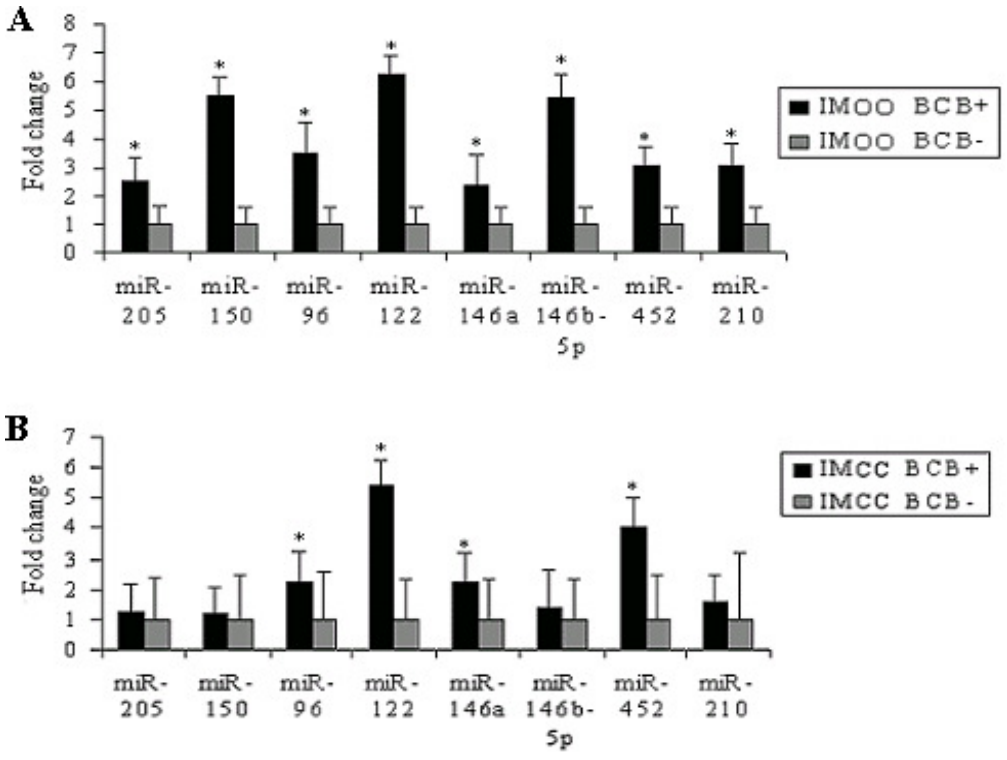


Figure 4.6: The relative abundance of selected miRNAs in oocytes (A) and cumulus cells (B) derived from bovine COCs screened by brilliant cresyl blue staining as growing (BCB-) and fully grown (BCB+) oocytes. The fold change in transcript abundance was calculated taking the oocytes and cumulus cells from the BCB- group as calibrator and their expression level set as one following normalization by endogenous

control U6 RNA. Significant differences ($p \leq 0.05$) between oocytes (BCB+ and BCB-) and cumulus cells (from BCB+ and BCB- COCs) in the relative abundance of specific miRNAs are indicated by star (*)

4.8 Effect of oocyte and cumulus cell factors for the expression of miRNAs

In order to elucidate whether the oocyte (as source of oocyte-secreted factors) is required for the expression of miRNAs in the surrounding cumulus cells and the vice versa, comparative analysis of oocytes matured with or without cumulus cells and cumulus cells matured with or without the oocyte cytoplasm were performed using the eight miRNAs. As shown in figure 4.7, the relative abundance of the majority of miRNAs was found to be higher ($p \leq 0.05$) in oocytes matured without the surrounding cumulus cells except for miR-96. On the other hand, a 16-fold increase in the relative abundance of miR-210 was observed in cumulus cells matured without the oocyte cytoplasm compared to those matured in the presence of oocyte cytoplasm. However, no significant differences were found in the relative abundance of miR-452 in these experimental groups.

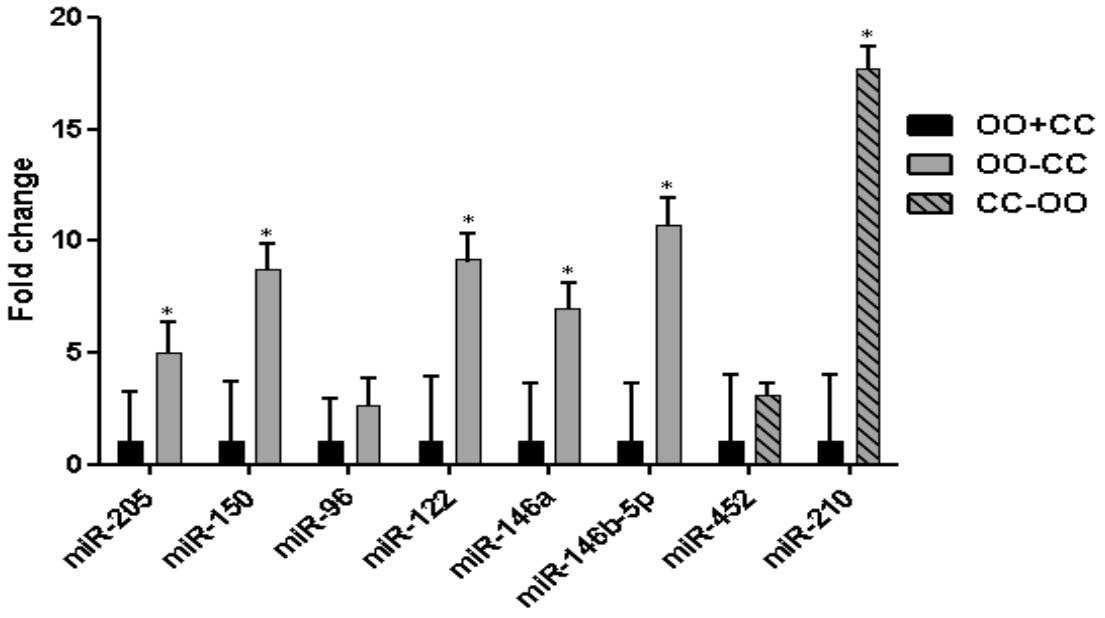


Figure 4.7: Relative expression level of miRNAs in oocytes matured with (OO+CC) or without (OO-CC) the surrounding cumulus cells and in cumulus cells matured with (CC+OO) or without (CC-OO) the oocyte cytoplasm. Samples with the highest

normalized value were used as a calibrator. Statistical significance ($p \leq 0.05$) between oocytes or cumulus cells is indicated by star (*)

4.9 Spatio-temporal distribution of miR-205 and miR-210 in ovarian sections, cumulus oocyte complexes and early preimplantation embryonic stages

In situ hybridization was performed to examine the cell type distribution of miR-205 and miR-210 enriched in oocyte and cumulus cells, respectively in section of bovine follicles (primary, secondary, early antral and tertiary follicles). A strong fluorescence signal of miR-205 was detected in the oocyte cytoplasm compared to the surrounding cumulus cells and other ovarian cells during the later stages of follicular development namely: early antral and tertiary follicles (Figure 4.8.1B, 1A). However, the expression level of miR-205 was not detectable in the oocyte cytoplasm at primary and secondary follicles (Figure 4.8.1C, 1D). On the other hand, miR-210 was found to be highly abundant in cumulus and granulosa cells compared to the oocyte cytoplasm in all stages of follicular development. Differences in miR-210 signal intensity between oocyte and surrounding cells was clear at the later stages of follicular development (early antral and tertiary follicles). Moreover, miR-210 was found to be localized in a single layer of cumulus cells of primary follicles (Figure 4.8.2D) and in multiple layers of cumulus cells in secondary, early antral and tertiary follicles (Figure 4.8.2C, B, A). While the oocyte cytoplasm in the primary follicle showed no miR-210 expression (Figure 4.8.2D), slight signals were detected in oocyte cytoplasm of the secondary, early antral and tertiary follicle stages (Figure 4.8.2C, B, A). The nuclear U6 RNA which was used as positive control in this study was detected in all follicular sections being localized in the nucleus (Figure 4.8.3A, B).

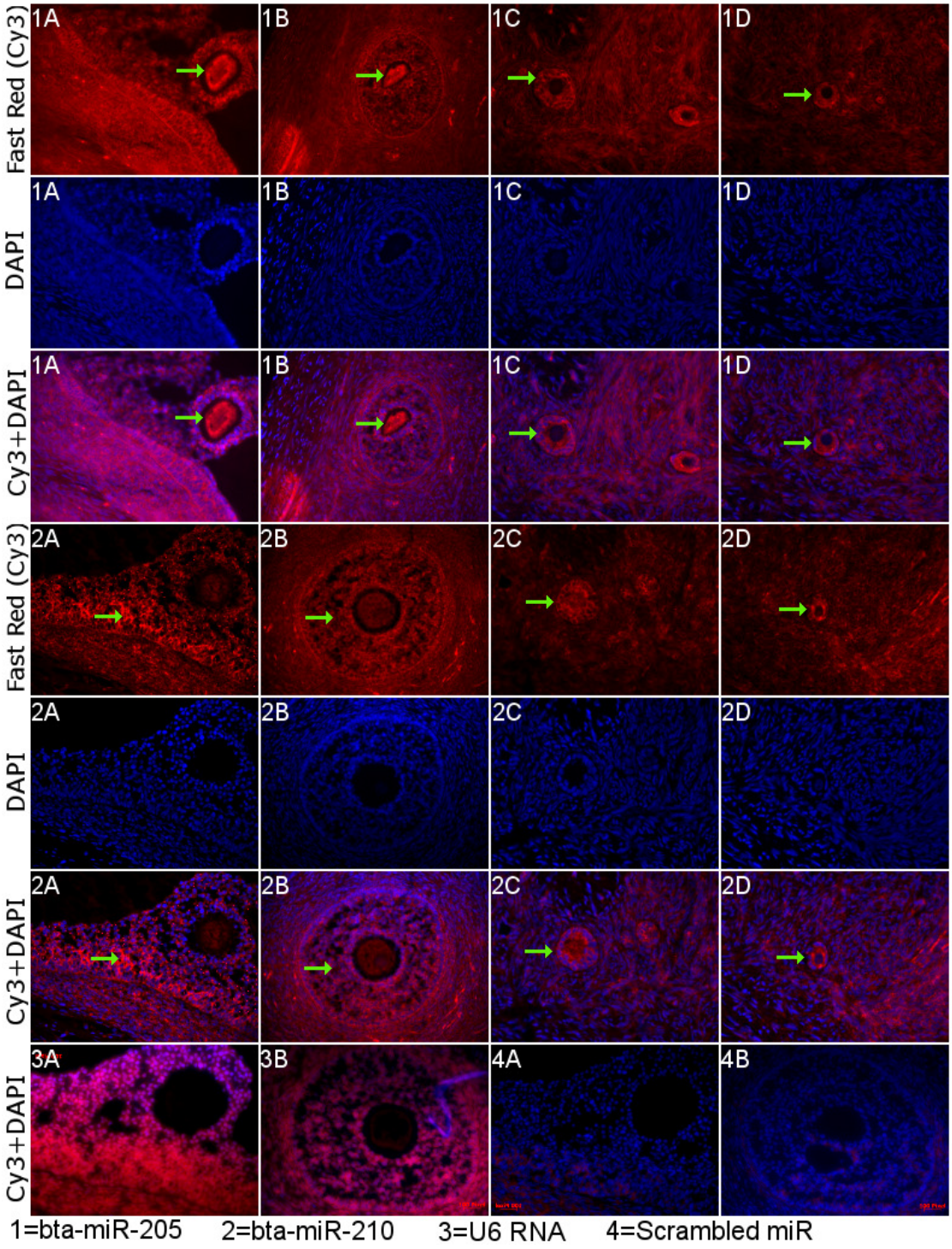


Figure 4.8: In situ detection of miR-205 and 210 in bovine follicular cryo-sections. Ovarian follicular cryo-sections were in situ hybridized with 3'-digoxigenin labeled locked nucleic acid (LNA) microRNA probes of miR-205 (1), miR-210 (2), U6 RNA (3) and scrambled miRNA (4). Cells with higher expression are marked by arrows. A, B, C and D represent tertiary, early antral, secondary and primary follicle, respectively

Cumulus oocyte complexes and *in vitro* produced bovine post fertilization developmental stages (zygote, 2-cell, 4-cell, 8-cell and blastocyst) were used to investigate the cellular distribution of these miRNAs in early stages of embryo development. Both 2- and 3-dimensional presentations showed that miR-205 was detected in oocytes and all stages of development with a reduced signal between 8-cell and blastocyst stages compared to the signal detected at oocyte, zygote, 2-cell and 4-cell stages (Figure 4.9). However, miR-210 is detected in cumulus cells of the COC and at all stages of development but without noticeable difference in signal intensity between the developmental stages.

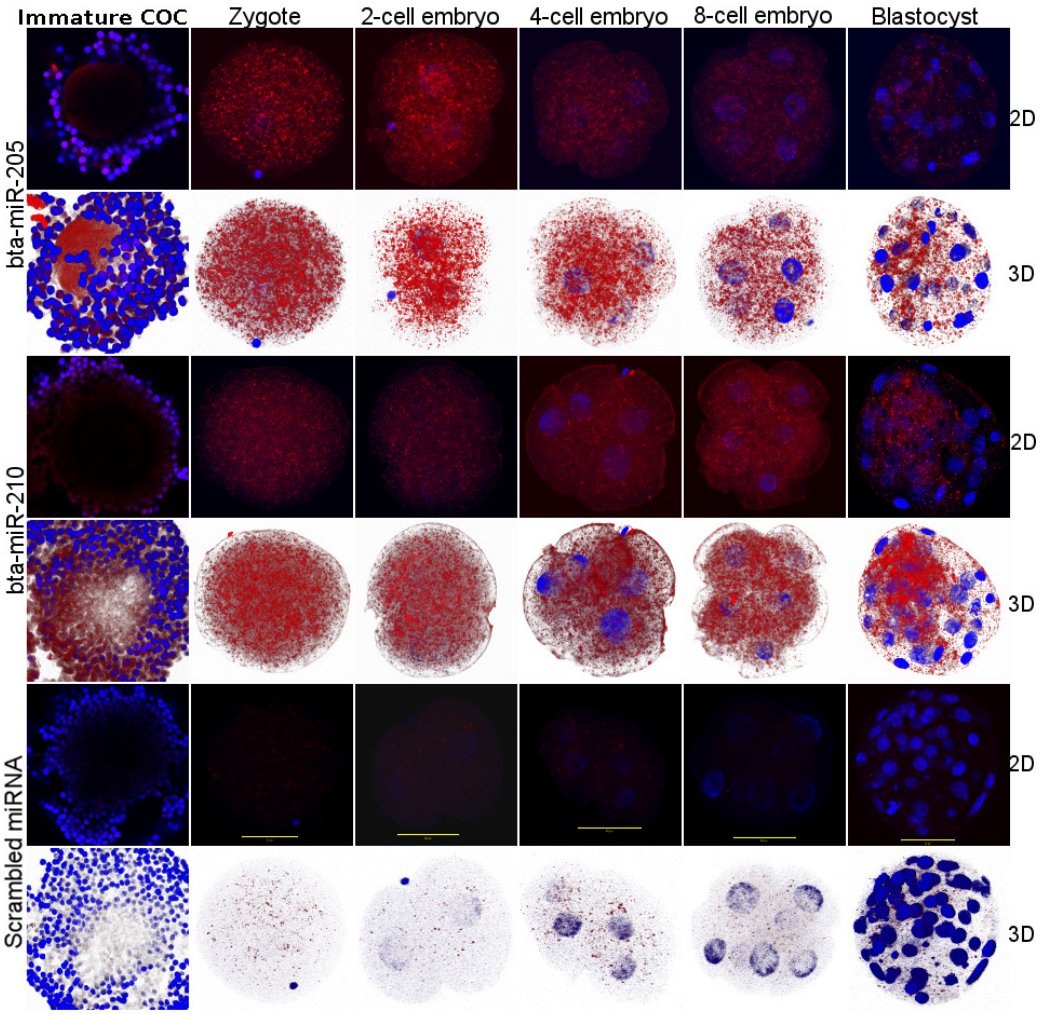


Figure 4.9: In situ detection of miR-205 and 210 in whole mount cumulus oocyte complexes and preimplantation embryo stages. Whole mount COCs and bovine preimplantation embryo stages were *in situ* hybridized with 3'-digoxigenin labeled

locked nucleic acid (LNA) microRNA probes of miR-205, miR-210 and scrambled miRNA. Hybridization signal and nucleus of the COCs and embryos were visualized by Fast Red (Cy-3) and DAPI, respectively. Images from at least 20 different layers or positions (Z positions) of single embryo were used to construct 3 dimensional (3D) image, where and 2 dimensional (2D) images represented here from only one position. Scale bar denotes 50 µm

5 Discussion

5.1 Identification of miRNAs expressed in oocyte and their surrounding cumulus cells before and after *in vitro* maturation

Mammalian oocytes, cumulus cells and other follicular cells are known to have distinct transcript profile and efforts are being made to correlate this profile or the expression of specific marker genes with oocyte developmental competence (Hamel et al. 2008; Ouandaogo et al. 2011; Robert et al. 2001). Recent study showed large scale of gene expression in oocyte and their companion CCs at germinal vesicle and metaphase II stages (Regassa et al. 2011). Furthermore, a distinct set of proteins were found by comparative analysis of bovine germinal vesicle oocytes and their surrounding cumulus cells (Memili et al. 2007; Peddinti et al. 2010). However, the identities and the role of post-transcriptional regulator miRNAs in bovine cumulus oocyte complex are poorly understood. MicroRNAs are reported to regulate the activity of about 30% of the protien coding genes in human (Lewis et al. 2005). As most microRNA sequences are conserved across related animal species (Griffiths-Jones et al. 2006; Niwa and Slack 2007) and due to the similarity in oocyte biology and many aspects of ovarian follicular dynamics between human and bovine species (Biggers et al. 1967; Buccione et al. 1990). In the present study we have used a human cell development and differentiation miRNA PCR array to compare the miRNA expression profile of oocytes and cumulus cells at GV and MII stages and distinct set of miRNAs were identified being enriched either in oocytes or cumulus cells, and from this set eight miRNAs were selected for future characterization. The result showed that oocytes at both GV and MII stages were enriched with large number of miRNAs under investigation compared to their surrounding cumulus cells. On the contrary recent studies showed higher level of protien expression in cumulus cell compared to GV oocyte (Memili et al. 2007; Peddinti et al. 2010). Study of the dynamic nature miRNAs expression in oocytes or cumulus cells before and after maturation revealed that miRNAs accumulation increases with maturation in vitro. This is in agreement with the dynamic pattern of miRNA expression in mouse oocytes during oogenesis, where many miRNAs have been detected in the matured dicer^{+} oocyte (Tang et al. 2007).

MicroRNAs are known to be expressed in developmental time and tissue specific manner (Plasterk 2006). The data in the present study and also by Tesfaye et al. (2009) showed that bovine oocyte maturation involves a wave of expression of miRNAs in oocytes and cumulus cells with distinct cell specific. The overall dynamics of miRNA expression during bovine oocyte maturation found in this study is different from the global mRNA transcript pattern between GV and MII in bovine and human oocytes reported by other studies (Assou et al. 2006; Fair et al. 2007; Mamo et al. 2011). This may suggest the potential role of those miRNA in regulation of transcripts and protein storage turnover through acceleration of deadenylation and decay of maternal mRNA (Giraldez et al. 2006).

5.2 Expression profile of selected miRNAs and their targets in preimplantation embryo and localization of two selected miRNAs

The embryonic genome has almost no impact on the earliest steps of the developmental program and the early mammalian embryo development is dependent on the maternal stores of mRNA and protein before the activation of embryonic genome. The transition from maternal-to-zygotic regulation is the first major change, which occurs after fertilization and comprises a dramatic reprogramming of gene expression that is essential for the development of most organisms. As mRNA, microRNAs play essential roles in gene regulation during early development (Castro et al. 2010; Choi et al. 2007; Giraldez et al. 2006; Martello et al. 2007). The miRNAs suppress translation in a sequence-specific manner and facilitate shifts in gene expression during developmental transitions, involvement of regulatory maternal miRNAs in maternal-zygotic transition was evidenced in mouse (Murchison et al. 2007; Tang et al. 2007), zebrafish (Giraldez et al. 2006), xenopus laevis and drosophila (Iovino et al. 2009; Lund et al. 2009). In the present study, the expression profiling of selected miRNAs in early stages of bovine preimplantation embryo showed various pattern of expression. Especially miRNAs enriched in oocytes showed a typical maternal expression profile in which expression was higher at earlier stages of development and drastically reduced between 8-cells and blastocyst stages (Figure 4.5). This is an additional evidence for dependence of early bovine embryo development on maternally inherited miRNAs, which are known to regulate the degradation of maternal mRNA or protein as observed in mouse and bovine

embryos (Lykke-Andersen et al. 2008; Tripurani et al. 2011), until the major activation bovine embryonic genome at 8-16 cell stage (Barnes and First 1991; Memili and First 1999; Telford et al. 1990). In addition, the same expression trend has been revealed in recent study where miR-424 and miR-10b are higher in GV and MII oocyte stage and early embryo until maternal-zygotic transition, but tend to decreased at morula and blastocyte stage (Tripurani et al. 2010a). Similarly, miR-196a in bovine which increased near eight cell stage and declines at morula and blastocyte stages of embryogenesis (Tripurani et al. 2011).

The predicted targets of selected miRNAs are reported to be involved in various biological process and molecular pathway which are vital for oocyte maturation. The GnRH signaling and G-protien coupled receptor signaling are dominant pathways for oocyte enriched miRNA targets (Figure 4.3A). The GnRH signaling controls biosynthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that in turn regulate development and activity of the ovaries (Jeong and Kaiser 2006). Gonadotropin-releasing hormone acts via G-protein coupled receptors (Gq/G11) lead to activation of phospholipase C (PLC, apparently PLC β1) which hydrolyzed phosphatidylinositol 1,4,5 bisphosphat (PIP2) to inostiol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). IP3 bind to receptors in the endoplasmic reticulum to release Ca^{2+} transiently from these intracellular stores and this elicits a rapid spike of LH release. Also, DAG activates protien kinase Cs (PKCs) that phosphorylate protien involved in the more sustained release of LH and gonadotrophin biogenesis (Kraus et al. 2001; McArdle et al. 2002; Millar 2006).

Comparative analysis of differentially expressed miRNAs and their predicted target genes during preimplantation embryo development revealed a reciprocal expression pattern for miR-205 and PLCB1 transcript, miR-96 and ITPR1 transcript, miR-146a and IRAK1 transcripts (Figure 4.5). This may show the possible mRNA degrading mode-of-action of the miRNAs in the post transcriptional regulation of the corresponding target genes. The PLCB1 gene is important element of the phosphoinostide pathway this pathway act principally by generating the release calcium. The nuclear phosphoinositide cycle in mouse oocytes seems to be essentially regulated by the PLCB1 isoform, microinjected into the GV, the anti-PLCB1 induced an inhibition of both nuclear and

cytoplasmic Ca^{2+} oscillations and meiosis resumption (Avazeri et al. 2000; Avazeri and Lefevre 1998). The functional role of ITPR1 (Target of miR-96) in operating Ca^{2+} release in oocytes at fertilization has been demonstrated in the golden hamster, mouse and frogs (Miyazaki et al. 1992; Runft et al. 1999; Xu et al. 1994). In human oocytes, the type I $InsP_3R$ are prominently expressed in oocytes, zygotes and embryos and are dynamically redistributed through maturation, fertilization and early embryogenesis (Goud et al. 1999). In addition, the expression of CCNG1 gene which may play role as a positive modulator of cell cycle in ovarian follicular development and oocyte maturation (Liu et al. 2006) remained constant throughout the developmental stages irrespective of the expression profile of its targeting miR-122. Similarly, miRNAs enriched in cumulus cells (miR-452 and miR-210) and their corresponding predicted target mRNA transcripts showed similar expression pattern in which all are detected in a relatively lower level in all stages of development without any defined profile.

The data of the present study in the relationship between the expression profile of selected miRNAs and their targets showed various scenario, this different relationship in expression profile of miRNA and target mRNA reflects the existing challenge in target identification because of varying outcome of binding of miRNAs to their target genes which might not result in alternation of target gene expression level (Brennecke et al. 2005; Grimson et al. 2007). Rather, the interaction may be induced translational repression which appears to be cap and poly A-tail dependent during initiation step (Humphreys et al. 2005; Pillai et al. 2005). Localization of miR-205 and miR-210 in early stages of bovine preimplantation embryos has validated the expression profile obtained by quantitative real time PCR and suggests the potential involvement of these miRNAs in early embryogenesis.

5.3 Expression analysis of selected miRNAs in growing and fully grown oocytes

The quality of oocytes or developmental competence plays an important role in a proper embryo development, it is acquired during folliculogenesis as the oocyte grows and during the period of oocyte maturation (Mermillod et al. 1999). Several morphological, cellular and molecular criteria have been proposed to evaluate oocyte quality or competence (Wang and Sun 2007). Morphological evaluation is usually applied to the

whole COC, whereas compact, continuous multilayer cumulus investment and bright, homogeneous ooplasm are considered as a sign of immature oocyte quality (Blondin and Sirard 1995). Molecular markers of oocyte quality could also be identified in cumulus cells or in follicular fluid. For instance, the rate of apoptosis in bovine cumulus cells before (Feng et al. 2007; Zeuner et al. 2003) or during *in vito* maturation (Ikeda et al. 2003) and several potential cumulus cell genes (HAS2, TNFAIP6,PTGS2 , GREM1, INHBA, BTC, CD44 and EGFR) identified as marker for human and bovine oocyte competence (Assidi et al. 2008; McKenzie et al. 2004; Caixeta et al., 2009). Brilliant cresyl blue (BCB) staining has been proposed as an easy and vital way to evaluate glucose 6-phosphate dehydrogenase (G6PDH) activity in oocytes (Alm et al. 2005), where, there is negative correlation between G6PDH activity level and oocyte quality. Moreover, BCB staining before IVM did not affect IVP and this making BCB staining method an appropriate way to evaluate oocyte quality. The gene expression profiles of fully growing (competent) oocytes (BCB+) were markedly different from those of growing (non competent) ones (BCB−) and this associated the characteristic process in each one where in BCB+ oocytes, the majority of expressed genes are associated with regulation of the cell cycle, transcription and translation, while BCB− oocytes encoded genes controlling ATP synthesis mitochondrial electron transport and calcium ion binding (Torner et al. 2008). A range of miRNA regulated proteins were identified during oocyte maturation or growth in Drosophila (Nakahara et al., 2005) and mouse (Tang et al. 2007). Similarly, we have confirmed the differential expression of miRNAs during oocyte growth by investigating the expression of selected miRNAs in growing (BCB-) and fully grown (BCB+) oocytes. Almost all selected miRNAs were found to increase in their abundance in fully grown oocytes compared to their growing counterparts. During oocyte growth, the synthesis of RNA is increased and reaches a peak at the beginning of follicular antrum formation (Moor et al. 1998; Wassarman and Kinloch 1992). So this may be explain the trend of miRNA in growing and fully growing oocyte where at growing stage the oocyte synthesis and accumulate mRNA and protein for future using during development. This trend was further validated by the increasing intensity of miR-205 signal in oocyte cytoplasm during their growth from primary follicle to tertiary follicle (Figure 4.8). However, except miR-96, miR-122, miR-146a and miR-452 the expression of selected miRNAs in cumulus cells derived from BCB+ and BCB- COCs showed no significant difference.

5.4 Relative expression level of selected miRNAs in presence or absence of oocyte or cumulus cell after maturation

While paracrine growth factors secreted by oocytes are involved in expansion of cumulus cells number and function, regulation of follicular cell function and pre- and post-ovulatory events (Gilchrist et al. 2001), the cumulus cell support oocyte metabolic processes such as glycolysis and amino acid uptake and regulate meiotic progression (Buccione et al. 1990; Tanghe et al. 2002). So far limited number of oocyte secreted factors (OSFs), are known to regulate cumulus and granulosa cell function. These include GDF9 and BMP15 which have a profound effect on the expression of genes in cumulus cells and enhance oocyte developmental competence (Hussein et al. 2006). Moreover, the presence or absence of oocyte and cumulus cells factors during bovine oocyte maturation led to transcriptome profile changes in both cell types (Regassa et al. 2011). Similarly, in the present study the presence of cumulus cells during oocyte maturation and vice versa was found to be important in the regulation of miRNAs expression in either of the two cell types, except for cumulus enriched miRNAs, all oocyte enriched miRNAs were found to be up-regulated in the absence of the surrounding cumulus cells during maturation. According to the fact that removal of cumulus cells at the beginning of in vitro oocyte maturation or shortly before fertilization resulted in detrimental effect on oocyte maturation, fertilization and subsequent development (Fatehi et al. 2002; Hussein et al. 2006; Zhang et al. 1995) and based on our results, we suggest that these miRNAs may involve in the regulation of genes associated with impaired nuclear maturation of oocytes due to absence of cumulus cell factors. On the other hand, Hussein et al. (2005) showed that removal of oocyte from cumulus oocyte complex led to increased the cumulus cell apoptosis compared with COC and the level of apoptosis in cumulus cells without oocyte was reversed when co-culture with oocyte. Interestingly in the present study, the absence of oocyte factors due to removal of oocyte from COC led to increase the expression of miR-210 in cumulus cells compared to those matured with oocyte cytoplasm. This is in agreement with the inhibitor effect of some miRNAs to the apoptosis, and miR-210 is one of these miRNA which decrease production of pro-apoptotic caspases (Wang and Lee 2009: Sirotkin et al. 2010). The summarized hypothesis in figure 5.1 on dependency of the two cell types to each other for miRNA expression needs further

investigation in order to identify the specific oocyte or cumulus cells factors and the mode of transcriptional regulation of the miRNAs.

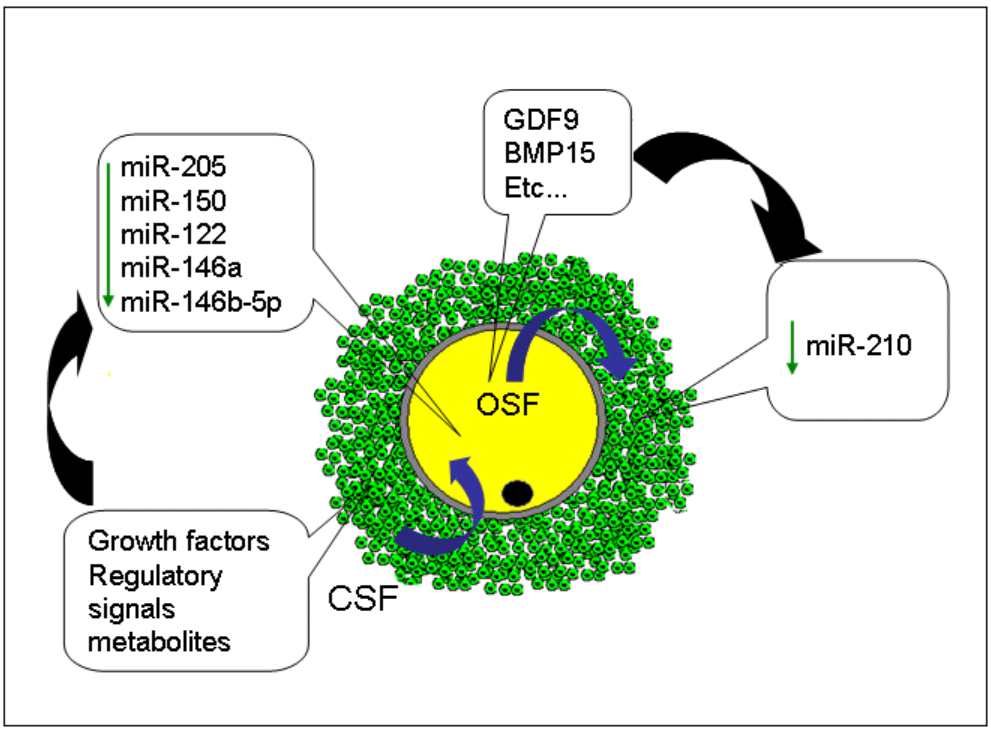


Figure 5.1: Hypothetical illustration depicting the dependency of bovine oocyte and cumulus cells in the relative abundance of miRNAs transcribed from their genome on the presence or absence of cumulus cell factor (CSF) and oocyte secreting factor (OSF), respectively. Green arrows indicate reduced level of miRNA expression. The inward and outward blue arrows indicate the effect of CSF and OSF on oocyte and cumulus cell development, respectively

5.5 Localization of miR-205 and miR-210 in ovarian sections

Mammalian folliculogenesis is a complex process through which primordial follicles develop into preovulatory follicles. During this time, oocyte increase in size accompanied by the proliferation and differentiation of their surrounding granulosa and theca cells. For this, spatio-temporal expressions of hundreds of genes have been shown to be essential (Matzuk et al. 2002; Roy and Matzuk 2006). The role of miRNAs have been evidenced in the ovaries through impaired function of corpus luteum and infertility in Dicer knockout mice (Otsuka et al. 2008). As observed by the genes, the results in the

present study have evidenced the spatio-temporal expression of miRNAs during bovine follicular development. Differences in the signal intensity in localization of miR-205 and miR-210 were found between oocytes and surrounding cumulus cells as early as the primary follicles stage and persist until the matured follicle. So, the miRNA may have importance in regulating genes involved in follicular development. In agreement with other study showed that blocking of miRNA as (miR-21) through ovarian bursal injection with a blocking LNA-21, led to decrease in the ovulation rate (Carletti et al. 2010). Similarly, the effect targeted deletion of Dicer (the miRNA processing key proteins) on ovulation rate (Hong et al. 2008; Nagaraja et al. 2008). As miR-210, which its signal intensity was higher in cumulus and granulosa cells in all stages of follicular development. Recent study revealed that the levels of miR-210 decreased in 64% of ovarian carcinomas and the authors suggested that this miRNA have tumor suppressive function (Dahiya and Morin 2010). However, the exact role of these non coding RNAs during bovine follicular development needs to be the focus of future research.

In conclusion, to the best of our knowledge, here we reported for the first time the classes of miRNA enriched in bovine oocytes or the surrounding cumulus cells during oocyte maturation which is a step forward in identifying miRNAs required for oocyte-cumulus communication. Moreover, we have also evidenced the dynamic degradation of miRNAs during oocyte maturation and interdependency of oocyte and cumulus cell on the activity of the miRNAs. Furthermore, the potential role of maternal miRNAs in early bovine embryo development could be depicted from their expression dynamics during preimplantation stage period. However, further functional studies on those differentially expressed miRNAs is required to understand the key role of miRNAs during oocyte maturation and their subsequent role during early embryonic development.

6. Summary

MicroRNAs are post transcriptional regulator that influence expression of hundreds of genes in numerous biological processes including development. The present study was preformed to identify the presence and expression of miRNAs in oocyte and their surrounding cumulus cells during maturation where the bidirectional communication between both cell types is important during oocyte maturation for the acquisition of developmental competence and subsequent embryonic and fetal development. For this, a total of three replicate pools each containing 1600 oocytes and the corresponding cumulus cells from both immature and matured COCs were used for total RNA isolation using miRNeasy® Mini kit. The isolated total RNA was fractionated into small and large RNA using RT^2 qPCR-Grade miRNA isolation kit. From each oocyte and cumulus cell groups, 50 ng of small RNA was used for reverse transcription using RT^2 miRNA first strand kit. The resulting cDNA from small RNA was used as template to profile 88 human miRNAs related to cell development and differentiation using RT profiler PCR array. The data was analyzed using comparative threshold cycle (Ct) method after normalization with endogenous control RNAs (SNORD44, SNORD47, SNORD48 and U6). The result of PCR array revealed that a total of 47 and 51 miRNAs to be highly abundant in immature and matured oocyte, respectively compared to their corresponding cumulus cells, while 8 and 6 miRNAs were enriched in cumulus cells compared to immature and matured oocytes, respectively. Based on expression intensity, six oocyte enriched miRNAs (miR-205, miR-150, miR-96, miR-122, miR-146a and miR-146b-5p) and two cumulus cell enriched miRNAs (miR-452 and miR-210) were selected for further characterization. These selected miRNAs were quantified during preimplantation embryo developmental stages (Zygote, 2-cell, 4-cell, 8-cell, morula and blastocyste) using quantitative real time PCR. The result revealed that all oocyte specific miRNAs were found to be highly abundant in early stages of embryo development and decreased after 8-cell until the blastocyst stage following a typical maternal transcript profile. Similar expression pattern has been observed during in situ localization of one of selected miRNA (miR-205) using 3'-Digoxigenin (DIG) labeled, LNA-modified oligonucleotide ISH probes in preimplantation embryo stages, in which signals were higher until 4-cell stage and reduced between 8-cell and blastocyst. However, the expression of miR-210 and miR-452 showed no defined profile in

preimplantation embryo stages. To identify the predicted targets of eight selected miRNAs, we used *in silico* analysis based on four target prediction softwares and we found that 854 and 234 genes are predicted to be targets of selected miRNAs enriched in oocyte and cumulus cells, respectively. Furthermore, from these targets one gene for each miRNA was selected to further analysis in preimplantation embryo stages. Some targets as (PLCB1, ITPR1 and IRAK1) targets of (miR-205, miR-96 and miR-146a), respectively have been showed an opposite expression profile compared to the profile observed by their targeting miRNAs, while other target genes such as CALML4 and TRAF6 transcripts showed a similar expression profile as their targeting miRNAs.

The expression profile of the selected eight miRNAs was investigated in oocyte and cumulus cells from cumulus oocyte complexes of varying competence separated based on brilliant cresyl blue (BCB) staining. Results showed that all studied miRNAs are found to be highly abundant ($p \leq 0.05$) in fully grown (competent) oocytes (BCB+) compared to their growing (BCB-) (non competent) counterparts. Moreover, to reveal the effect of oocyte or cumulus cells factors on the expression of miRNA in both cell types during maturation, we used three different groups (cumulus oocyte complex matured as intact (COC), oocyte matured without cumulus cells (DO), and cumulus cells matured without oocyte (OOX)). The result of quantification showed that miR-205, miR-150, miR-122, miR-146a, miR-146b-5p and miR-452 were found to be abundant at higher level ($p \leq 0.05$) in oocyte matured without cumulus cells (DO) compared to those matured in the presence of cumulus cells (COC). On the other hand, the expression of miR-205, miR-150 and miR-122 in cumulus cell was higher in presence of oocyte cytoplasm during maturation, while 16 fold increases in relative abundance of miR-210 was observed in oocyteoctimized cumulus cells (OOX). Finally, in situ localization of miR-205 and miR-210 in ovarian follicle revealed a spatio-temporal expression during follicular development and support the notion that miRNAs may have role during folliculogenesis. Collectively, these results demonstrated that bovine oocyte and cumulus cells have distinct set of miRNA, which may have role in the physiology of the two cell types and their communication to each other. However, the exact role of these miRNAs in oocyte maturation in particular and early embryonic development in general needs further investigation.

7. Zusammenfassung

MicroRNAs sind post transkriptionelle Regulatoren, die die Expression von hunderten Genen beeinflussen, und an einer Vielzahl von biologischen Prozessen einschließlich der Entwicklung beteiligt sind. Die vorliegende Studie wurde durchgeführt, um die Präsenz sowie die Expression von miRNAs in Eizellen und ihren umliegenden Kumuluszellen während der Maturation zu untersuchen. Die bi-direktionelle Kommunikation zwischen den beiden Zelltypen ist endscheidend während der Eizellenreife für den Erwerb der Eentwicklungskompetenz und anschließenden embryonalen und fetalen Entwicklung. Dazu wurden insgesamt drei replizierte Pools von jeweils 1600 Eizellen und den ent-sprechenden Kumuluszellen separat von unreifen und reifen Kumuluseizellen-komplexen (COCs) verwendet, um miRNA mittels des miRNeasy® mini Kit zu isolieren. Die isolierte totale RNA wurde mit Hilfe des RT² qPCR-Grade miRNA Isolationskits in kleine und große RNA unterteilt. Von jeder Gruppe von Eizellen und Kumuluszellen wurden 50ng small-RNA verwendet, um eine reverse Transkription mit dem RT² miRNA first strand Kit durchzuführen. Die daraus resultierenden cDNA von small-RNA wurde verwendet, um mit dem SYBER-green basierender real time PCR System Expressionsprofile von 88 humanen miRNAs zu erfassen. Diese humanen miRNAs sind relevant für die Zellentwicklung und -differenzierung. Die Auswertung der Daten erfolgte, nach Normalisierung mit endogenen Kontroll-RNAs (SNORD44, SNORD47, SNORD48 und U6), auf Grundlage der komparativen Ct Methode. Das Ergebnis des PCR Arrays zeigte daß insgesamt 47 bzw. 51 miRNAs unterschiedlich zwischen immaturen bzw. reifen Eizellen und ihren umgebenden Kumuluszellen exprimiert waren, während acht bzw. sechs miRNAs in Kumuluszellen von unreifen bzw. unreifen Eizellen angereichert waren. Basierend auf den Expressionsintensitäten von sechs in Eizellen (miR-205, -150, -122, -96, -146a und 146b-5p) und zwei in Kumuluszellen angereicherten miRNAs (miR-452 und miR-210), wurden diese für eine weitere Charakterisierung in Präimplantationsstadien von Embryonen ausgewählt.

Die ausgewählten miRNAs wurden während den Präimplantionsentwicklunsstadien der Embryonen (Zygote, 2-Zell-, 4-Zell-, 8-Zell-Stadien, Morula und Blastozyste) mit quantitativer real time PCR quantifiziert. Die Ergebnisse zeigten, dass alle eizellen-

spezifischen-miRNAs in den frühen Stadien der Embryonalentwicklung deutlich erhöht exprimiert waren und nach dem 8-Zell-Stadium im expressionsuniveau sanken. Bis hin zum Blastozystenstadium folgten die embryonen den typischen maternalen Expressionsmustern. Vergleichbare Ergebnisse wurden bei der *in situ* Lokalisierung von miR-205 mit Hilfe von 3'Digoxigenin (DIG) gelabelten, LNA-modifizierten Oligonukleotide ISH Probes in Embryonen des Präimplantationsstadiums erzielt, bei denen das Signal bis zum 4-Zellstadium höher war und sich zwischen dem 8-Zell- und Blastozystenstadium reduzierte. Leider zeigten die ausgewählten miRNAs miR-210 und miR-452 kein definiertes Profil im Präimplantationsstadium von Embryonen.

Um mögliche Zielgene dieser acht ausgewählten miRNAs zu identifizieren, wurden *in silico* Analysen mittels vier verschiedener Online-Platformen zur „Target-Prediction" durchgeführt. So konnten 854 bzw. 234 Gene für die ausgewählten miRNAs vorhergesagt werden, die in Eizellen und Kumuluszellen exprimiert waren. Basierend auf diesen Analysen wurden Expressionsprofile von möglichen Zielgenen der miRNAs ermittelt. Diese zeigten, dass einige Zielgene (PLCB1, ITPR1 und IRAK1) ein reverses Expressionsprofil im Vergleich zu den beobachteten Profilen ihrer miRNAs (miR-205, miR-96 und miR-146a) aufwiesen, während andere Zielgene wie die Transkripte von CALML4 und TRAF6 ein vergleichbares Expressionsprofil wie ihre Ziel-miRNAs hatten.

Die Genexpressionsanalyse von acht ausgewählten miRNAs wurde in noch wachsenden wie auch in ausgewachsenen Eizellen, die mittels Brillantkresylblau (BCB)-Färbung separiert wurden, sowie ihren umgebenden Kumuluszellen durchgeführt. Diese Untersuchung zeigte, dass alle ausgewählten miRNAs signifikant verschieden exprimiert waren ($p \leq 0.05$), beim Vergleich von ausgewachsenen (kompetenten) Eizellen (BCB+) mit entsprechenden noch wachsenden (nicht kompetenten) Eizellen (BCB-). Um ferner den Effekt der Eizelle oder der Kumuluszelle an sich auf die Expression von miRNA in beiden Zelltypen während der Maturation aufzuzeigen, wurden drei verschiedene Gruppen (intakter Kumuluseizellenkomplex nach Reifung (COC), reife Eizellen ohne umliegenden Kumuluszellen (DO) und gereifte Kumuluszellen ohne Eizelle (OOX)) für weitere Untersuchungen verwendet. Die Ergebnisse der Quantifizierung zeigten, dass die miRNAs miR-205, miR-150, miR-122,

miR-146a, miR-146b-5p und miR-452 in gereiften Eizellen ohne Kumuluszellen (DO) stark angereichert ($p \leq 0.05$) waren verglichen mit gereiften Eizellen mit umliegenden Kumuluszellen (COC). Andererseits waren die Expressionen von miR-205, miR-150 und miR-122 in Kumuluszellen höher in der Gegenwart von Eizellenzytoplasma, während eine 16-fache relative Anreicherung von miR-210 in Eizellen mit oocytesektomies Kumuluszellen (OOX) beobachtet werden konnte. Schließlich zeigte die *in situ* Lokalisierung von miR-205 und miR-210 eine raum-zeitliche Expression während der Follikelentwicklung und unterstützt die Annahme, dass miRNAs eine Rolle bei der Follikulogenese spielen. Insgesamt verdeutlichen diese Ergebnisse, dass bovine Eizellen und Kumuluszellen über eine ausgeprägte Gruppe an miRNA verfügen, die möglicherweise eine Rolle in der Physiologie der beiden Zelltypen sowie ihrer Kommunikation miteinander spielt. Jedoch sind weitere Untersuchungen notwendig, um die Bedeutung der miRNAs insbesondere in reifen Eizellen und in der frühen embryonalen Entwicklung tiefer zu klären.

8 References

Aboobaker AA, Tomancak P, Patel N, Rubin GM, Lai EC (2005): Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development. Proc Natl Acad Sci USA 102: 18017-22

Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM (2001): Intercellular communication via connexion 43 gap junctions is required for ovarian folliculogenesis in the mouse. Dev Biol 233: 258-70

Ademokun A, Turner M (2008): Regulation of B-cell differentiation by microRNAs and RNA-binding proteins. Biochem Soc Trans 36: 1191-3

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: 463-71

Ajduk A, Malagocki A, Maleszewski M (2008): Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca2+ oscillations. Reprod Biol 8: 3-22

Albertini DF (1992): Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. Mutat Res 296: 57-68

Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ (2001): Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 121: 647-53

Aleman LM, Doench J, Sharp PA (2007): Comparison of siRNA-induced off-target RNA and protein effects. RNA 13: 385-95

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: 2194-205

Armstrong DT, Xia P, de Gannes G, Tekpetey FR, Khamsi F (1996): Differential effects of insulin-like growth factor-I and follicle-stimulating hormone on proliferation and differentiation of bovine cumulus cells and granulosa cells. Biol Reprod 54: 331-8

Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H (2008): MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27: 2128-36

Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA (2008): Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. Biol Reprod 79: 209-22

Assou S, Anahory T, Pantesco V, Le Carrour T, Pellestor F, Klein B, Reyftmann L, Dechaud H, De Vos J, Hamamah S (2006): The human cumulus-oocyte complex gene-expression profile. Hum Reprod 21: 1705-19

Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Reme T, Dechaud H, De Vos J, Hamamah S (2008): A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod 14: 711-9

Atef A, Francois P, Christian V, Marc-Andre S (2005): The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. Mol Reprod Dev 71: 358-67

Avazeri N, Courtot AM, Pesty A, Duquenne C, Lefevre B (2000): Cytoplasmic and nuclear phospholipase C-beta 1 relocation: role in resumption of meiosis in the mouse oocyte. Mol Biol Cell 11: 4369-80

Avazeri N, Lefevre B (1998): Phospholipase C1, the PLC isoform present at the nuclear level, regulates the IP3-dependent Ca2+ oscillations responsible for mouse oocyte germinal vesicle breakdown. Biology of the cell 90: 125-125(121)

Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE (2005): Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122: 553-63

Baker TG, Hunter RHF (1978): Interrelationships between the oocyte and somatic cells with in the Graafian follicle of mammals. Ann Biol Anim Bioch Biophys 18: 419-426

Bao B, Garverick HA (1998): Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 76: 1903-21

Barnes FL, First NL (1991): Embryonic transcription in in vitro cultured bovine embryos. Mol Reprod Dev 29: 117-23

Bartel DP (2004): MicroRNAs genomics, biogenesis, mechanism, and function. Cell 116: 281-97

Bartel DP (2009): MicroRNAs target recognition and regulatory functions. Cell 136: 215-33

Behm-Ansmant I, Izaurralde E (2006): Quality control of gene expression: a stepwise assembly pathway for the surveillance complex that triggers nonsense-mediated mRNA decay. Genes Dev 20: 391-8

Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC (2007): Mammalian mirtron genes. Mol Cell 28: 328-36

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ (2003): Dicer is essential for mouse development. Nat Genet 35: 215-7

Bettegowda A, Smith GW (2007): Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. Front Biosci 12: 3713-26

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W (2006): Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 125: 1111-24

Bhojwani S, Alm H, Torner H, Kanitz W, Poehland R (2007): Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. Theriogenology 67: 341-5

Biemar F, Zinzen R, Ronshaugen M, Sementchenko V, Manak JR, Levine MS (2005): Spatial regulation of microRNA gene expression in the Drosophila embryo. Proc Natl Acad Sci USA 102: 15907-11

Biggers JD, Whittingham DG, Donahue RP (1967): The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci USA 58: 560-7

Blondin P, Sirard MA (1995): Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 41, 54–62

Bodin L, Di Pasquale E, Fabre S, Bontoux M, Monget P, Persani L, Mulsant P (2007): A Novel Mutation in the Bone Morphogenetic Protein 15 Gene Causing

Defective Protein Secretion Is Associated with Both Increased Ovulation Rate and Sterility in Lacaune Sheep Endocrinology 148: 393-400

Borchert GM, Lanier W, Davidson BL (2006): RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13: 1097-101

Braw-Tal R, Yossefi S (1997): Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. J Reprod Fertil 109: 165-71

Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003): bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 113: 25-36

Brennecke J, Stark A, Russell RB, Cohen SM (2005): Principles of microRNA-target recognition. PLoS Biol 3: e85

Brinster RL (1971): Oxidation of pyruvate and glucose by oocytes of the mouse and rhesus monkey. J Reprod Fertil 24: 187-91

Buccione R, Schroeder AC, Eppig JJ (1990): Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod 43: 543-7

Bueno MJ, Perez de Castro I, Malumbres M (2008): Control of cell proliferation pathways by microRNAs. Cell Cycle 7: 3143-8

Bushati N, Cohen SM (2007): microRNA functions. Annu Rev Cell Dev Biol 23: 175-205

Cai X, Hagedorn CH, Cullen BR (2004): Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-66

Caixeta ES, Ripamonte P, Franco MM, Junior JB, Dode MA (2009): Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. Reprod Fertil Dev 21: 655-64

Camaioni A, Hascall VC, Yanagishita M, Salustri A (1993): Effects of exogenous hyaluronic acid and serum on matrix organization and stability in the mouse cumulus cell-oocyte complex. J Biol Chem 268: 20473-81

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: 167-79

Carleton M, Cleary MA, Linsley PS (2007): MicroRNAs and cell cycle regulation. Cell Cycle 6: 2127-32

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

Carletti MZ, Fiedler SD, Christenson LK (2010): MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. Biol Reprod 83: 286-95

Carthew RW, Sontheimer EJ (2009): Origins and Mechanisms of miRNAs and siRNAs. Cell 136: 642-55

Castro FO, Sharbati S, Rodriguez-Alvarez LL, Cox JF, Hultschig C, Einspanier R (2010): MicroRNA expression profiling of elongated cloned and in vitro-fertilized bovine embryos. Theriogenology 73: 71-85

Ceci M, Gaviraghi C, Gorrini C, Sala LA, Offenhauser N, Marchisio PC, Biffo S (2003): Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature 426: 579-84

Chalfie M, Horvitz HR, Sulston JE (1981): Mutations that lead to reiterations in the cell lineages of C. elegans. Cell 24: 59-69

Chang CL, Wang HS, Soong YK, Huang SY, Pai SY, Hsu SY (2011): Regulation of oocyte and cumulus cell interactions by intermedin/adrenomedullin 2. J Biol Chem 286: 43193-203

Chang H, Brown CW, Matzuk MM (2002): Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 23: 787-823

Chekulaeva M, Filipowicz W (2009): Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol 21: 452-60

Chen L, Russell PT, Larsen WJ (1993): Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol Reprod Dev 34: 87-93

Chen L, Zhang H, Powers RW, Russell PT, Larsen WJ (1996): Covalent linkage between proteins of the inter-alpha-inhibitor family and hyaluronic acid is mediated by a factor produced by granulosa cells. J Biol Chem 271: 19409-14

Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R (2007): MicroRNA silencing through RISC recruitment of eIF6. Nature 447: 823-8

Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R (2005): TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436: 740-4

Cheng C, Bhardwaj N, Gerstein M (2009): The relationship between the evolution of microRNA targets and the length of their UTRs. . BMC Genomics 10: 431

Chian RC, Okuda K, Niwa K (1995): Influence of cumulus cells on in vitro fertilization of bovine oocytes derived from in vitro maturation. Animal Reproduction Science 38: 37-48

Chivukula RR, Mendell J T (2008): Circular reasoning: microRNAs and cell-cycle control.Trends Biochem Sci 33(10): 474-81

Choi WY, Giraldez AJ, Schier AF (2007): Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science 318: 271-4

Cillo F, Brevini TA, Antonini S, Paffoni A, Ragni G, Gandolfi F (2007): Association between human oocyte developmental competence and expression levels of some cumulus genes. Reproduction 134: 645-50

Cloonan N, Brown MK, Steptoe AL, Wani S, Chan WL, Forrest AR, Kolle G, Gabrielli B, Grimmond SM (2008): The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. Genome Biol 9: R127

Colonna R, Mangia F (1983): Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes. Biol Reprod 28: 797-803

Coutinho LL, Matukumalli LK, Sonstegard TS, Van Tassell CP, Gasbarre LC, Capuco AV, Smith TP (2007): Discovery and profiling of bovine microRNAs from immune-related and embryonic tissues. Physiol Genomics 29: 35-43

Cox JF, Hormazabal J, Santa Maria A (1993): Effect of the cumulus on in vitro fertilization of bovine matured oocyte. Theriogenology 40: 1259-l 267

Creighton CJ, Benham AL, Zhu H, Khan MF, Reid JG, Nagaraja AK, Fountain MD, Dziadek O, Han D, Ma L, Kim J, Hawkins SM, Anderson ML, Matzuk MM, Gunaratne PH (2010): Discovery of novel microRNAs in female reproductive tract using next generation sequencing. PLoS One 5: e9637

Crosby IM, Osborn JC, Moor RM (1981): Follicle cell regulation of protein synthesis and developmental competence in sheep oocytes. J Reprod Fertil 62: 575-82

Crozet N (1989): Nucleolar structure and RNA synthesis in mammalian oocytes. J Reprod Fertil Suppl 38: 9-16

Cui XS, Zhang DX, Ko YG, H. KN (2009): Aberrant epigenetic reprogramming of imprinted microrna-127 and rtl1 in cloned mouse embryos. Biochem Biophys Res Commun 379: 390-394

Cullen BR (2004): Transcription and processing of human microRNA precursors. Mol Cell 16: 861-5

Czech B, Zhou R, Erlich Y, Brennecke J, Binari R, Villalta C, Gordon A, Perrimon N, Hannon GJ (2009): Hierarchical rules for Argonaute loading in Drosophila. Mol Cell 36: 445-56

Dahiya N, Morin PJ (2010): MicroRNAs in ovarian carcinomas. Endocr Relat Cancer. 17(1): F77–F89

Dalbies-Tran R, Mermillod P (2003): Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. Biol Reprod 68: 252-61

Dammer EB, Sewer MB (2008): Phosphorylation of CtBP1 by cAMP-dependent protein kinase modulates induction of CYP17 by stimulating partnering of CtBP1 and 2. J Biol Chem 283: 6925-34

Davis E, Caiment F, Tordoir X, Cavaille J, Ferguson-Smith A, Cockett N, Georges M, Charlier C (2005): RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. Curr Biol 15: 743-9

de Matos DG, Furnus CC, Moses DF (1997): Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. Biol Reprod 57: 1420-5

Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004): Processing of primary microRNAs by the Microprocessor complex. Nature 432: 231-5

DeRenzo C, Seydoux G (2004): A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends Cell Biol 14: 420-6

Derry MC, Yanagiya A, Martineau Y, Sonenberg N (2006): Regulation of poly (A)-binding protein through PABP-interacting proteins. Cold Spring Harb Symp Quant Biol 71: 537-43

Diaz FJ, O'Brien MJ, Wigglesworth K, Eppig JJ (2006): The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion. Dev Biol 299: 91-104

Ding XC, Grosshans H (2009): Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J 28: 213-22

Donahue RP, Stern S (1968): Follicular cell support of oocyte maturation: production of pyruvate in vitro. J Reprod Fertil 17: 395-8

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM (1996): Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383: 531-5

Downs SM, Schroeder AC, Eppig JJ (1986): Developmental Capacity of Mouse Oocytes Following Maintenance of Meiotic Arrest In Vitro. Gamete Research 15: 305-316

Dragovic RA, Ritter LJ, Schulz SJ, Amato F, Armstrong DT, Gilchrist RB (2005): Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. Endocrinology 146: 2798-806

Dragovic RA, Ritter LJ, Schulz SJ, Amato F, Thompson JG, Armstrong DT, Gilchrist RB (2007): Oocyte-secreted factor activation of SMAD 2/3 signaling enables initiation of mouse cumulus cell expansion. Biol Reprod 76: 848-57

Driancourt MA (1991): Follicular dynamics in sheep and cattle. Theriogenology 35: 55–68

Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM (1998): The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12: 1809-17

Easow G, Teleman AA, Cohen SM (2007): Isolation of microRNA targets by miRNP immunopurification. RNA 13: 1198-204

Edery I, Humbelin M, Darveau A, Lee KA, Milburn S, Hershey JW, Trachsel H, Sonenberg N (1983): Involvement of eukaryotic initiation factor 4A in the cap recognition process. J Biol Chem 258: 11398-403

Ehrenberg M, Hauryliuk V, Po¨yry TAA (2007): Translation termination, the prion [PSI], and ribosomal recycling. In: M. B. Mathews, N. Sonenberg, and J. W. B. Hershey, Eds, Translational Control in Biology and Medicine. ColdSpring Harbor Laboratory Press, New York: 173-196

Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM (1999a): Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol 13: 1035-48

Elvin JA, Yan C, Matzuk MM (2000): Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. Proc Natl Acad Sci USA 97: 10288-93

Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM (1999b): Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13: 1018-34

Eppig JJ (1976): Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. J Exp Zool 198: 375-82

Eppig JJ (1979): FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. Nature 281: 483-4

Eppig JJ (1991): Intercommunication between mammalian oocytes and companion somatic cells. Bioessays 13: 569-74

Eppig JJ (2001): Oocyte control of ovarian follicular development and function in mammals. Reproduction 122: 829-38

Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK (2005): Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. Biol Reprod 73: 351-7

Eppig JJ, Viveiros MM, Marin Bivens C, De La Fuente R (2004): Regulation of Mammalian Oocyte Maturation. In: PCK. Leung, EY. Adashi, editors, The Ovary. San Diego, CA: Elsevier AcademicPress: 113-129

Eppig JJ, Wigglesworth K, Pendola F, Hirao Y (1997): Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol Reprod 56: 976-84

Erickson BH (1966): Development and senescence of the postnatal bovine ovary. J Anim Sci 25: 800-5

Erickson GF, Shimasaki S (2001): The physiology of folliculogenesis: the role of novel growth factors. Fertil Steril 76: 943-9

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP (2006): miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3: 87-98

Esquela-Kerscher A, Slack FJ (2006): Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-69

Eulalio A, Huntzinger E, Izaurralde E (2008a): Getting to the root of miRNA-mediated gene silencing. Cell 132: 9-14

Eulalio A, Huntzinger E, Izaurralde E (2008b): GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol 15: 346-53

Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, Fauser M, Izaurralde E (2009): Deadenylation is a widespread effect of miRNA regulation. RNA 15: 21-32

Eulalio A, Rehwinkel J, Stricker M, Huntzinger E, Yang SF, Doerks T, Dorner S, Bork P, Boutros M, Izaurralde E (2007): Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev 21: 2558-70

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates JR, 3rd, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N (2009): Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell 35: 868-80

Fabian MR, Sonenberg N, Filipowicz W (2010): Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-79

Fair T (2003): Follicular oocyte growth and acquisition of developmental competence. Anim Reprod Sci 78: 203-16

Fair T, Carter F, Park S, Evans AC, Lonergan P (2007): Global gene expression analysis during bovine oocyte in vitro maturation. Theriogenology 68 Suppl 1: S91-7

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M (1997a): Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryol (Berl) 195: 327-36

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M (1997b): Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. Mol Reprod Dev 46: 208-15

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

Fair T, Hyttel P, Greve T, Boland M (1996): Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. Mol Reprod Dev 43: 503-12

Fang Z, Rajewsky N (2011): The impact of miRNA target sites in coding sequences and in 3'UTRs. PLoS One 6: e18067

Fatehi AN, Zeinstra EC, Kooij RV, Colenbrander B, Bevers MM (2002): Effect of cumulus cell removal of in vitro matured bovine oocytes prior to in vitro fertilization on subsequent cleavage rate. Theriogenology 57: 1347-55

Feng WG, Sui HS, Han ZB, Chang ZL, Zhou P, Liu DJ, Bao S, Tan JH (2007): Effects of follicular atresia and size on the developmental competence of bovine

oocytes: a study using the well-in-drop culture system. Theriogenology 67, 1339– 1350

Ferguson EL, Sternberg PW, Horvitz HR (1987): A genetic pathway for the specification of the vulval cell lineages of Caenorhabditis elegans. Nature 326: 259-67

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA (2009): Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology 71: 836-48

Fiedler SD, Carletti MZ, Hong X, Christenson LK (2008): Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells. Biol Reprod 79: 1030-7

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008): Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9: 102-14

Friedman RC, Farh KK, Burge CB, Bartel DP (2009): Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105

Fukuda T, Yamagata K, Fujiyama S, Matsumoto T, Koshida I, Yoshimura K, Mihara M, Naitou M, Endoh H, Nakamura T, Akimoto C, Yamamoto Y, Katagiri T, Foulds C, Takezawa S, Kitagawa H, Takeyama K, O'Malley BW, Kato S (2007): DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. Nat Cell Biol 9: 604-11

Fukui Y (1990): Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine oocytes matured in vitro. Mol Reprod Dev. 26: 40-6

Fukui Y, Sakuma Y (1980): Maturation of bovine oocytes cultured in vitro: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. Biol Reprod 22: 669-73

Fulka JJ, First, NL, Moor, R.M (1998): Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. Mol Hum Reprod. Mol Hum Reprod 4: 41–9

Fülöp C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, Day AJ, Salustri A, Hascall VC, Glant TT, Mikecz K (2003): Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. Development 130: 2253-61

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O (2000): Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25: 279-83

Gangaraju VK, Lin H (2009): MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10: 116-25

Germani A, Bruzzoni-Giovanelli H, Fellous A, Gisselbrecht S, Varin-Blank N, Calvo F (2000): SIAH-1 interacts with alpha-tubulin and degrades the kinesin Kid by the proteasome pathway during mitosis. Oncogene 19: 5997-6006

Geshi M, Takenouchi N, Yamauchi N, Nagai T (2000): Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. Biol Reprod 63: 1730-4

Gilchrist RB, Lane M, Thompson JG (2008): Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 14: 159-77

Gilchrist RB, Ritter LJ, Armstrong DT (2001): Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation. Dev Biol 240: 289-98

Gilchrist RB, Ritter LJ, Armstrong DT (2004): Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 82-83: 431-46

Gilchrist RB, Thompson JG (2007): Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. Theriogenology 67: 6-15

Gingras AC, Raught B, Sonenberg N (1999): eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68: 913-63

Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF (2006): Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312: 75-9

Glazov EA, Cottee PA, Barris WC, Moore RJ, Dalrymple BP, Tizard ML (2008): A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Res 18: 957-64

Gonzalez G, Behringer RR (2009): Dicer is required for female reproductive tract development and fertility in the mouse. Mol Reprod Dev 76: 678-88

Gordo AC, He CL, Smith S, Fissore RA (2001): Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. Mol Reprod Dev 59: 106-14

Gordon I (2003a): The Bovine Oestrous Cycle and Associated Events. In: I. Gordon, Editors, Laboratory Production of Cattle Embryos Cambridge: CAB International / Cambridge University Press: 42-78

Gordon I (2003b): Maturing the Bovine Oocyte. In: I. Gordon, editor, Laboratory Production of Cattle Embryos. Cambridge: CAB International / Cambridge University Press: 114-116

Gosden RG, Bownes M (1995): Cellular and molecular aspects of oocyte development. In: J.G. Grudzinskas and J.L. Yovich, Editors, Gametes-The Oocyte. Cambridge Reviews in Human Reproduction, Cambridge University Press, Cambridge: 23-53

Goud PT, Goud AP, Van Oostveldt P, Dhont M (1999): Presence and dynamic redistribution of type I inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during in-vitro maturation, fertilization and early cleavage divisions. Mol Hum Reprod 5: 441-51

Gougeon A (1996): Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17: 121-55

Grazul-Bilska AT, Reynolds LP, Redmer DA (1997): Gap junctions in the ovaries. Biol Reprod 57: 947-57

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R (2004): The Microprocessor complex mediates the genesis of microRNAs. Nature 432: 235-40

Griffiths-Jones S (2004): The microRNA Registry. Nucleic Acids Res 32: D109-11

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34: D140-4

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008): miRBase: tools for microRNA genomics. Nucleic Acids Res 36: D154-8

Grifo JA, Tahara SM, Morgan MA, Shatkin AJ, Merrick WC (1983): New initiation factor activity required for globin mRNA translation. J Biol Chem 258: 5804-10

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007): MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91-105

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC (2001:) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106: 23-34

Grün D, Wang YL, Langenberger D, Gunsalus KC, Rajewsky N (2005): microRNA target predictions across seven Drosophila species and comparison to mammalian targets. PLoS Comput Biol 1: e13

Gu S, Jin L, Zhang F, Sarnow P, Kay MA (2009): Biological basis for restriction of microRNA targets to the 3' untranslated region in mammalian mRNAs. Nat Struct Mol Biol 16: 144-50

Guil S, Caceres JF (2007): The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. Nat Struct Mol Biol 14: 591-6

Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, Sirard MA (2008): Identification of differentially expressed markers in human follicular cells associated with competent oocytes. Hum Reprod 23: 1118-27

Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN (2004): The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18: 3016-27

Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN (2006): Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125: 887-901

Han L, Witmer PD, Casey E, Valle D, Sukumar S (2007): DNA methylation regulates MicroRNA expression. Cancer Biol Ther 6: 1284-8

Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM (2004): Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod 70: 900-9

Haouzi D, Assou S, Mahmoud K, Hedon B, De Vos J, Dewailly D, Hamamah S (2009): LH/hCGR gene expression in human cumulus cells is linked to the expression of the extracellular matrix modifying gene TNFAIP6 and to serum estradiol levels on day of hCG administration. Hum Reprod 24: 2868-78

Hashimoto S, Saeki K, Nagao Y, Minami N, Yamada M, Utsumi K (1998): Effects of cumulus cell density during in vitro maturation of the developmental competence of bovine oocytes. Theriogenology 49: 1451-63

Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M, Hsueh AJ (1999): Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 140: 1236-44

He X, He L, Hannon GJ (2007): The guardian's little helper: microRNAs in the p53 tumor suppressor network. Cancer Res 67: 11099-101

Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C, Junemann C, Niepmann M (2008): microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J 27: 3300-10

Herbert TP, Proud CG (2007): Regulation of transulation elongation and cotransulational protein targeting pathway. In: M. B.T. Mathews, N.Sonenberg, J. Hershey, eds,Translational Control in Biology and Medicine Cold Spring Harbor Laboratory Press 48: 601-624

Hong X, Luense LJ, McGinnis LK, Nothnick WB, Christenson LK (2008): Dicer1 is essential for female fertility and normal development of the female reproductive system. Endocrinology 149: 6207-12

Horvitz HR, Sulston JE (1980): Isolation and genetic characterization of cell-lineage mutants of the nematode Caenorhabditis elegans. Genetics 96: 435-54

Hosoe M, Shioya Y (1997): Distribution of cortical granules in bovine oocytes classified by cumulus complex. Zygote 5: 371-6

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

Houbaviy HB, Murray MF, Sharp PA (2003): Embryonic stem cell-specific MicroRNAs. Dev Cell 5: 351-8

Hulshof SCJ, Bevers MM, van der Donk HA, van den Hurk R (1992): The isolation and characterization of preantral follicles from foetal bovine ovaries. In: Proceedings of the 12th International Congress on Animal Reproduction. 1: 336-338

Humphreys DT, Westman BJ, Martin DI, Preiss T (2005): MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci USA 102: 16961-6

Hussein TS, Froiland DA, Amato F, Thompson JG, Gilchrist RB (2005): Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. J Cell Sci 118: 5257-68

Hussein TS, Thompson JG, Gilchrist RB (2006): Oocyte-secreted factors enhance oocyte developmental competence. Dev Biol 296: 514-21

Hutvagner G, Simard MJ (2008): Argonaute proteins: key players in RNA silencing. Nat Rev Mol Cell Biol 9: 22-32

Hutvagner G, Zamore PD (2002): A microRNA in a multiple-turnover RNAi enzyme complex. Science 297: 2056-60

Hyttel P (1987): Bovine cumulus-oocyte disconnection in vitro. Anat Embryol Berl 176: 41-44

Hyttel P, Fair T, Callesen H, Greve T (1997): Oocyte growth, capacitation and final maturation in cattle. Theriogenology 47 23-32

Imataka H, Gradi A, Sonenberg N (1998): A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J 17: 7480-9

Iovino N, Pane A, Gaul U (2009): miR-184 has multiple roles in Drosophila female germline development. Dev Cell 17: 123-33

Jackson RJ, Standart N (2007): How do microRNAs regulate gene expression? Sci STKE 2007: re1

Jeong KH, Kaiser UB (2006): Gonadotropin-releasing hormone regulation of gonadotropin biosynthesis and secretion. In:J.D. Neill, editors, Knobil and Neill's Physiology of Reproduction 3rd edition. Elsevier, Amsterdam: 1635-1726.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004): Human MicroRNA targets. PLoS Biol 2: e363

Jopling CL, Schutz S, Sarnow P (2008): Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. Cell Host Microbe 4: 77-85

Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005): Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309: 1577-81

Jovanovic M, Hengartner MO (2006): miRNAs and apoptosis: RNAs to die for. Oncogene 25: 6176-87

Juengel JL, McNatty KP (2005): The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 11: 143-60

Kaivo-oja N, Jeffery LA, Ritvos O, Mottershead DG (2006): Smad signalling in the ovary. Reprod Biol Endocrinol 4: 21

Kapp LD, Lorsch JR (2004): The molecular mechanics of eukaryotic translation. Annu Rev Biochem 73: 657-704

Kawahara Y, Zinshteyn B, Chendrimada TP, Shiekhattar R, Nishikura K (2007): RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. EMBO Rep 8: 763-9

Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Orom UA, Lund AH, Perrakis A, Raz E, Agami R (2007): RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell 131: 1273-86

Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E (2007): The role of site accessibility in microRNA target recognition. Nat Genet 39: 1278-84

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH (2001): Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 15: 2654-9

Khvorova A, Reynolds A, Jayasena SD (2003): Functional siRNAs and miRNAs exhibit strand bias. Cell 115: 209-16

Kim VN (2005): MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6: 376-85

Kimura N, Totsukawa K, Sato E (2006): Significance of Mammalian Cumulus-Oocyte Complex Matrix in Oocyte Meiotic Maturation Review of the Synthetic Control and Possible Roles of Hyaluronan (HA) and HA-binding Protein. J. Mamm. Ova Res. 23: 36-51

Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT, Mourelatos Z (2007): An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell 129: 1141-51

Knight PG, Glister C (2001): Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 121: 503-12

Kölle S, Dubielzig S, Reese S, Wehrend A, Konig P, Kummer W (2009): Ciliary transport, gamete interaction, and effects of the early embryo in the oviduct: ex vivo analyses using a new digital videomicroscopic system in the cow. Biol Reprod 81: 267-74

Kraus S, Naor Z, Seger R (2001): Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. Arch Med Res 32: 499-509

Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N (2005): Combinatorial microRNA target predictions. Nat Genet 37: 495-500

Kruip TAM, Cran, DG, Van Beneden, TH, Dieleman, SJ (1983): Structural changes in bovine oocytes during final maturation in vivo Gamete Res 8: 29-4.

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M (2005): Silencing of microRNAs in vivo with 'antagomirs'. Nature 438: 685-9

Kuchenbauer F, Mah SM, Heuser M, McPherson A, Ruschmann J, Rouhi A, Berg T, Bullinger L, Argiropoulos B, Morin RD, Lai D, Starczynowski DT, Karsan A, Eaves CJ, Watahiki A, Wang Y, Aparicio SA, Ganser A, Krauter J, Dohner H, Dohner K, Marra MA, Camargo FD, Palmqvist L, Buske C, Humphries RK (2011): Comprehensive analysis of mammalian miRNA* species and their role in myeloid cells. Blood 118: 3350-8

Kuhn DE, Martin MM, Feldman DS, Terry AV, Jr., Nuovo GJ, Elton TS (2008): Experimental validation of miRNA targets. Methods 44: 47-54

Kumar NM, Gilula NB (1996): The gap junction communication channel. Cell 84: 381-8

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001): Identification of novel genes coding for small expressed RNAs. Science 294: 853-8

Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T (2003): New microRNAs from mouse and human. RNA 9: 175-9

Lai EC (2002): Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet 30: 363-4

Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M, Ritvos O (1998): A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 78: 135-40

Lam X, Gieseke C, Knoll M, Talbot P (2000): Assay and importance of adhesive interaction between hamster (Mesocricetus auratus) oocyte-cumulus complexes and the oviductal epithelium. Biol Reprod 62: 579-88

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001): An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 858-62

Lau P, Verrier JD, Nielsen JA, Johnson KR, Notterpek L, Hudson LD (2008): Identification of dynamically regulated microRNA and mRNA networks in developing oligodendrocytes. J Neurosci 28: 11720-30

Lee R, Feinbaum R, Ambros V (2004a): A short history of a short RNA. Cell 116: S89-92, 1 p following S96

Lee RC, Ambros V (2001): An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862-4

Lee RC, Feinbaum RL, Ambros V (1993): The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843-54

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003): The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415-9

Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002): MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21: 4663-70

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN (2004b): MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23: 4051-60

Lehmann U, Hasemeier B, Christgen M, Muller M, Romermann D, Langer F, Kreipe H (2008): Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 214: 17-24

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

Lewis BP, Burge CB, Bartel DP (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003): Prediction of mammalian microRNA targets. Cell 115: 787-98

Li R, Norman RJ, Armstrong DT, Gilchrist RB (2000): Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells. Biol Reprod 63: 839-45

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK (1997): Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91: 197-208

Lim JM, Liou SS, Hansel W (1996): Intracytoplasmic glutathione concentration and the role of beta-mercaptoethanol in preimplantation development of bovine embryos. Theriogenology 46: 429-39

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: 769-73

Lingel A, Simon B, Izaurralde E, Sattler M (2004): Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nat Struct Mol Biol 11: 576-7

Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, Johnson JM, Cummins JM, Raymond CK, Dai H, Chau N, Cleary M, Jackson AL, Carleton M, Lim L (2007): Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 27: 2240-52

Liu HC, Tang Y, He Z, Rosenwaks Z (2010): Dicer is a key player in oocyte maturation. J Assist Reprod Genet 27: 571-80

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ (2004): Argonaute2 is the catalytic engine of mammalian RNAi. Science 305: 1437-41

Liu X, Fortin K, Mourelatos Z (2008): MicroRNAs: biogenesis and molecular functions. Brain Pathol 18: 113-21

Liu ZH, Zhang H, He YP, Zhang JH, Yue LM (2006): Cyclin G1 expressing in mouse ovary and relating to follicular development. Sichuan Da Xue Xue Bao Yi Xue Ban 37: 893-7

Llave C, Xie Z, Kasschau KD, Carrington JC (2002): Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297: 2053-6

Long D, Lee R, Williams P, Chan CY, Ambros V, Ding Y (2007): Potent effect of target structure on microRNA function. Nat Struct Mol Biol 14: 287-94

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U (2004): Nuclear export of microRNA precursors. Science 303: 95-8

Lund E, Liu M, Hartley RS, Sheets MD, Dahlberg JE (2009): Deadenylation of maternal mRNAs mediated by miR-427 in Xenopus laevis embryos. RNA 15: 2351-63

Lussier JG, Matton P, Dufour JJ (1987): Growth rates of follicles in the ovary of the cow. J Reprod Fertil 81: 301-7

Lykke-Andersen K, Gilchrist MJ, Grabarek JB, Das P, Miska E, Zernicka-Goetz M (2008): Maternal Argonaute 2 is essential for early mouse development at the maternal-zygotic transition. Mol Biol Cell 19: 4383-92

Lytle JR, Yario TA, Steitz JA (2007): Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci USA 104: 9667-72

Ma J, Flemr M, Stein P, Berninger P, Malik R, Zavolan M, Svoboda P, Schultz RM (2010): MicroRNA activity is suppressed in mouse oocytes. Curr Biol 20: 265-70

Ma JB, Ye K, Patel DJ (2004): Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature 429: 318-22

Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD (2008): Dicer1 is required for differentiation of the mouse male germline. Biol Reprod 79: 696-703

Machlin ES, Sarnow P, Sagan SM (2011): Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. Proc Natl Acad Sci USA 108: 3193-8

Majid S, Dar AA, Saini S, Yamamura S, Hirata H, Tanaka Y, Deng G, Dahiya R (2010): MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. Cancer 116: 5637-49

Mamo S, Carter F, Lonergan P, Leal CL, Al Naib A, McGettigan P, Mehta JP, Evans AC, Fair T (2011): Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: potential molecular markers of oocyte maturation. BMC Genomics 12: 151

Maroney PA, Yu Y, Fisher J, Nilsen TW (2006): Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol 13: 1102-7

Martello G, Zacchigna L, Inui M, Montagner M, Adorno M, Mamidi A, Morsut L, Soligo S, Tran U, Dupont S, Cordenonsi M, Wessely O, Piccolo S (2007): MicroRNA control of Nodal signalling. Nature 449: 183-8

Massague J (2000): How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1: 169-78

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ (2002): Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296: 2178-80

McArdle CA, Franklin J, Green L, Hislop JN (2002): Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. J Endocrinol 173: 1-11

McCallie B, Schoolcraft WB, Katz-Jaffe MG (2010): Aberration of blastocyst microRNA expression is associated with human infertility. Fertil Steril 93: 2374-82

McGrath SA, Esquela AF, Lee SJ (1995): Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 9: 131-6

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM (2004): Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum Reprod 19: 2869-74

McNatty KP, Moore LG, Hudson NL, Quirke LD, Lawrence SB, Reader K, Hanrahan JP, Smith P, Groome NP, Laitinen M, Ritvos O, Juengel JL (2004): The oocyte and its role in regulating ovulation rate: a new paradigm in reproductive biology. Reproduction 128: 379-86

Meister A (1983): Selective modification of glutathione metabolism. Science 220: 472-7

Memili E, First NL (1999): Control of gene expression at the onset of bovine embryonic development. Biol Reprod 61: 1198-207

Memili E, Peddinti D, Shack LA, Nanduri B, McCarthy F, Sagirkaya H, Burgess SC (2007): Bovine germinal vesicle oocyte and cumulus cell proteomics. Reproduction 133: 1107-20

Mermillod P, Oussaid B, Cognié Y (1999): Aspects of follicular and oocyte maturationthat affect the developmental potential of embryos. J Reprod Fertil Suppl 54, 449-460

Merrick WC (2004): Cap-dependent and cap-independent translation in eukaryotic systems. Gene 332: 1-11

Millar RP (2006): Gonadotrophin releasing horrmone, In: A. j. kastin,editor, handbook of bilogicaly active peptied. Elsevier acadamic press, USA: 637-644

Mineno J, Okamoto S, Ando T, Sato M, Chono H, Izu H, Takayama M, Asada K, Mirochnitchenko O, Inouye M, Kato I (2006): The expression profile of microRNAs in mouse embryos. Nucleic Acids Res 34: 1765-71

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: 811-22

Miyazaki S, Yuzaki M, Nakada K, Shirakawa H, Nakanishi S, Nakade S, Mikoshiba K (1992): Block of Ca2+ wave and Ca2+ oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science 257: 251-5

Moor RM, Dai Y, Lee C, Fulka JJr (1998): Oocyte maturation and embryonic failure. Hum Reprod Update 4: 223-36

Mori T, Amano T, Shimizu H (2000): Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. Biol Reprod 62: 913-9

Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G (2002): miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev 16: 720-8

Mtango NR, Potireddy S, Latham KE (2008): Oocyte quality and maternal control of development. Int Rev Cell Mol Biol 268: 223-90

Mtango NR, Potireddy S, Latham KE (2009): Expression of microRNA processing machinery genes in rhesus monkey oocytes and embryos of different developmental potentials. Mol Reprod Dev 76: 255-69

Mukhopadhyay D, Asari A, Rugg MS, Day AJ, Fulop C (2004): Specificity of the tumor necrosis factor-induced protein 6-mediated heavy chain transfer from inter-alpha-trypsin inhibitor to hyaluronan: implications for the assembly of the cumulus extracellular matrix. J Biol Chem 279: 11119-28

Murchison EP, Hannon GJ (2004): miRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr Opin Cell Biol 16: 223-9

Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, Hannon GJ (2007): Critical roles for Dicer in the female germline. Genes Dev 21: 682-93

Nagaraja AK, Andreu-Vieyra C, Franco HL, Ma L, Chen R, Han DY, Zhu H, Agno JE, Gunaratne PH, DeMayo FJ, Matzuk MM (2008): Deletion of Dicer in somatic cells of the female reproductive tract causes sterility. Mol Endocrinol 22: 2336-52

Nakahara K, Kim K, Sciulli C, Dowd SR, Minden JS, Carthew RW (2005): Targets of microRNA regulation in the Drosophila oocyte proteome. Proc Natl Acad Sci USA 102: 12023-8

Nielsen CB, Shomron N, Sandberg R, Hornstein E, Kitzman J, Burge CB (2007): Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. RNA 13: 1894-910

Nilsen TW (2007): Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet 23: 243-9

Niwa R, Slack FJ (2007): The evolution of animal microRNA function. Curr Opin Genet Dev 17: 145-50

Nottrott S, Simard MJ, Richter JD (2006): Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol 13: 1108-14

Obernosterer G, Martinez J, Alenius M (2007): Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. Nat Protoc 2: 1508-14

Ohsugi M, Adachi K, Horai R, Kakuta S, Sudo K, Kotaki H, Tokai-Nishizumi N, Sagara H, Iwakura Y, Yamamoto T (2008): Kid-mediated chromosome compaction ensures proper nuclear envelope formation. Cell 132: 771-82

Okada C, Yamashita E, Lee SJ, Shibata S, Katahira J, Nakagawa A, Yoneda Y, Tsukihara T (2009): A high-resolution structure of the pre-microRNA nuclear export machinery. Science 326: 1275-9

Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC (2007): The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell 130: 89-100

Okamura K, Phillips MD, Tyler DM, Duan H, Chou YT, Lai EC (2008): The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. Nat Struct Mol Biol 15: 354-63

Olena AF, Patton JG (2010): Genomic organization of microRNAs. J Cell Physiol 222: 540-5

Olsen PH, Ambros V (1999): The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 216: 671-80

Orom UA, Nielsen FC, Lund AH (2008): MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30: 460-71

Osada H, Takahashi T (2007): MicroRNAs in biological processes and carcinogenesis. Carcinogenesis 28: 2-12

Otsuka F, Yamamoto S, Erickson GF, Shimasaki S (2001): Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276: 11387-92

Otsuka M, Zheng M, Hayashi M, Lee JD, Yoshino O, Lin S, Han J (2008): Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. J Clin Invest 118: 1944-54

Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, De Vos J, Hamamah S (2011): Human Cumulus Cells Molecular Signature in Relation to Oocyte Nuclear Maturity Stage. PLoS ONE 6(11): e27179

Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, Zhang X, Song JS, Fisher DE (2008): Chromatin structure analyses identify miRNA promoters. Genes Dev 22: 3172-83

Pang RTK, Liu W, Leung CON, Ye T, Kwan PCK, Lee K, Yeung WSB (2011): miR-135A Regulates Preimplantation Embryo Development through Down-Regulation of E3 Ubiquitin Ligase Seven in Absentia Homolog 1A (SIAH1A) Expression PLoS ONE 6 |: e27878

Parker R, Song H (2004): The enzymes and control of eukaryotic mRNA turnover. Nat Struct Mol Biol 11: 121-7

Parrish JJ, Susko-Parrish J, Winer MA, First NL (1988): Capacitation of bovine sperm by heparin. Biol Reprod 38: 1171-80

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000): Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408: 86-9

Peddinti D, Memili E, Burgess SC (2010): Proteomics-based systems biology modeling of bovine germinal vesicle stage oocyte and cumulus cell interaction. PLoS One 5: e11240

Pedersen T, Peters H (1968): Proposal for a classification of oocytes and follicles in the mouse ovary. J Reprod Fertil 17: 555-7

Perreault SD, Barbee RR, Slott VL (1988): Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev Biol 125: 181-6

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, Hellen CU (2001): Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA 98: 7029-36

Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CU (1998): A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev 12: 67-83

Petersen CP, Bordeleau ME, Pelletier J, Sharp PA (2006): Short RNAs repress translation after initiation in mammalian cells. Mol Cell 21: 533-42

Picton H, Briggs D, Gosden R (1998): The molecular basis of oocyte growth and development. Mol Cell Endocrinol 145: 27-37

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W (2005): Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309: 1573-6

Pillai RS, Bhattacharyya SN, Filipowicz W (2007): Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 17: 118-26

Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R (2008): MicroRNA-373 induces expression of genes with complementary promoter sequences. . Proc Natl Acad Sci USA 105: 1608-13

Plasterk RH (2006): Micro RNAs in animal development. Cell 124: 877-81

Rajewsky N (2006): microRNA target predictions in animals. Nat Genet 38 Suppl: S8-13

Ralph JH, Telfer EE, Wilmut I (1995): Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. Mol Reprod Dev 42: 248-53

Regassa A, Rings F, Hoelker M, Cinar U, Tholen E, Looft C, Schellander K, Tesfaye D (2011): Transcriptome dynamics and molecular cross-talk between bovine oocyte and its companion cumulus cells. BMC Genomics 12: 57

Rehwinkel J, Behm-Ansmant I, Gatfield D, Izaurralde E (2005): A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA 11: 1640-7

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000): The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403: 901-6

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002): Prediction of plant microRNA targets. Cell 110: 513-20

Richter JD (2008): Think you know how miRNAs work? Think again. Nat Struct Mol Biol 15: 334-6

Ro S, Park C, Song R, Nguyen D, Jin J, Sanders KM, McCarrey JR, Yan W (2007): Cloning and expression profiling of testis-expressed piRNA-like RNAs. RNA 13: 1693-702

Robert C, Gagne D, Bousquet D, Barnes FL, Sirard MA (2001): Differential display and suppressive subtractive hybridization used to identify granulosa cell messenger rna associated with bovine oocyte developmental competence. Biol Reprod 64: 1812-20

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. Genome Res 14: 1902-10

Rosenkrans CF, Jr., First NL (1994): Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J Anim Sci 72: 434-7

Roy A, Matzuk MM (2006): Deconstructing mammalian reproduction: using knockouts to define fertility pathways. Reproduction 131: 207-19

Ruby JG, Jan CH, Bartel DP (2007): Intronic microRNA precursors that bypass Drosha processing. Nature 448: 83-6

Runft LL, Watras J, Jaffe LA (1999): Calcium release at fertilization of Xenopus eggs requires type I IP (3) receptors, but not SH2 domain-mediated activation of PLCgamma or G(q)-mediated activation of PLCbeta. Dev Biol 214: 399-411

Saito K, Ishizuka A, Siomi H, Siomi MC (2005): Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. PLoS Biol 3: e235

Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P, Salvatori G, Mahoney DJ, Day AJ, Siracusa G, Romani L, Mantovani A (2004): PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. Development 131: 1577-86

Salustri A, Yanagishita M, Hascall VC (1989): Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J Biol Chem 264: 13840-7

Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB (2008): Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. Science 320: 1643-7

Schmitter D, Filkowski J, Sewer A, Pillai RS, Oakeley EJ, Zavolan M, Svoboda P, Filipowicz W (2006): Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. Nucleic Acids Res 34: 4801-15

Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003): Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199-208

Seggerson K, Tang L, Moss EG (2002): Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev Biol 243: 215-25

Seitz H, Ghildiyal M, Zamore PD (2008): Argonaute loading improves the 5' precision of both MicroRNAs and their miRNA* strands in flies. Curr Biol 18: 147-51

Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008): Widespread changes in protein synthesis induced by microRNAs. Nature 455: 58-63

Senger PL (1997): Embryogenesis of the pituitary gland and the male or female reproductive system. In: Pathways to Pregnancy and Parturition. Current Conception Inc., 1st edition: 58-76

Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004): The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25: 72-101

Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004): The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25: 72-101

Shiraishi K, Okada A, Shirakawa H, Nakanishi S, Mikoshiba K, Miyazaki S (1995): Developmental changes in the distribution of the endoplasmic reticulum and inositol 1,4,5-trisphosphate receptors and the spatial pattern of Ca2+ release during maturation of hamster oocytes. Dev Biol 170: 594-606

Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, Yu M, Vandenberg SR, Ginzinger DG, James CD, Costello JF, Bergers G, Weiss WA, Alvarez-Buylla A, Hodgson JG (2008): miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med 6: 14

Simon AM, Goodenough DA, Li E, Paul DL (1997): Female infertility in mice lacking connexin 37. Nature 385: 525-9

Singh B, Zhang X, Armstrong DT (1993): Porcine oocytes release cumulus expansion-enabling activity even though porcine cumulus expansion in vitro is independent of the oocyte. Endocrinology 132: 1860-2

Singh SK, Pal Bhadra M, Girschick HJ, Bhadra U (2008) MicroRNAs--micro in size but macro in function. FEBS J 275: 4929-44

Sirard MA, Blondin P (2006): oocyte maturation and IVF in cattle. Anim Reprod Sci 42: 417-426

Sirotkin AV, Ovcharenko D, Mlyncek M (2010): Identification of protein kinases that control ovarian hormone release by selective siRNAs. J Mol Endocrinol 44: 45-53

Slezak-Prochazka I, Durmus S, Kroesen B, van den Berg A (2010): MicroRNAs, macrocontrol: Regulation of miRNA processing. RNA.16 (6):1087-95

Smitz JE, Cortvrindt RG (2002): The earliest stages of folliculogenesis in vitro. Reproduction 123: 185-202

Sonenberg N, Rupprecht KM, Hecht SM, Shatkin AJ (1979): Eukaryotic mRNA cap binding protein: purification by affinity chromatography on sepharose-coupled m7GDP. Proc Natl Acad Sci USA 76: 4345-9

Standart N, Jackson RJ (2007): MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev 21: 1975-82

Stricker SA (2006): Structural reorganizations of the endoplasmic reticulum during egg maturation and fertilization. Semin Cell Dev Biol 17: 303-13

Sugiura K, Pendola FL, Eppig JJ (2005): Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. Dev Biol 279: 20-30

Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ (2007): Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. Development 134: 2593-603

Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R (2010): MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr Biol 20: 271-7

Sun G, Yan J, Noltner K, Feng J, Li H, Sarkis DA, Sommer SS, Rossi JJ (2009): SNPs in human miRNA genes affect biogenesis and function. RNA 15: 1640-51

Sun TT, Chung CM, Chan HC (2011): Acrosome reaction in the cumulus oophorus revisited: involvement of a novel sperm-released factor NYD-SP8. Protein Cell 2: 92-8

Sunkar R, Zhu JK (2004): Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 16: 2001-19

Sutovsky P, Schatten G (1997): Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol Reprod 56: 1503-12

Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG (2003a:) Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. Reproduction 126: 27-34

Sutton ML, Gilchrist RB, Thompson JG (2003b): Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. Hum Reprod Update 9: 35-48

Sutton-McDowall ML, Gilchrist RB, Thompson JG (2010) :The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 139: 685-95

Taganov KD, Boldin MP, Baltimore D (2007): MicroRNAs and immunity: tiny players in a big field. Immunity 26: 133-7

Tang F, Kaneda M, O'Carroll D, Hajkova P, Barton SC, Sun YA, Lee C, Tarakhovsky A, Lao K, Surani MA (2007): Maternal microRNAs are essential for mouse zygotic development. Genes Dev 21: 644-8

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: 414-24

Tatemoto H, Sakurai N, Muto N (2000): Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. Biol Reprod 63: 805-10

Taylor DJ, Frank J, Kinzy TG (2007): Structure and Function of the Eukaryotic Ribosome and Elongation Factors. In: M. B.T. Mathews, N.Sonenberg, J. Hershey, eds,Translational Control in Biology and Medicine. Cold Spring Harbor Laboratory Press 48: 601-624

Telford NA, Watson AJ, Schultz GA (1990): Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 26: 90-100

Tesfaye D, Regassa A, Rings F, Ghanem N, Phatsara C, Tholen E, Herwig R, Un C, Schellander K, Hoelker M (2010): Suppression of the transcription factor MSX1 gene delays bovine preimplantation embryo development in vitro. Reproduction 139: 857-70

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: 665-77

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: 197-212

Tripurani SK, Lee KB, Wang L, Wee G, Smith GW, Lee YS, Latham KE, Yao J (2010b): A novel functional role for the oocyte-specific transcription factor newborn ovary homeobox (NOBOX) during early embryonic development in cattle. Endocrinology 152: 1013-23

Tripurani SK, Lee KB, Wee G, Smith GW, Yao J (2011): MicroRNA-196a regulates bovine newborn ovary homeobox gene (NOBOX) expression during early embryogenesis. BMC Dev Biol 11: 25

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

Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006): Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20: 515-24

Vallee M, Gravel C, Palin MF, Reghenas H, Stothard P, Wishart DS, Sirard MA (2005): Identification of novel and known oocyte-specific genes using complementary DNA subtraction and microarray analysis in three different species. Biol Reprod 73: 63-71

van den Hurk R, Zhao J (2005): Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63: 1717-51

Van Montfoort APA, Geraedts JPM, Dumoulin JCM, Stassen APM, Evers JLH, Ayoubi TAY (2008): Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. Mol Hum Reprod. 14: 157-168

van Wezel IL, Rodgers RJ (1996): Morphological characterization of bovine primordial follicles and their environment in vivo. Biol Reprod 55: 1003-11

Vanderhyden BC (1993): Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). J Reprod Fertil 98: 219-27

Vanderhyden BC, Armstrong DT (1989): Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. Biol Reprod 40: 720-8

Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM (2002): Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. Mol Endocrinol 16: 1154-67

Vasudevan S, Steitz JA (2007): AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 128: 1105-18

Vasudevan S, Tong Y, Steitz JA (2007): Switching from repression to activation: microRNAs can up-regulate translation. Science 318: 1931-4

Vella MC, Choi EY, Lin SY, Reinert K, Slack FJ (2004): The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. Genes Dev 18: 132-7

Vitt UA, Hayashi M, Klein C, Hsueh AJ (2000): Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol Reprod 62: 370-7

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: 619-28

Wang B, Yanez A, Novina CD (2008): MicroRNA-repressed mRNAs contain 40S but not 60S components. Proc Natl Acad Sci USA 105: 5343-5348.

Wassarman PM, Kinloch RA (1992): Gene expression during oogenesis in mice. Mutat Res 296: 3-15

Webster RJ, Giles KM, Price KJ, Zhang PM, Mattick JS, Leedman PJ (2009): Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. J Biol Chem 284: 5731-41

Wells SE, Hillner PE, Vale RD, Sachs AB (1998): Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell 2: 135-40

Wienholds E, Plasterk RH (2005): MicroRNA function in animal development. FEBS Lett 579: 5911-22

Wightman B, Ha I, Ruvkun G (1993): Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-62

Wilfred BR, Wang WX, Nelson PT (2007): Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. Mol Genet Metab 91: 209-17

Witkos TM, Koscianska E, Krzyzosiak WJ (2011): Practical Aspects of microRNA Target Prediction. Curr Mol Med 11: 93-109

Wang Y, Lee CG (2009): MicroRNA and cancer—Focus on apoptosis. J Cell Mol Med 13:12–23.

Wang Q, Sun QY (2007): Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reprod Fertil Dev 19, 1-12

Wongsrikeao P, Kaneshige Y, Ooki R, Taniguchi M, Agung B, Nii M, Otoi T (2005): Effect of the removal of cumulus cells on the nuclear maturation, fertilization and development of porcine oocytes. Reprod Domest Anim 40: 166-70

Wu L, Belasco JG (2008): Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell 29: 1-7

Wu L, Fan J, Belasco JG (2006): MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci USA 103: 4034-9

Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M (2005): Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434: 338-45

Xu S, Linher-Melville K, Yang BB, Wu D, Li J (2011b): Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatase. Endocrinology 152: 3941-51

Xu YW, Wang B, Ding CH, Li T, Gu F, Zhou C (2011a): Differentially expressed micoRNAs in human oocytes. J Assist Reprod Genet 28: 559-66

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS (1995): Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 53: 951-7

Xu Z, Kopf GS, Schultz RM (1994): Involvement of inositol 1,4,5-trisphosphate-mediated Ca2+ release in early and late events of mouse egg activation. Development 120: 1851-9

Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15: 854-66

Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K (2006): Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol 13: 13-21

Yao G, Yin M, Lian J, Tian H, Liu L, Li X, Sun F (2010a): MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. Mol Endocrinol 24: 540-51

Yao N, Yang BQ, Liu Y, Tan XY, Lu CL, Yuan XH, Ma X (2010b): Follicle-stimulating hormone regulation of microRNA expression on progesterone production in cultured rat granulosa cells. Endocrine 38: 158-66

Yekta S, Shih IH, Bartel DP (2004): MicroRNA-directed cleavage of HOXB8 mRNA. Science 304: 594-6

Yi R, Qin Y, Macara IG, Cullen BR (2003): Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17: 3011-6

Yin L, Chung CM, Huo R, Liu H, Zhou C, Xu W, Zhu H, Zhang J, Shi Q, Wong HY, Chen J, Lu Y, Bi Y, Zhao C, Du Y, Ma M, Cai Y, Chen WY, Fok KL, Tsang LL, Li K, Ni Y, Chung YW, Zhou Z, Sha J, Chan HC (2009): A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. Cell Mol Life Sci 66: 900-8

Yoshida M (1993): Role of glutathione in the maturation and fertilization of pig oocytes in vitro. Mol Reprod Dev 35: 76-81

Yoshino O, McMahon HE, Sharma S, Shimasaki S (2006): A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. Proc Natl Acad Sci USA 103: 10678-83

Yu Z, Jian Z, Shen S, Purisima E, Wang E (2007): Global analysis of microRNA target gene expression reveals that miRNA targets are lower expressed in mature mouse and Drosophila tissues than in the embryos Nucl. Acids Res. 35 152-164

Zekri L, Huntzinger E, Heimstadt S, Izaurralde E (2009): The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol Cell Biol 29: 6220-31

Zeng Y, Cullen BR (2004): Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. Nucleic Acids Res 32: 4776-85

Zeuner A, Muller K, Reguszynski K, Jewgenow K (2003): Apoptosis within bovine follicular cells and its effect on oocyte development during in vitro maturation. Theriogenology 59, 1421–1433

Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA (1995): Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development in vitro. Mol Reprod Dev 40: 338-44

Zhang Y, Verbeek FJ (2010): Comparison and integration of target prediction algorithms for microRNA studies. J Integr Bioinform 7

Zhao S, Liu MF (2009): Mechanisms of microRNA-mediated gene regulation. Sci China C Life Sci 52: 1111-6

Zhao Y, Srivastava D (2007): A developmental view of microRNA function. Trends Biochem Sci 32: 189-97

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