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The role of cellular and extracellular MicroRNAs in bovine follicular development and as potential indicators of early pregnancy

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Dedicated

To my parents and my son

Abstract

MicroRNAs (miRNAs), a class of short non-coding RNA molecules, emerged as major regulators of ~60% of all protein encoding genes. These molecules play an important role in mammalian follicular growth and embryonic development by modulating the expression of genes in follicular somatic cells and early embryos at post-transcriptional level. In addition to these cellular miRNAs, extracellular miRNAs found in virtually all biological fluids are getting great attention as potential diagnostic markers for diseases and various physiological conditions. The present study was designed to characterize the expression profile and functional role of cellular miRNAs in bovine granulosa cells and assessing the potential use of extracellular miRNAs in bovine early pregnancy diagnosis. For this, three experiments were conducted.

In order to characterize specific miRNAs expression patterns, next-generation sequencing analysis in granulosa cells of preovulatory dominant and subordinate follicles obtained at day 19 of the estrous cycle revealed a total of 315 and 323 known miRNAs to be expressed, respectively. Among these, 64 miRNAs were found to be differentially expressed, of which 34 miRNAs including the miR-183-96-182 cluster were preferentially enriched, while the remaining 30 miRNAs were suppressed in preovulatory dominant follicles compared to the subordinate follicles counterpart. Moreover, the negative correlation in the expression patterns of the miR-183-96-182 cluster and its validated pro-apoptotic target gene, FOXO1 in preovulatory dominant follicles further substantiates the regulatory role of miRNAs in ovarian function during the follicular phase of the bovine estrous cycle.

Functional analysis of the miR-183-96-182 cluster miRNAs in cultured granulosa cells showed that enrichment or inhibition of this miRNA cluster resulted in suppression or increased FOXO1 mRNA and protein, respectively. Concomitantly, overexpression of the miRNA cluster increased the rate of cell proliferation, decreased the proportion of cells under the G_0/G_1 arrest and increased the percentage of cells under the S phase. Even though inhibition of the miR-183-96-182 cluster slowed down the rate of cell proliferation, no measurable changes in the cell cycle status were observed. Furthermore, selective knockdown of FOXO1 mRNA using siRNA showed similar phenotypes as observed in the overexpression of the miRNA cluster experiment.

In the third experiment, miRNA PCR array platform was used to determine the expression signature of extracellular miRNAs in serum samples from pregnant and non-pregnant cows at day 19 and 24 post-insemination. Results showed that a total of 302 and 316 miRNAs were detected in day 19 pregnant and non-pregnant cows, respectively. Similarly, 356 and

325 miRNAs were detected in day 24 pregnant and non-pregnant cows, respectively. Comparative expression analysis revealed 8 and 23 differentially expressed miRNAs in day 19 and 24 pregnant cows, respectively. Interestingly, 1 miRNA (miR-433) and 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p, and miR-323a-3p) homologous to the human pregnancy-associated C14MC miRNA cluster were among the differentially expressed miRNAs in day 19 and 24 pregnant cows, respectively. The predicted target genes of the differentially expressed extracellular miRNAs were found to be involved in pathways important in pregnancy implantation like the adherens junction and ECM-interaction.

Taken all together, the present study demonstrates the functional involvement of miRNAs in the later stage of bovine follicular development by coordinately targeting key genes essential in determining the follicular fate. Moreover, this study characterizes unique expression signatures of extracellular miRNAs that could be potential indicators of early pregnancy in dairy cows.

Kurzfassung

MicroRNAs (miRNAs), eine Klasse von kurzen nicht-kodierenden RNA-Molekülen, gelten als Hauptregulatoren von ~60% aller Protein-kodierenden Gene. Diese Moleküle spielen eine wichtige Rolle beim Follikelwachstum und der embryonalen Entwicklung von Säugetieren, indem sie die Expression von Genen in follikulären somatischen Zellen und frühen Embryonen auf der posttranskriptionellen Ebene modulieren. Zusätzlich zu diesen zellulären miRNAs haben extrazelluläre miRNAs, die in praktisch allen biologischen Flüssigkeiten gefunden werden, als potentielle diagnostische Marker für Krankheiten und verschiedene physiologische Zustände große Aufmerksamkeit erlangt. In der vorliegenden Studie wurden dazu Expressionsprofile und die funktionelle Rolle dieser zellulären miRNAs in Rinder-Granulosazellen charakterisiert und der mögliche Einsatz von extrazellulären miRNAs in der Rinder-Frühträchtigkeitsdiagnose beurteilt. Dazu wurden drei Experimente durchgeführt.

Zur Charakterisierung der miRNA-Expressionsmuster konnten mittels einer „Next-Generation Sequenzanalyse“ in Granulosazellen von präovulatorisch dominanten und subordinierten Follikeln vom Tag 19 des Östruszyklus insgesamt 315 bzw. 323 bekannte miRNAs identifiziert werden. Von diesen waren 64 miRNAs differentiell exprimiert, davon zeigten 34 miRNAs einschließlich des miR-183-96-182-Clusters eine Hochregulation, während die restlichen 30 miRNAs in präovulatorischen dominanten Follikeln im Vergleich zum subordinierten Follikel runterreguliert waren. Darüber hinaus bestätigt die negative Korrelation in den Expressionsmustern des miR-183-96-182-Clusters und seines validierten pro-apoptotischen Zielgens FOXO1 in präovulatorisch dominanten Follikeln die regulatorische Rolle von miRNAs während der follikulären Phase des Östruszyklus des Rindes.

Die funktionelle Analyse der miR-183-96-182-Cluster-miRNAs in kultivierten Granulosazellen zeigte, dass die Hoch- oder Runterregulation dieses miRNA-Clusters zu einer Suppression bzw. Erhöhung der Expression von FOXO1-mRNA bzw. Protein führte. Gleichzeitig erhöhte die Überexpression des miRNA-Clusters die Zellproliferationsrate, verringerte den Anteil der Zellen während dem G_0/G_1-Zellzyklusarrest und erhöhte den Prozentsatz der Zellen während der S-Phase. Obwohl die Hemmung der Expression des miR-183-96-182-Clusters die Geschwindigkeit der Zellproliferation verlangsamte, wurden keine messbaren Veränderungen im Zellzyklusstatus beobachtet. Der selektive Knockdown von FOXO1 unter Verwendung von siRNA zeigte ähnliche Phänotypen, wie sie bei der Überexpression des miRNA-Cluster-Experiments beobachtet wurden.

Im dritten Experiment wurde eine miRNA PCR-Array-Plattform verwendet, um die Expressions-Signatur von extrazellulären miRNAs in Serumproben von trächtigen und nicht-trächtigen Kühen am Tag 19 und 24 nach der Insemination zu erstellen. Es konnten in Proben vom Tag 19 insgesamt 302 und 316 miRNAs nachgewiesen wurden. ?Am Tag 24 wurden 356 und 325 miRNAs identifiziert. Die Vergleichs-Expressionsanalyse ergab 8 sowie 23 differentiell exprimierte miRNAs am Tag 19 bzw. 24 bei trächtigen Kühen. Interessanterweise waren eine miRNA (miR-433) und 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p und miR-323a-3p) homolog zu dem humanen schwangerschafts-assoziierten C14MC-miRNA-Cluster exprimiert. Es wurde festgestellt, dass die vorhergesagten Zielgene der differentiell exprimierten extrazellulären miRNAs an Signalwegen beteiligt sind, die für die Trächtigkeitsimplantation, wie die Adhärens-Junction und die ECM-Interaktion, wichtig sind.

Zusammenfassend zeigt die vorliegende Studie die funktionelle Beteiligung von miRNAs im späteren Stadium der Entwicklung der Rinderfollikel durch koordinierte Ansteuerung von wichtigen Genen, die für die Bestimmung der follikulären Entwicklung wesentlich sind. Diese Studie zeigt einzigartige Expressionssignaturen von extrazellulären miRNAs, die potentielle Indikatoren für die frühe Trächtigkeit bei Milchkühen sein könnten.

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List of abbreviations

A	Adenine
Ago	Argonaute
AMH	Anti-Mullerian hormone
ANOVA	Analysis of variance
ART	Assisted reproductive techniques
ATP	Adenosin triphosphate
BMP	Bone morphogenetic proteins
BNC	Binucleate cells
bp	Base pair
bta	*Bos taurus*
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CCK-8	Cell counting kit-8
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
COC	Cumulus-oocyte-complex
COH	Controlled ovarian hyperstimulation
Cq	Quantification cycle
Ct	Cycle threshold
CTBP1	C-terminal binding protein-1
C14MC	Chromosome 14 microRNA cluster
C19MC	Chromosome 19 microRNA cluster
DCP2	Decapping protein 2
ddH_2O	Double distilled water
DF	Dominant follicle
DGCR8	DiGeorge critical region 8
DMEM	Dulbecco modified Eagle medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
eCG	Equine chorionic gonadotropin
EGA	Embryonic genome activation
ERα	Estrogen Receptor alpha

EtBr	Ethidium bromide
FASL	Fas ligand
FBS	Fetal bovine serum
FDR	False discovery rate
FF	Follicular fluid
FOXO1	Forkhead box protein O1
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF	Growth differentiation factor
gDNA	Genomic Deoxyribonucleic acid
GnRH	Gonadotropin releasing hormone
GO	Gene ontology
GPCRs	G protein–coupled receptors
hCG	Human chorionic gonadotropin
HDL	High -density lipoprotein
hsa	*Homo sapiens*
ICM	Inner cell mass
IFN-τ	Interferon tau
IM	Intramuscular
ISG	IFN-τ-stimulated genes
IU	International Unit
IVC	In vitro culture
KEGG	Kyoto Encyclopaedia of Genes and Genomes
Kb	Kilobase
KL	Kit-ligand/stem cell factor
KO	Knockout
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LE	Luminal epithelium
LNA	Locked nucleic acids
MAPK	Mitogen activated protein kinase

MET	Maternal-to-embryo transition
miRNA	MicroRNA
miR-183	MicroRNA-183
miR-182	MicroRNA-182
miR-96	MicroRNA-96
miRISC	MicroRNA induced silencing complex
mmu	*Mus musculus*
mRNA	Messenger RNA
MRP	Maternal recognition of pregnancy
MZT	maternal-to-zygotic-transition
n	Number of samples
NGS	Next-generation sequencing
OD	Optical density
OT	Oxytocin
OTR	Oxytocin receptor
PBS	Phosphate buffer saline
PCA	Principal Component analysis
PCR	Polymerase chain reaction
PGF2α	Prostaglandin F2-alpha
PGR	Progesterone receptor
PI	Propidium Iodide
PI3K	Phosphatidylinositide 3-kinase
Pri-miRNAs	Primary microRNAs
Pre-miRNAs	Precursor microRNAs
qRT-PCR	Quantitative real time polymerase chain reaction
r	Correlation coefficient
RISC	RNA-induced silencing complex
rno	*Rattus norvegicus*
RNAse	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of mean
SF1	Steroidogenic factor 1

siRNAs	Short interfering RNAs
SNP	Single nucleotide polymorphism
SREBP1	Sterol regulatory element binding transcription factor 1
SREBP2	Sterol regulatory element binding transcription factor 2
stRNAs	small temporal RNAs
5s rRNA	5s Ribosomal RNA
TBST	Tris-buffered saline with Tween 20
TC	Theca cells
TE	Trophectoderm
TGF-ß	Transforming growth factor beta superfamily
TIMP3	TIMP metallopeptidase inhibitor 3
TNF- α	Tumor necrosis factor-alpha
TRBP	TAR RNA-binding protein2
TSS	Transcription start site
U	Uracil
3´-UTR	3´-Untranslated region
UV	Ultra violet
U6 snRNA	U6 small non-coding small nuclear RNA
WNT4	Wingless type MMTV integration site family member 4
xg	Gravity
XRN1	Exoribonuclease 1
ZP	Zona pellucida
ZP3	Zona pellucida sperm-binding protein 3

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Chapter 1
General introduction

1.1 Bovine follicular development

Mammalian ovaries contain large amount of developmentally arrested primordial follicles reserve, where each follicle encompasses a quiescent oocyte arrested at prophase of meiosis I surrounded by a single layer of flattened pre-granulosa cells (Fortune *et al.* 1998). A cow is born with approximately 133,000 primordial follicles reserve that gradually deplete during the cow´s reproductive lifespan (Erickson 1966). Throughout the reproductive lifespan of a cow, primordial follicles progressively leave the arrested pool and continue to grow into primary follicles. The transition of primordial follicles into growing primary follicles is accompanied by the increase in the size of the oocyte, transformation of pre-granulosa cells from flattened to proliferating cuboidal cells and increase the number of granulosa cells forming a single layer around the growing oocyte (Fortune *et al.* 2000). Following this, growing follicles increase in size by adding a secondary layer of granulosa cells, deposition of material needed for zona pellucida (ZP) assembly, formation of cortical granule in oocyte cytoplasm and initiation of oocyte RNA synthesis (Driancourt 1991). Subsequently, growing follicles become responsive to gonadotropin (Fair 2003). During the transition of primordial follicles into primary and then to secondary follicles, a distinct layer of theca cells are organized around the growing follicle to interact with granulosa cells and cell-to-cell interaction is established between theca and granulosa cells (Parrott & Skinner 2000). It has been reported that granulosa cells secreted kit-ligand/stem cell factor (KL) could be responsible for recruiting theca cells from undifferentiated stromal cells during follicular recruitment (Parrott & Skinner 1997). During the course of subsequent phases of growth, the number of granulosa cells and the diameter of oocyte increase and the formation of the ZP is induced (Braw-Tal & Yossefi 1997). As follicles attain hundreds of granulosa cells, follicular transition to tertiary or antral follicles begins through the formation of theca externa, theca interna and basal lamina (Driancourt 1991). Finally, several fluid-filled patches of cavities merge and form a single bigger antral cavity filled with follicular fluid (Smitz & Cortvrindt 2002).

1.1.1 Bovine follicular waves

In bovine, follicular development occurs in a wave-like fashion with an average of two to three growth waves emerge per estrous cycle (Fortune 1994). The reason behind cows exhibiting either 2 or 3-waves is unclear. It was shown that there is no difference between lactating cows and nulliparous heifers in the proportion of 2 or 3-waves per estrous cycle (Wolfenson *et al.* 2004). Moreover, the number of waves per estrous cycle is not associated

with the breed and age of the cow (Adams *et al.* 2008). The bovine estrous cycle consists two distinct phases: the luteal and follicular phases. The luteal phase is the period which follows the ovulation and the formation of the corpus luteum (CL). It is the longer phase of the estrous cycle with an average duration of 14-18 days. Whereas, the follicular phase is the period between luteolysis (regression of the CL) and ovulation. It is the shortest phase of the estrous cycle with an average duration of 4-6 days. The emergence of a new follicular wave is preceded by a sharp rise of serum follicle stimulating hormone (FSH) concentration (Adams *et al.* 1992). On the day of ovulation (day 0), the first follicular wave emerges, while the second wave emerges 9-10 days after ovulation in cows with a two-wave pattern of the estrous cycle. Whereas, in cows, exhibiting three-wave pattern of follicular growth, the second and third waves emerge 8-9 and 15-16 days after ovulation, respectively (Mapletoft *et al.* 2002). Only the dominant follicle (DF) of the last follicular wave is destined to ovulate leaving the remaining follicles to undergo regression (Ginther & Kot 1994). In a three-wave pattern, each follicular wave lasts on average 5-7 days and is accompanied by recruitment, selection and dominance phases (Lussier *et al.* 1987). The mechanism and regulation of recruitment, selection and dominance during follicular waves are briefly discussed below. Figure 1.1 illustrates follicular wave dynamics in bovine.

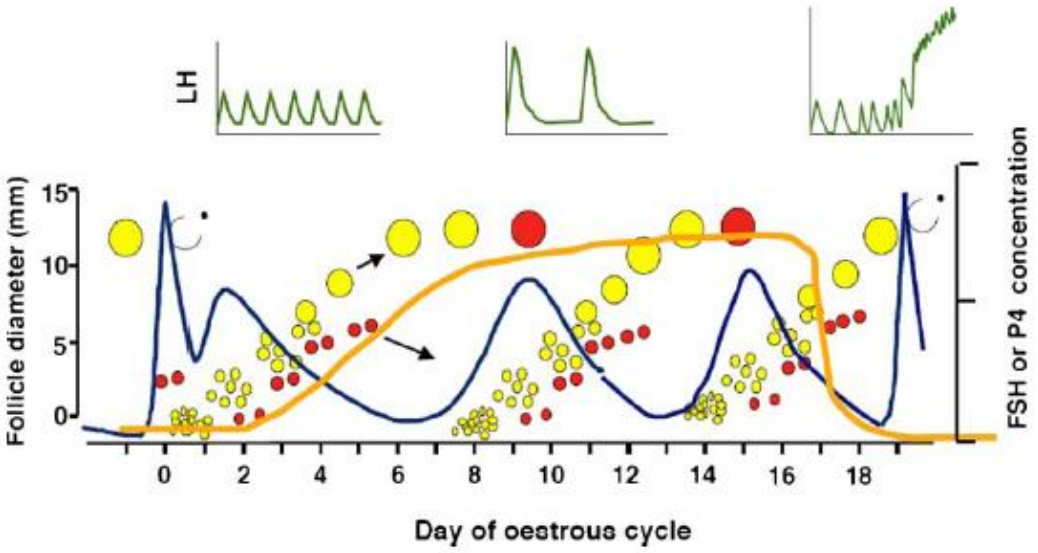

Figure 1.1: Schematic presentation of bovine follicular waves. Every 28 days, two or three follicular waves emerge preceded by a transient increase of FSH secretion (dark blue line). The relative size of follicles is indicated on the y-axis and healthy growing follicles and atretic follicles are shaded with yellow and red colors, respectively. A functional corpus luteum remains until the emergence of a wave during the follicular phase of the estrous cycle leading to elevated secretion of progesterone (yellow line). The frequency and

amplitude of the luteinizing hormone (LH) secretion are indicated in the top panel of the figure. A surge in LH and FSH during the follicular phase induces ovulation of the dominant follicle. Adapted from Forde *et al.* (2011).

1.1.1.1 Follicular recruitment

Follicular recruitment is a phenomenon where cohorts of small antral follicles emerge as a result of the increasing FSH level in the circulation. Follicular recruitment emerges after the regression of the DF in the first and second waves or after ovulation of preovulatory DF at the third wave. Regression or ovulation of the DF decline estrogen and inhibin synthesis and the decrease in the amount of estrogen and inhibin in circulation sends a negative feedback to the pituitary gland and result in FSH secretion increment which serves as a survival factor for small antral follicles (Armstrong & Webb 1997). If the rise in FSH is inhibited, the emergence of new follicular waves is delayed or even inhibited (Fortune 1994). Contrary to this, administration of exogenous FSH increases the number of recruited follicles in dose-dependent manner (Adams *et al.* 1993). Each wave recruits about 3-6 follicles to reach up to 4-5 mm of diameter (Fortune *et al.* 2001), of which one or two are selected to grow further and reach dominance.

1.1.1.2 Follicular selection and dominance

Following the recruitment of cohorts of small antral follicles, the future dominant and largest subordinate follicles are selected to develop further while the growth of the remaining follicles reduced (Fortune 1993; Chun *et al.* 1996). The growth of the DF and largest subordinate follicles progress in similar rate until they reach a stage called deviation, a point at which both follicles start to differ in their rate of growth (Ginther *et al.* 1997). The selected follicle increase in diameter until it acquires the capability to produce adequate estradiol and inhibin to suppress the elevated FSH level. The lower FSH concentration has an inhibitory effect on the emergence of new cohorts of smaller follicles. Once the selected follicle attained a diameter of > 5 mm, it acquires the capacity to suppress FSH secretion (Gibbons *et al.* 1999). As the diameter of follicles increase, the amount and concentration of estradiol production also increase accordingly. It was indicated that after deviation, the concentration of estradiol in the follicular fluid of the largest follicle is higher than the remaining small follicles and even the second-largest subordinate follicle (Bodensteiner *et al.* 1996). The suppression of FSH after deviation provokes atresia in the remaining subordinate antral follicles (Ginther *et al.* 2000). During selection of DF, a two-way functional coupling

between FSH and follicles is established, where the dominant follicles inhibit secretion of FSH through estradiol and inhibin production and on the other hand, the follicles depend on FSH for their growth (Ginther *et al.* 2001). After the deviation, the functional coupling remains limited between the DF and FSH, and it restricts the level of FSH to basal level, which helps the DF grow. During the dominance phase of follicular development, luteinizing hormone (LH) plays a critical role (Ginther *et al.* 2001). As the diameter of the DF surpass 8 mm, granulosa cells acquire LH receptors and make a transition from FSH dependent to LH dependent, which enables the DF to survive under basal FSH level (Mihm *et al.* 2006). Even though, basal level of FSH is required for the development of the DF during the dominance stage (Turzillo & Fortune 1993), the indispensable role of LH in dominance was further confirmed in heifers immunized for gonadotropin releasing hormones (GnRH) where administration of exogenous LH appeared to be necessary for the DF to attain ovulatory size (Crowe *et al.* 2001).

1.1.1.3 Ovulation and corpus luteum development

The DF of the last follicular wave prepares itself to ovulate through functional changes. For instance, declining the intrafollicular estradiol to progesterone ratio (Mihm *et al.* 2006). Moreover, the shift in the pulse frequency of hypothalamus affects the GnRH and promotes the release of LH (Nicol *et al.* 2008). The increased level of estradiol in circulation positively affects the GnRH to increase the release of LH which induces the preovulatory LH surge and cause the ovulation of the DF (Wiltbank *et al.* 2002). The preovulatory LH surge stimulates the luteinization of granulosa and theca cells (Smith *et al.* 1994). Gap junction within the cumulus cells during the preovulatory stage facilitates detachment of the cumulus-oocyte-complex (COC) from the follicular wall and remains freely floating inside the follicular fluid. In addition, the growing oocyte produces a factor that stimulates the production of hyaluronic acid by the surrounding cumulus cells (Salustri *et al.* 1990). The production of hyaluronic acid by cumulus cells in turn results in expansion of the cumulus cells.

Following the LH surge, the progesterone concentration increases which leads to increase in the expression of the progesterone receptor in granulosa cells of the preovulatory follicles (Cassar *et al.* 2002). Immediately after ovulation, the residues of the follicular wall cells (granulosa and theca cells) turn into CL which functions as a transient endocrine gland, which secretes predominantly progesterone. Progesterone is indispensable in pregnancy establishment and maintenance. Moreover, progesterone is necessary for the negative

feedback mechanism to suppress the release of pituitary gonadotropins during the luteal phase of the estrous cycle. Following ovulation, the increment in progesterone secretion reduces the pulse of LH but increase its amplitude.

1.1.2 Role of gonadotropins in follicular development

Gonadotropins are hormones that govern the estrous cycle released from the anterior pituitary gland and include the LH and FSH (Mark & Michael 2013). The estrous cycle is tightly regulated by the release of hormones from the hypothalamus (GnRH), pituitary (LH and FSH), ovaries (progesterone, estradiol and inhibin) and the uterine gland (prostaglandin F2α) (Roche 1996). The GnRH mediates the secretion of LH and FSH through its action on the anterior pituitary (Schally *et al.* 1971). During the follicular phase of the bovine estrous cycle, the level of progesterone declines to the basal level due to the regression of CL. Conversely, estradiol concentration increase due to the active growth of preovulatory follicle, which induces the surge of the GnRH from the surge center of the hypothalamus. Once the GnRH is transported into the anterior pituitary gland, it binds with the G protein–coupled receptors (GPCRs) which activate the release of LH and FSH from the cytoplasm of the gonadotropin cells (Weck *et al.* 1998). After the ovulation of the DF, the level of progesterone starts to rise due to the differentiation of the residual granulosa and theca cells of the ovulating DF into progesterone secreting luteal cells. The LH is the major hormone responsible for the differentiation of the granulosa and theca cells into luteal cells (Milvae *et al.* 1996). Figure 1.2 illustrates the feedback mechanisms of gonadotropins during follicular development.

1.2 Bovine embryonic development

Successfully ovulated and developmentally competent oocyte surrounded by cumulus cells is destined to undertake series of physiologically important processes in the maternal endometrium. The following section will briefly discuss the physiological processes of fertilization, early cleavage and implantation of embryos.

1.2.1 Sperm capacitation and fertilization

Following ovulation, the COC is released into the bursal cavity and then to the infundibulum where it is directed towards the oviduct through the exterior cilia covering the exterior surface of the infundibulum. Transportation of the COC towards the ampulla where

fertilization takes place is facilitated by the increased ciliation of the luminal epithelial cells as a result of elevated level of estradiol prior to ovulation (Gaddum-Rosse & Blandau 1976).

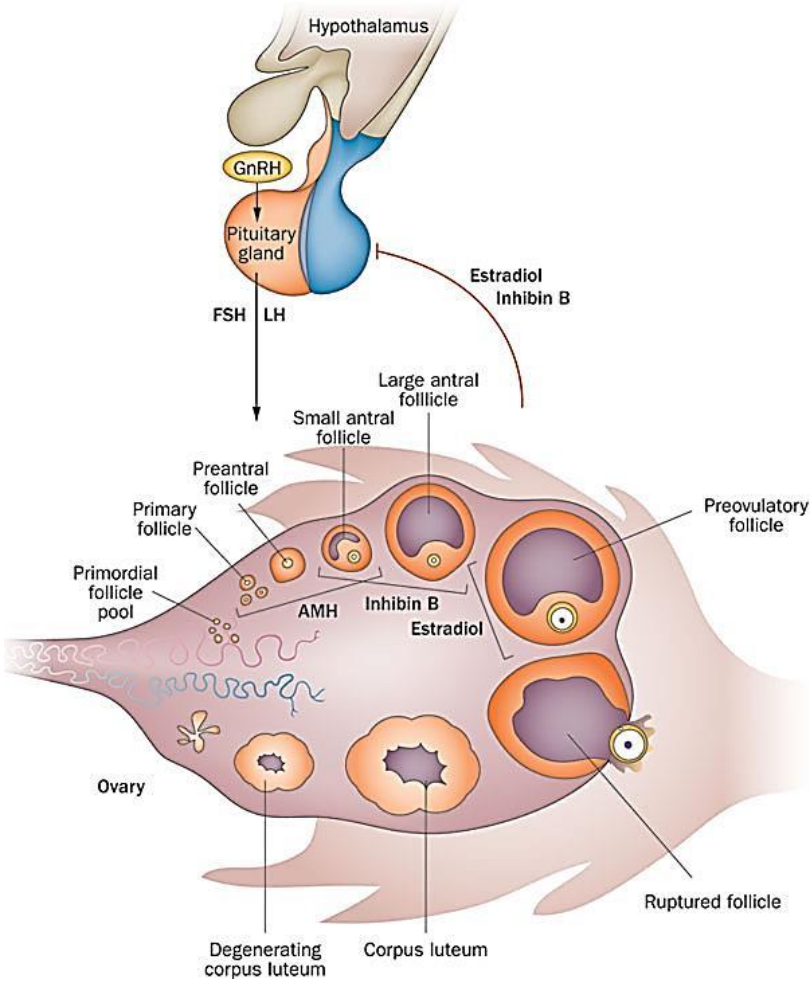

Figure 1.2: Dynamics of follicular recruitment, dominance, ovulation and feedback mechanism of gonadotropins. Follicles are continuously recruited from the primordial follicle reserve from which limited numbers of follicles are selected under the influence of FSH. Finally, one follicle ovulates under the influence of LH and the remaining follicles undergo atresia. The release of FSH and/or LH from the pituitary is governed by the negative feedback mechanism. The dominant follicle secretes more estradiol and inhibin B that inhibits the release of FSH from the pituitary, FSH-dependent growth of small follicles and the emergence of a new follicular wave. The reduction of estradiol and inhibin as a result of regression or ovulation of the DF increases the level of FSH and LH. Adapted from Visser *et al.* (2012).

Finally, the fully-grown oocyte undergoes meiotic maturation and prepares itself to interact with sperm. Following insemination or copulation, spermatozoa deposited in the female reproductive tract go through obligatory maturational processes in the female genital tract through processes termed as "capacitation" and "acrosome reaction". The capacitation process includes series of functional, biochemical and biophysical modifications induced by the interactions between the sperm cells and the epithelial tubal cells that increase the competence of the spermatozoa to fertilize the oocyte (Baldi *et al.* 2000). It involves cAMP-dependent protein tyrosine phosphorylation mediated removal of inhibitory effects of the decapacitation factors and rearrangements of lipid and protein (Visconti & Kopf 1998). Moreover, changes in the pH and intracellular Ca^{+2} further facilitate the capacitation process by improving motility of the spermatozoa. Only capacitated sperm cells are physiologically able to undergo acrosome reaction upon interaction with the ZP of the oocyte. During the acrosome reaction, the outer acrosomal membrane fuses with the plasma membrane that leads to the formation of multiple vesicles. Acrosome-reacted sperm interacts with the ZP through the zona pellucida sperm-binding protein 3 (ZP3) and penetrate into the perivitelline space through the sperm head. Following penetration of the sperm head, the plasma membrane of the oocyte fuses with the equatorial segment which leads to the disappearance of spermatozoa nuclear membrane (Yanagimachi 1988). The fusion of the sperm and oocyte cytoplasm leads to the activation of the oocyte by releasing the cortical granules and resumption of meiosis. After the release of cortical granules, the ZP undergoes chemical changes that prevent further penetration of sperms (polyspermy) through a process called "zona block". The resumption of meiosis in oocyte leads to the exclusion of the second polar body and formation of female pronucleus. Decondensation of the male pronucleus is important to make chromosomal pairing up with the female pronucleus. Finally, male and female pronuclei come to close proximity and fuse and the first mitotic division starts (Yanagimachi 1988). The processes of sperm capacitation and acrosome reaction are summarized in figure 1.3.

1.2.2 Early embryonic cleavage and embryonic genome activation

Following the fusion of male and female pronuclei, the zygote proceeds with series of mitotic cleavages where each cleavage results in two daughter blastomeres and progressively attain 2-, 4-, 8-, 16-cell embryo. The embryo continues to cleave and gives rise to a solid ball of cells termed the "morula". During each cleavage within the ZP, there is an

increase in the number of cells as opposed to the cytoplasmic total mass, which remains constant.

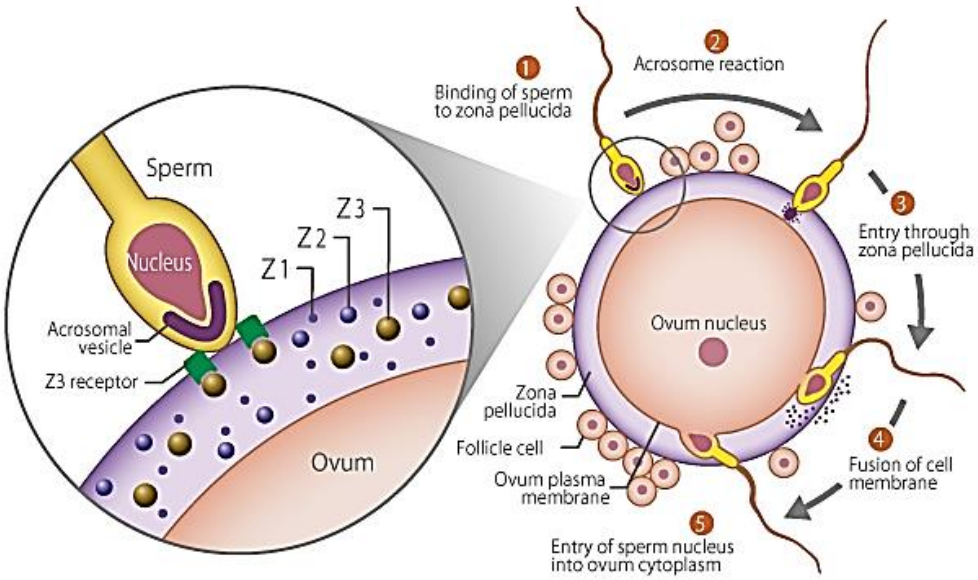

Figure 1.3: Initial processes of sperm capacitation, acrosome reaction and fertilization. Capacitated sperm binds with the ZP and reacts with the ZP3 receptors found on the ZP of the oocyte (**1**) and acrosome reaction is initiated through the fusion of the outer acrosomal membrane with the plasma membrane of the sperm cell and creates pores to allow acrosomal enzymes to pass through (**2**). The sperm membrane penetrates through the ZP (**3**) and the plasma membranes of the oocyte and the sperm start to fuse (**4**) and the nucleus of the spermatozoa is released into the oocyte (**5**) and start the process of decondensation to facilitate chromosomal recombination between the male and female pronuclei. Adapted from (http://csls-text.c.u-tokyo.ac.jp/inactive/12_07.html).

The outer cells of the morula are more compacted than the inner cells and that leads to the development of gap junction in the inner cells and tight junction in the outer cells. The development of tight junction in the outer cells of the morula compromises their permeability. Subsequently, water and Na^+ start to diffuse towards the embryo and a fluid-filled blastocoel cavity and a blastocyst with a recognizable cavity and two distinct cell population; the inner cell mass (ICM) and trophectoderm (TE) are formed (Magnuson *et al.* 1978). The outer cells of the blastocysts, trophoblast cells, will develop into the fetal component of placenta and the ICM will give rise to the body of the embryo through differentiating into two distinct cells, the endoderm and mesoderm (Schlafer *et al.* 2000). As

the blastocyst continue to divide, the amount of fluid in the blastocoel increase, the trophoblastic cells start to secrete proteolytic enzymes to weaken the ZP and increase pressure pulses by the blastocyst. Finally, the ZP ruptures and the blastocyst hatches to become free-floating in the uterus of the cow.

Early stages of bovine embryo development are controlled by maternal transcripts and proteins loaded into the oocyte during oogenesis. As the embryo progress to develop, the maternal-to-zygotic-transition (MZT) is trigged by degrading a subset of maternal transcripts and initiation of transcription of the embryonic genome which is referred as embryonic genome activation (EGA) (Tadros & Lipshitz 2009). Degradation of maternal transcripts through deadenylation inducing proteins binding to the 3´-untranslated region (UTR) of genes (Schier 2007) and subsequent transcription of the embryonic genome increases the number of embryo-derived transcripts (Sirard 2010). The timing of the EGA is species-dependent and in bovine it is suggested to start between 8- and 16-cell stages, while the main EGA is preceded with minor activation of the embryonic genome at 2- to 4-cell stage (Memili *et al.* 1998). During the minor EGA, some transcripts associated with cell cycle, cell proliferation and metabolism are activated (Kanka *et al.* 2012). The processes of embryonic cleavages and timing of genome activation in bovine embryos are illustrated in figure 1.4.

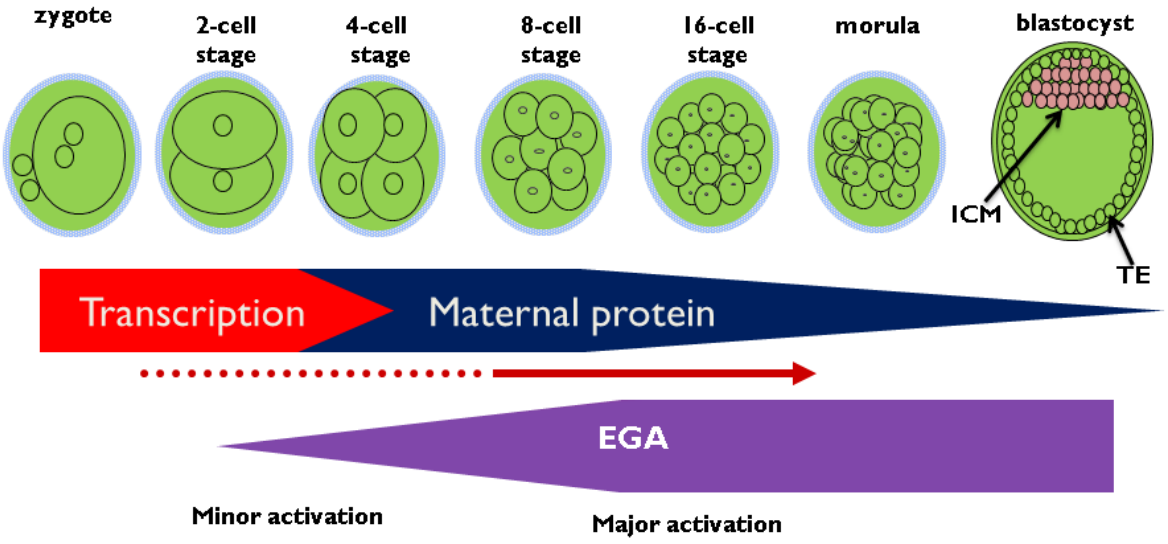

Figure 1.4: Early embryonic cleavages and activation of the embryonic genome. The bovine zygote undergoes series of mitotic divisions in the oviduct, which results in increasing the number of blastomeres until it reaches the morula stage. The morula enters the uterus and proceeds to form blastocyst comprising the blastocoel, ICM and TE. The

embryo utilizes maternal transcripts and proteins until the minor genome activation, which starts at 2-cell stage, and the major genome activation after the 8- to 16-cell stage.

1.2.3 Maternal recognition and implantation of pregnancy

The hatched blastocyst continues to expand and remains free-floating and autonomous in the uterus. In order the pregnancy to survive under the hostile maternal endometrium, luteal regression should be blocked by biochemical signals released from the growing embryo. The phenomenon in which the embryo secretes an interferon tau (IFN-τ) protein as signaling molecule is known as the maternal recognition of pregnancy (MRP) (Roberts *et al.* 1999). The antiviral, anti-proliferative and immunomodulatory characteristics of IFN-τ signify its additional role in protecting the conceptus from viral infection and modulating maternal immune system (Roberts 1989). In addition, progesterone plays important roles during pregnancy establishment by initiating the window of endometrial receptivity, avoiding abortion by inducing non-contractility of myometrium and protecting the embryo from maternal immune system insults (Soloff *et al.* 2011). During pregnancy, progesterone is mainly produced by the CL and its regression during the later stage of the gestation period will not compromise the pregnancy as the placenta attains the capability to produce sufficient amount of progesterone from the maternal cholesterol (Senger 2005). The mechanism in which the IFN-τ acts on the endometrium is through oxytocin (OT)-dependent reduction of prostaglandin F2α (PGF2α) synthesis. The production of OT by bovine CL stimulates endometrial cells to synthesize PGF2α, which requires coupling of the OT with the oxytocin receptor (OTR). As a result, the IFN-τ acts on the endometrial cells to prevent the synthesis of OTR, which later prevents the luteolysis to sustain the lifespan of CL and elevate production of progesterone. In addition, IFN-τ enhances the expression of several genes referred as IFN-τ-stimulated genes (ISG). The ISG are implicated in regulating uterine receptivity and conceptus development (Spencer *et al.* 2007). The mechanisms involved in maternal pregnancy recognition are illustrated in figure 1.5.

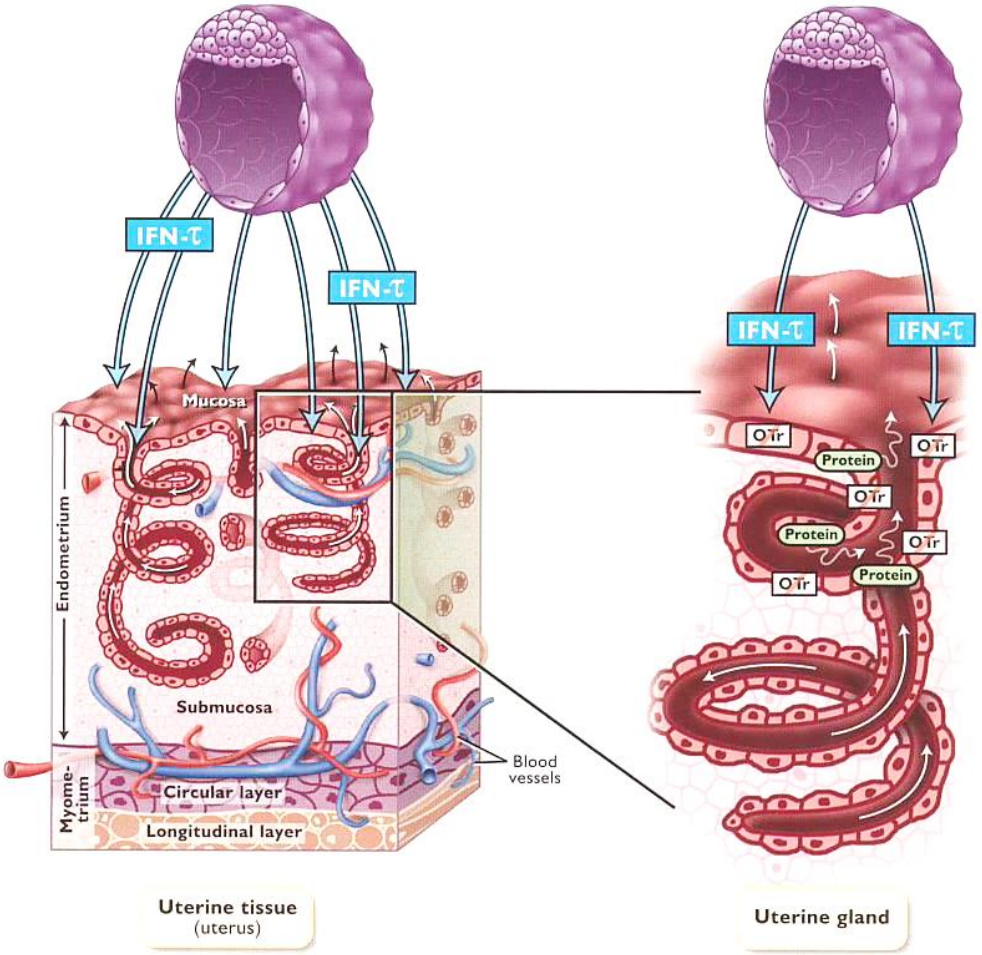

Figure 1.5: Maternal recognition of pregnancy. The trophoblastic cells of hatched bovine blastocyst secrete IFN-τ, which acts on the endometrial cells of the uterus to inhibit the production of OTR. The inhibition of OTR inhibits oxytocin-mediated the stimulation of PGF2α and prevent the regression of the corpus luteum. Moreover, IFN-τ promotes the production of proteins from the uterine glands that nourishes the embryo. Adapted from Senger (2005).

Implantation of conceptus comprises several phases. It starts with the hatching of the blastocyst by shedding the ZP and adjusting the orientation of the trophectoderm to create precontact and apposition with the endometrial luminal epithelium (LE). Following this, the trophoblast papillae will be formed and extended into the uterine glands and firm adhesion between trophectoderm and the endometrial LE is established. Several adhesion molecules are secreted by endometrial LE to facilitate adhesion. Finally, mononuclear trophoblast cells start to differentiate into binucleate cells (BNC) and migrate to fuse with the LE (Guillomot 1995). Figure 1.6 illustrates the processes of blastocyst implantation in maternal endometrium.

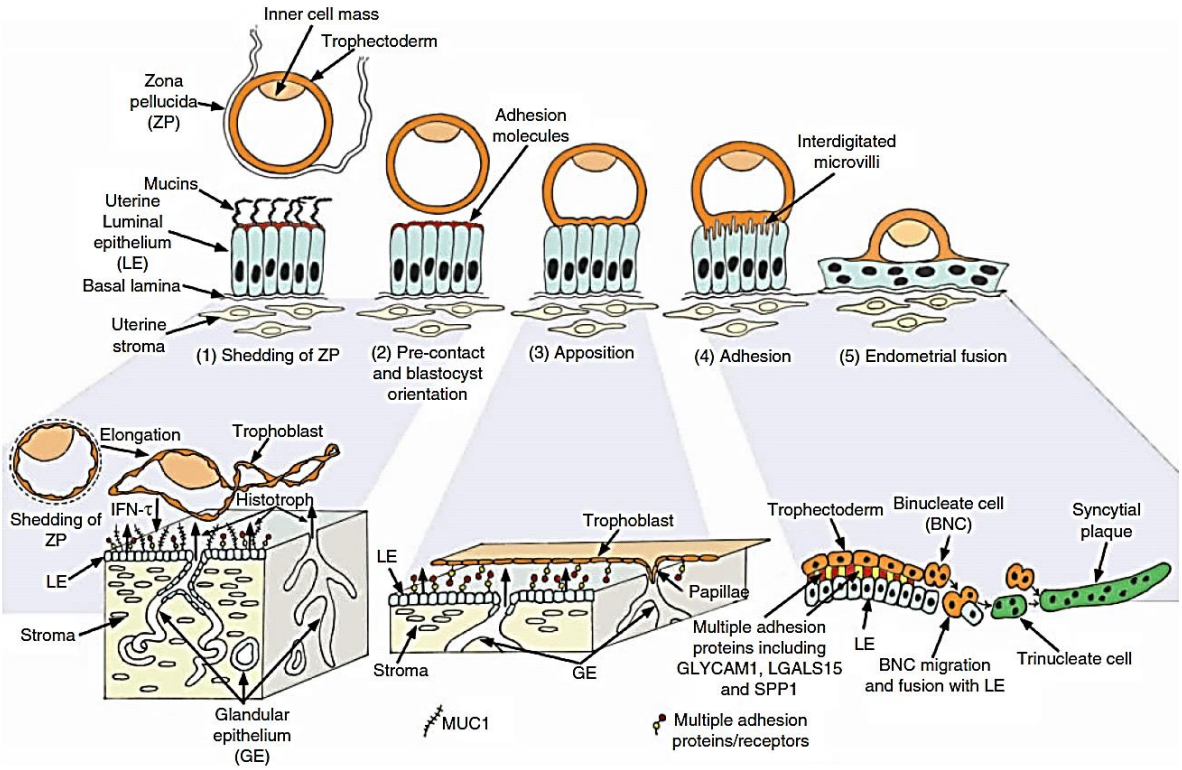

Figure 1.6: Implantation of blastocyst in the maternal endometrium. Implantation of the blastocyst starts with shedding of the ZP (phase 1) followed by precontact orientation (phase 2), where the conceptus migrates towards uterine horn ipsilateral of the corpus luteum. During the apposition stage (phase 3), elongation of blastocyst forms a filamentous conceptus with trophoblast papillae and a transient attachment of trophectoderm to endometrial LE is formed. Adhesion of the trophectoderm to the endometrial LE is formed with the several adhesion proteins are involved (phase 4). Finally, the binucleate cells differentiate from mononuclear trophoblast cells and create trinucleate cells to facilitate endometrial fusion (phase 5). Adapted from Spencer *et al.* (2007).

1.3 Transcriptional regulation of follicular and embryonic development

Bovine follicular development requires appropriate expression of multitude of genes at different stages. During early folliculogenesis, stage-specific expression of oocyte-specific genes like Figla, Nobox, Kit and Ntrk2 and genes expressed in the surrounding somatic cells such as Foxl2, Kitl and Ngf have been reported (Choi & Rajkovic 2006). During the formation of primordial follicles, Figla, Nobox, Pten, Foxo3A NGF genes are shown to be

involved. Similarly, βFGF, GDF9 and BMP4 are shown to be involved in the transition of primordial follicles into primary follicles (Monget *et al.* 2012).

During the course of follicular development, bi-directional communication between the oocyte and the surrounding cumulus cells, granulosa cells and theca cells are essential (Eppig 2001). Several extracellular signaling molecules are involved in the bi-directional dialogue. The transforming growth factor-beta (TGF-ß) superfamily are among the key intraovarian regulators implicated in this dialogue. The TGF-ß super family are expressed in oocyte and somatic cells in stage-dependent manner playing key regulatory role during folliculogenesis (Findlay *et al.* 2002).

Transcriptome analysis of oocytes and cumulus cells matured in the presence or absence of the respective companion cells was performed using microarray technique. Results showed that arrays of genes are exclusively expressed in either the oocyte and /or cumulus cells. This signifies the molecular cross-talk between oocyte and the surrounding cumulus cells (Regassa *et al.* 2011). Similarly, transcriptome of cumulus cells derived from in vitro and in vivo matured COCs was analyzed using customized cDNA microarray. It was revealed that 6 genes were differentially expressed between cumulus cells of the two groups (Tesfaye *et al.* 2009a). In the same study, it was reported that genes associated with cumulus cell expansion (TNFAIP6) and oocyte maturation (INHBA and FST) were enriched in cumulus cells of in vivo matured COCs and cumulus cells of in vitro matured COCs are enriched with genes associated with stress response including members of the heat shock protein; HSPA5 and HSP90AB1. This could support the notion that the environment at which the COC are mature determines the developmental competence of the oocyte due to the fact that the cumulus cells serve as mediators of the interactions between the oocyte and the surrounding environment (Sutton *et al.* 2003; Sirard *et al.* 2007).

The transition of follicles from primordial into early antral follicles is accompanied by oocyte enlargement, formation of ZP and proliferation of granulosa cells. Members of the TGF-ß superfamily are implicated in primordial follicle activation. Among the family members, the bone morphogenetic proteins (BMP), the BMP-4 and BMP-7 are expressed in stromal/theca cells and are involved in primordial-to-primary follicle transition and enhancement of follicular survival (Nilsson & Skinner 2003). Similarly, the GDF-9, BMP-15, and BMP-6 are selectively expressed in oocytes of primordial follicles (Elvin *et al.* 2000). It was shown that mice null for GDF-9 are infertile with arrested folliculogenesis at primary stage (Carabatsos *et al.* 1998), signifying the role of GDF-9 in follicular progression. Conversely, the Anti-Mullerian Hormone (AMH) plays an inhibitory role in primordial follicle initiation (Durlinger

et al. 2002). Interestingly, mouse exposed to AMH in vitro showed reduced number of growing follicles (Durlinger *et al.* 2002). However, targeted deletion of AMH increase the rate of primordial follicle recruitment, which resulted in early depletion of follicular reserve (Durlinger *et al.* 1999).

As the follicle progress through the antral stage of development, granulosa and theca cells continue to proliferate and the oocyte increases its diameter. At this stage of development, follicles are under the influence of FSH (Knight & Glister 2006). Traditionally, gene expression analysis on follicular development was performed by studying the expression pattern of candidate genes. This has limited our understanding with regard to the global pictures of transcriptome dynamics in various stages of follicular development. However, the recent advancements in microarray and high throughput sequencing technology have provided powerful methodological advantages for large-scale transcriptomic study of bovine COC, granulosa and theca cells derived from follicles at various stages development.

Analysis of granulosa cell transcriptome of healthy small (< 5 mm) and large (> 10 mm) antral follicles revealed that the expression of genes in small follicles are more heterogeneous than in the large follicles, and as the follicles increase in size the expression of genes become more uniform (Hatzirodos *et al.* 2014b). In addition, the study showed that more numbers of pathways were activated in larger follicles than the small counterpart. This could be associated with the pronounced impact of LH on granulosa cells of larger follicles. The LH surge poses a great deal of transcriptional changes in granulosa cells of preovulatory follicles. Temporal expression of genes in granulosa cells of healthy small growing antral follicles before the induction of LH surge, 6 and 22 hours post-LH surge revealed an interesting pattern in the enrichment of genes. Pre-LH surge granulosa cells are enriched with genes involved in biological processes related to cell division, development and proliferation. Whereas genes enriched in granulosa cells in response to LH surge are involved in biological processes related to stimulus response, vascularization, and lipid synthesis. Interestingly, later response of granulosa cells to LH-surge was found to be associated with protein localization and intracellular transport which could be involved in the process of granulosa cell luteinization (Gilbert *et al.* 2011).

The transcriptional regulation of follicular atresia could be understood by profiling the transcriptome of granulosa cells from healthy and atretic follicle. Comparative analysis of bovine granulosa cells from healthy and atretic follicles revealed that healthy follicles are more heterogeneous than the atretic counterpart, implying higher variability in the follicular growth outcomes in health follicles as opposed to the atretic follicles, which are destined to

follicular demise (Hatzirodos *et al.* 2014a). Moreover, the same study reported that the transcription factor TP53 and its apoptosis-associated downstream genes were activated in the atretic follicles, and pathways like TGF-ß signaling pathway and tumor necrosis factor-alpha (TNF-α) pathway were among the top-ranking pathways. In an attempt to determine the transcriptional regulation of deviation in cattle, the transcriptome of granulosa cells derived from pre-deviation and during the onset of deviation during the follicular wave were analyzed and 83 genes were differentially expressed and that could be associated with deviation during follicular wave in cattle (Li *et al.* 2016). Interestingly, one of the genes enriched during the onset of deviation was CYP19A1; a member of the cytochrome p450 superfamily responsible estrogen biosynthesis and this could be attributed to the increased potential of estradiol production of follicles with growing diameter and after deviation (Beg & Ginther 2006).

The aforementioned dynamically regulated and highly orchestrated processes of follicular recruitment, selection of dominant follicles and subsequent ovulation or removal of anovulatory follicles through atresia and the formation and regression of corpus luteum are coordinately governed by endocrine and paracrine factors. It is evidenced that these factors are regulated by the expression and interaction of multitude of genes expressed in a spatiotemporal manner in different follicular cells (Bonnet *et al.* 2008). It is evident that understanding the posttranscriptional regulatory mechanisms of these important genes in various follicular cells would significantly widen our understanding on the genetic regulation of bovine follicular development.

1.4 MicroRNAs as posttranscriptional gene regulators

MicroRNAs (miRNAs); a class of single-stranded, small non-coding RNA molecules, 19-25 nucleotides in length emerged as regulators of gene expression at post-transcriptional level (Bartel 2004). It was first discovered as small temporal RNA (stRNA) that regulates post-embryonic developmental transition in *C. elegans* and named as lin-4. The lin-4 encodes for a 22 nucleotides non-coding RNA with partial complementarity to 7 conserved sites in the 3´-UTR of the nuclear protein-encoding host gene, the lin-14 (Lee *et al.* 1993). Downregulation of lin-14 gene at the end of first larval stage helps the second larval stage to proceed. For this, the presence of an intact 3´-UTR of the lin-14 mRNA and a functional lin-4 are required to repress its protein expression (Lee *et al.* 1993). The discovery of lin-4 and its impact on the translation inhibition pioneered the discovery of new mechanism in the

regulation of genes during development. The let-7 was the second miRNA to be discovered, in which the let-7 gene encodes a 21 nucleotides-long miRNA, which controls the transition of the 4th larval stage into adult in *C. elegans* (Reinhart *et al.* 2000). Identification of lin-4 and let-7 was achieved through the conventional forward genetics approach, where loss-of-function mutations on the miRNAs arrest the development timing of larvae of *C. elegans* (Lee *et al.* 1993; Reinhart *et al.* 2000). Using the forward genetics approach, two additional miRNAs, the bantam locus which encodes for miRNA (Hipfner *et al.* 2002) and miR-14 (Xu *et al.* 2003) were identified in *D. melanogaster*. Moreover, the miRNA lys-6, which controls neural asymmetry in *C. elegans,* was also discovered using the conventional forward genetics approach (Johnston & Hobert 2003). Nevertheless, due to the smaller size of miRNAs and their resistance to mutagenesis to bring mutation in the seed region, the number of miRNAs identified using the forward genetics approach is very limited. Moreover, the functional redundancy of several miRNAs makes phenotype-based identification of individual miRNAs became challenging (Abbott *et al.* 2005). However, advancements in cDNA library preparation and sequencing, microarray technology, qRT-PCR and deep sequencing have facilitated the identification of thousands of miRNAs from various species. Thus, the "miRBase", an online repository for published microRNA sequences and annotations contains 28,645 hairpin and 35,828 mature miRNA sequences, from 223 species (miRbase Release 21: June 2014, http://www.mirbase.org).

1.4.1 Biogenesis of miRNAs

The biogenesis of miRNAs is a multi-step process, which involves transcription of miRNA genes in nucleus followed by series of maturational processes in the cytoplasm. It starts with the transcription of miRNA genes mediated by RNA polymerase II (Pol II) or occasionally by RNA polymerase III (Pol III), which gives rise to the primary miRNAs (pri-miRNAs) transcript (Lee *et al.* 2003; Lee *et al.* 2004). Based on how the pri-miRNAs are further processed into mature miRNAs, there are two broader classes of miRNA biogenesis pathways; canonical and non-canonical ones. During the canonical pathway, the pri-miRNA is endonucleolytically cleaved by a microprocessor complex composed of the RNase II enzyme Drosha and DiGeorge critical region 8 protein (DGCR8) and gives rise to a 60-70 nucleotides long miRNA precursor (pre-miRNA) in the nucleus (Gregory *et al.* 2004). Drosha has two domains of RNase and it cleaves the 5′ and 3′ arms of the pri-miRNA by using the DGCR8 as stability enhancer and molecular ruler to precisely determine the site of cleavage (Han *et al.* 2006). The pre-miRNA contains 2 nucleotides overhang at the 3′ end,

which is important for DGCR8 binding and Drosha cleavage (Zeng & Cullen 2005). Conversely, the non-canonical miRNA biogenesis skips the Drosha-mediated cleavage of pre-miRNA. Instead, the pre-miRNA is generated through splicing of introns from mRNA transcript by splicing machinery (Ruby *et al.* 2007). Following the nuclear processing, in both canonical and non-canonical pathways, the pre-miRNAs are exported into the cytoplasm by Exportin-5 and Ran-GTP co-factor (Yi *et al.* 2003). In the cytoplasm, the pre-miRNA is further cleaved by another RNase III Dicer coupled with double-stranded TAR RNA-binding protein2 (TRBP) and generates a 22-nucleotide long miRNA-duplex (Hutvagner *et al.* 2001). Following the Dicer-mediated cleavage, the miRNA-duplex binds with Argonaute (Ago) protein and separated into functional guide and passenger strands. The guide strand is incorporated with RNA-induced silencing complex (RISC), whereas the passenger strand is degraded (Hutvagner & Zamore 2002). Even though it is assumed that both strands of the miRNA duplex could give rise to mature miRNAs, only one strand is incorporated in the RISC complex leaving the other strand to degrade. The thermodynamic stability of the base pairs at the end of the duplex determines the functional asymmetry, where the strand with less stable base pairs at its 5´end is loaded into the RISC complex (Schwarz *et al.* 2003). Figure 1.7 summarizes the steps in miRNAs biogenesis.

MicroRNA genes are distributed throughout all chromosomes in the genome either as a single miRNA or as a cluster of several miRNAs, and about 50% of all known miRNAs are localized in clusters (Lagos-Quintana *et al.* 2001) and transcribed as polycistronic primary miRNA transcripts (Lee *et al.* 2002). The relatedness of miRNAs in a given cluster could be associated with the fact that they arise from gene duplication. According to their genomic organization, miRNAs are broadly classified as intergenic and intragenic (intronic and exonic). Intergenic miRNAs do not overlap with other genes and are transcribed independently of the host gene´s promoter (Olena & Patton 2010).

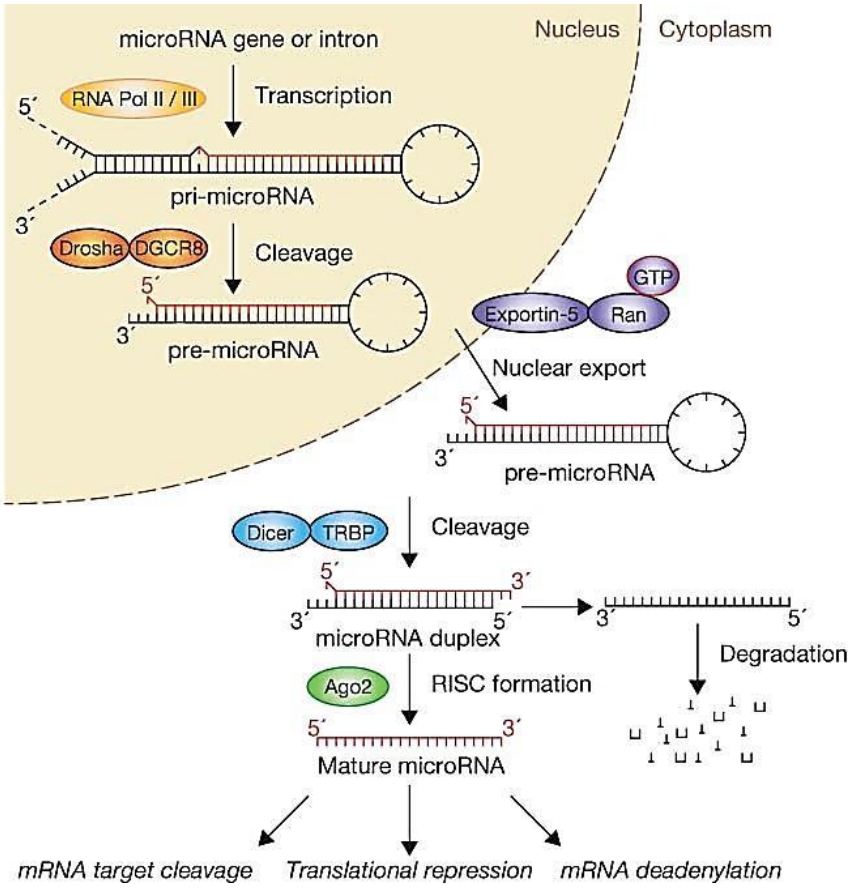

Figure 1.7: MicroRNA biogenesis pathway. The canonical pathway of miRNA biogenesis starts with the transcription of primary miRNA transcripts (pri-miRNA) and cleaved into pre-miRNA with the help of the Drosha–DGCR8 microprocessor complex in the nucleus. The pre-miRNA is exported from nucleus to the cytoplasm and further processed by Dicer and matured miRNA is loaded into RISC and silence target gene through either mRNA degradation or translational repression. Adapted from Winter *et al.* (2009).

Whereas, the intragenic miRNAs are transcribed from either the intronic or exonic regions of genes and utilize the transcriptional units of the host genes (Lagos-Quintana *et al.* 2003). Intronic miRNAs reside within the intronic regions of either protein-coding or non-protein coding RNAs. Interestingly, transcription of the host gene and an intronic miRNA can coincide and be co-regulated (Bartel 2004). In some cases, non-canonical miRNA biogenesis pathway could give rise to mitrons; where the intron of the host gene gives rise to pre-miRNA with splice site on either side (Ruby *et al.* 2007). Exonic miRNAs are also found overlapping with exon and intron of non-protein coding gene and transcribed by the

promoter of the host gene while the maturation pathway excludes the function of the host gene (Rodriguez *et al.* 2004). The genomic contexts of miRNA transcription are indicated in figure 1.8.

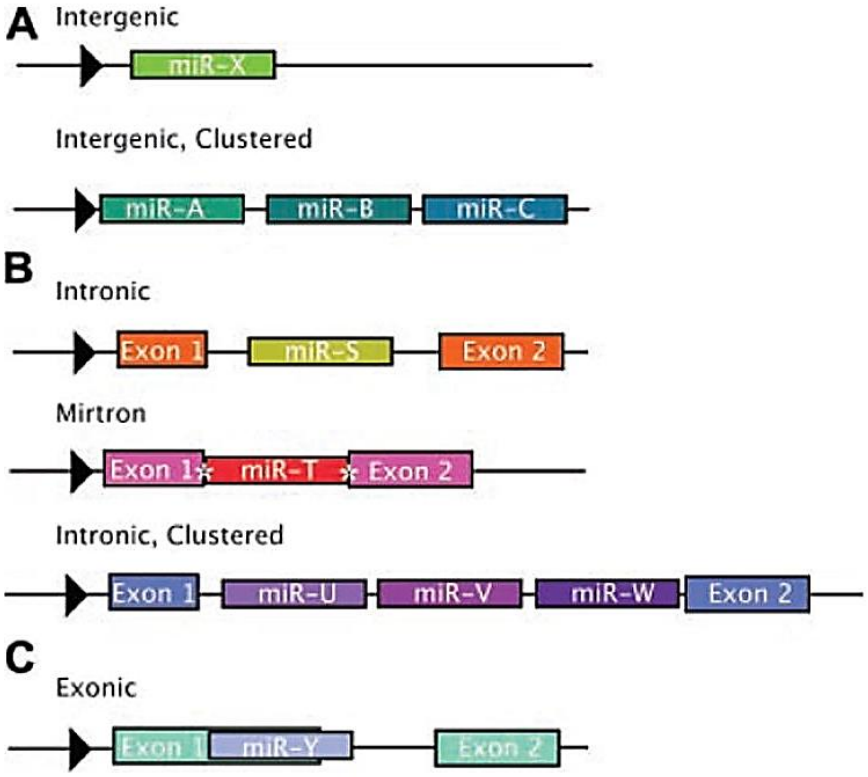

Figure 1.8: Genomic location of miRNAs. MicroRNAs are transcribed from distinct intergenic (**A**), intronic (**B**) or exonic (**C**) regions of the genome either as individual or cluster of multiple miRNAs. Adapted from Olena & Patton (2010).

1.4.2 Mechanisms of miRNA-mediated post-transcriptional gene silencing

The formation of miRNA-induced silencing complexes (miRISCs) through the association of miRNAs with the Ago protein family helps miRNAs exert their gene regulatory functions. The miRISCs complexes mediate silencing of target mRNAs with partial or full sequence complementary to the miRNAs (Ameres & Zamore 2013). In animals, the target genes have partial sequence complementarity with the miRNAs, and degradation through catalytically active Ago proteins is precluded, and Ago proteins recruit partner proteins like the GW182 proteins to facilitate gene silencing. A target gene is silenced either by repressing the translation or through accelerated degradation of the mRNA (Fabian & Sonenberg 2012). Enzymes involved in cellular mRNA decay pathway are involved in catalyzing miRNA target gene degradation. The mRNA decay pathway, starts with deadenylation of mRNA through

PAN2–PAN3 and CCR4–NOT deadenylase complexes (Wahle & Winkler 2013). This results in a significant reduction of the length of the poly(A) tail. Following the deadenylation, mRNAs will be decapped with the help of decapping protein 2 (DCP2) coupled with additional cofactors (Jonas & Izaurralde 2013). Finally, the major cytoplasmic endonuclease enzyme exoribonuclease 1 (XRN1) will degrade the deadenylated and decapped mRNAs. The mRNA decay machinery is efficiently utilized by the miRISCs by directly recruiting the GW182 proteins. The GW182 proteins are involved in miRISC mediated gene silencing by binding to the W motifs of the Ago proteins and to multitude of other silencing complexes (Chekulaeva *et al.* 2009). In addition to the mRNA degradation, miRNAs are also involved in translational repression. To determine the contribution of miRNAs in translational repression in cells, global ribosome profiling analysis indicated that miRISCs predominantly act through mRNA degradation. Whereas, translational repression accounts for only 2-26% of endogenous target gene repression (Guo *et al.* 2010; Eichhorn *et al.* 2014). MicroRNAs repress translation in a cap-dependent manner, in which mRNAs lacking functional 5´-cap structure are repressed by endogenous miRNAs. It was shown that addition of cap-binding complex eIF4F in mouse stimulated ribosomal recruitment and antagonized miRNA-mediated silencing (Mathonnet *et al.* 2007). Mechanisms of miRNA-mediate gene silencing are briefly illustrated in figure 1.9.

MicroRNAs interact with the 3´UTR of their target mRNA according to the Watson-Crick base pairing principle. Unlike in plants, where most miRNAs interact with target mRNAs in perfect complementarity (Jones-Rhoades *et al.* 2006), animal miRNAs base pair with their target imperfectly. Experimental and bioinformatics analysis have identified several rules with regard to the miRNA-mRNA interactions.

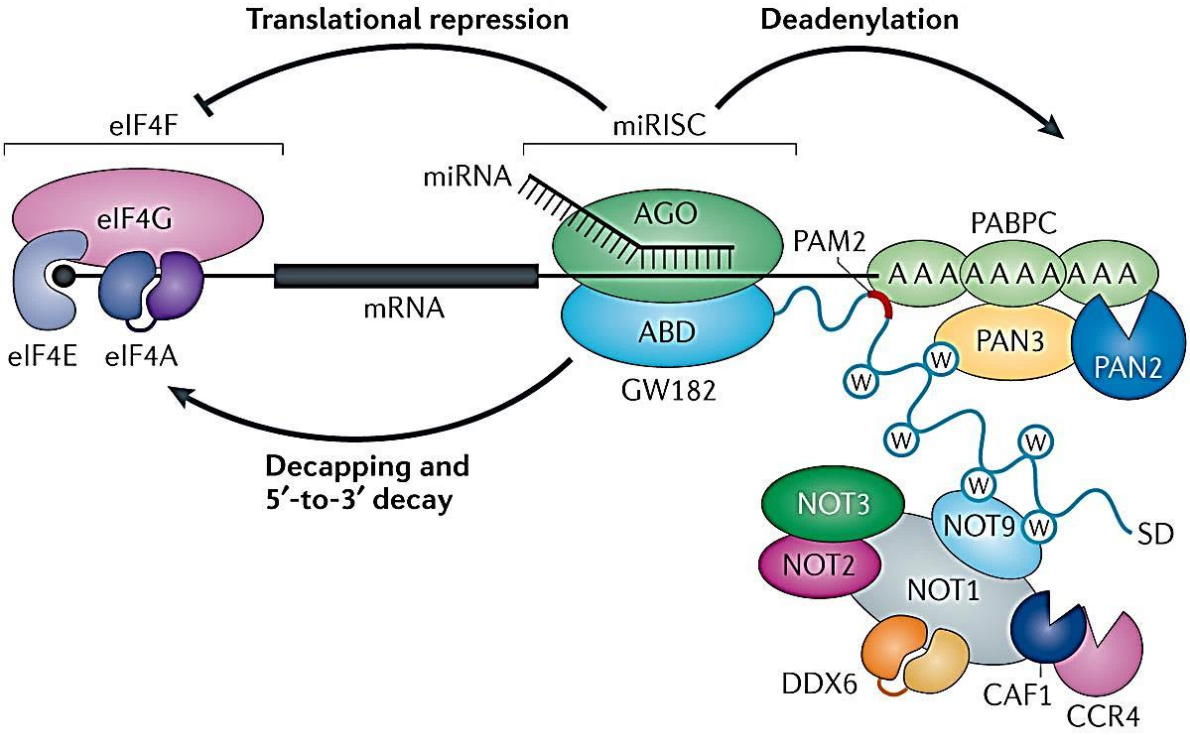

Figure 1.9: Mechanism of miRNA-mediated gene silencing. MicroRNA bound to the Ago protein in miRISC recognizes binding sites within the 3′-UTR region of the target gene. The poly-A tail of the target mRNA is deadenylated, decapped and rapidly degraded by endo nuclease enzyme XRN1. MicroRNAs also repress translation by interfering with the activity and assembly of eIF4F complex. Adapted from Jonas & Izaurralde (2015).

A perfect miRNA-mRNA base pairing between the 3′ UTR region of the target mRNA and the 'seed' region of miRNAs (nucleotide position 2 to 7) in the 5′ end of mature miRNAs have better target repression ability. Whereas, mismatches and GU pairs in the seed region greatly affects the repression efficiency. Moreover, a residue at position 1 and A or U at position 9 of the miRNA improve site efficiency (Brennecke *et al.* 2005). It is also noted that reasonable complementarity of the 3′ of miRNAs is necessary to stabilize the miRNA-mRNA interactions. In the presence of suboptimal base pairing in the seed region, good base pairing in the position 13-16 of the miRNA help stabilize the interaction (Lewis *et al.* 2006). In addition, several other factors like AU-rich neighborhood, the length of the 3' UTRs, position in relation to the poly(A) tail and multiple copies of binding sites can influence the efficacy of miRNA target recognition and accessibility of the binding sites (Grimson *et al.* 2007). Figure 1.10 describes mechanisms of miRNA target gene recognition.

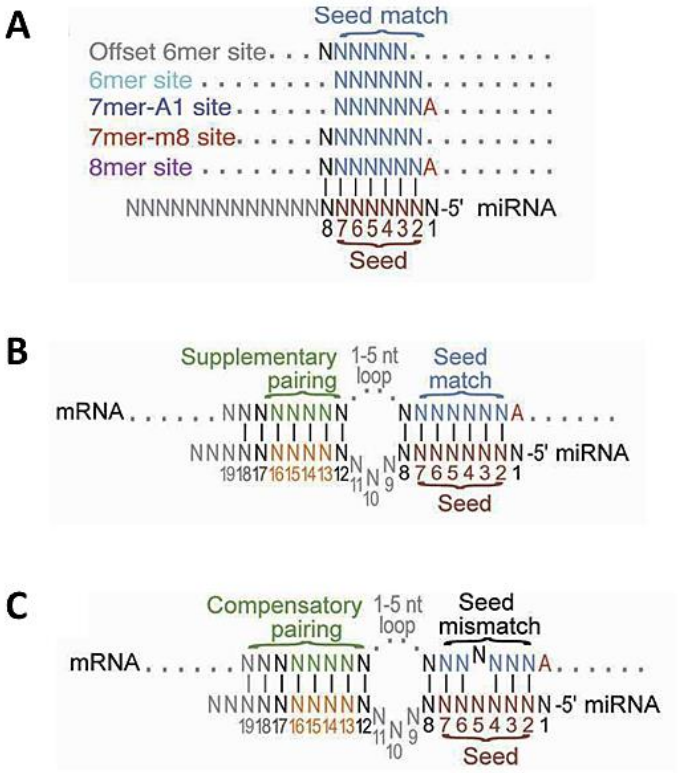

Figure 1.10: MicroRNA target recognition principles. The seed region (nucleotides 2–7) of a miRNA share six contiguous Watson–Crick matches to the target mRNA and the offset 6mer contains six contiguous matches to nucleotides 3–8 (**A**). Preferential pairing to the 3′ end of the miRNA can occur either as a supplementary pairing (**B**) or as a compulsory pairing (**C**). Modified from Friedman *et al.* (2009).

The expression of miRNAs is cell- or tissue- specific and could be under the influence of hormonal profile and/or stage of development making functional studies of miRNAs very challenging (Sood *et al.* 2006). The function of miRNAs in one cell or tissue might not be identical in another cells or tissues. In addition, the functional redundancy of miRNAs, targeting of multiple genes by a single miRNA and a single gene being targeted by several miRNAs further complicate functional study of miRNAs. The functional involvement miRNAs in follicular and embryonic development are discussed below.

1.4.3 Involvement of miRNAs in ovarian function and embryo development

Several studies have reported that miRNAs are expressed in ovaries of several mammalian species including in human (Liang *et al.* 2007), mice (Ro *et al.* 2007), cow (Hossain *et al.* 2009), goat (Ling *et al.* 2014), sheep (Di *et al.* 2014) and pig (Li *et al.* 2011). Involvement of

miRNAs in ovarian function was first verified in mouse, where Dicer1 knockout (KO) mice were embryonic lethal (Bernstein *et al.* 2003) and mice mutant for Dicer1 alleles ($Dicer^{d/d}$) were infertile due to failure to form corpus luteum (Otsuka *et al.* 2008). Moreover, conditional inactivation of Dicer1 in mouse ovarian granulosa cells, oviductal and uterine cells reduced the rate of ovulation, shorten the uterine horns and leads to the formation of oviductal cysts (Nagaraja *et al.* 2008). Thus, Identification of miRNAs in ovaries of various mammalian species shifted the research focus towards deciphering their regulatory role in follicular cells and oocyte during various stages of follicular development.

1.4.3.1 MicroRNAs in mammalian follicular development

MicroRNAs are implicated in primordial follicle formation and maintenance. Study on neonatal mouse identified three miRNAs (miR-143, miR-145, and miR-376a) enriched in ovarian tissue (Xu. *et al.* 2011). Moreover, the study showed that mouse ovaries transfected with miR-376a resulted in primordial follicle increment and reduced oocyte apoptosis through the downregulation of PCNA and other pro-apoptotic genes (BAX, TNF and TNFR-2). Conversely, the expression of the anti-apoptotic BCL2 and oocyte survival genes; Pard6a, Lhx8 increased upon miR-376a overexpression in mouse neonatal ovaries (Zhang *et al.* 2014). Moreover, the miR-143 is identified to be selectively expressed in mouse pre-granulosa cells and inhibition of miR-143 reduced the expression of genes related to cell cycle and proliferation of pre-granulosa cells (Zhang *et al.* 2013). Similarly, miR-145 regulate follicular activation in neonatal mouse by repressing the expression of the Zona pellucida sperm-binding protein genes (Zp1, Zp2, and Zp3), which in turn activate the TGF-ß signaling pathway by increasing the expression of TGFBR2 (Yang *et al.* 2013). During follicular recruitment and selection of the dominant follicles miRNAs are indicated to play important regulatory roles. Comparative analysis of miRNAs in large vs small and large healthy vs large atretic bovine follicles showed that a total of 523 miRNAs were expressed in both follicular categories and miR-144, miR-202, and miR-873 were highly abundant in large healthy follicles compared to the large atretic follicles counterpart. In addition, the study suggested miR-873 as a promising candidate miRNA with regulatory role during follicular selection (Sontakke *et al.* 2014). The association of miRNAs with follicular selection and ovulation was also examined in horse by comparing dominant vs. luteinized follicles (Schauer *et al.* 2013). In that study, it was found that 6 miRNAs (miR-132, miR-212 miR-21, miR-145, miR-224, and miR-378) to be differentially expressed.

1.4.3.2 MicroRNAs in granulosa cell functions

Proliferation, differentiation and apoptosis of granulosa cells are essential for normal follicular development, oocyte maturation and follicular atresia. Growing number of studies have indicated that miRNAs are involved in granulosa cell functions (Sirotkin *et al.* 2009; Carletti *et al.* 2010; Yin *et al.* 2014). The granulosa cells are widely studied type of ovarian cells with regard to the role of miRNAs in ovarian function. Interestingly, majority of the miRNA functional studies widely use an in vitro granulosa cell culture model through loss-and-gain of function to modulate the levels of selected miRNAs (reviewed in Maalouf *et al.* (2016)). Several miRNAs are indicated to be involved in granulosa cell proliferation. For instance, primary human granulosa cells transfected with miR-15a inhibitor and mimic. It was revealed that the expression of cell proliferation marker genes (MAPK/ERK1, 2 and PCNA) increased upon inhibition of miR-15a. Contrary to this, overexpression of miR-15a resulted in opposite effect (Sirotkin *et al.* 2014). Another study showed that, overexpression of miR-224 induced granulosa cell proliferation in a TGF-β1-dependent manner by targeting the SMAD4 gene. Whereas, inhibition of miR-224 suppressed granulosa cells proliferation induced by TGF-ß1 treatment (Yao *et al.* 2010). Treatment of mouse granulosa cells with activin A decreased the expression of miR-181a and proliferation of granulosa cell was reduced upon miR-181a overexpression through CCND2 and PCNA downregulation, signifying the interplay between miR-181a and acvr2a in regulating granulosa cells proliferation (Zhang *et al.* 2013). Granulosa cells treated with TGF-β1 reduced the expression of miR-320 (Yao *et al.* 2010) and overexpression of miR-320 in mouse granulosa cells inhibited cell proliferation by targeting E2F1 and SF-1. Interestingly, miR-380 suppressed proliferation of granulosa cells indirectly through miR-320 transactivation (Yin *et al.* 2014).

Throughout the stages of ovarian follicular development, the majority of the follicles undergo atresia triggered by granulosa cells apoptosis. Accumulated evidence is available signifying the role of miRNAs in granulosa cells apoptosis and determining the fate of follicular cells towards ovulation or atresia. In one of the early miRNA functional study, miR-21 as one of the three highly induced miRNAs in murine granulosa cells upon administration of an ovulatory dose of LH, induced granulosa cell apoptosis upon its inhibition (Carletti *et al.* 2010). In the same study, in vivo anti-miR-21 treated ovary resulted in reduced rate of ovulation compared to the untreated contralateral ovary. Similarly, SMAD7 is directly targeted by miR-92a where the expression of miR-92a was lower in porcine atretic follicles and overexpression of miR-92a in vitro prevented granulosa cell apoptosis by targeting

SMAD7 (Liu *et al.* 2014b). On the other hand, let-7g induces apoptosis in pig granulosa cells by targeting the TGFBR1 gene and downregulating the TGF-ß signaling pathway (Zhou *et al.* 2015). Similarly, miR-26b promotes porcine granulosa cells apoptosis and induces follicular atresia by targeting SMAD4 and ATM (Lin *et al.* 2012; Liu *et al.* 2014a). Inhibition of INHBB by miR-34a promoted apoptosis in porcine granulosa cells (Tu *et al.* 2014).

According to the two-cell-two-gonadotropin theory, luteinizing hormone stimulates thecal cells to produce androgens, and follicle-stimulating hormone stimulates granulosa cells to produce estrogens from androgens (Barnes *et al.* 2000). Growing evidences indicate that miRNAs act as regulators of steroidogenesis (Schauer *et al.* 2013; Toms *et al.* 2015). The effect of miRNAs on the release of major ovarian steroid hormones was determined by transfecting human primary granulosa cells with 80 different human pre-miRNAs constructs, and the release of progesterone was inhibited by 36 miRNAs. Whereas, 10 miRNAs promoted progesterone release (Sirotkin *et al.* 2009). MiR-378 decreased ovarian estradiol production by targeting aromatase. However, overexpression of the aromatase 3'-UTR in granulosa cells enhanced aromatase expression at protein level, possibly mediated by the binding of miR-378 within this region, thereby reducing the binding of this miRNA to the endogenous aromatase 3'-UTR (Xu *et al.* 2011). In a separate study, the progesterone receptor (PGR) was targeted by miR-378-3p which leads to decrease the protein and mRNA levels and its downstream target genes; ADAMTS1, CTSL1, and PPARG, which are involved in follicular maturation and remodeling (Toms *et al.* 2015). A recent study showed that level of miR-378 in the follicular cells was correlated with the level of estradiol, the estradiol: progesterone [E:P] ratio and CYP19A1 (Donadeu *et al.* 2016). The release of estradiol from human and murine granulosa cells was inhibited by miR-34a and miR-320, respectively (Sirotkin *et al.* 2009; Yin *et al.* 2014). However, miR-320 could stimulate testosterone and progesterone in murine ovaries. Interestingly, miR-383 has been shown to act as a positive regulator of estradiol production in mouse granulosa cells by inhibiting RBMS1, which leads to c-Myc inactivation and steroidogenesis (Yin *et al.* 2014). Moreover, miR-132 is involved in the cAMP signaling pathway and promotes estradiol synthesis via the translational repression of Nurr1 in mouse ovarian granulosa cells and significantly induce expression of CYP19A1 through NURR1; a direct target that suppresses CYP19A1 expression (Wu *et al.* 2015b). Similarly, miR-133b is involved in FSH-induced estrogen production and reported to regulate Foxl2 and inhibit Foxl2-mediated transcriptional repression of StAR and CYP19A1 to promote estradiol production (Dai *et al.* 2013).

1.4.3.3 MicroRNAs during oocyte maturation

Continuous interaction between the oocyte and the surrounding cumulus cells is essential for appropriate oocyte maturation (Hawkins & Matzuk 2010). MicroRNAs are implicated to play essential roles in oocyte maturational processes. The potential role of miRNAs during oocyte maturation was first evidenced in Dicer KO mouse oocytes, which showed defects in meiotic spindle formation and caused meiotic arrest with severe defects in chromosome congression (Murchison *et al.* 2007). MicroRNAs are preferentially expressed in immature and in vitro mature bovine oocytes (Tesfaye *et al.* 2009b). Moreover, the relative abundance of miRNAs in bovine oocyte and the surrounding cumulus cells during in vitro maturation showed interdependency between the two cell types in the sets of miRNAs expressed in either of them during oocyte maturation, signifying the fact that oocyte-cumulus interaction affect the expression of miRNAs in either of the cells (Abd El Naby *et al.* 2013). In the same study, it was also noted that oocyte enriched miRNAs; miR-205, miR-150, miR-122, miR-96, miR-146a and miR-146b-5p showed maturation time-dependent reduction in their expression pattern. The number of miRNAs identified in human cumulus cells was greater compared with the metaphase II (MII) oocytes counterpart, of which let-7b, let- 7c and miR-21 were the three highly abundant cumulus cell specific miRNAs. Contrary to the cumulus cells, the MII oocyte were enriched with only 3 miRNAs, of which miR-184 and miR-10a were highly abundant (Assou *et al.* 2013). Interestingly, miRNAs specifically enriched in MII oocyte target genes associated with transcriptional regulation and cell cycle. Whereas, miRNAs enriched in cumulus cells affect genes involved in extracellular matrix and apoptosis pathways (Assou *et al.* 2013).

1.4.3.4 MicroRNAs in early embryonic development

During the preimplantation period, the embryonic genome is activated to further support embryonic development in the later stages of cleavage. The maternal mRNAs are completely degraded by maternal factors or through de novo miRNA transcription that induces deadenylation of mRNAs (Iwasaki & Siomi 2014). Following the embryonic genome activation, maternal miRNAs could gradually decay and the embryonic miRNAs are activated (Lee *et al.* 2014). MicroRNA expression in mouse embryos during the stage of oocyte-to-zygote transition has shown interesting pattern where clusters of miRNAs have distinct maternal, maternal-zygotic and zygotic expression pattern (Tang *et al.* 2007). For instance, the let-7 family miRNAs tend to be the most abundant maternal miRNAs accounting for a third of total miRNAs in the oocyte (Tam *et al.* 2008), and their abundance sharply decline

after the zygotic genome activation with no sign of restoration in early embryos. It was also reported that the degradation of maternal miRNAs between the zygotic and 2-cell stage was followed by de novo transcription of miRNAs after the 2-cell stages (Tang *et al.* 2007). The miR-290 cluster miRNAs were found to be the most pronounced zygotic miRNAs in mouse embryos starting the 2-cell stage and accumulate throughout the preimplantation period (Zeng & Schultz 2005). The miR-17-92 cluster miRNAs were expressed in a maternal-zygotic pattern, in which their abundance remains higher in both the oocyte and preimplantation embryo, with a slight decline during the oocyte-to-zygote transition at 2-cell stage. The expression patterns miRNA during the oocyte-to-zygote transition is illustrated in figure 1.11.

MicroRNAs are implicated in regulating the embryonic genome activation and maternal mRNA degradation. For instance, miR-430 is expressed during the onset of zygotic transcription in zebrafish and targets several maternal mRNAs by accelerating the deadenylation and clearance of maternal mRNAs during early embryonic stage (Giraldez *et al.* 2006). Similarly, the linear increment in the expression of miR-130a and miR-21 between zygote and 8-cell stage embryo leads to significant reduction of these miRNAs and the SRFS3 gene after α-amanitin treatment suggested the important role of miRNAs in maternal-to-embryo transition (MET) (Mondou *et al.* 2012). In bovine preimplantation embryos, the expression of oocyte enriched miRNAs; miR-205, miR-150, miR-96, miR-122, miR-146a and miR-146b-5p appeared to be highly abundant until the 4-cell stage embryos and drastically reduced after the major embryonic genome activation at 8-cell and through the blastocyst stage (Abd El Naby *et al.* 2013). The involvement of miRNAs in early lineage differentiation of bovine blastocysts was deciphered by comparing the expression pattern miRNAs in early and hatched blastocysts. It was shown that miR-127, miR-130a, miR-155, miR-196a, miR-203, miR-28, miR-29c, and miR-376a were upregulated in hatched blastocysts, whereas, miR-135a, miR-218, miR-335, and miR-449b were significantly downregulated in hatched bovine embryos. Moreover, functional validation revealed that miR-218 is expressed in the ICM and regulates the expression of NANOG through the FGF signaling (Goossens *et al.* 2013). Involvement of miRNAs in embryonic cell fate determination was observed in mouse blastocysts and it was shown that miR-93 is highly expressed in differentiating primitive endoderm and trophectoderm (Foshay & Gallicano 2009). In that study, it was shown that miR-93 and miR-17-5p were highly abundant in the mesoderm of gastrulating embryos. Interestingly, these miRNAs regulate STAT3; a gene

known to regulate embryonic stem cells, playing important role in determining the fate and differentiation of early embryonic cells.

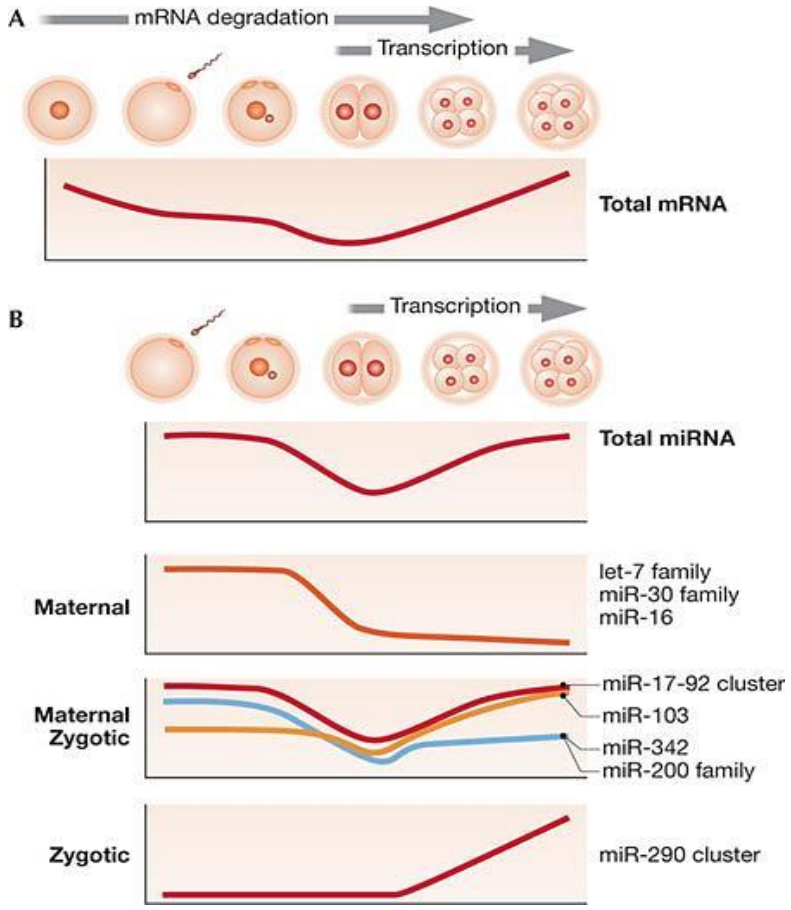

Figure 1.11: Expression dynamics of miRNAs during oocyte-to-zygote transition. The maternal total mRNA degrades after fertilization and decline until the activation of the embryonic transcription at 2-cell stage (**A**). The expression dynamics of total miRNA population has similar pattern like the mRNA expression dynamics. However, certain clusters of miRNAs exhibit maternal (let-7 family, miR-30 family and miR-16), maternal-zygotic (miR-17-92 cluster, miR-103, miR-342 and miR-200 family) and zygotic (miR-290 cluster) expression pattern (**B**). Adapted from Svoboda & Flemr (2010).

1.4.4 Extracellular miRNAs

While the majority of the miRNAs are found intracellularly, tremendous numbers of miRNAs have been reported to circulate in the extracellular environment and biological fluids and these miRNAs are termed as extracellular miRNAs. Extracellular miRNAs are detected in almost all biological body fluids including serum, plasma, saliva, tears, urine, breast milk, colostrum, peritoneal fluid, cerebrospinal fluid, bronchial lavage, seminal fluid

and follicular fluid (Chen *et al.* 2008; Chim *et al.* 2008; Weber *et al.* 2010; da Silveira *et al.* 2012; Sohel *et al.* 2013).

1.4.4.1 Characteristics of extracellular miRNAs

Extracellular miRNAs are endowed with unique characteristics, which help them withstand the hostile extracellular environment. There is high Ribonuclease (RNase) and Deoxyribonuclease (DNase) activity in the extracellular environment, which degrades cellular miRNAs and other RNA species within few seconds. However, extracellular miRNAs were found to be remarkably stable under the high RNase conditions. Addition of synthetic miRNAs to serum or plasma resulted in rapid degradation, indicating the vulnerability of exogenous miRNAs to the high RNase activity in body fluids (Mitchell *et al.* 2008). It was also noted that exposure of extracellular miRNAs to high temperature, extreme pH, extended storage time and multiple freeze-thaw cycles did not affect their stability (Turchinovich *et al.* 2011). For instance, incubation of serum at room temperature for 24 h and 10 times freeze-thaw cycles did not affect the detectability of extracellular miRNAs using qRT-PCR (Chen *et al.* 2008; Mitchell *et al.* 2008).

1.4.4.2 Mechanisms of release of miRNAs into extracellular environment

The relative stability and impermeability of extracellular miRNAs to RNase led to the emergence of a hypothesis that they are encapsulated in membrane vesicles (exosomes and shedding vesicles). The hypothesis is further reinforced by the detection of miRNAs in purified human peripheral blood microvesicles (Hunter *et al.* 2008). Similarly, exosomes release by cultured cells were confirmed to contain functional miRNAs (Valadi *et al.* 2007). The assumption that miRNAs are released into the extracellular space in vesicle-mediated pathway was challenged following the findings which demonstrated that the vast majority of the extracellular miRNAs are released in vesicle-free and associated with the Ago protein in both blood plasma and cell-conditioned media (Arroyo *et al.* 2011; Turchinovich *et al.* 2011). Interestingly, under normal cell culture condition, ~99% of the release miRNAs are vesicle-free and associated with Ago protein (Turchinovich *et al.* 2011). The stability of the Ago proteins could contribute to the stability of the associated miRNAs in the nuclease-rich environment. In addition to the vesicle-mediated and Ago protein-coupled release of miRNAs into the extracellular space, lipoprotein-associated release of miRNAs has been reported (Vickers *et al.* 2011). In the same study, it was shown that high-density lipoprotein (HDL) and low-density lipoprotein (LDL) derived from human blood plasma shuttle

considerable amount of miRNAs into circulation. Moreover, cellular apoptotic bodies are released into the extracellular environment encapsulating variety of molecules including miRNAs (Zernecke *et al.* 2009). A hypothetical model describing the packaging and releasing mechanisms of miRNAs into the extracellular environment is indicated in figure 1.12.

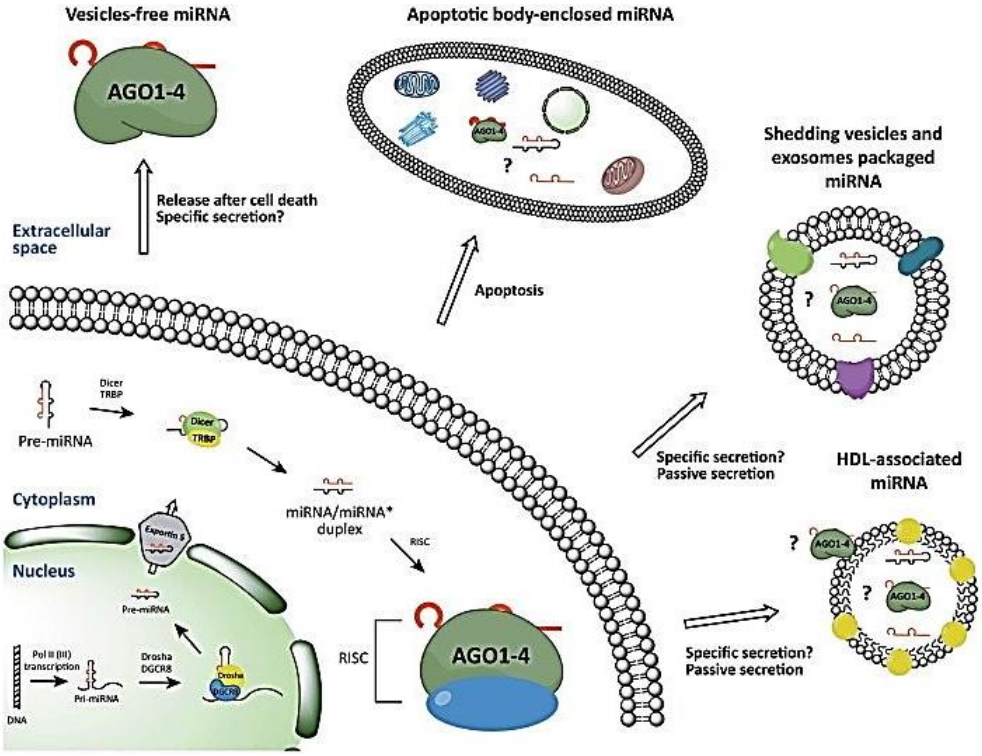

Figure 1.12: Packaging and release of miRNAs into the extracellular environment. Mature miRNAs are release into the extracellular environment through different releasing pathways. Extracellular miRNAs can be released either through membranous vesicle-mediated (apoptotic bodies, shedding vesicles and exosomes), through vesicle-free and associated with AGO protein and through incorporation with HDL particles. Adapted from Turchinovich *et al.* (2011).

1.4.5 Extracellular miRNAs in follicular cell-to-cell communication

Ovarian follicular development and oocyte maturation are well-coordinated processes and mediated by a constant exchange of signals between ovarian somatic cells and the oocytes (Zuccotti *et al.* 2011). These exchanges of signals are made possible through the establishment of gap junctions between the oocyte and the surrounding somatic cells (Gilchrist *et al.* 2008). Mutual communication between the oocyte and cumulus cells is achieved through secretion of both oocyte and cumulus cells factors and are shown to affect

the relative abundance of mRNAs (Regassa *et al.* 2011) and miRNAs (Abd El Naby *et al.* 2013) in each cell type. The oocyte-secreted factors, which pass through the gap junctions include ions, metabolites and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control the development of the oocyte (Gilchrist *et al.* 2008). In addition to the direct communication, extracellular vesicles circulating in the follicular fluid could facilitate the transfer of signals between the different follicular cell types.

1.4.5.1 Follicular extracellular miRNAs and their association with oocyte developmental competence

Mammalian follicular fluid consists of a complex mixture of nucleic acids, proteins, metabolites and ions, which are known to be secreted by the oocyte, granulosa cells, theca cells and blood plasma components that diffuse to the follicular fluid via theca capillaries (Revelli *et al.* 2009; Rodgers & Irving-Rodgers 2010). The follicular fluid creates a suitable microenvironment for the growth and development of oocytes and its biochemical composition varies depending on the physiological status of the follicle. Alteration in the follicular fluid composition can be associated with the oocyte quality and thus, the follicular fluid components may provide useful diagnostic information on oocyte developmental competence and ovarian functions (Revelli *et al.* 2009).

The composition of the follicular fluid, including the miRNA signature could reflect the physiological status of the ovary in general and follicular microenvironment in particular, which in turn might determine the developmental competence of the enclosed oocyte. Tremendous numbers of miRNAs have been found to circulate in follicular fluid (Donadeu & Schauer 2013; Sohel *et al.* 2013). The expression of miRNAs in microvesicles and exosomes isolated from follicular fluid varied between young and old mares and could be associated with the age-related decline of ovarian functions (da Silveira *et al.* 2012). In the same study, it was also reported that the miRNAs identified in follicular fluid were also present within surrounding granulosa and cumulus cells. In a similar study, age related differences in the expression of circulating miRNAs in follicular fluid of younger and older women were observed. It was shown that miR-21-5p, miR-134, miR-190b and miR-99b-3p were differentially expressed in follicular fluid of young women (Diez-Fraile *et al.* 2014).

The size and concentration of vesicles in which miRNAs are encapsulated in the follicular fluid between different sized follicles has been investigated (Navakanitworakul *et al.* 2016). In the same study, no significant differences were observed in the size of the extracellular vesicles between small, medium and large follicles. However, the concentration of

extracellular vesicles decreased progressively as the size of follicles increase. Interestingly, miRNAs enriched in exosomes of follicular fluid of small follicle were found to be associated with cell proliferation. Whereas, miRNAs abundant in large follicle were found to be related to inflammatory response pathways (Navakanitworakul *et al.* 2016).

The developmental competence of oocyte is a key predictor for the successful in vitro development of embryo. Thus, the current methods of assessing the developmental competence of oocyte remain subjective and in some cases invasive. Most of the techniques rely on the morphological, ultrastructural and metabolic criteria and often lead to poor correlation with the developmental competence of oocyte (Sirard & Assidi 2013). Extracellular miRNAs expressed in follicular fluid are implicated to be associated with the developmental competence of the enclosed oocyte. Comparative analysis of miRNAs enclosed in exosomes derived from follicular fluid of growing oocyte and fully-grown oocyte showed unique expression signature. Interestingly, exosome enclosed miRNAs of follicular fluid can either be released or up taken by neighboring granulosa cells (figure 1.12) and could alter the expression of target mRNA in granulosa cells (Sohel *et al.* 2013).

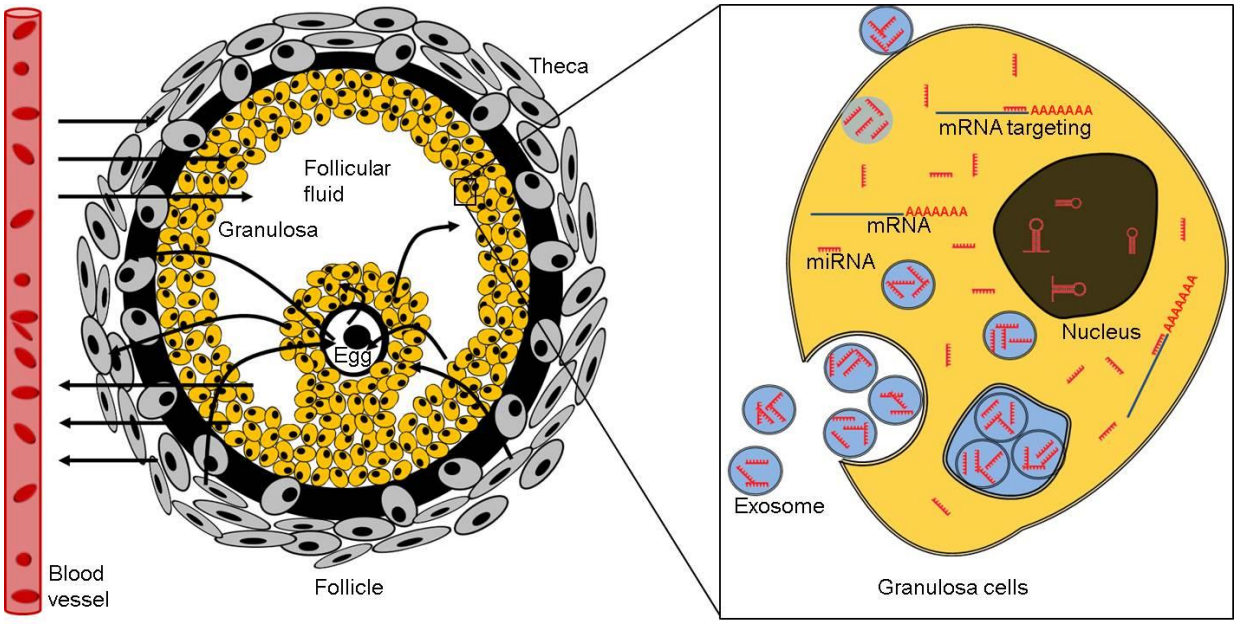

Figure 1.13: Potential role of exosomes as carriers of molecular messages in follicular miRNA environment. Extracellular miRNAs in cell-to-cell communication, where miRNAs released from either the oocyte or follicular cells (granulosa cells, cumulus cells and theca calls) into follicular fluid through exosome-mediated pathway are up taken by neighboring cells. Adapted from Tesfaye *et al.* (2017).

Among the assisted reproductive techniques (ART), estrous synchronization and controlled ovarian hyper-stimulation (COH) are widely used to improve the reproductive performance of dairy cattle. Even though the COH helps cattle grow multiple follicles through the administration of supraphysiological level of gonadotropins, it is also implicated to have negative effect on the development competence and quality of oocytes, embryo quality and endometrium receptivity (Santos *et al.* 2010). Moreover, the number of embryos reached the blastocyst stage in stimulated heifers was lower compared to the unstimulated counterparts, in which stress related genes were highly abundant in embryos developed in the oviduct of stimulated heifers (Gad *et al.* 2011). In line with this, the impact of ovarian hyperstimulation on the expression pattern circulatory miRNAs in follicular fluid and blood plasma was assessed using a miRNA PCR array (Noferesti *et al.* 2015). It was revealed that the follicular fluid and blood plasma of hyper-stimulated heifers showed differential expression of 57 and 21 miRNAs, respectively. In that study it was also showed that differentially expressed miRNAs in follicular fluid of hyper-stimulated heifers are involved in pathway like the TGF-ß signaling pathway, MAPK signaling pathway and oocyte meiosis.

1.4.5.2 Circulatory miRNAs as indicators of pregnancy establishment

Mammalian embryonic development and pregnancy establishment are accompanied by implantation where the placenta is developed from the trophoblast cells of the trophectoderm (Cross *et al.* 1994). The placenta is a miRNA-enriched organ and its function is affected by miRNAs through their role in proliferation, invasion and migration of trophoblast cells (Fu *et al.* 2013; Morales-Prieto *et al.* 2014). Some placenta-specific miRNA clusters are exclusively expressed during pregnancy. Among the placenta-specific miRNA clusters, the C19MC (transcribed from chromosome 19 of human genome) and the C14MC (transcribed from chromosome 19 of human genome) miRNA cluster were found to be associated with pregnancy (Morales-Prieto *et al.* 2013). The presence of pregnancy-related miRNAs in the circulation of pregnant women makes them valuable tools as non-invasive biomarkers for pregnancy diagnosis. This fact is further supported by the presence of large quantities of exosomes in the circulation of pregnant women compared to the non-pregnant counterpart (Toth *et al.* 2007). Plasma samples of pregnant women were enriched with 16 and 5 miRNAs, which belong to the C19MC and C14MC, respectively as the pregnancy progressed to the third trimester. Interestingly, the expression of these miRNAs decreased after delivery which signifies their association with pregnancy (Miura *et al.* 2010).

In domestic animals especially in dairy cows, detection of pregnancy as early as the first 3 weeks has tremendous economic importance by increasing the amount of milk produced by shortening the inter-calving intervals and possibility to rebreed non-pregnant cows in the next cycle (Arbel *et al.* 2001). Comparative analysis of extracellular miRNAs in plasma samples derived from day 24 pregnant and non-pregnant identified 11 miRNAs (miR-454, let-7f, miR-30c, let-7d, miR-151-5p, miR-374b, miR-631, miR-29b, miR-30b-5p, miR-26a and miR-103) upregulated and 5 miRNAs (miR-101, miR-141, miR-29c, miR-339a and miR-29a) downregulated in plasma samples of pregnant cows (Ioannidis & Donadeu 2016).

1.5 Rationale and objectives of the study

As explained above, several studies supported the notion that miRNAs are important gene regulators at posttranscriptional level, which are implicated in various pathophysiological conditions of bovine reproduction. The majority of the studies focused on determining specific functions of a single or group of miRNAs in vitro culture conditions. Thus, genome-wide expression profiling of cellular miRNAs in different follicular cells and extracellular miRNAs in maternal circulation would give a comprehensive overview of the physiological condition under scrutiny.

1.5.1 Hypothesis

Granulosa cells of preovulatory dominant and subordinate follicles exhibit unique miRNA expression signatures, which could be associated with the survival of the follicles. Moreover, miRNAs in circulation could differentiate cows according to the pregnancy status and be used as potential indicators of early pregnancy.

1.5.2 Objectives of the study

The aim of the present study was to determine the expression profile and functional role of cellular and extracellular miRNAs in bovine follicular development and as potential indicators of early pregnancy. The specific objectives of each chapter of the PhD thesis are indicated below.

Chapter 2: To determine the expression pattern of miRNAs in bovine granulosa cells of preovulatory dominant and subordinate follicles derived from day 19 of the estrous cycle and decipher their involvement in ovulation and follicular atresia.

Chapter 3: To determine the functional role of miR-183-96-182 cluster miRNAs in bovine granulosa cell function by regulating the expression of FOXO1 transcription factor and its downstream transcripts.

Chapter 4: To determine the expression signature of extracellular miRNAs in serum of pregnant and non-pregnant cows at day 19 and 24 post-insemination.

Chapter 2

MicroRNA Expression Profile in Bovine Granulosa Cells of Preovulatory Dominant and Subordinate Follicles during the Late Follicular Phase of the Estrous Cycle

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Abstract

In bovine, ovarian follicles grow in a wave-like fashion with commonly 2 or 3 follicular waves emerge per estrous cycle. The dominant follicle of the follicular wave which coincides with the LH-surge becomes ovulatory leaving the subordinate follicles to undergo atresia. These physiological processes are controlled by timely and spatially expressed genes and gene products, which in turn are regulated by post-transcriptional regulators. MicroRNAs; class of short non-coding RNA molecules are one of the important posttranscriptional regulators of genes associated with various cellular processes. Here we investigated the expression pattern of miRNAs in granulosa cells of bovine preovulatory dominant and subordinate follicles during the late follicular phase of the bovine estrous cycle using Illumina miRNA deep sequencing. In addition to 11 putative novel miRNAs, a total of 315 and 323 known miRNAs were detected in preovulatory dominant and subordinate follicles, respectively. Moreover, in comparison with the subordinate follicles, a total of 64 miRNAs were found to be differentially expressed in preovulatory dominant follicles, of which 34 miRNAs including the miR-132 and miR-183 clusters were significantly enriched and 30 miRNAs including the miR-17-92 cluster, bta-miR-409a and bta-miR-378 were significantly downregulated in preovulatory dominant follicles. In-silico pathway analysis revealed that canonical pathways related to oncogenesis, cell adhesion, cell proliferation, apoptosis, and metabolism were significantly enriched by the predicted target genes of differentially expressed miRNAs. Furthermore, Luciferase reporter assay analysis showed that one of the differentially regulated miRNAs; the miR-183 cluster miRNAs were validated to target the 3′-UTR of *FOXO1* gene. Moreover, *FOXO1* was highly enriched in granulosa cells of subordinate follicles in comparison with the preovulatory dominant follicles demonstrating reciprocal expression pattern with miR-183 cluster miRNAs. In conclusion, the presence of distinct sets of miRNAs in granulosa cells of preovulatory dominant and subordinate follicles supports the potential role of miRNAs in post-transcriptional regulation of genes involved in bovine follicular development during the late follicular phase of the estrous cycle.

Keywords: Bovine, Folliculogenesis, miRNAs, Gene regulation

Introduction

Bovine follicular development is a highly orchestrated, periodic and gonadotropin-dependent process which starts with the activation of resting follicles gradually leading to the growth

and development of a preovulatory follicle accompanied by sequential differentiation of oocyte and the surrounding somatic cells (Knight & Glister 2001). In cattle, recruitment of growing follicle, selection, and growth of leading follicles, ovulation of the preovulatory dominant follicle and degeneration of anovulatory subordinate follicles undertake in a wave-like fashion with typically 2 or 3 follicular waves per estrous cycle (Fortune *et al.* 1991; Adams 1999). The first follicular wave emerges soon after ovulation, whereas the second and third follicular waves start to emerge 8-9 and 15-16 days post ovulation, respectively (Adams *et al.* 2008). The dominant follicle of the wave which coincides with a sharp rise of luteinizing hormone (LH-surge) becomes ovulatory, while the remaining follicles of both the first and second waves eventually undergo follicular atresia (Adams *et al.* 2008).

Follicular recruitment, selection, dominance, and ovulation are tightly regulated by endocrine and paracrine hormones, among which the follicle stimulating hormone (FSH) and luteinizing hormone (LH) play crucial roles (Hunter *et al.* 2004). Throughout the estrous cycle granulosa cells undergo several developmental changes. During the early stage of the cycle, granulosa cells of small growing follicles secrete inhibin and acquire follicle stimulating hormone receptors (*FSHR*). Granulosa cells of dominant follicles acquire LH receptors (*LHCGR*) and secrete more estradiol than the subordinate follicles, triggering the LH surge that allows it to develop into preovulatory follicle. These synergistically coordinated actions of hormones induce tremendous morphological reorganization and functional changes in the oocyte and its companion somatic cells, which lead to cellular differentiation and give rise to a developmentally competent oocyte.

Cellular differentiation that occurs during follicular development are regulated by the expression and interaction of multitude of genes in a spatio-temporal manner in different compartment of the follicle: granulosa cells (Hatzirodos *et al.* 2014a; Hatzirodos *et al.* 2014b), theca cells (Hatzirodos *et al.* 2014c), follicular fluid and oocyte (Bonnet *et al.* 2008). Nevertheless, understanding the post-transcriptional regulation of genes in follicular cells at different stages of follicular development would help us to understand the molecular mechanisms underlying follicular recruitment, selection, ovulation and follicular atresia in more depth.

MicroRNAs (miRNAs) are a class of endogenous short non-coding RNA molecules, ~22-nucleotides (nt) long that post-transcriptionally regulate expression of genes by either degrading the mRNA or translational repression (Bartel 2004). Previously, we have shown the presence of distinct set of miRNAs in bovine ovary (Hossain *et al.* 2009) and spatio-temporal expression of miRNAs in bovine oocytes, cumulus cells and preimplantation

embryos (Tesfaye *et al.* 2009; Abd El Naby *et al.* 2013). Interestingly, both bovine oocyte and cumulus cells were found to be interdependent on each other for signals affecting the activity of miRNAs (Abd El Naby *et al.* 2013).

Similarly, the involvement of miRNAs in mouse LH-induced luteinization and ovulation was demonstrated in mural granulosa cells after LH/hCG stimulation (Fiedler *et al.* 2008). In addition, the association of selected candidate miRNAs with follicular selection, maturation and ovulation in mare granulosa cells and follicular fluid derived from ovulatory dominant, largest subordinate and anovulatory dominant follicles has been demonstrated by Schauer *et al.* (2013). Despite the fact that follicular development in mammalian species is regulated by spatio-temporal expression of genes (Bonnet *et al.* 2008), little is known about the abundance of larger set of miRNAs in follicular cells.

Recently, we reported the expression pattern of miRNAs in granulosa cells of subordinate and dominant follicles and the possible association with follicular recruitment, selection and dominance during the early luteal phase of bovine estrous cycle (Salilew-Wondim *et al.* 2014). Nonetheless, information on the abundance of regulatory miRNAs in granulosa cells of bovine preovulatory dominant and subordinate follicles during the follicular phase of the estrous cycle is missing. Thus, we aimed to examine differences in miRNA abundance in bovine granulosa cells derived from preovulatory dominant and subordinate follicles obtained at day 19 of the bovine estrous cycle. Moreover, the potential role of miRNAs in ovulation and follicular atresia is elucidated by predicting significantly enriched canonical pathways by predicted target genes of differentially expressed miRNAs.

Materials and Methods

Experimental animals and estrous synchronization

Handling of experimental animals was in accordance with the 1972 German law of rules and regulations of animal protection. The experiment was licensed by the animal welfare office of the University of Bonn with proposition number 84–02.05.20.12.075. Healthy Simmental heifers (n=7) aged between 15 and 20 months and weight range of 380 to 500 kg were used for this study. Animals were housed in the same herd and farm with free-stall barn fitted with slotted floors and cubicles lined with rubber mats. Heifers were synchronized according to standard synchronization protocols as previously described (Salilew-Wondim *et al.* 2010; Salilew-Wondim *et al.* 2014). Briefly, all experimental heifers were pre-synchronized by intramuscular (IM) administration of 500 mg of prostaglandin 2-alpha

($PGF_{2\alpha}$)-analogue; cloprostenol (Estrumate, Munich, Germany) twice within 11 days. Two days after each of the $PGF_{2\alpha}$ treatments animals received 0.02 mg of gonadotropin releasing hormone (GnRH)-analogue; buserelin (Receptal; Intervet, Boxmeer, The Netherlands). Common signs of oestrus were visually monitored three times a day. The day at which animals exhibit oestrus was considered as day 0. Finally, synchronized heifers were slaughtered 19 days after the onset of oestrus using a standard scarifying procedure at a local slaughter house.

Ovary collection and follicle categorization

Ovaries were retrieved and immediately transported in thermos flask containing warm (37°C) physiological saline solution (0.9% NaCl) solution. Upon arrival, ovaries were repeatedly washed with warm (0.9% NaCl), rinsed in 70% ethanol for 30 seconds and washed again three times with warm saline solution. Ovaries were visually inspected for the presence of one bigger preovulatory dominant follicle and several other subordinate follicles. Ovary samples of 2 cows lacking preovulatory dominant follicles were excluded from the experiment. Follicles were carefully isolated from ovaries by dissecting using scissors and forceps and were categorized according to their external surface diameter as previously described in Fortune *et al.* (1991) and Ireland *et al.* (1980) with minor modifications. Briefly; follicles with external surface diameter of 12 mm and above were categorized as preovulatory dominant and follicles with surface diameter ≤ 11 mm were considered as subordinate.

Granulosa cells, theca cells, cumulus-oocyte-complex and follicular fluid collection

Each of the retrieved preovulatory dominant and subordinate follicles was dissected into two halves and follicular contents were released into a sterile plastic petri dish. Cumulus-oocyte-complexes (COC) were recovered, transferred into separate tubes and stored -80°C for further analysis. Follicular fluid samples devoid of COC were transferred into 15 ml sterilized falcon tubes and centrifuged at 750*xg* for 7 minutes to a separate the granulosa cells pellet from follicular fluid supernatant. Follicular fluid samples were transferred into separate tube and stored in -80°C until further use. Granulosa cells pellets were washed twice with PBS (without Ca^{2+} and Mg^{2+}) and stored in -80°C for further analysis.

Similarly, theca cell layers were gently scraped from both the preovulatory dominant and the subordinate follicles as previously described (Allegrucci *et al.* 2003) with minor

modification. Briefly; follicle halves were examined under dissecting microscope and theca cell layers (theca interna and theca externa) were gently peeled away from the basal membrane using forceps. Theca cell samples were repeatedly washed with PBS (without Ca^{2+} and Mg^{2+}) to avoid granulosa cells contamination. Finally, theca cell samples were transferred into 0.65 ml sterilized tubes containing RNAlater solution (Sigma-Aldrich, Steinheim, Germany) and stored in -20°C until further processing.

Total RNA isolation and quality control

Total RNA enriched with miRNAs was isolated from granulosa cells, theca cells and follicular fluid samples of both preovulatory dominant and subordinate follicles using the miRNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instruction. On-column DNA digestion was performed to remove genomic DNA contamination using RNase-free DNase (Qiagen GmbH, Hilden, Germany). Similarly, total RNA from COC was isolated using AllPrep DNA/RNA Micro Kit (Qiagen GmbH, Hilden, Germany) following manufacturer´s protocol. RNA concentration and integrity were assessed by NanoDrop 8000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Finally, total RNA samples of granulosa cells were pooled to generate biological triplicates and used for library preparation and miRNA deep sequencing. RNA samples from theca cell, COC, and follicular fluid were used to determine the expression pattern of selected differentially expressed miRNAs in granulosa cells of preovulatory dominant follicles.

Purity of isolated granulosa cells

The purity of isolated granulosa cell was assessed by analzsing the presence of granulosa cell-specific marker gene (*FSHR*) and absence of theca cell specific marker gene (*CYP17A1)*. For this, specific primers targeting *FSHR, CYP17A1* and *GAPDH* were designed using primer3web version 4.0.4 (http://bioinfo.ut.ee/primer3/) (Table 2. S1). Complementary DNA (cDNA) was synthesized using First Strand cDNA Synthesis Kit (Thermo scientific, MA, and USA) according to manufacturer´s instruction. Briefly; 1 µg of total RNA samples isolated from granulosa cells was mixed with 1 µl of oligo$(dT)_{18}$ were incubated at 65 °C for 5 minutes. Nine µl of master mix (4 µl of 5X reaction buffer, 1 µl RiboLock RNase inhibitor, 2 µl of 10 mM dNTP mix and 2 µl of M-MuLV Reverse Transcriptase) was added to the RNA template and incubated at 37 °C for 60 minutes. Reactions were terminated by heating

at 75 °C for 5 minutes. Finally, polymerase chain reaction (PCR) was set with thermocycling conditions of pre-incubation at 95°C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C (*FSHR* and *GAPDH*) and 57 °C (*CYP17A1*) for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR product was mixed with loading buffer and loaded into 2% agarose gel stained with Ethidium bromide (EtBr) and visualized under UV on Gel Doc XR+ imaging system (BIO-RAD, München, Germany) to detect the presence or absence of gene specific bands.

Library preparation and miRNA deep sequencing

MicroRNA library preparation and miRNA deep sequencing were performed by a commercial company GATC BioTech AG (Konstanz, Germany) according to the Illumina small RNA sample preparation protocol. One µg of miRNA enriched total RNA samples from granulosa cells were subjected to construction of tagged miRNA sequencing libraries using TruSeq Small RNA Sample Prep Kit according to manufacturer's instructions. Briefly; specific 3´and 5´ RNA adapters (Table 2.S1) were ligated to each end of the RNA template followed by purification of the 1st and 2nd adapter ligation products. The 3´ RNA adapter is modified in a way to capture miRNAs and other small RNA species in the sample. Single stranded cDNA was synthesized by reverse transcription using RT primers (S1 Table). CDNA samples were amplified by PCR using specific primers (Table 2.S1). PCR products were gel purified and band fraction size range of 140-160 nucleotides were excised using a clean scalpel. Finally, single read clusters were generated and sequencing was performed on Illumina HiSeq 2000 in single read mode with read length of 50 bases. Base-calling, data filtering, and index sorting were performed by the CASAVA Pipeline version 1.8.0. Raw FASTQ sequence reads of 50 nucleotides length were obtained.

Sequence quality control and pre-processing

FASTQ files were subjected to preliminary sequence quality control procedures using FASTQC version 0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Per base sequence quality and per sequence quality scores were thoroughly inspected. The 5´ adapter, 3´ adapter, RT primers, PCR primers and their corresponding reverse complementary sequences were trimmed. Moreover, sequence reads with Phred score lower than 18 and sequence reads shorter than 18 bp after trimming were removed from all the data sets using both Cutadapt (Martin 2012) (https://code.google.com/p/cutadapt/) and Seqtk tools (https://github.com/lh3/seqtk). The raw FASTQ files and processed CSV files

have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series with accession number GSE56002.

Sequence read alignment and detection of bovine miRNAs

Detection of both known and novel miRNAs was elucidated using the miRDeep2.0.0.5 software package (Friedlander *et al.* 2012). The *Bos taurus* genome release 72 was downloaded from ensemble genome browser (ftp://ftp.ensembl.org/pub/release-72/fasta/bos_taurus/dna/) and indexed with Bowtie 1.3 (Langmead *et al.* 2009) (http://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.1.0/). Moreover, the FASTA file of all matured bovine miRNA, precursor bovine miRNAs and miRNAs of other species (human, mouse and rat) were downloaded from miRBase database (release 20: June 2013) (http://mirbase.org/ftp.shtml). Sequence reads were mapped to the bovine reference genome and aligned sequence reads were blasted against both mature and precursor miRNAs of bovine, human, mouse and rat.

Prediction of novel miRNAs

Novel miRNAs and their respective read counts were inferred using miRDeep2 software package as described in Friedlander *et al.* (2008). MiRDeep2 predicts the probability of unannotated sequence being novel miRNA-based on the genomic context which surrounds the sequence and the capability of the sequence to fold into hairpin structure with low free energy (Friedlander *et al.* 2012). Secondary structure of miRNA precursor was predicted using RNAfold (Hofacker *et al.* 1994) and minimum free energy algorithm (Gruber *et al.* 2008)

Data normalization and differential expression of miRNAs

The workflow of miRDeep2 was followed to generate the expression data of all known miRNAs. Raw expression data was normalized as previously described (Salilew-Wondim *et al.* 2014). Differences in the number of reads in each sample were normalized using DESeq2 by generating a "hypothetical reference" with read count equals to the geometric mean of read count of all samples. DESeq2 uses a negative binomial distribution model to count on the biological and technical variability among samples. Finally, the normalized read count of each miRNA in each sample was obtained by dividing the read count each miRNA to the geometric mean of all samples. Analysis of differentially expressed miRNAs was designed in a way to evaluate the differences in miRNA expression in granulosa cells derived from

preovulatory dominant and their subordinate follicles counterparts. Differential expression of miRNAs was calculated from read count data using the DESseq2 of the R package as described in Anders & Huber (2010). MicroRNAs with $\log_2$ fold change differences ≥ 1, *p*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.1 were considered as statistically significantly differentially expressed. PermutMatrix was used for clustering analysis and heat map generation of differentially expressed miRNAs (Caraux & Pinloche 2005)

MicroRNA target gene prediction and functional annotation

The interaction between differentially expressed miRNAs and their target mRNA was predicted using miRecords; a widely used web-based database to predict animal miRNA-target mRNA interactions (Xiao *et al.* 2009). Target genes that were predicted by at least 4 target prediction algorithms within miRecords were filtered for further analysis. For miRNAs whose target genes were not available in miRecords were searched in miRDB (Wang 2008) and targetscan cow release 6.2 (Friedman *et al.* 2009). Following this, lists of predicted target genes of individual miRNAs were imported to DAVID Bioinformatics systems; a freely available bioinformatics tool (http://david.abcc.ncifcrf.gov/). Gene ontology (GO) and canonical pathways significantly enriched by the predicted target genes of each miRNAs were identified. Canonical pathways were identified from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Ogata *et al.* 1999).

Validation of candidate miRNAs using qPCR

Nine differentially expressed candidate miRNAs identified by NGS (bta-miR-132, bta-miR-212, bta-miR-21-3p, bta-miR-96, bta-miR-182, bta-miR-221, bta-miR-335, bta-miR-708 and bta-miR-214) were randomly selected for quantitative real-time PCR (qPCR) validation. Furthermore, the relative abundance of these candidate miRNAs was assessed in theca cells, COC and follicular fluid. For this, first strand cDNA was synthesized from equal amount of total RNA input using miRCURY LNA™ Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) according to the manufacturer´s instruction. CDNA templates were 40X diluted using nuclease-free water and 4 µl of diluted cDNA template was mixed with 5 µl of ExiLENT SYBR Green Master mix and 1 µl of PCR primer mix (Exiqon, Vedbaek, Denmark). QPCR amplification was performed in a StepOnePlus Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA) with the thermocycling conditions of initial heating at 95°C for 10 minutes followed by 40 cycles of amplification step at 95°C for 10 s and 60°C for 1 min. Melting curve analysis was performed to assess specific amplification of primers.

Plasmid construction

To experimentally validate the in silico target gene prediction of differentially expressed miRNAs, we selected the miR-183 cluster miRNAs (bta-miR-183, bta-miR-182 and bta-miR-96); which are predicted to target the Forkhead box protein O1 (*FOXO1*) gene. For this, we constructed plasmid DNA containing part of the 3′-UTR of bovine *FOXO1* gene harbouring the putative miRNA binding sites for bta-miR-183, bta-miR-182 and bta-miR-96. The 3´-UTR DNA fragment was amplified from genomic DNA of bovine granulosa cell using specific primers (Table 2.S1). Simultaneously, mutant *FOXO1* 3´-UTR constructs with mutations on target recognition sites of miR-183, miR-182 and miR-96 were generated. The wild-type and mutant *FOXO1* 3´-UTR were cloned between the *SacI*/*XhoI* restriction sites downstream of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The presence of miRNA binding sites in the plasmid constructs was confirmed by sequencing before and after cloning.

Luciferase reporter assay

Primary granulosa cells were cultured in a 24-well plate as previously described (Spanel-Borowski *et al.* 1994). Sub-confluent (70-80% confluency), cultured cells were co-transfected with *FOXO1* wild-type or mutant 3´-UTR reporter constructs and bta-miR-183-182-96 miRNA mimics using lipofectamine 2000 (Invitrogen, Darmstadt, Germany). Cells were lysed 24 hours post transfection and activity of firefly and Renilla Luciferase were determined using Dual-Glo luciferase assay kit (Promega, Madison, WI, USA). Data was calculated as the ratio of Firefly luciferase to Renilla activity.

Quantification of genes targeted by miR-183 cluster using qPCR

Primers of the predicted target genes; *FOXO1* was designed from the reference mRNA sequence using primer3 program (Table 2.S1). Total RNA isolated from granulosa cells of preovulatory dominant and subordinate follicles was used to synthesize cDNA as described above. Then, a PCR master mix comprising of 7.4 µl ddH2O, 0.3 µl of forward primer, 0.3 µl of reverse primer and 10 µl of 1x SYBR Green I master mix (Bio-Rad) was mixed with 2 µl of cDNA template to make a reaction volume of 20 µl. Finally, thermocycling conditions were set to 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Melting curve analysis was performed to assess specific amplification of primers.

Statistical analysis

Expression data of selected candidate miRNAs generated by qPCR in granulosa cells, theca cells, cumulus-oocyte-complex and follicular fluid derived from preovulatory dominant and subordinate follicles was analyzed using the comparative threshold cycle (Ct) method (Livak & Schmittgen 2001). Expression data was normalized against the geometric mean of the expression of 2 endogenous reference miRNAs; 5*s* Ribosomal RNA (*5s* rRNA) and *U6* small non-coding small nuclear RNA (*U6* snRNA). Similarly, expression of target mRNA in granulosa cells of preovulatory dominant and subordinate was analysed using the comparative threshold cycle (Ct) method (Livak & Schmittgen 2001). Expression data of target mRNA was normalized against the expression of *GAPDH*. Two-tailed student´s *t*-test was performed to discover statistical differences in the mean expression value between treatment groups and statistical significance was defined at p-value ≤ 0.05. GraphPad prism 5 (GraphPad, San Diego, CA) was used to plot the relative expression of selected miRNAs and mRNA. QPCR was performed in biological triplicates and relative expression values are presented as mean ± SD of normalized Ct values.

Results

A total of 5 preovulatory dominant and 76 subordinate follicles were obtained from 5 experimental animals. The mean surface diameter of retrieved preovulatory dominant follicles (15.4 ± 3.68 mm) was significantly different ($p < 0.001$) compared to the mean surface diameter of subordinate follicles (6.53 ± 0.99 mm). Moreover, analysis on the purity of isolated granulosa cells from these two follicular categories showed a strong presence of granulosa cell-specific marker gene (*FSHR*) and a very negligible level of theca cell specific marker gene (*CYP17A1*) in all samples (Figure 2.S1).

Characterization of miRNA deep sequencing data

To investigate the involvement of miRNAs in bovine follicular development specifically during the preovulatory stage, 6 miRNA sequencing libraries were generated using granulosa cells derived from preovulatory dominant and subordinate follicles. Libraries were sequenced using the Illumina HiSeq2000 small RNA deep sequencing technology and 50 bases long sequence reads were generated. Accordingly, 8.2 and 8.9 million reads were obtained from libraries of the preovulatory dominant and subordinate follicles, respectively. After filtering low-quality reads and empty adaptors, the mean quality read of the biological

triplicates was 2.4 and ~3 million in preovulatory dominant and subordinate follicle libraries, respectively. Quality filtered sequence reads were used for detection of known annotated and prediction of novel miRNAs. From all reads which passed the quality control criteria, 663,338 reads in preovulatory dominant and 928,373 in subordinate follicles were mapped to the bovine reference genome, comprising 27.6 and 31.4% of the total quality reads obtained, respectively. Furthermore, 343,221 reads in preovulatory dominant and 467,028 in subordinate follicles were found to be similar with known bovine miRNAs reported in miRBase release 20 (Table 2.1).

Table 2.1: Summary of sequence reads alignment to bovine reference genome and known miRNAs annotated in miRBase

Group	Sample ID*	Total number of QC reads	Number of mapped reads €	Mapped reads (%)	Reads Aligned to known miRNAs	Aligned to Known miRNAs £ (%)
Dominant follicle	D1	1,925,662	599,377	31.1	345,689	57.7
	D2	1,967,061	392,924	20.0	255,260	65.0
	D3	3,161,472	997,715	31.6	428,716	43.0
Subordinate follicle	S1	3,069,606	861,596	28.1	459,794	53.4
	S2	2,895,393	939,835	32.5	520,377	55.4
	S3	2,922,775	983,688	33.7	420,913	42.8

*: D1, D2, D3 denote for biological triplicates of preovulatory dominant follicles and S1, S2, S3 denote for biological triplicates of subordinate follicles

€: Number of quality filtered reads aligned to bovine reference genome release 72.

£: Proportion of mapped sequence reads aligned to known annotated miRNAs in miRBase release 20

MicroRNAs expressed in granulosa cells of preovulatory dominant and subordinate follicles

MicroRNAs with at least 1 read count in at least two of the three biological replicates were considered as detected. Accordingly, a total of 315 and 323 known bovine miRNAs were detected in preovulatory dominant and subordinate follicles, respectively of which 287 miRNAs were commonly detected in both sample groups. However, 28 miRNAs including bta-miR-96 and bta-miR-122 were found to be specific to preovulatory dominant. While, 36 miRNAs including bta-miR-409a and bta-miR-449b were unique to subordinate follicles.

The abundance of detected miRNAs showed a broader range both in the preovulatory dominant and subordinate follicles. Among which, bta-miR-26a and bta-miR-10b were the

two most abundantly expressed miRNAs with a read count of 49000 and 28169 in preovulatory dominant and 77,730 and 62,390 in subordinate follicles; accounting for 22.5 and 30% of the sequence reads aligned to known miRNAs, respectively. Similarly, three isoforms of the let-7 family (bta-let-7a-5p, bta-let-7f, and bta-let-7i) comprised 8.5 and 7.3% of sequence reads aligned to known miRNAs in preovulatory dominant and in subordinate follicles, respectively. Nevertheless, the overwhelming majority of the detected miRNAs in both libraries had less than 50 read counts (Figure 2.S2). Among the top 10 abundantly expressed miRNAs in each group, 7 miRNAs (bta-miR-26a, bta-miR-10b, bta-let-7a-5p, bta-let-7f, bta-let-7i, bta-miR-27b and bta-miR-191) were commonly expressed in both preovulatory and subordinate follicles (Table 2.2). List of all detected miRNAs with their normalized read count is indicated in (Table 2.S2).

Table 2.2: List of Top 10 highly abundantly expressed miRNAs in granulosa cells of preovulatory dominant and subordinate follicles

Preovulatory dominant follicle		Subordinate follicle	
miRNA ID	**Average read count §**	**miRNA ID**	**Average read count§**
bta-miR-26a	48999.00	bta-miR-26a	77730.33
bta-miR-10b	28168.67	bta-miR-10b	62390.00
bta-miR-202	12209.00	bta-miR-92a	13653.33
bta-let-7a-5p	10838.33	bta-let-7f	13331.00
bta-let-7f	9595.33	bta-miR-27b	13194.67
bta-miR-22-3p	8710.33	bta-miR-99b	12241.33
bta-let-7i	8695.67	bta-let-7a-5p	11003.67
bta-miR-21-5p	8695.33	bta-let-7i	9734.67
bta-miR-27b	8476.67	bta-miR-191	8563.00
bta-miR-191	7700.33	bta-miR-143	8397.67

§: The arithmetic mean of read counts of biological triplicates

Expression pattern of miR-#-5p and miR-#-3p arms

We found that majority of the detected miRNAs to be derived from the miR-#-5p arm of miRNA precursor. In the sequencing libraries, there were 36 and 39 miRNAs derived from the miR-#-3p arm of the miRNA precursor in preovulatory dominant and subordinate

follicles, respectively. Interestingly, 26 miR-#-5p/3p duplexes were detected, among them, 22 duplexes were commonly expressed in both the dominant and subordinate follicles (Table 2.3). The expression of the majority of the miR-#-3p arms is lower than their corresponding miR-#-5p arm. For instance, the expression of the 3p arm of bta-let-7a in dominant and subordinate follicles was 27 and 34 reads, respectively. Whereas, the expression of its corresponding 5p arm in dominant and subordinate follicles was 10,838 and 11,004 reads, respectively. MicroRNAs like miR-151-5p and miR-126-5p exhibited a similar level of expression as their corresponding 3p arms. However, the expression level of miR-22-3p both in dominant and subordinate follicles was higher than miR-22-5p. Moreover, miR-199a-5p/3p and miR-2313-5p/3p duplexes were exclusively expressed in preovulatory dominant follicles and absent in subordinate follicles. A representative miRNA precursor (bta-mir-126) with functional 5p and 3p arms is shown in figure 2.S3

Table 2.3: Expression pattern of miRNA-duplexes in preovulatory dominant and subordinate follicle libraries

miRNA-Duplex*	Read count in Preovulatory Dominant follicles		Read count in Subordinate follicles	
	miR-#-5p	miR-#-3p	miR-#-5p	miR-#-3p
bta-let-7a	10838	27	11004	34
bta-mir-21	8695	399	4662	120
bta-mir-151	1465	1457	1971	2187
bta-mir-99a	687	12	1981	13
bta-mir-423	654	388	1081	806
bta-mir-424	460	69	87	12
bta-mir-6119	395	7	1015	20
bta-mir-425	164	9	143	10
bta-mir-199a	123	244	0	0
bta-mir-129	83	60	3	1
bta-mir-17	50	13	157	15
bta-mir-2483	35	7	17	10
bta-mir-126	27	23	74	56
bta-mir-22	20	8710	2	1792
bta-mir-1388	17	1	29	3
bta-mir-362	11	2	15	1
bta-mir-345	6	30	3	15
bta-mir-2313	5	118	0	0
bta-mir-503	5	8	4	4
bta-mir-193a	2	5	2	4
bta-mir-2320	2	2	2	1
bta-mir-2284t	1	5	3	14
bta-mir-411c	1	2	1	1
bta-mir-545	1	1	1	1
bta-mir-455	0	0	2	8
bta-mir-9	0	0	2	1

*: miRNA-duplex is the 5p and 3p arm of a single miRNA precursor

Differentially expressed miRNAs in granulosa cells of preovulatory dominant follicles

Comparison of the level of expression of all detected miRNAs was determined by calculating the ratio of the normalized expression values of each miRNA in both follicles. It

was observed that majority of the detected miRNAs are equally expressed in both preovulatory and subordinate follicles (Figure 2.1).

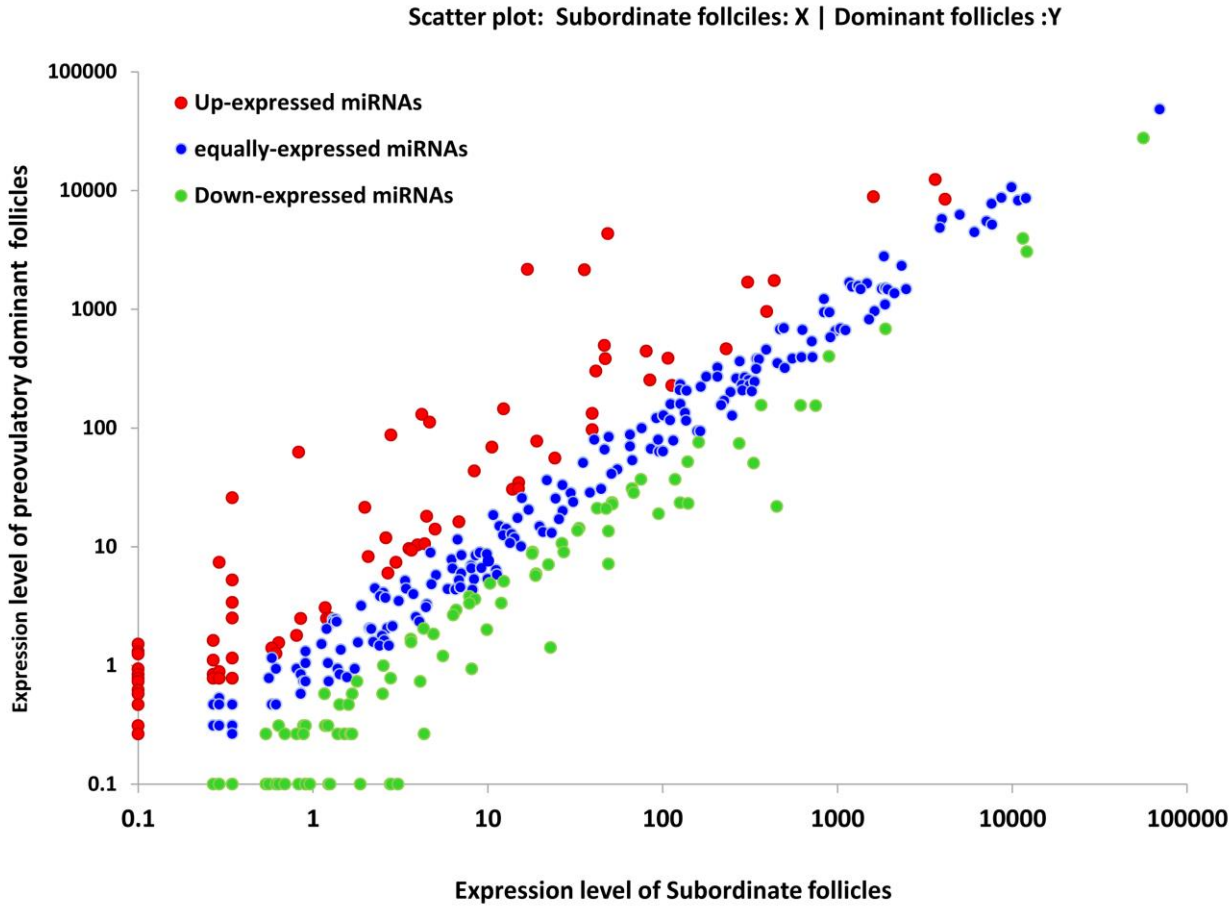

Figure 2.1: Scatter plot of miRNA expression level in preovulatory dominant and subordinate follicles. The expression level of preovulatory dominant and subordinate follicles is indicated in Y and X axis, respectively. Up-expressed miRNAs; with ratio ≥ 2 are labeled with red points. Equally expressed miRNAs; with 0.5 < ratio ≤ 2 are labeled with blue points. Down-expressed miRNAs; with ratio ≤ 0.5 are labeled with green points. Ratio= Normalized expression level in preovulatory dominant follicles / Normalized expression level in subordinate follicles.

Differential expression analysis revealed 64 miRNAs to be significantly differentially expressed between preovulatory dominant and subordinate follicles. In comparison with subordinate follicles, 34 matured miRNAs including miR-132 cluster (bta-miR-132 and bta-miR-212) and miR-183 cluster (bta-miR-183, bta-miR-182 and bta-miR-96) were significantly enriched in preovulatory dominant follicles (Table 2.4). While, the expression level of 30

other matured miRNAs including bta-miR-409a, bta-miR-335, bta-miR-378, and bta-miR-17-5p were significantly reduced in preovulatory dominant follicles (Table 2.5). The Log_2 fold change values in preovulatory dominant follicles range from 7.03 (bta-miR-183) up to -16.4 (bta-miR-409a). The hierarchical clustering of all and top 20 differentially expressed miRNAs in granulosa cells derived from the two follicular categories is described in figure 2.2. The majority of the differentially expressed miRNAs were detected in both follicular categories. However, 2 miRNAs (bta-miR-96 and bta-miR-375) enriched in preovulatory dominant follicles were uniquely detected in granulosa cells of preovulatory dominant follicles. Similarly, 3 miRNAs (bta-miR-1434, bta-miR-4099 and bta-miR-2344) were detected only in granulosa cells of subordinate follicles.

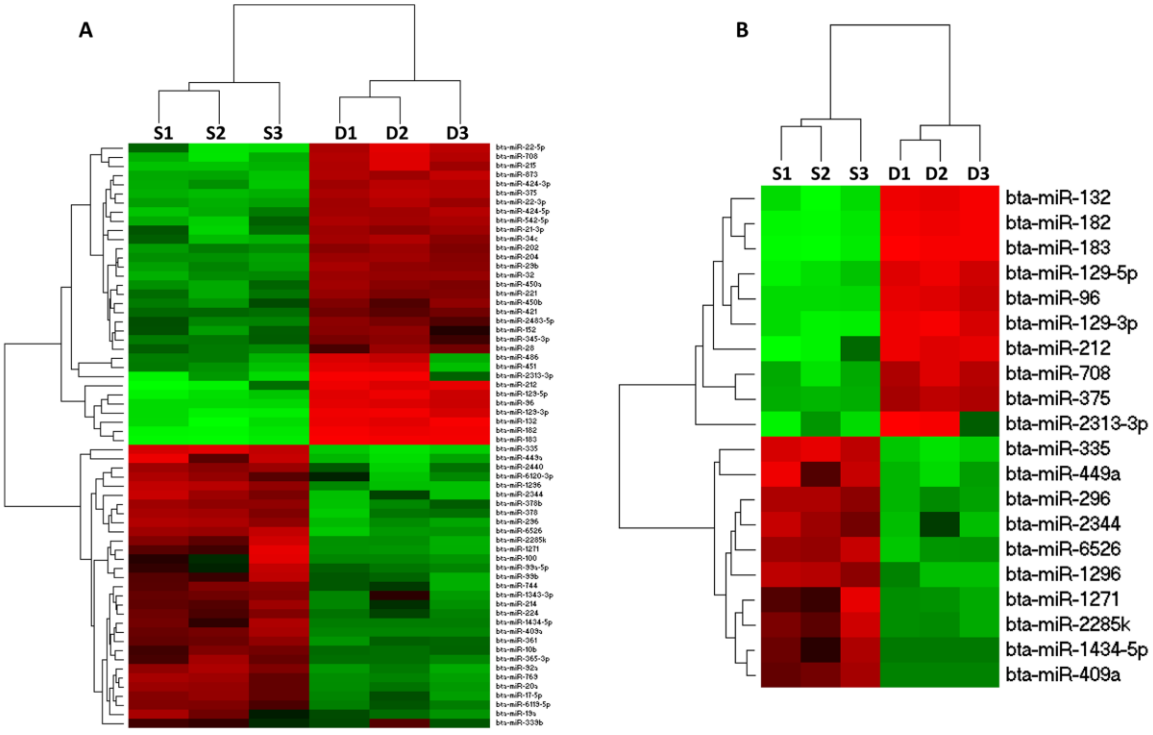

Figure 2.2: Hierarchical clustering of differentially expressed miRNAs in granulosa cells of preovulatory dominant and subordinate follicles. Heat map of all differentially expressed miRNAs in preovulatory dominant follicles (**A**) and 20 top miRNAs differentially expressed in preovulatory dominant follicles (**B**). Red and green blocks represent up and down regulated miRNAs, respectively. Legend: S1-S3 subordinate follicle triplicates and D1-D3 preovulatory dominant follicle triplicates.

Table 2.4: List of miRNAs up-regulated in granulosa cells of preovulatory dominant follicles

miRNAs ID	Fold change	*p*-value	FDR
bta-miR-183	7.03	< 0.0001	< 0.0001
bta-miR-182	6.49	< 0.0001	< 0.0001
bta-miR-96	6.42	< 0.0001	< 0.0001
bta-miR-129-3p	6.13	< 0.0001	< 0.0001
bta-miR-132	5.91	< 0.0001	< 0.0001
bta-miR-129-5p	5.00	< 0.0001	< 0.0001
bta-miR-2313-3p	4.96	< 0.0001	< 0.0001
bta-miR-212	4.67	< 0.0001	< 0.0001
bta-miR-375	4.62	0.0007	0.0073
bta-miR-708	3.56	< 0.0001	< 0.0001
bta-miR-215	3.42	< 0.0001	< 0.0001
bta-miR-22-5p	3.37	< 0.0001	0.0005
bta-miR-451	3.03	0.0003	0.0034
bta-miR-873	2.86	< 0.0001	< 0.0001
bta-miR-424-3p	2.69	< 0.0001	< 0.0001
bta-miR-424-5p	2.47	< 0.0001	< 0.0001
bta-miR-486	2.46	0.0006	0.0061
bta-miR-22-3p	2.46	< 0.0001	< 0.0001
bta-miR-542-5p	2.40	< 0.0001	< 0.0001
bta-miR-34c	2.15	0.0057	0.0422
bta-miR-29b	2.06	0.0011	0.0099
bta-miR-32	2.03	< 0.0001	< 0.0001
bta-miR-204	2.00	< 0.0001	< 0.0001
bta-miR-21-3p	1.85	< 0.0001	0.0006
bta-miR-202	1.77	< 0.0001	< 0.0001
bta-miR-450a	1.75	< 0.0001	< 0.0001
bta-miR-221	1.58	< 0.0001	< 0.0001
bta-miR-450b	1.28	< 0.0001	0.0002
bta-miR-28	1.27	0.0003	0.0028
bta-miR-152	1.20	0.0052	0.0393
bta-miR-2483-5p	1.19	0.0063	0.0457
bta-miR-345-3p	1.13	0.0125	0.0760
bta-miR-339b	1.02	0.0005	0.0056
bta-miR-421	1.01	< 0.0001	< 0.0001

Fold change values are in Log_2 scale

Table 2.5: List of miRNAs down-regulated in granulosa cells of preovulatory dominant follicles

miRNAs ID	FC	*p*-value	FDR
bta-miR-409a	-16.43	0.0097	0.0642
bta-miR-1434-5p	-16.39	0.0143	0.0847
bta-miR-335	-4.35	< 0.0001	< 0.0001
bta-miR-449a	-3.88	0.0001	0.0011
bta-miR-2344	-3.48	0.0073	0.0512
bta-miR-1296	-2.78	< 0.0001	< 0.0001
bta-miR-1271	-2.72	< 0.0001	0.0009
bta-miR-6526	-2.60	< 0.0001	< 0.0001
bta-miR-296	-2.45	< 0.0001	< 0.0001
bta-miR-2285k	-2.33	< 0.0001	0.0003
bta-miR-100	-2.29	0.0007	0.0073
bta-miR-378	-2.01	< 0.0001	< 0.0001
bta-miR-92a	-1.99	< 0.0001	< 0.0001
bta-miR-769	-1.90	< 0.0001	< 0.0001
bta-miR-378b	-1.85	< 0.0001	0.0005
bta-miR-2440	-1.77	0.0160	0.0939
bta-miR-20a	-1.70	< 0.0001	< 0.0001
bta-miR-6120-3p	-1.64	0.0114	0.0713
bta-miR-99b	-1.55	0.0011	0.0106
bta-miR-99a-5p	-1.47	0.0027	0.0217
bta-miR-17-5p	-1.45	< 0.0001	0.0003
bta-miR-365-3p	-1.33	0.0108	0.0682
bta-miR-19a	-1.27	0.0166	0.0956
bta-miR-214	-1.25	0.0106	0.0682
bta-miR-361	-1.24	< 0.0001	0.0001
bta-miR-744	-1.21	0.0057	0.0422
bta-miR-6119-5p	-1.17	< 0.0001	< 0.0001
bta-miR-1343-3p	-1.11	0.0024	0.0206
bta-miR-224	-1.05	0.0017	0.0153
bta-miR-10b	-1.03	< 0.0001	< 0.0001

Fold change values are in Log_2 scale

Prediction of novel bovine miRNAs

Novel miRNAs detected in at least one of the three biological replicates with at least 1 read count are reported. A total of 11 novel miRNAs were predicted by miRDeep2 software, of which 7 were commonly predicted in both the preovulatory dominant and subordinate follicles. However, 3 and 1 predicted novel miRNAs were unique for preovulatory dominant and subordinate follicles, respectively (Table 2.6). Sequence homology search was made for

all the predicted novel miRNAs using the basic local alignment search tool (BLASTN) application against all known annotated matured miRNAs in miRBase release 20. It was shown that no known homologous miRNAs was aligned to all the predicted novel miRNAs. Genomic context analysis of the predicted novel miRNAs revealed that 6 novel miRNAs were transcribed from intergenic region, 4 from intronic region of transcripts (*PDCL2, AFF1, RGS22, and TEX14*) and 1 other from exonic regions of the *MAGED1* gene (Table 2.6). A representative readout of a predicted novel miRNAs is shown in figure 2.3.

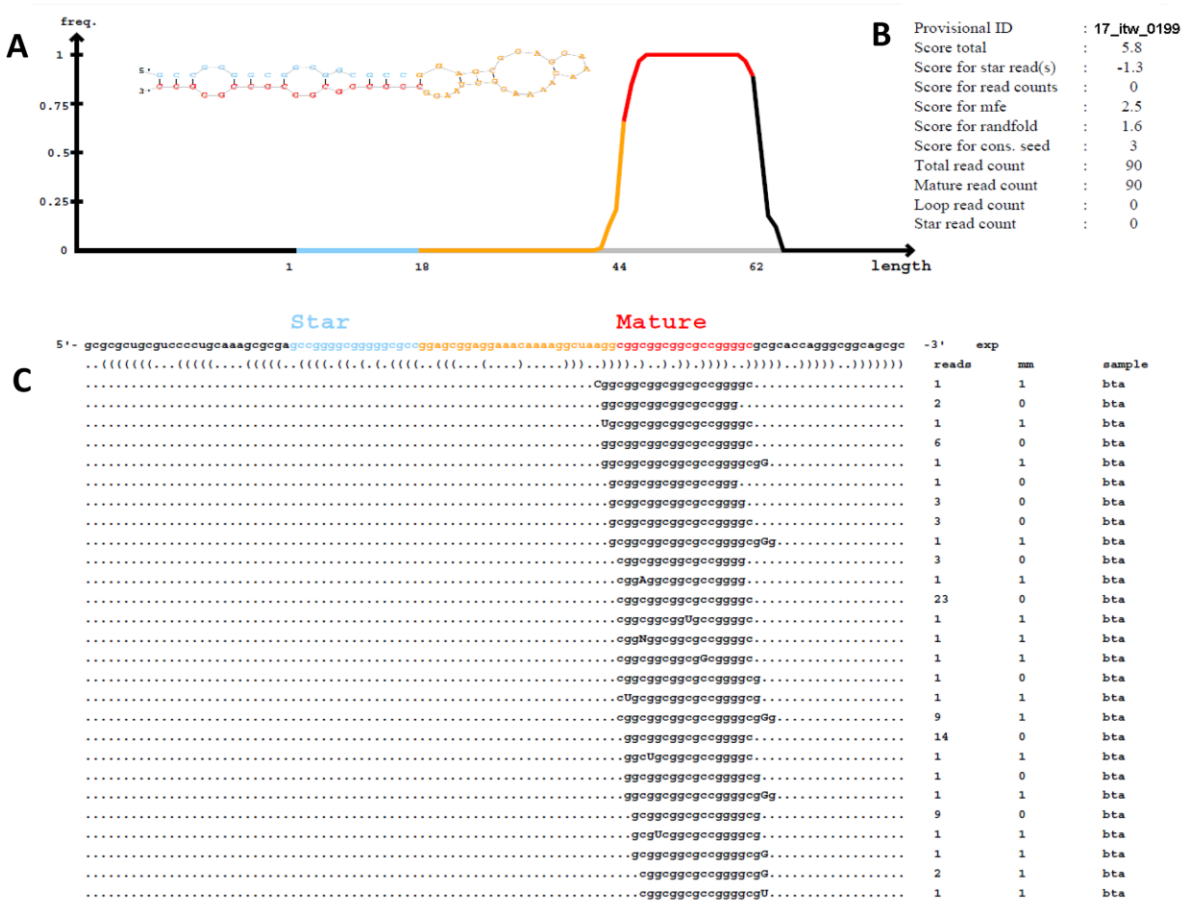

Figure 2.3: Graphic illustration of a representative predicted novel miRNA by miRDeep2. The primary miRNA hairpin with both mature and star miRNAs highlighted with red and blue colors, respectively (**A**). MiRDeep2 scores and provisional ID are shown (**B**). The consensus matured miRNA sequence and other isomiRs and their corresponding read counts are indicated. Mismatched nucleotides of isomiRs with the miRNAs hairpin are written in capital letter (**C**).

Table 2.6: Predicted novel miRNAs expressed in granulosa cells of preovulatory dominant and/or subordinate follicles

Provisional miRNA ID	Matured miRNA Sequence	genomic coordinates and strand of miRNA precursors	Average read count in PDF	Average read count in SF	Genomic region of novel miRNAs and overlapping transcript
4_itw_0216	gggcgcgcgccgcggcu	4:89824112..89824190:-	-	323	Intergenic
X_itw_0135	ccggggccgcgguuccgc	X:62080875..62080910:-	1282	-	Intergenic
X_itw_0174	cccgugaucuggccaaaccc	X:95711630..95711673:-	17.5	-	Exonic, MAGED 1
6_itw_0271	caaaaaguucguccagauuuuu	6:72695823..72695883:+	13.5	-	Intronic, PDCL 2
9_itw_0214	cccgcggggcgcgcgccug	9:25842847..25842909:-	314	2580.3	Intergenic
4_itw_0009	gguggcgggggggagguc	4:114121560..114121624:-	149	93.5	Intergenic
16_itw_0200	cggcggcggcgccggggc	16:26800409..26800490:+	62	26.5	Intergenic
17_itw_0199	cggcggcggcgccggggcgcg	17:72247500..72247567:+	51.5	46.5	Intergenic
6_itw_0083	uaaaaguuugguuggguuuuu	6:103797778..103797838:+	20.7	18	Intronic, AFF1
14_itw_0227	gggggggggccggggcc	14:66538774..66538818:+	7.5	20.5	Intronic, RGS22
19_itw_0090	ggagaggacaccgucugagugg	19:9830946..9830993:-	12	73	Intronic, TEX14

Target gene prediction, gene ontology, and pathways enriched by differentially expressed miRNAs

To understand the functional involvement of differentially expressed miRNAs in bovine follicular development, target genes of each differentially expressed miRNAs were predicted and used to determine the most significantly enriched canonical pathways. Gene ontology (GO) analysis of predicted target genes of differentially expressed miRNAs revealed that biological processes associated with transcription regulation, regulation of cell proliferation and cell death were among the highly enriched GO terms. Representative GO terms enriched by predicted target genes are indicated in (Table 2.S3). Apart from this, a total of 64 canonical pathways were enriched by the predicted target genes of differentially expressed miRNAs. Pathways important in oncogenesis (pathways in cancer and endometrial cancer), cell adhesion (Axon guidance, Focal adhesion and Gap junctions), cell proliferation (MAPK signaling pathway, Wnt signaling pathway, and cell cycle), cell survival (TGF-β signaling pathway) and metabolism (GnRH and insulin signaling pathway) were among the pathways enriched by both up and downregulated miRNAs.

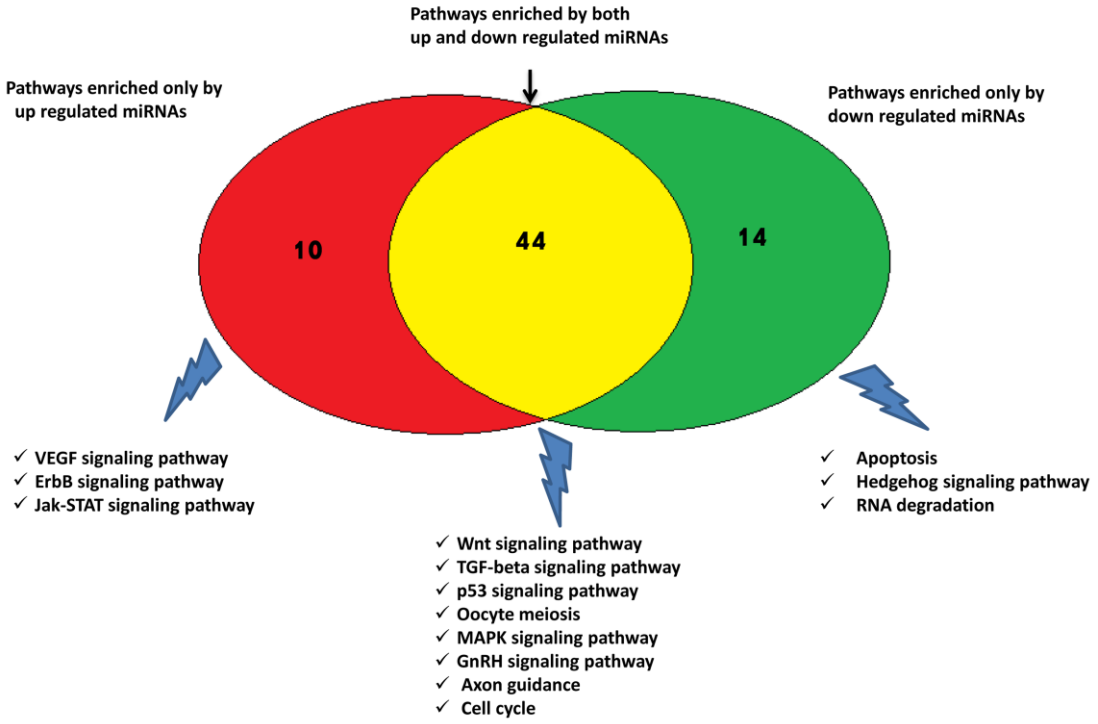

Figure 2.4: Venn diagram showing the number of canonical pathways enriched by predicted target genes of differentially expressed miRNAs. Representative pathways enriched by predicted target of only upregulated miRNAs (red), only down regulated miRNAs (green) and both up and downregulated miRNAs (yellow) are shown.

Pathways like VEGF signaling pathway, ErbB signaling pathways and Jak-STAT signaling pathway were enriched only by miRNAs upregulated in preovulatory dominant follicles. Interestingly, apoptosis pathway, RNA degradation pathway and Hedgehog signaling pathway were enriched only by downregulated miRNAs in preovulatory follicles (Figure 2.4). A representative list of pathways known to be involved in ovarian follicular development along with the list of miRNAs predicted to modulate is indicated in table 2.S4.

Validation of candidate miRNAs using qPCR

Nine representative differentially expressed miRNAs were randomly selected to validate their expression in granulosa cells of preovulatory dominant and subordinate follicles using qPCR. As shown in figure 2.5, the qPCR result was in agreement with the Illumina deep sequencing result.

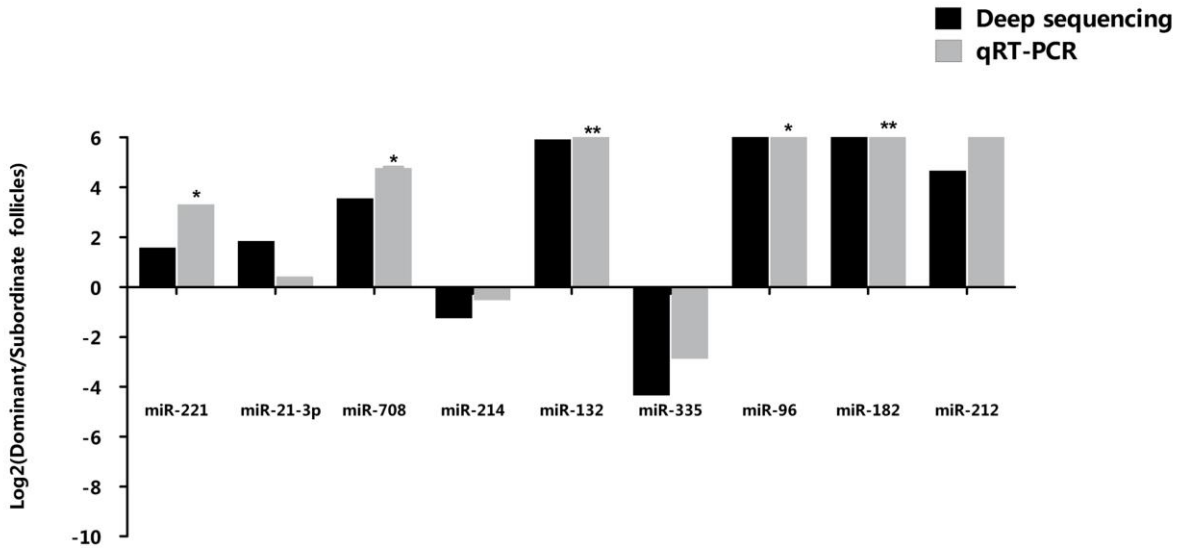

Figure 2.5: QPCR validation of selected candidate miRNAs differentially expressed between preovulatory dominant and subordinate follicles. The relative abundance of individual miRNAs is presented as the ratio of preovulatory dominant and subordinate follicles. The mean expression value of target miRNA was normalized against the expression of endogenous control U6 snRNA and 5s rRNAs. The normalized miRNA expression ($2^{-\Delta CT}$) in preovulatory dominant follicle was divided by the normalized miRNA expression in subordinate follicle and Log_2 transformed. MiRNAs with Log_2 ratio > 0 are up-regulated in preovulatory dominant follicles and miRNAs with Log_2 ratio < 0 are down-regulated in preovulatory dominant follicles compared to the subordinate follicles counterparts. Statistical significance of each miRNA are represented by asterisks; *, $p<0.05$ and **, $p<0.01$

Expression of candidate miRNAs in theca cell, COC, and follicular Fluid

The relative abundance of selected candidate miRNAs was determined in theca cells, COC and follicular fluid derived from preovulatory dominant and subordinate follicles where the corresponding granulosa cells were used for deep sequencing. Results showed that the relative abundance of miR-132 cluster (bta-miR-132 and bta-miR-212) and member of the miR-183 cluster (bta-miR-182, and bta-miR-96) was higher in theca cells, COC and follicular fluid of the preovulatory dominant follicles compared to the subordinate follicles counterparts (Figure 2.6).

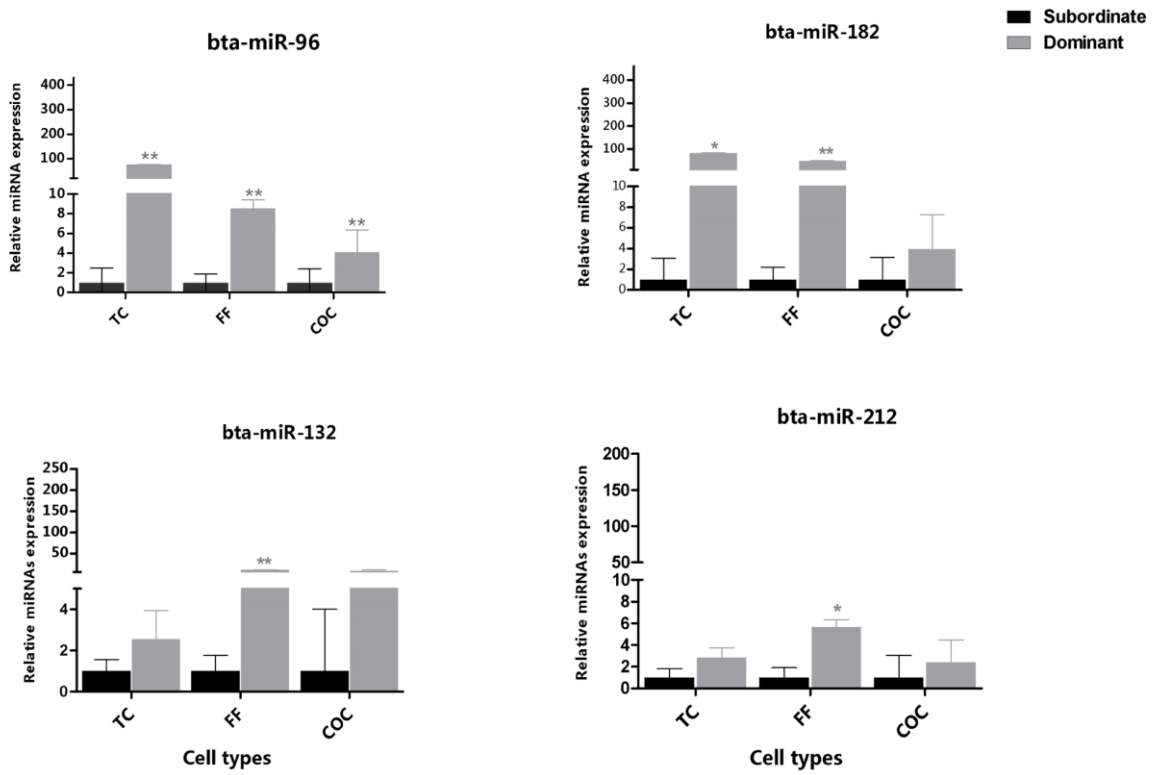

Figure 2.6: Expression pattern of members of the miR-183 cluster (bta-miR-182 and bta-miR-96) and miR-132 cluster (bta-miR-132 and bta-miR-212) in companion follicular cells of both preovulatory dominant and subordinate follicles using qPCR. The mean expression value of target miRNA was normalized against the expression of endogenous control U6 snRNA and 5s rRNAs. Relative expression values were calculated using ΔΔCT method. Error bars represent SD of ΔΔCT values. Statistical significance of each miRNA are represented by asterisks; *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$

Legend: TC= Theca cells COC= cumulus-oocyte-complex FF= follicular fluid

On the contrary, the relative abundance of bta-miR-335 was higher in theca cells, COC and follicular fluid of subordinate follicles compared to the preovulatory dominant follicle. Moreover, the follicular fluid of subordinate follicles was highly enriched with bta-miR-708, bta-miR-221, bta-miR-21-3p, bta-miR-335 and bta-miR-214 compared to the follicular fluid derived from dominant follicles (Figure 2.6 and 2.7).

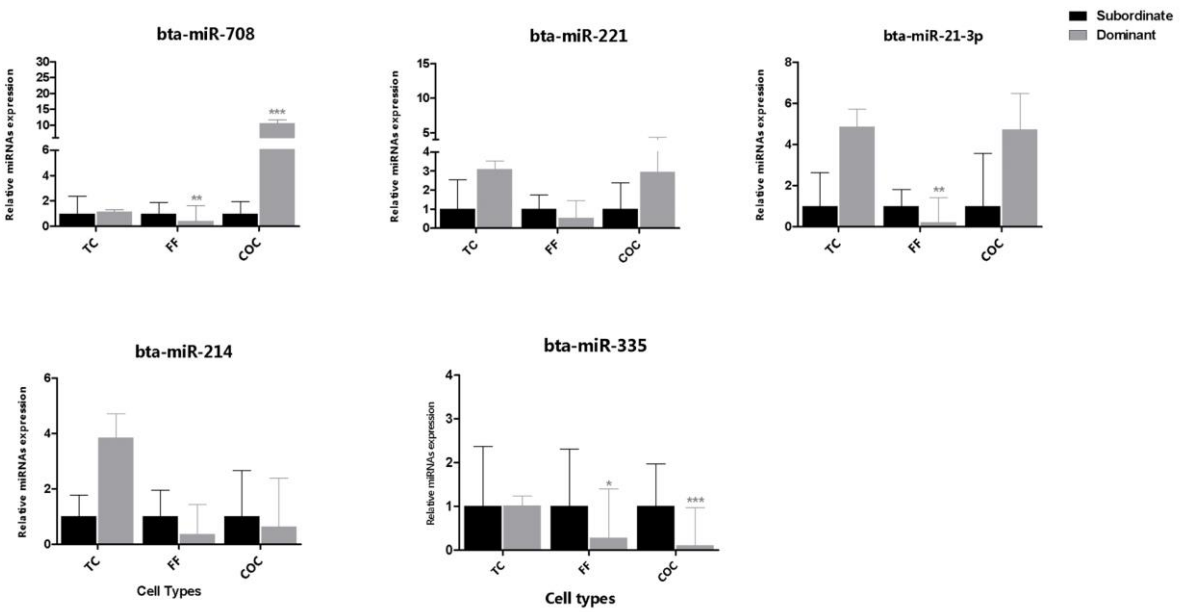

Figure 2.7: Expression pattern of bta-miR-708, bta-miR-221, bta-miR-21-3p, bta-miR-214 and bta-miR-335 in companion follicular cells of both preovulatory dominant and subordinate follicles using qPCR. The mean expression value of target miRNA was normalized against the expression of endogenous control U6 snRNA and 5s rRNAs. Relative expression values were calculated using ΔΔCT method. Error bars represent SD of ΔΔCT values. Statistical significance of each miRNA are represented by asterisks; *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$

Legend: TC= Theca cells COC= cumulus-oocyte-complex FF= follicular fluid

Validation of gene targeted by candidate miRNAs

All members of the miR-183 cluster (bta-miR-183, bta-miR-96 and bta-miR-182) were the top three miRNAs highly enriched in granulosa cells of preovulatory dominant follicles. To find genes targeted by miR-183 cluster members, we used an online miRNA target gene prediction database; targetscan (http://www.targetscan.org/). All members the miR-183 cluster are predicted to co-ordinately target the 3´-UTR of bovine *FOXO1* gene (Figure 2.8). The 3,365 nucleotides long 3'-UTR of bovine *FOXO1* transcript contains binding sites for several miRNAs. To experimentally validate whether miR-183 cluster target the 3´-UTR of

bovine *FOXO1* gene, we cloned a section of the 3′-UTR containing the putative miRNA binding sites into a dual luciferase reporter vector. We showed that the luciferase firefly activity was significantly reduced upon co-transfection the *FOXO1* 3′-UTR plasmid construct with miR-183 cluster miRNA mimics (Figure 2.8). In contrast, co-transfection of miR-183 cluster miRNA mimics with *FOXO1* 3′-UTR mutant construct had no significant effect on the firefly luciferase activity (Figure 2.8).

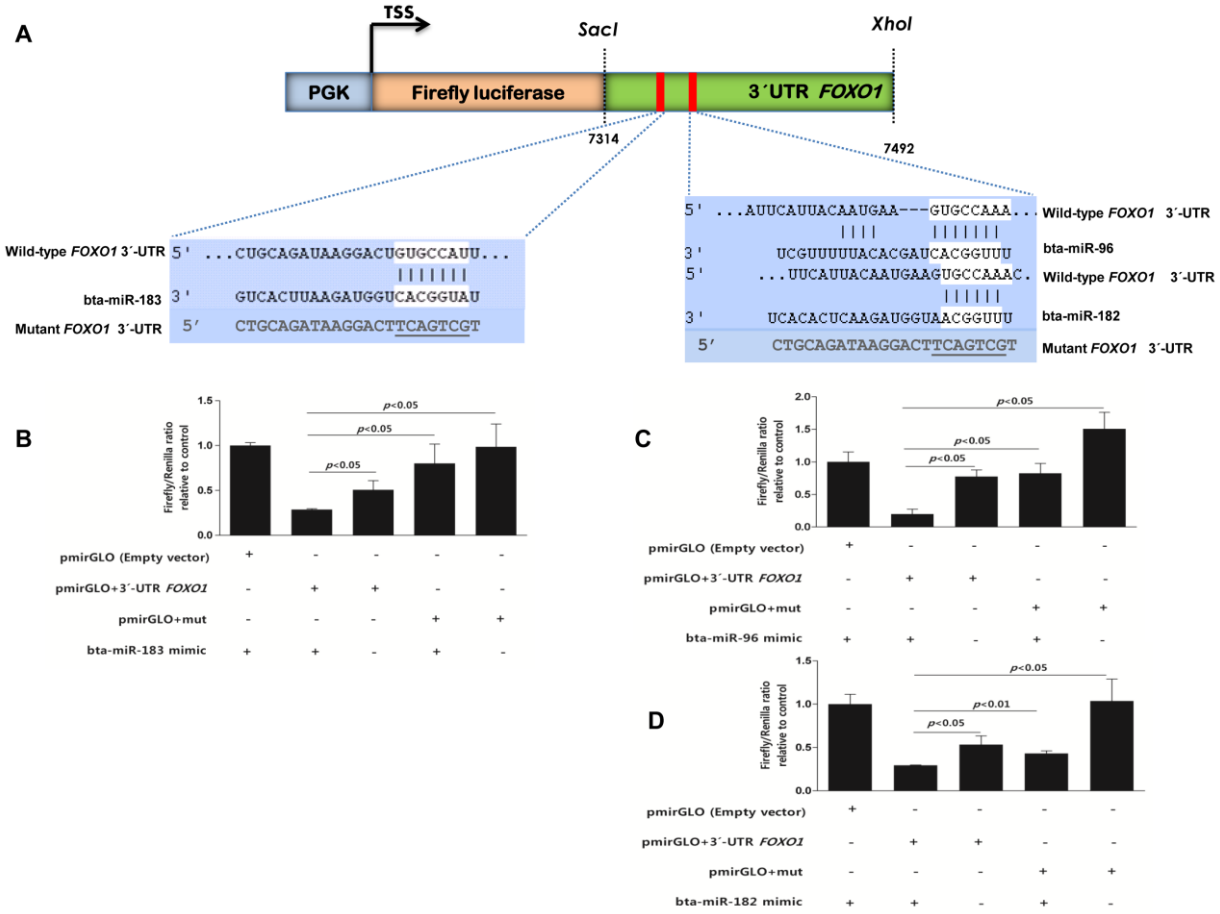

Figure 2.8: MiR-183 cluster miRNAs coordinately target FOXO1 mRNA in bovine granulosa cells. Putative miR-183 cluster binding sites and their genomic coordinates in the 3′-UTR of bovine *FOXO1* mRNA were designed. Plasmid with wild-type and mutant sequences (underlined) for miRNA binding were fused downstream the firefly luciferase gene between *SacI* and *XhoI* restriction sites. The PGK promoter and transcription start site (TSS) of are indicated (**A**). Granulosa cells were transfected with or without bta-miR-183 mimic (**B**), with or without bta-miR-96 mimic (**C**), with or without bta-miR-182 mimic (**D**). The activity of luciferase was significantly inhibited when the bovine *FOXO1* 3′-UTR with wild type of miRNA binding sites was co-transfected with all miR-183 cluster miRNA mimics. However, the activity of Luciferase was not affected when *FOXO1* 3′-UTR

with mutant sequences at the miRNA binding sites were co-transfected with or without miR-183 cluster miRNA mimics.

Interestingly, the qPCR result showed that the expression level of *FOXO1* was highly enriched in granulosa cells of subordinate follicles compared to the preovulatory dominant follicles (Figure 2.9) and the expression profile of *FOXO1* gene showed a reciprocal pattern to the expression of miR-183 cluster miRNAs.

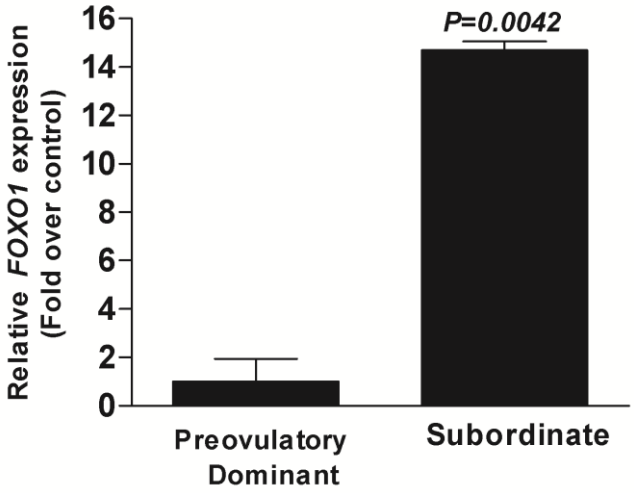

Figure 2.9: FOXO1 is enriched in granulosa cells of subordinate follicles. The relative expression of *FOXO1*; a validated target of miR-183 cluster was quantified in granulosa cells of preovulatory dominant and subordinate follicles using qPCR. The relative expression of target transcript was normalized to the *GAPDH*. Statistical significance between groups was analyzed by two-tailed student t-test. Expression data is presented as mean ± SD of normalized Ct value of biological triplicates.

Discussion

The aim of this study was to investigate the expression pattern of regulatory miRNAs in granulosa cells derived from bovine preovulatory dominant and subordinate follicles using the Illumina deep sequencing approach and consequently to identify their potential role in bovine follicular development during the late follicular phase of bovine the estrous cycle. MicroRNA deep sequencing quantifies the relative abundance of miRNAs by determining their frequencies in terms of read counts. Highly abundant miRNAs have higher likelihood of having higher read counts compared to miRNAs with lower abundance (t Hoen *et al.* 2008). Of the total detected miRNAs, only 28 and 36 miRNAs were found to be specific to

preovulatory and subordinate follicles, respectively while majority of the detected miRNAs (>80%) were commonly expressed between dominant and subordinate follicles. This implies that majority of the detected miRNAs may play a housekeeping role in maintaining normal physiological function in ovarian granulosa cells during the late follicular phase of the oestrus cycle. Interestingly, bta-miR-26a, bta-miR-10b and 3 isoforms of the let-7 family (bta-let-7a-5p, bta-let-7f, and bta-let-7i) were among the top 10 abundantly expressed miRNAs in granulosa cells of both preovulatory dominant and subordinate follicles. We previously showed that these miRNAs are abundantly expressed in bovine granulosa cells derived from both dominant and subordinate follicle during the early luteal phase of bovine estrous cycle (Salilew-Wondim *et al.* 2014). Similarly, Miles *et al.* (2012), applied cDNA libraries and massive parallel sequencing, showed members of the let-7 family to be abundantly expressed in bovine ovaries. Similarly, bta-miR-26a, let-7 family, bta-miR-10b and bta-miR-143 were among the top 10 abundantly expressed miRNAs in bovine ovarian and testicular tissues (Huang *et al.* 2011). Previous study identified that Let-7 family and bta-miR-143 are abundantly expressed in cDNA library cloned from bovine ovary signifying their potential role in bovine ovarian functions (Hossain *et al.* 2009).

In addition to the possibilities of quantifying the relative abundance of miRNAs, profiling the expression of miRNAs using next-generation sequencing has greater advantage over other array based miRNome profiling tools (microarray and PCR panels) and direct sequencing of cDNA clones for its capability to predict novel unannotated miRNAs (Friedlander *et al.* 2008). Currently, there are 35,828 matured miRNAs discovered from 223 species deposited in the latest release of miRbase (release 21.0 June, 2014). The numbers of discovered bovine miRNAs are limited to 793, compared with 2,588 in human and 1,915 in mouse. We showed that 50-56% the sequence reads mapped to the reference bovine genome was mapped to known bovine miRNAs signifying that the sequencing libraries are predominantly enriched with miRNAs. Moreover, 11 novel miRNAs have been predicted from the sequence reads which were not mapped to reported matured miRNAs. In agreement with previous results (Glazov *et al.* 2008), part of the remaining sequence reads that were not aligned to known miRNAs may represent novel miRNAs or other class of regulatory RNAs.

During miRNA biogenesis, a miRNA precursor is clipped by an endonuclease enzyme Drosha and exported to the cytoplasm for further processing by Dicer and give rise to a double stranded miRNA-duplex. The thermodynamic stability of each end of the miRNA-duplex determines the biologically active strand that can be incorporated into the miRNA-induced silencing complex (miRISC) while the other strand is considered to be inactive and

subsequently be degraded (O'Toole *et al.* 2006). Similarly, the asymmetric stability of the Dicer cleavage miRNA-duplexes suggests to determine the miRNA arm choice (Hutvagner 2005). However, there are growing evidence indicating both arms of a miRNA precursor could be functional by targeting the same gene co-ordinately (Yang *et al.* 2013) and/or can have different targeting properties and different biological functions (Marco *et al.* 2012). Results demonstrated functional 3p and 5p arms of certain miRNA precursors. For instance, the 5p and 3p arms of mir-17 precursor were found to be expressed in both the preovulatory and subordinate follicles with slightly higher expression of the 5p arms. Both miR-17-5p and miR-17-3p have been implicated in co-ordinately targeting the *TIMP* metallopeptidase inhibitor 3 (*TIMP3*) gene and induce growth and invasion of prostate tumour (Yang *et al.* 2013). We found the 3p arm of mir-22 to be highly abundant compared to its corresponding 5p arm with relatively higher abundance in preovulatory dominant follicles and it also reported to inhibits the Estrogen signaling pathways by inhibiting the expression of Estrogen Receptor alpha (*ERα*) mRNA (Pandey & Picard 2009). This is further supported by the fact that there a sharp decline in estradiol concentration in the circulation during the preovulatory period (Komar *et al.* 2001). Thus, it can be assumed that the 3p arm of bta-mir-22 precursor is functionally more relevant than the corresponding 5p arm during the late follicular phase of preovulatory stage of bovine estrous cycle.

Differential expression of certain miRNAs in different stages of bovine follicular developments and cell types may provide valuable insight into their potential role in folliculogenesis in stage manner. Both bta-miR-21-3p and bta-miR-21-5p are enriched in granulosa cells of preovulatory dominant follicle with 3.6 and 2.1-folds higher, respectively. Moreover, bta-miR-21-5p was among the top 10 miRNAs abundantly expressed in preovulatory dominant follicles with average read counts of 8,695. Upregulation of miR-21 in mouse luteinizing mural granulosa cells was reported following human chorionic gonadotropin (hCG) treatment (Carletti *et al.* 2010). Increased cell apoptosis was observed in mural granulosa cells transfected with miR-21 targeting LNA oligonucleotide (miR21-LNA) evidencing the critical role of miR-21 in preventing apoptosis of granulosa cells of preovulatory follicle following the LH surge. Moreover, in-vivo experiments demonstrated that there was significant reduction in ovulation rate and subsequently lowered the number of cumulus-oocyte-complex recovered in oviducts of miR21-LNA inhibitor treated ovaries compared to the untreated controls. This implies that bta-miR-21 plays important role in regulating bovine follicular development and preventing apoptosis of granulosa cells by targeting the 3′-UTR of pro-apoptotic genes in preovulatory dominant follicles.

Similarly, the miR-132 family (bta-miR-132 and bta-miR-212) was among the robustly expressed miRNAs in granulosa cells of preovulatory dominant follicles with 60 and 26-fold higher, respectively compared to the subordinate follicles counterparts. Bta-miR-132 and bta-miR-212 are transcribed from intergenic region of chromosome 19 of the bovine genome and have the same seed region. Fiedler *et al.* (2008) demonstrated that upregulation of both miR-132 and miR-212 in mouse preovulatory granulosa cells following the induction of ovulatory dose of LH/hCG. Inhibiting the expression of both miR-132 and miR-212 using LNA inhibitor showed increased the C-terminal binding protein-1 (*CTBP1*) protein levels. Interestingly, *CTBP1* regulates adrenal steroidogenesis by periodically interacting with steroidogenic factor 1 (*SF1*), which in turn regulates the transcription of *CYP17* gene (Fiedler *et al.* 2008).

We showed bta-miR-378 to be 4-folds lower in granulosa cells of preovulatory dominant follicles in comparison with the subordinate ones. MiR-378 targets the 3´-UTR of aromatase gene *(CYP19A)*; a gene responsible for estradiol biosynthesis in granulosa cells and inhibition of miR-378 in vitro resulted in an increased estradiol production implying aromatase gene is post-transcriptionally regulated by the action of miR-378 (Xu *et al.* 2011). Thus, downregulation of miR-378 in preovulatory dominant follicles may suggest increased level of aromatase gene. Similarly, bta-let-7f as one of the eight let-7 family isoforms found to be highly abundant both in dominant and subordinate follicles with slightly higher expression in subordinate follicles. The let-7f is reported as a tumour suppressor miRNA in breast cancer cells and further validated to target *CYP19A* gene (Shibahara *et al.* 2012).

Members of the miR-183 cluster; bta-miR-96, bta-miR-182 and bta-miR-183 are transcribed from intergenic region of chromosome 4 of the bovine genome. All members of the miR-183 cluster were found to be the top 3 highly enriched miRNAs in granulosa cells of preovulatory dominant follicles with fold regulation of 130.7, 89.9 and 85.7, respectively. We showed that these list of conserved miRNAs target the 3´-UTR *FOXO1* gene; a transcription factor which induces expression of genes involved in apoptosis, glucose metabolism, cell cycle progression and differentiation (Myatt *et al.* 2010). Similarly the expression of the apoptotic *FOXO1* gene showed marked reduction in granulosa cells preovulatory dominant follicles showing opposite expression pattern with miR-183 cluster miRNAs. This could signify that enrichment of *FOXO1* in subordinate follicles could facilitate the activation of pro-apoptotic downstream target genes which in turn play role in follicular atresia. In consistence with the finding, Shi and LaPolt (2003) showed decreased expression of *FOXO1* protein in granulosa cells of healthy preovulatory follicles compared to the follicles

undergoing atresia. Similarly, increased *FOXO1* expression in mouse granulosa cells has been implicated with accumulation of reactive oxygen species (ROS) (Shen *et al.* 2012). Following the LH surge, numerous genes related to inflammation are expressed in preovulatory follicles, which lead to massive recruitment of ROS, macrophages, and neutrophils from inflammatory cells (Richards *et al.* 2002). Depletion of both neutrophils and macrophages can reduce ovulation in rat and mouse ovaries (Brannstrom *et al.* 1995; Van der Hoek *et al.* 2000). Thus, further *in-vitro* functional study is needed to confirm whether the miR-183 cluster in granulosa cells of preovulatory dominant follicles co-ordinately suppress the expression of *FOXO1* and other downstream pro-apoptotic genes and prevent apoptosis of granulosa cells. Furthermore, the role of miR-183 cluster in ROS accumulation in granulosa cells is yet to be determined.

Expression of selected miRNAs in theca cell, COC showed that the relative abundance of the member of the miR-183 and miR-132 cluster is higher in preovulatory dominant follicles. This supports the crosstalk between granulosa cells and other companion cells within the follicle. Furthermore, increased miRNAs level in follicular fluids could further be supported by the fact that there are significant number of circulatory miRNAs in bovine follicular fluid carried by exosomes and have potential role in cell-to-cell communication in follicular microenvironment (Sohel *et al.* 2013).

Pathways known to be involved in ovarian functions and hormonal regulation namely; Axon guidance, MAPK signalling pathway, Wnt signaling pathways, TGF-β signaling pathway, GnRH signaling pathways and progesterone-mediated oocyte maturation were among the highly enriched canonical pathways enriched by predicted target genes of differentially expressed miRNAs. For instance, the Wnt signaling pathway is enriched by 10 up-regulated and 10 down-regulated miRNAs and is known to be involved in mammalian reproduction including follicular development, ovulation, formation and regression of Paramesonephric duct (Kobayashi *et al.* 2011). Similarly, the *Wnt-4* gene regulates the function of ovarian granulosa cells in rodent ovary in stage-specific expression of specific *Wnt/Fz* genes (Hsieh *et al.* 2002). Interestingly, ErbB signalling pathway was enriched by only target genes of miRNAs upregulated in preovulatory dominant follicles (bta-miR-129-5p, bta-miR-221, bta-miR-339b and bta-miR-96) and suggests possible involvement of these miRNAs in modulating ErbB gene family during bovine follicular development. Similarly, apoptosis pathway was enriched by only target genes of miRNAs down-regulated in preovulatory dominant follicles (bta-miR-1271, bta-miR-17-5p and bta-miR-365-3p). This signifies enrichment of specific miRNAs in subordinate follicles could trigger apoptosis pathways by post-transcriptionally regulating the

balance between pro and anti-apoptotic genes to determine the fate of follicular cells and cause follicular atresia.

Conclusion

The spatio-temporal expression of miRNAs in granulosa cells during the follicular phase of the estrous cycle supports the potential role of miRNAs in post-transcriptional regulation of genes involved in bovine follicular development, mainly ovulation of a preovulatory dominant follicle and regression of anovulatory subordinate follicles. In addition to the differential expression of miRNAs, the present study identified cluster of miRNAs which are abundantly expressed in granulosa cells of both preovulatory and regressing follicles signifying their housekeeping role during follicular development. The information we provided here may be helpful in deciphering the molecular mechanism of bovine follicular ovulation and atresia. Further *In-vitro* experiment is required to fully understand the specific functional role of classes or cluster of miRNAs during various stages of the follicular development in general and the follicular phase of the estrous cycle in particular.

Acknowledgements

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Chapter 3

MicroRNA-183-96-182 Cluster Regulates Bovine Granulosa Cell Proliferation and Cell Cycle Transition by Coordinately Targeting *FOXO1*

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ABSTRACT

Large-scale expression profiling of micro-RNAs (miRNAs) in bovine granulosa cells from dominant and subordinate follicles on Day 19 of the estrous cycle revealed enriched micro-RNA-183-96-182 cluster miRNAs in preovulatory dominant follicles that coordinately regulate the forkhead box protein O1 (FOXO1) gene. However, little is known about the role of this cluster in bovine granulosa cell function. We used an in vitro granulosa cell culture model to investigate this role. Granulosa cells aspirated from small growing follicles (3–5 mm in diameter) were cultured in Dulbecco modified Eagle medium/F-12 medium supplemented with fetal bovine serum and transfected with locked nucleic acid-based miRNA mimics, inhibitors, and corresponding negative controls. Overexpression of the miRNA cluster resulted in suppression of *FOXO1* mRNA and protein, whereas inhibition of the cluster increased expression of *FOXO1* mRNA. Overexpression also increased the relative rate of cell proliferation, whereas inhibition slowed it down. Similarly, the proportion of cells under G_0/G_1 arrest declined, whereas the ratio of cells in S phase increased in response to miR-183-96-182 overexpression. Selective knockdown of *FOXO1* mRNA using anti-FOXO1 small interfering RNA increased the rate of granulosa cell proliferation, decreased the proportion of cells under G_0/G_1 arrest, and increased the proportion of cells in the S-phase of cell cycle. Our data suggest that miR-183-96-182 cluster miRNAs promote proliferation and G_1/S transition of bovine granulosa cells by coordinately targeting *FOXO1*, suggesting a critical role in granulosa cell function.

Keywords: Cell cycle, Cell proliferation, *FOXO1,* Granulosa cell, miR-183-96-182 cluster

INTRODUCTION

Bovine ovarian follicular growth is a complex process where cohort follicles of various sizes undergo a series of maturational waves accompanied by dramatic changes in the shape and function of granulosa cells (Robker & Richards 1998). Among the factors involved in regulating follicular development, the pituitary gonadotropic hormones (mainly follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) are known to play critical roles in controlling ovarian functions by triggering several downstream pathways (Makker *et al.* 2014). This triggering action, in turn, leads to various morphological and functional changes in follicular cells (Hirshfield 1991). These sequential changes in the morphology and function granulosa cells during various stages of follicular development are tightly regulated

by the expression of multitude of genes (Douville & Sirard 2014; Hatzirodos *et al.* 2014b) and intraovarian growth factors and steroids (Hirshfield 1991).

The wingless-type MMTV integration site family member 4 (WNT4) and members of the transforming growth factor β (TGF-β) superfamily are among the major genes involved in follicular growth and granulosa cell functions (Boyer *et al.* 2010; Fenwick *et al.* 2013). Transcription factors like the forkhead box (FOXO) transcription factor have emerged as important regulators of mammalian ovarian functions (Uhlenhaut & Treier 2011). The FOXO genes work coordinately with the phosphatidylinositide 3-kinase (PI3K)/AKT signaling pathway for their activation (Hannenhalli & Kaestner 2009). Translational modifications regulate the activity of FOXO with non-phosphorylated (Zhang *et al.* 2013) FOXO binding to FOXO-recognized elements (FRE) within the promoter regions of target genes to trans-activate or repress the expression of downstream transcripts (Shen *et al.* 2014). Phosphorylation of FOXO proteins causes its nuclear exclusion and subsequent degradation in the cytoplasm, which leads to inhibition of FOXO-mediated transcription (Huang & Tindall 2007). The FOXO genes are involved in primordial follicle activation, oocyte maintenance, and granulosa cell proliferation and differentiation (Makker *et al.* 2014). Based on these facts, it is predicted that the large set of genes that play a significant role in mammalian follicular development and granulosa cell proliferation and differentiation, in one way or another be regulated transcriptionally or post-transcriptionally.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that have emerged as regulatory molecules involved in modulating the expression of genes at the post-transcriptional level. This occurs by either degrading the messenger RNA (mRNA) or by suppressing protein translation (Ying *et al.* 2006). In addition to the expression profiling miRNAs in follicular cells (Salilew-Wondim *et al.* 2014; Gebremedhn *et al.* 2015), there is growing evidence of the involvement of miRNAs in various important cellular physiological processes, including granulosa cell proliferation (Yao *et al.* 2010; Yan *et al.* 2012; Zhang *et al.* 2013), cell cycle transition (Jiang *et al.* 2015), apoptosis (Carletti *et al.* 2010; Liu *et al.* 2014; Rui *et al.* 2015; Zhou *et al.* 2015), hormone biosynthesis (Wu *et al.* 2015), oocyte developmental competence and cell-to-cell communication (da Silveira *et al.* 2012; Sohel *et al.* 2013), and ovarian disorders like polycystic ovary syndrome (Hossain *et al.* 2013).

We have demonstrated differential expression of miRNAs between granulosa cells of subordinate and dominant follicles obtained on Day 3, day 7 (early luteal phase) (Salilew-Wondim *et al.* 2014), and Day 19 (late follicular phase) (Gebremedhn *et al.* 2015) of the bovine estrous cycle. Among the various clusters, the microRNA-183-96-182 (miR-183-96-

182) cluster miRNAs were found to be the top three most significantly upregulated in granulosa cells of preovulatory dominant follicles compared to their subordinate counterparts (Gebremedhn *et al.* 2015). In silico analysis of the miR-183-96-182 cluster miRNAs revealed an array of predicted target genes that are known to be involved in various physiological processes, including apoptosis, the cell cycle, and metabolism.

A wet-laboratory dual-luciferase reporter assay technique confirmed that all miRNAs of the miR-183-96-182 cluster coordinately target the *FOXO1* gene (Gebremedhn *et al.* 2015). In the same study, as opposed to the expression of the miRNA cluster, we showed that *FOXO1* mRNA is highly enriched in granulosa cells of bovine subordinate follicles compared to their preovulatory dominant counterparts. Thus, further understanding of the molecular crosstalk between the miRNA cluster and the *FOXO1* transcription factor and the phenotypic changes in bovine granulosa cell function are needed. This information would widen our understanding of the role of miRNAs in regulating important transcription factors in bovine folliculogenesis. We aimed to decipher the functional role of the miR-183-96-182 cluster miRNAs in granulosa cells using an *in vitro* loss-and-gain functional analysis. The results provide evidence of the regulatory role of these miRNAs in bovine granulosa cell proliferation and cell cycle transition by targeting the *FOXO1* gene as a transcriptional factor, which subsequently regulates the expression of other downstream transcripts.

MATERIALS AND METHODS

Ovarian Sample Collection and Granulosa Cell Isolation

Bovine ovaries were collected from a local abattoir and transported in vacuum flask containing warm physiologic saline solution (0.9% NaCl) to the laboratory. Afterwards, ovarian samples were processed as previously described by Gebremedhn *et al.* (2015). Briefly, upon arrival, ovaries were washed twice with warm (37°C) phosphate buffer saline without Ca2+/Mg2+ (PBS^{-}). They were then rinsed in 70% warm ethanol for 30 sec, followed by washing three times with PBS^{-}. Granulosa cells were aspirated from small healthy growing follicles (3- to 5-mm diameter) using 20-gauge sterile needles (B-Braun, Melsungen, Germany) and transferred into a 15-ml sterilized tube (Falcon; Thermo Fisher Scientific, Dreieich, Germany) containing warm PBS^{-}.

The cumulus-oocyte-complexes (COC) were left to settle down at the bottom of the tube. The upper suspension of follicular fluid with floating granulosa cells was carefully transferred into 15-ml tubes and centrifuged at 750*xg* for 7 min. The supernatant follicular fluid was removed, and granulosa cell pellets were resuspended in red blood cell lysis buffer

for 1 min. Next, the pellets were washed with Dulbecco modified Eagle medium/F-12 Ham culture medium (DMEM/F-12 Ham; Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). After granulosa cells were centrifuged briefly and washed with PBS^{-}, samples were resuspended in DMEM/F-12 Ham culture medium supplemented with 10% FBS. Cell viability was assessed using trypan blue (Sigma-Aldrich) exclusion.

Granulosa Cell Culture

Isolated granulosa cells were seeded at 2 $x10^5$ cells per well in a tissue culture-treated 24-well plate (Starlab, Hamburg, Germany) in DMEM/F-12 Ham supplemented with 10% FBS, 1% penicillin-streptomycin (Sigma-Aldrich), and 1% fungizone (Sigma-Aldrich). Cells were incubated at 37^{O}C in a humidified chamber with 5% CO2 atmosphere. Culture medium was replaced with fresh medium every 48 h. To determine the effect of plating on the expression of the miR-183-96-182 cluster miRNAs and gene markers, cultured granulosa cells were harvested using 0.25% trypsin-EDTA (Sigma-Aldrich) at 24, 48, 96, and 144 h after being plated. Freshly isolated granulosa cells were snap frozen immediately and used as controls for timed expression patterns of miRNAs and genes.

Locked Nucleic Acid-Oligonucleotide Transfection

To determine the role of miR-183-96-182 cluster miRNAs in bovine granulosa cells, an in vitro gain-and-loss of function experiment was performed. The experiment was done using Locked Nucleic Acid (LNA)-mediated oligonucleotide miRNA mimics, mimic negative control, inhibitors, and inhibitor negative control (Exiqon, Vedbæk, Denmark). Oligonucleotide transfection was performed in subconfluent (75–80%) plated granulosa cells, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent in Opti-MEM I reduced-serum medium (Invitrogen).

According to the manufacturers' instructions, 75 nM miRNA mimic, inhibitor, or corresponding negative control was added to each well of the 24-well plate. After 24 h of incubation, the culture medium was replaced with fresh medium. The efficiency of the LNA-oligonucleotide transfection in either decreasing or increasing the expression of target miRNAs was assessed 48 h later by using quantitative real-time PCR (qPCR). Similarly, the sponge effect of miRNA inhibition was assessed using qPCR. In this phenomenon, inhibition of one miRNA in a cluster results in a decrease in the expression of another miRNA within the cluster.

RNA Interference

Two antisense LNA GapmeRs (Exiqon) targeting bovine FOXO1 mRNA (FOXO1-short interfering RNAs [siRNAs]) and negative control siRNA (NCsiRNA) (Table 3.S1; supplemental data are available online at www.biolreprod.org) were used for targeted knockdown of bovine FOXO1mRNA. For this, $2x10^5$ cells were seeded onto a 24-well plate. Sub confluent cells were transiently transfected with pooled 75 nM FOXO1-siRNAs or NC-siRNA, using Lipofectamine 2000 (Invitrogen) in Opti-MEM I reduced-serum medium. Twenty-four hours post transfection, the culture medium was replaced with fresh medium. Cells were trypsinized 48 h post transfection and used for subsequent molecular and cellular phenotype analyses.

Cell Proliferation Assay

We evaluated the effect of overexpression or inhibition of miR-183-96-182 cluster miRNAs and selective degradation of FOXO1 mRNA on the proliferation rate of granulosa cells. A 96-well plate was seeded with $2x10^4$ cells, and subconfluent cells were transfected with 75 nM miR-183-96-182 cluster miRNA mimics or mimic negative control, miRNA inhibitors, or inhibitor negative control, and FOXO1-siRNAs or NC-siRNA. Cell viability was measured using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technology, Kumamoto, Japan) at 48, 72, 96, and 120 h post-transfection according to the manufacturer's instructions. Briefly, 10 µl of CCK-8 was added to each well, and plates were incubated for 3 h at 37°C in 5% CO_2 atmosphere. The optical density (OD) of released formazan dye was measured as an indicator of the number of living cells at a wavelength of 450 nm, using a microplate reader (BioTek Instruments Inc, Friedrichshall, Germany). Blank OD measurements were obtained from wells containing only culture medium and used for normalization. The increase in viable cell number relative to the first measurement at 48 h post-transfection (given an arbitrary value of 1) was used to calculate the relative proliferation of cells.

Cell Cycle Assay

We next determined the role of miR-183-96-182 cluster miRNAs and the FOXO1 gene in granulosa cell cycle regulation. Cultured granulosa cells were transfected with 75 nM miR-183-96-182 cluster miRNA mimics or mimic negative control, miRNA inhibitors or inhibitor negative control, and FOXO1-siRNAs or NC-siRNA. Cells were trypsinized 48 h later and collected in a 15-ml Falcon tube, followed by centrifugation at 7503g for 5 min and being washed twice with 1xPBS^-. A minimum of ~$1x10^6$ cells were fixed in ice-cold 70% ethanol at

4°C overnight. Cells were then centrifuged briefly, and the cell pellets were washed twice with 500 µl of 1xPBS^{-}. Cells were stained with 50 µg/ml of propidium iodide (PI) and 50 µg/ml of RNase. Cells were then incubated at 37^{O}C for 30 min and processed using an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Cell cycle distribution was analyzed using ModFit LT software (http://www.vsh.com/products/mflt/index.asp).

Western Blot Analysis

Whole-cell protein lysate was prepared from granulosa cell samples collected 48 h after transfection, using 1X passive lysis buffer (Promega, Madison, WI). Protein concentration was determined using the Bradford method (Bradford 1976). Equal amounts (30 µg) of protein lysate from each sample were electrophoresed in 8–10% gradient SDS-PAGE and transferred onto nitrocellulose membrane (Whatman-protran; Sigma-Aldrich). Membranes were blocked in 1X Roti-Block blocking solution (Carl Roth, Karlsruhe, Germany) at room temperature for 1 h. They were then incubated overnight at 48C with diluted primary antibodies of polyclonal rabbit anti-FOXO1 (1:150 dilution; code sc-11350; Santa Cruz Biotechnology, Dallas, TX) or with diluted mouse monoclonal anti-ß-actin (1:1000 dilution; Santa Cruz Biotechnology) as an internal control.

After being washed three times with diluted Tris-buffered saline with Tween 20 (13 TBST), the membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit (1:5000 dilution) or goat-anti-mouse (1:5000 dilution) secondary antibodies for 2 h at room temperature. Subsequently, membranes were washed three times with 1X TBST, and specific signals were visualized with enhanced chemiluminescence, using Clarity Western ECL substrate (Bio-Rad, Munich, Germany). Images were acquired using Quantity One 1-D software (Bio-Rad) and a Gel Doc XRS+ imaging system (Bio-Rad).

RNA Isolation and qPCR

Total RNA enriched with miRNA was isolated from all granulosa cell samples by using the miRNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. On-column DNA digestion was performed to remove genomic DNA contamination by using RNase-free DNase (Qiagen). Total RNA samples were quantified using a NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A 1-µg portion of total RNA sample from each treatment group was reverse-transcribed to cDNA by using First-Strand cDNA synthesis kit (Thermo Fisher Scientific) with oligo $(dT)_{18}$ primers.

Gene-specific primers were designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/). For semi-quantitative PCR, thermocycling consisted of preincubation at 95°C for 5 min, then 40 cycles of 30-sec denaturation at 95°C, 30-sec of annealing at 55°C (GAPDH), and 57°C (FSHR and LHR) for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min, and maintenance at 4°C. The specificity of each primer was confirmed by sequencing the PCR products and BLAST analysis of the sequence results. Finally, 10 µl of PCR product was mixed with 5 µl of loading buffer, loaded onto 2% agarose gel stained with ethidium bromide, and visualized under UV light, using Gel Doc XRS+ imaging system (Bio-Rad).

Similarly, qPCR experiments were performed using a reaction volume of 20 µl with 7.4 µl of ddH_2O, 0.3 µl of forward primer, 0.3 µl of reverse primer, 10 µl of 13 SYBR Green I Master Mix (Bio-Rad), and 2 µl of cDNA template. Thermocycling conditions consisted of preheating at 95°C for 3 min, followed by 40 cycles of amplification at 958C for 15 sec, and 1 min at 608C. The specificity of PCR amplification was determined by melting curve analysis generated at the end of each qPCR run. Due to the stable expression of *GAPDH* in granulosa cells of various stages (before and after LH surge) (Douville & Sirard 2014), the expression of *GAPDH* was used as an internal control to normalize the expression of genes of interest.

Candidate miRNAs were quantified as described previously (Salilew-Wondim *et al.* 2014b; Gebremedhn *et al.* 2015). Briefly, cDNA was synthesized from 80 ng of miRNA-enriched total RNA; using a miRCURY LNA™ Universal cDNA synthesis kit (Exiqon) according to the manufacturer´s instructions. The resulting cDNA was 40x diluted and used for qPCR analysis of candidate miRNAs using ExiLENT SYBR Green Master mix (Exiqon). Thermal cycling conditions were preheating at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 10 sec and 60°C for 1 min. The specificity of each miRNA amplification was evaluated by melting curve analysis. The geometric mean of the expression of U6 small noncoding small nuclear RNA (snRNA) and 5S ribosomal RNA was used to normalize the expression values of candidate miRNAs. qPCR data were analyzed using the comparative cycle threshold (C_t) method (Livak & Schmittgen 2001). The list of primers used in the experiment is available in Table 3.S2.

Statistical Analysis

Statistical analysis was performed using Prism version 5 software (GraphPad, LaJolla, CA). Data are mean ± SEM biological replicates. Statistical differences in mean expression values of two treatment groups were compared using a two-tailed Student t-test. For time-course

expression analysis, the normality of expression data was first assessed using a D'Agostino and Pearson omnibus normality test for the residual values of the normalized expression values. Equality of variance was evaluated using the Bartlett test. Statistical differences among means were then analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett post-hoc test. Statistical significance was defined as a *P* value of ≤ 0.05.

RESULTS

Expression of MiR-183-96-182 Cluster miRNAs in Bovine Granulosa Cells Increased While FOXO1 mRNA Decreased Upon Plating

We determined whether *in vitro* granulosa cell plating affected the identity of granulosa cells and transformed them into luteal cells. To achieve this, the presence of *FSHR* and *LHR* was determined at different time points during plating as marker genes of granulosa and luteal cells, respectively. We also examined the expression of ovulatory genes that are known to be induced by *LH* surge, namely *PTX3* and *PTGS2* across plating time. *FSHR* was detected in granulosa cells before and after plating. *LHR* was not detected in any of the times before and after plating of granulosa cells (Figure 3.S1-A). Similarly, expression levels of *PTX3* and *PTGS2* were not induced during the culture period, as shown in figure 3.S1-B.

The relative abundance of miR-183-96-182 cluster miRNAs in granulosa cells was analyzed in cultures collected at 24, 48, 96, and 144 h post plating. Results revealed that the relative expression of all mature miRNAs of the cluster increased with time, reaching significant levels starting at 48 h after plating (Figure 3.1A–C). Interestingly, *FOXO1* expression was significantly reduced during the culture period (Figure 3.1D), indicating a reciprocal expression pattern with the miRNA cluster.

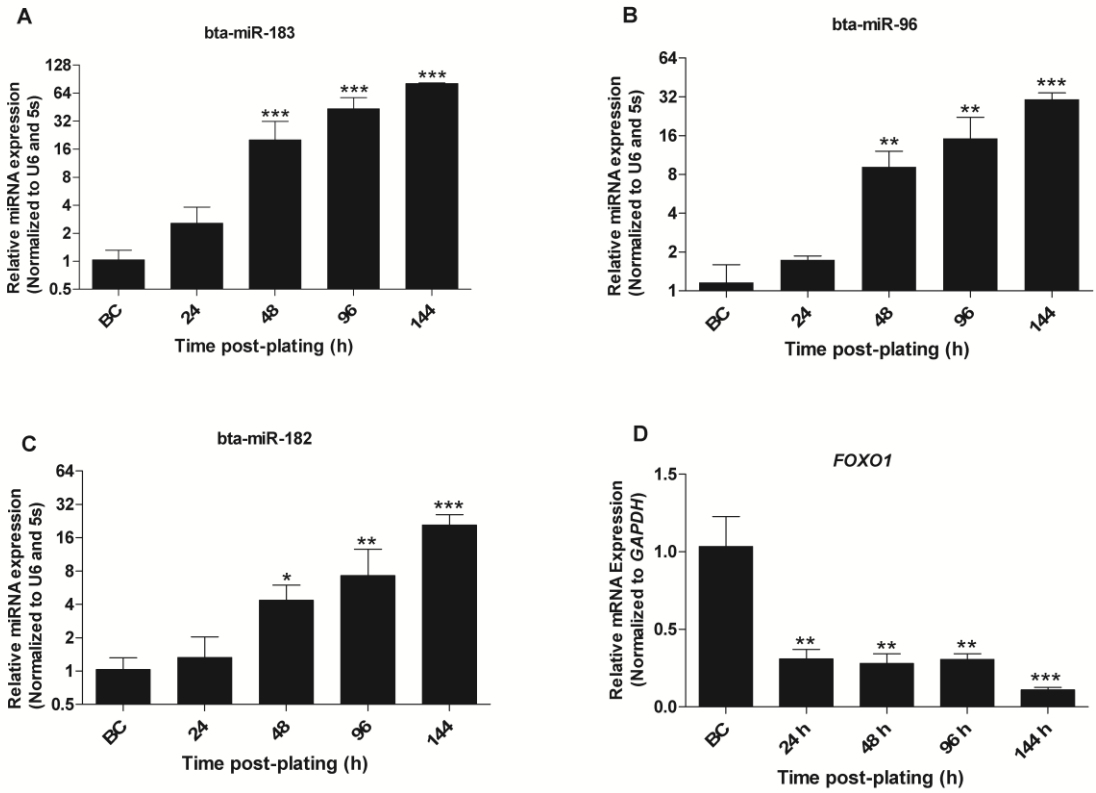

Figure 3.1: Expression of miR-183-96-182 cluster miRNAs increased in response to in vitro culture. Effect of granulosa cells culture on the expression of miR-183 (**A**), miR-96 (**B**), miR-182 (**C**). Expression pattern of FOXO1 mRNA in response to in vitro culture (**D**). The Y-axis indicates relative expression of miRNAs or mRNA, and the X-axis indicates the time post-plating (before culture [BC], 24, 48, 96, and 144 h post plating). Expression values of target miRNAs and mRNA were normalized to those of the geometric mean of U6 small noncoding small nuclear RNA (U6 snRNA) and 5s ribosomal RNA (5s rRNA) and GAPDH, respectively. One-way ANOVA followed by a Dunnett post-hoc test was used to detect statistical differences. Data are mean ± SEM of n=3 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

LNA-Mediated Inhibition of Individual Members of MiR-183-96-182 Cluster Resulted in Sponge Effect in Cultured Granulosa Cells

To modulate the expression of the miR-183-96-182 cluster miRNAs in vitro, we utilized LNA-mediated knockdown of components of the miRNAs both individually and pooled. Interestingly, inhibition of individual miRNA resulted in a sponge effect (Figure 3.2A–C), in which transfection of cells with miR-183 inhibitor caused a significant reduction in the expression of all three miRNAs of the miRNA cluster. Similarly, inhibition of miR-96 led to a measurable reduction in the level of all three miRNAs. However, inhibition of miR-182

reduced the expression of miR-183 and caused no significant reduction in miR-96 expression level. Transfection using pooled inhibitors resulted in a coalescent reduction in the expression of all three miRNAs.

To determine the specificity of the LNA-oligo nucleotide miRNA inhibitors, we checked for the expression of miR-335, which does not belong to the miR-183-96-182 cluster and has no sequence homology. Inhibition of targeted miRNAs using either individual or pooled miRNA inhibitors of the miR-183-96-182 cluster had no significant impact on the expression of miR-335 (Figure 3.2D).

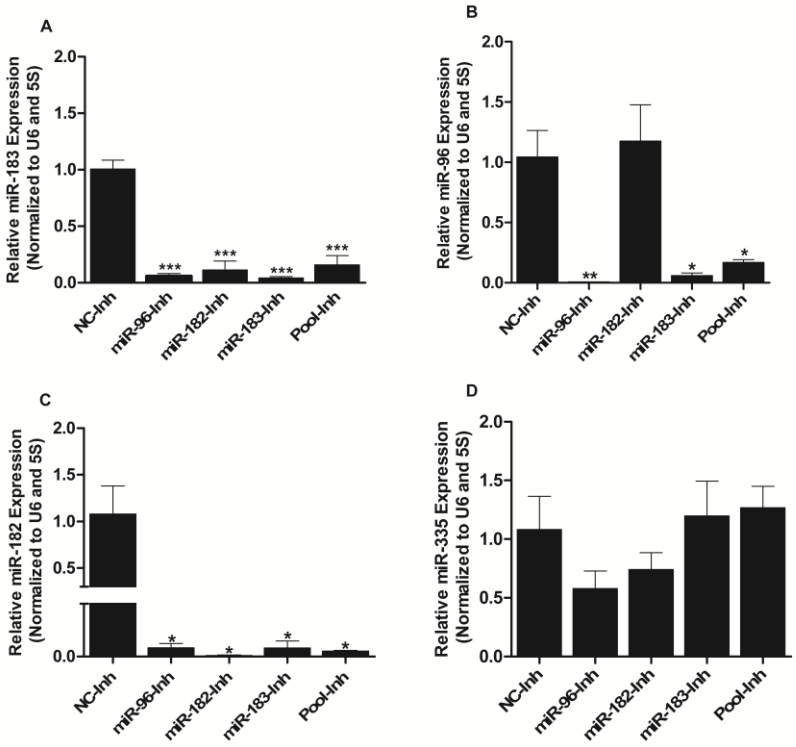

Figure 3.2: Sponge effect was observed upon inhibition of individual miRNAs of the miR-183-96-182 cluster. LNA-based miRNA inhibitors effectively inhibited individual miRNAs of the miR-183-96-182 cluster and resulted in a sponge effect (**A**, **B**, **C**). Inhibitions of either individual or pooled miRNAs of the miR-183-96-182 cluster did not produce the sponge effect on nonmember miRNA of the cluster (**D**). Expression values of target miRNAs were normalized to those of the geometric mean of U6 small noncoding small nuclear RNA (U6 snRNA) and 5s ribosomal RNA (5s rRNA). Two-tailed Student t-test was used to detect statistical differences. Data are mean ± SEM of n =3 (*P<0.05, **P <0.01, ***P<0.001).

In contrast to the sponge effect observed upon transfection of granulosa cells with miRNA inhibitors, transfection of granulosa cells with miRNA mimics showed a selective increment in the expression of the specific miRNAs on the cluster. Subsequently, cells

transfected with pooled miRNA mimics showed increased expression of all miRNAs in the cluster (Figure 3.3A–C).

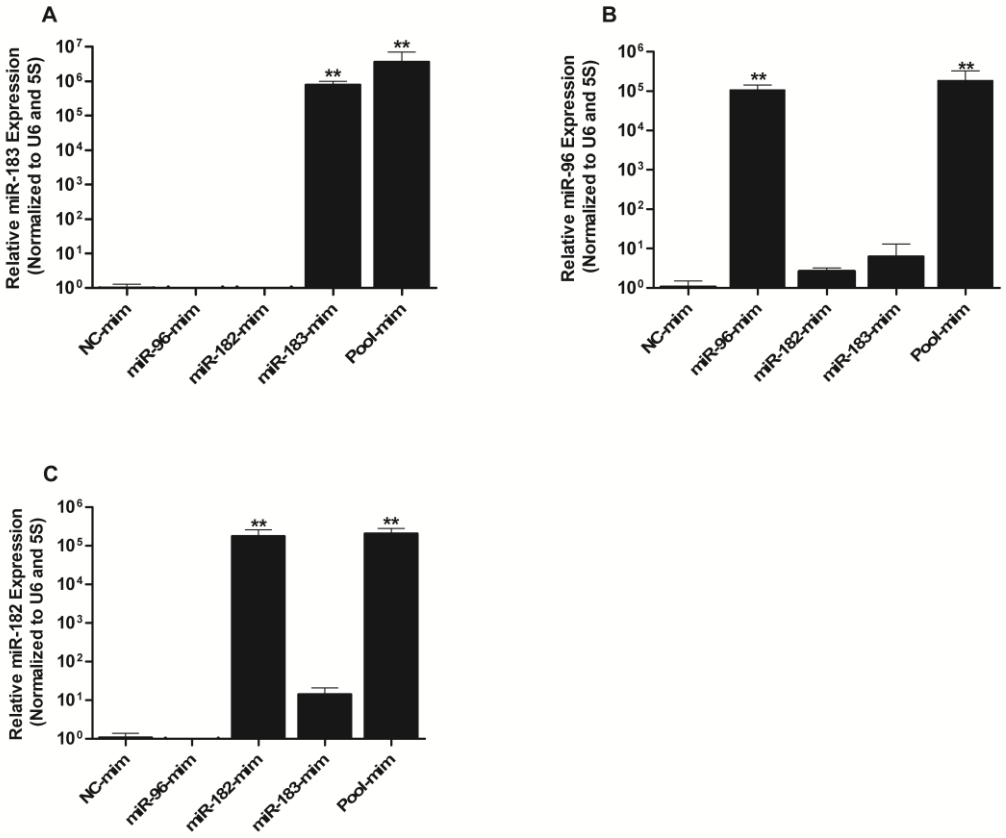

Figure 3.3: Transfection of granulosa cells with individual miRNA mimics of the miR-183-96-182 cluster selectively increased the expression of corresponding miRNAs (A–C). Expression values of target miRNAs were normalized to those of the geometric mean of U6 small noncoding small nuclear RNA (U6 snRNA) and 5s ribosomal RNA (5s rRNA). Two-tailed Student t-test was used to detect statistical differences. Data are mean ± SEM of n=3 (**$P < 0.01$).

MiR-183-96-182 Cluster miRNAs Regulate the Expression of FOXO1 and Subsequently its Pro-Apoptotic Downstream Transcript in Bovine Granulosa Cells In Vitro

We previously demonstrated that *FOXO1* is a target gene of all miR-183-96-182 cluster miRNAs (Gebremedhn *et al.* 2015). To further determine the role of miR-183-96-182 cluster miRNAs in modulating the expression of *FOXO1* in bovine granulosa cells in vitro, *FOXO1* mRNA and protein levels were measured in granulosa cells transfected with individual or pooled miRNA mimics, inhibitors, mimic negative control, and inhibitor negative control.

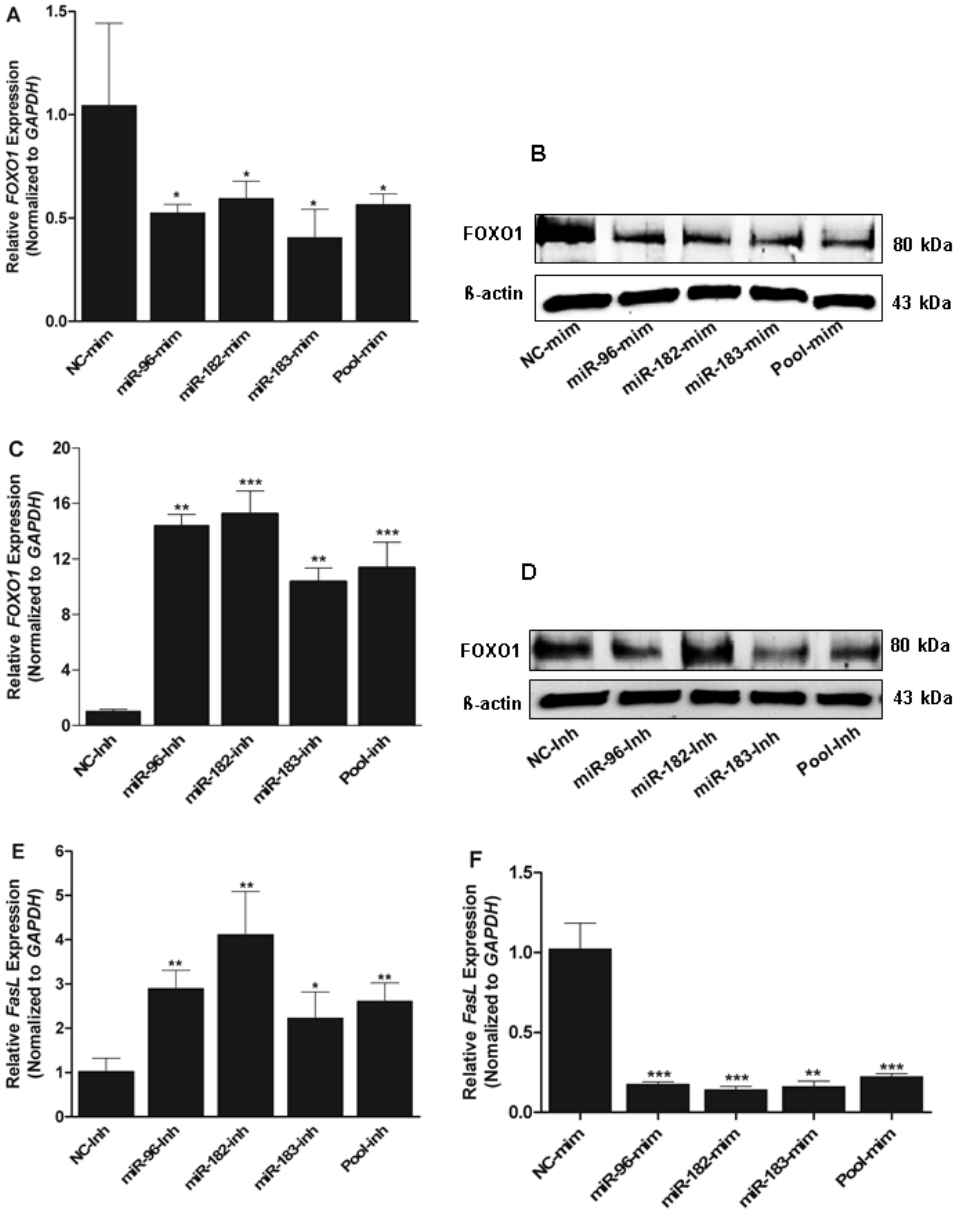

Figure 3.4: Modulation of miR-183-96-182 cluster miRNAs in granulosa cells led to a change in mRNA and protein of FOXO1 gene. Expression of *FOXO1* mRNA (**A**) and protein (**B**) decreased significantly upon overexpression of miR-183-96-182 cluster miRNAs. Inhibition of miR-183-96-182 cluster miRNAs significantly increased the expression of *FOXO1* mRNA (**C**) and FOXO1 protein expression (**D**). Expression of *FASL* mRNAs was decreased upon overexpression (**E**) and increased upon inhibition (**F**) of miR-183-96-182 cluster miRNAs. *GAPDH* was used as internal control, and two-tailed Student t-test was used to detect statistical differences. Data are mean ± SEM of n=3 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

QPCR analysis revealed that FOXO1 mRNA was significantly decreased in both the individual and the pooled miRNA mimic-transfected granulosa cells compared to granulosa cells transfected with mimic-negative control (Figure 3.4A). In agreement with the mRNA expression pattern, FOXO1 protein expression level was markedly decreased in granulosa cells treated with both individual and pooled miRNA mimics (Figure 3.4B).

Conversely, inhibition of either the individual or the cluster miRNAs using LNA-miRNA inhibitors resulted in sharp increment in *FOXO1* mRNA (Figure 3.4C). However, the increment in FOXO1 protein in granulosa cells transfected with miR-183-96-182 cluster inhibitors was not in full agreement with the increase in *FOXO1* mRNA (Figure 3.4D). *FOXO1* as a transcription factor regulates the expression of apoptosis-related genes, including the Fas ligand (*FASL*) (Cui *et al.* 2009). To further confirm whether the alteration in expression of *FOXO1* by the miR-183-96-182 cluster miRNAs could affect the expression of this *FOXO1* downstream gene, we checked the expression of *FASL*, a pro-apoptotic downstream gene of FOXO1. Interestingly, similar to the trend of *FOXO1*, the expression of *FASL* mRNA increased in granulosa cells transfected with miR-183-96-182 cluster inhibitors (Figure 3.4E). However, the expression decreased in granulosa cells transfected with miR-183-96-182 cluster mimics (Figure 3.4F).

Overexpression of MiR-183-96-182 Cluster Promotes Granulosa Cell proliferation

To assess the role of the miR-183-96-182 cluster in granulosa cell proliferation, cells were transfected with miRNA mimics, inhibitors, and corresponding negative controls. The relative rate of cell proliferation treated with the mimics was higher than that in the control group (Figure 3.5A). In contrast, the relative rate of granulosa cell proliferation transfected with the inhibitors was lower than that with the inhibitor control (Figure 3.5B).

Overexpression of MiR-183-96-182 Cluster Promotes G_1/S Cell Cycle Transition in Bovine Granulosa Cells

We further examined the effect of miR-183-96-182 cluster miRNAs in modulating cell cycle transition in granulosa cells. Granulosa cells transfected with the mimics showed reduction in the proportion of cells in G_0/G_1 arrest, as well as an increment in the percentage of cells in S phase compared to granulosa cells transfected with mimic-negative control (Figure 3.6). MiR-183 mimic caused a higher reduction in the proportion of cells in G_0/G_1 phase (~12%) and an increment of cells in S phase (10%) (Figure 3.6B), followed by pooled mimics (Figure 3.6E), miR-96, mimic (Figure 3.6C), and miR-182 mimic (Figure 3.6D).

This increased level of miRNAs in granulosa cells could enhance cell proliferation by promoting G_1/S cell cycle transition. In contrast, inhibition of miR-183-96-182 cluster miRNAs did not result in measurable changes in the cell cycle profiles of granulosa cells (Table 3.S3).

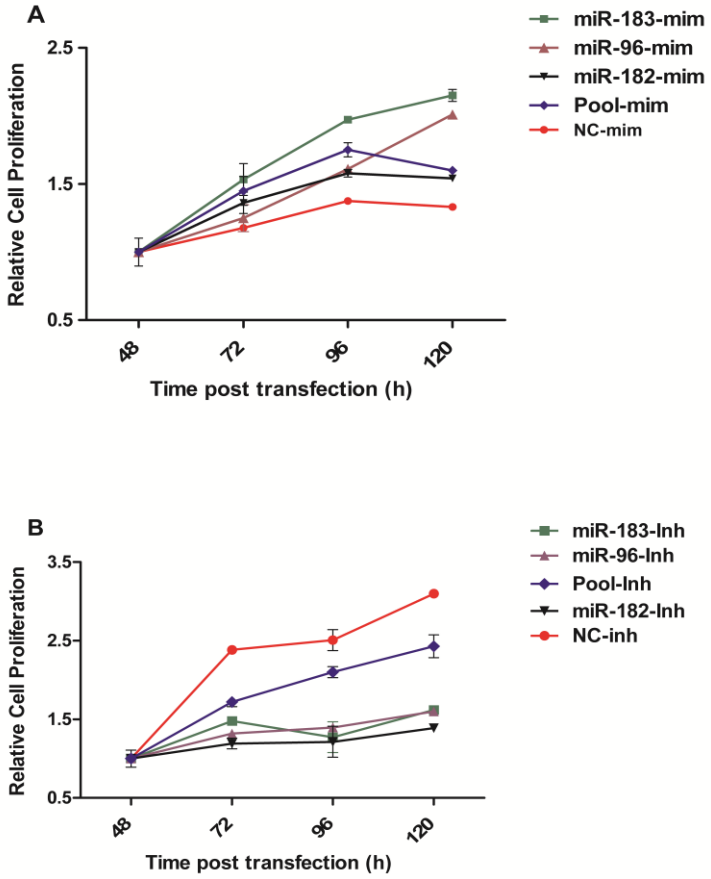

Figure 3.5: Inhibition of miR-183-96-182 cluster miRNAs reduced the rate of granulosa cell proliferation. Cell proliferation assay of granulosa cells transfected with miR-183-96-182 cluster mimics and mimic negative control (**A**), cells transfected with miR-183-96-182 cluster inhibitors and inhibitor negative control (**B**). The relative rate of cell proliferation is indicated on the Y-axis, and time post transfection is indicated on the X-axis. Data are mean ± SEM of n=3.

Selective knockdown of FOXO1 by siRNA Promotes Granulosa Cell Proliferation and Induces G_1/S Cell Cycle Transition

To determine the involvement of *FOXO1* in granulosa cell proliferation and cell cycle transition and to confirm the regulatory role of miRNAs in *FOXO1* expression, small interfering RNA (siRNA) technique was used to selectively knockdown the *FOXO1* mRNA in granulosa cells.

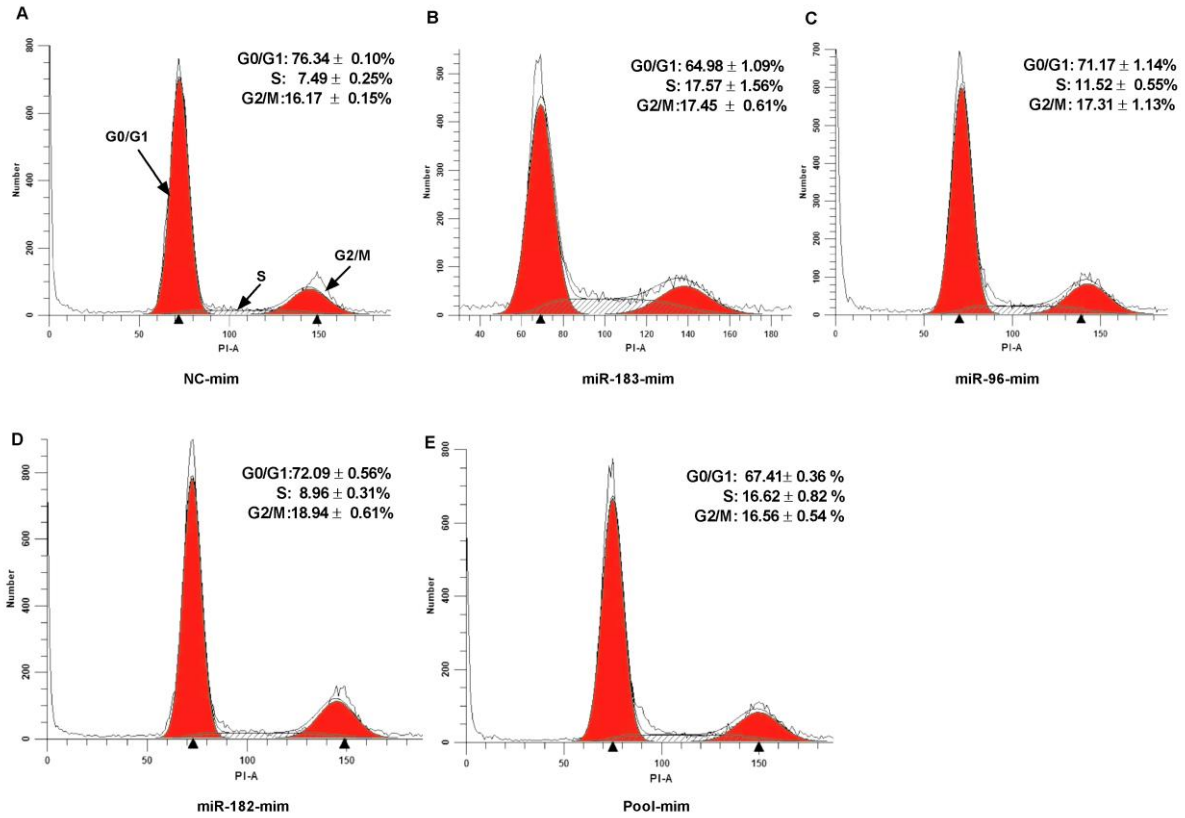

Figure 3.6: Overexpression of miR-183-96-182 cluster changes the cell cycle profile of granulosa cells. Representative histograms of flow cytometric analysis of granulosa cells transfected with miRNA mimic negative control (**A**), miR-183 mimics (**B**), miR-96 mimic (**C**), miR-182 mimic (**D**), and pooled mimics (**E**). The analyzed cell counts are indicated on the Y-axis, and the DNA content of cells detected by PI staining is indicated on the X-axis. Data are mean ± SEM of n = 3.

Granulosa cells transfected with FOXO1-siRNAs showed a significant reduction in *FOXO1* mRNA level (~72%) compared to that in cells treated with NC-siRNA (Figure 3.7A). In agreement with the mRNA expression, the FOXO1 protein markedly declined in granulosa cells transfected with FOXO1-siRNAs compared to that in the negative control (Figure 3.7B). The degradation of FOXO1 also resulted in reduced expression of downstream pro-apoptotic *FASL* mRNA (Figure 3.7C).

Similarly, results of cell proliferation assays showed a relative rate of cell proliferation in granulosa cells transfected with FOXO1-siRNAs that was increased compared to that in the negative control (Figure 3.7D), indicating an anti-proliferative role for FOXO1 in bovine granulosa cells. Interestingly, G_0/G_1 cell cycle arrest was markedly decreased in granulosa cells transfected with FOXO1-siRNA. These cells showed a proportion of cells in the S-

phase of the cell cycle that was increased (Figure 3.7E) compared to NC-siRNA transfected cells (Figure 3.7F).

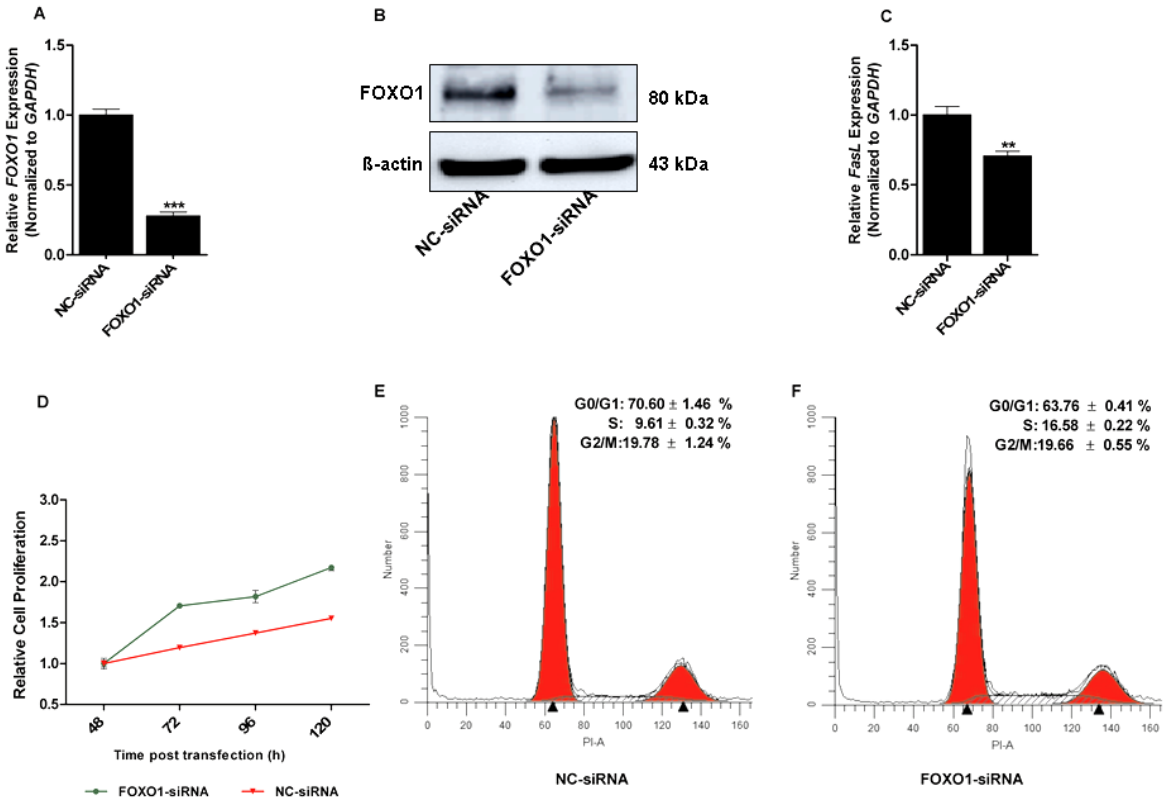

Figure 3.7: Targeted suppression of FOXO1 expression promotes cell proliferation and altered the cell cycle profile of granulosa cells. QPCR analysis of *FOXO1* mRNA levels in granulosa cells transfected with FOXO-siRNAs or NC-siRNA is shown (**A**). Western blot analyses of FOXO1 protein in granulosa cells transfected with FOXO-siRNAs or NC-siRNA (**B**). *FASL* mRNA levels in granulosa cells transfected with FOXO-siRNAs or NC-siRNA, as determined by qPCR (**C**). The relative rate of granulosa cell proliferation transfected with FOXO-siRNAs or NC-siRNA as determined by CCK-8 (**D**). Representative histograms of flow cytometric cell cycle analysis of cells transfected with NC-siRNA (**E**) and FOXO-siRNAs (**F**). *GAPDH* was used as an internal control for gene expression analysis, and two-tailed Student t-test was used to detect statistical differences in gene expression analysis. Data are mean ± SEM of n=3 experiments (**P < 0.01, ***P < 0.001).

DISCUSSION

Bovine folliculogenesis is a well-orchestrated biological process in which the granulosa cells produce steroid hormones, growth factors, and cytokines (Richards 1994; Toda *et al.* 2012). Large-scale transcriptome profiling of granulosa cells showed the expression and interaction of a multitude of genes and transcription factors in regulating granulosa cell function in various stages of follicular development (Douville & Sirard 2014; Hatzirodos *et al.* 2014a; Hatzirodos *et al.* 2014b). Recently, miRNAs have been shown to play regulatory roles in ovarian functions, including activation of primordial follicles, follicular recruitment, and granulosa cell functions (Maalouf *et al.* 2016). The aim of the present study was to decipher the functional role of miR-183-96-182 cluster miRNAs in bovine granulosa cells. We demonstrated that modulation these miRNAs leads to changes in the proliferative potential of granulosa cells and transition in the cell cycle profile through coordinated targeting of the *FOXO1* transcription factor. The regulatory role of the miRNA cluster in granulosa cell function through *FOXO1* could be confirmed by selective degradation of *FOXO1* mRNA in bovine granulosa cells.

The transcription of the cluster miRNAs increased while the expression of their target gene *FOXO1* decreased in response to plating. However, this trend of expression was not accompanied by luteinization of the granulosa cells during the *in vitro* culture, which was confirmed by the absence of *LHR* throughout the culture period. This absence of *LHR* could be associated with the follicular size from which the granulosa cells were aspirated, as the acquisition of *LHR* in bovine granulosa cells is correlated with the follicular diameter (Beg *et al.* 2001; Evans *et al.* 2004). Maintenance of the identity of granulosa cells during plating was validated by detection of *FSHR* throughout the culture period.

Even though the mechanisms that govern transcription of miRNAs are not fully understood, miRNAs are known to be transcribed either as individuals or in clusters (Lai *et al.* 2003). MicroRNA clusters are groups of miRNAs adjacently located on the same chromosome that are transcribed as one pre-miRNA transcript and further processed into individual mature miRNAs (Mathelier & Carbone 2013). The chromosomal span of miRNA clusters ranges from hundreds to several kilobase (kb) pairs and often shares a common promoter (Ryazansky *et al.* 2011). The miR-183-96-182 cluster is a class of conserved polycistronic miRNAs transcribed from a 4.6-kb-long intergenic region of bovine chromosome 4 of the bovine genome (Figure 3.S2-A). This cluster is evolutionarily conserved across mammalian species (Figure 3.S2-B). Members of this miRNA cluster are transcribed in the same direction from telomere to centromere and function synergistically

(Weeraratne *et al.* 2012). Studies have shown that the miR-183-96-182 cluster miRNAs are enriched in most breast cancers (Li *et al.* 2014), regulate oxidative stress-induced apoptosis (Tang *et al.* 2013), and inhibit invasion and metastasis in lung cancers (Kundu *et al.* 2015) by regulating the expression of several key genes.

The *LNA*-mediated inhibition of individual members of the miR-183-96-182 cluster resulted in a sponge effect (Figure 3.2A-C), which could be attributed to the partial sequence homology between miRNAs in the same cluster (Figure 3.S2-A). In addition to the conserved seed sequences, the identical directional transcription of individual miRNAs in the same cluster suggests their identical origin of a primary miRNA transcript that contributes to the sponge effect in miRNA knockdown experiments (Weeraratne *et al.* 2012; Tang *et al.* 2013). In addition to the sponge effect, high sequence homology between miRNAs in the cluster permits them to target similar genes and play a vital role by coordinately regulating key genes in cellular processes (Ebert *et al.* 2007; Dambal *et al.* 2015).

In bovine granulosa cells, overexpression of the miR-183-96-182 cluster miRNAs resulted in modulation of both mRNA and protein expression of FOXO1 (Figure 3.4A and B). This is attributed to the conserved seed sequence and co-expression of the cluster miRNAs, which resulted in co-targeting of *FOXO1* and subsequently affected the molecular pathways in the signaling axis (Hausser & Zavolan 2014). The abundance of FOXO1 protein after inhibition of the cluster was not in full agreement with the *FOXO1* mRNA expression (Figure 3.4C and D). The discrepancies could be due to the fact that binding sites of several other miRNAs reside within the 3′-untranslated region (UTR) of *FOXO1,* and inhibiting only the miR-183-96-182 cluster might not produce the desired increment in FOXO1 protein level.

The FOXO transcription factor family comprises *FOXO1*, *FOXO3*, *FOXO4*, and *FOXO6*. It is known to regulate genes involved in follicular growth (Liu *et al.* 2013) and numerous cellular processes, including proliferation, apoptosis, cell cycle arrest, cell differentiation, and metabolism (Barthel *et al.* 2005; Huang & Tindall 2007). *FOXO1* is abundantly expressed in granulosa cells of growing follicles (Liu *et al.* 2009), anovulatory subordinate follicles (Gebremedhn *et al.* 2015), and small atretic bovine follicles (Hatzirodos *et al.* 2014a), signifying its potential involvement in ovarian follicular development. Its involvement in the apoptotic process during follicular atresia is evidenced by the depletion and enrichment of *Foxo1* in rat granulosa cells of healthy antral follicles and small atretic follicles, respectively (Shi & LaPolt 2003).

Despite the fact that *FASL* is not among the conserved target genes of the miR-183-96-182 cluster miRNAs in bovine (Figure 3.S3), its expression was affected by modulation of the cluster miRNAs (Figure 3.4E and F) and by modulation of *FOXO1* mRNA (Figure 3.7C). This was in agreement with the observations by Shen *et al.* (2012). They showed that induction of *Foxo1* through oxidative stress in mouse granulosa cells induced the expression of its downstream transcripts *Fasl*, *Bim,* and *Trail,* which were associated with cell cycle arrest and apoptosis.

Proliferation of granulosa cells is ultimately important in follicular growth and the creation of a unique microenvironment for oocyte maturation (Maruo 1995). Accumulated evidence is available on the involvement of miRNAs in regulating granulosa cell proliferation (Yao *et al.* 2010; Yan *et al.* 2012; Zhang *et al.* 2013). In the present study, the rate of granulosa cell proliferation increased upon miR-183-96-182 cluster miRNAs overexpression, which is accompanied by downregulation of the anti-proliferative *FOXO1* gene. This is further supported by the shift in the proportion of cells from G_0/G_1 arrest to the S phase of the cell cycle.

Inhibition of miR-183-96-182 cluster miRNAs decreased the relative rate of granulosa cell proliferation. Similar results have been reported in breast cancer cells, where the enrichment of the miR-183-96-182 cluster miRNAs promotes cell proliferation, cell migration, and change in the cell cycle profile (Li *et al.* 2014). The rate at which granulosa cells proliferate was higher in cells where *FOXO1* mRNA was suppressed compared to the control group in the RNA interference experiment, which indicates an anti-proliferative role of *FOXO1*. Consistent with the present findings, another study on cultured granulosa cells indicated that *FOXO1* can regulate genes associated with cell proliferation and apoptosis (Park *et al.* 2005).

Selective degradation of *FOXO1* using siRNA in granulosa cells resulted in a decline in the proportion of cells under G_0/G_1 arrest and an increase of cells in the S-phase of the cell cycle. This suggests that overexpression of *FOXO1* in bovine granulosa cells maintains the cells in G_0/G_1 arrest and prevents cells from entering the S phase and progressing in the cell cycle. Mammalian FOXO proteins are involved in regulating the G_1/S phase cell cycle transition and commit the cells to cell cycle progression (Dijkers *et al.* 2000; Medema *et al.* 2000). This was further evidenced in mouse granulosa cells, where *Foxo1* was depleted and showed promoted cell cycle transition (Park *et al.* 2005). Taken together, enrichment of *FOXO1* in granulosa cells has an inhibitory role in cell cycle transition by preventing the induction of positive regulators of cell cycle.

In conclusion, the present study provides new insights into the role of miR-183-96-182 cluster miRNAs in coordinated regulation of the FOXO1 transcription factor in bovine granulosa cells. These miRNAs exert subsequent influence on bovine granulosa cell proliferation and cell cycle transition.

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Chapter 4

Maternal Serum Circulatory MicroRNAs as Potential Indicators of Early Pregnancy in Bovine

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Abstract

Pregnancy establishment in bovine is a complicated physiological process, which begins with the formation of zygote in the oviduct. After continuous cleavages, the embryo enters the uterine cavity. As the embryo grows in size and changes its shape into filamentous, the trophectoderm releases interferon tau (IFN-τ); a protein which functions as a maternal recognition molecule and prevents regression of the corpus luteum and pregnancy maintenance. Confirmation of pregnancy establishment at the earliest day post-insemination increases the reproduction efficiency of dairy cows by allowing non-pregnant cows to rebreed. Inaccuracies of the currently available pregnancy detection tools lead to decline in the profitability of the dairy industry. Thus, development of non-invasive biomarkers that can predict early pregnancy status of cows effectively could be proposed as alternative tools. MicroRNAs (miRNAs), a subclass of small non-coding RNAs are known to regulate genes involved in various pathophysiological conditions. Moreover, mature miRNAs are present in the circulation and have been associated with various pregnancy related disorders. The study aimed to profile the expression of circulatory miRNAs in maternal serum of pregnant and non-pregnant dairy cows. For this, lactating Holstein-Friesian cows were estrous synchronized and inseminated with frozen semen. Blood samples were taken 19 and 24 days post-insemination. RNA was isolated from pooled (4 animals/pool) serum samples of pregnant and non-pregnant cows and subjected to cDNA synthesis. The expression signature of circulatory miRNAs was analyzed using commercially available PCR array containing 748 mature miRNAs. Results showed that a total of 302 and 316 miRNAs were detected in day 19 pregnant and non-pregnant cows, respectively. Similarly, 356 and 325 miRNAs were detected in day 24 pregnant and non-pregnant cows, respectively. Principal component analysis separated pregnant and non-pregnant cows both at day 19 and 24 according to the miRNAs expression signature. Comparative expression analysis of miRNAs revealed that 8 and 23 miRNAs to be differentially expressed in the pregnant cows of day 19 and 24, respectively. Interestingly, 1 miRNA (miR-433) and 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p, and miR-323a-3p) homologous to the human pregnancy-associated C14MC miRNAs were among the differentially expressed miRNAs in day 19 and 24 pregnant cows, respectively. In addition, pathways important in pregnancy implantation; adherens junction and ECM-interaction were significantly enriched by target genes of differentially expressed miRNAs. In conclusion, the expression of miRNAs in maternal circulation could be associated with the pregnancy status and be indicators of early pregnancy in bovine.

Keywords: Early pregnancy, circulatory miRNAs, Bovine, Serum, PCR Array

Introduction

Pregnancy establishment in mammals is an evolutionarily conserved and complicated process which starts with the formation of zygote by the union of a developmentally competent oocyte and sperm in the oviduct. In bovine, after series of cleavages the embryo enters the uterine cavity and remains free-floating and autonomous (Forde *et al.* 2011). The free-floating embryo releases signals to prevent endometrial luteolytic activity and the regression of the corpus luteum to sustain the production of progesterone (Spencer *et al.* 2004). Around day 16 of the gestation period, the trophectoderm of an elongated conceptus synthesizes and secretes interferon tau (IFN-τ), a protein molecule which serves as maternal recognition signal of pregnancy and implantation by acting against the secretion of the PGF to prevent endometrial luteolytic activity and regression of the corpus luteum (Roberts *et al.* 1999; Mansouri-Attia *et al.* 2009). Failure of the embryo to secrete sufficient IFN-τ leads to pregnancy loss (Mann *et al.* 1999). At early stage of the pregnancy establishment, pregnancy losses are not detectable unless the animals show signs of estrous 21 days post-insemination. Therefore, identification of pregnant and non-pregnant cows at the earliest day post-insemination improves the reproductive performance of dairy cows by increasing rate of pregnancy and shortening the calving intervals (Fricke 2002). Currently, several pregnancy diagnosis tools including rectal palpation, milk progesterone test, ultrasonography and pregnancy-associated glycoproteins have been widely utilized. However, the sensitivity, specificity, and invasiveness of these pregnancy diagnosis aids are in some cases questionable, which could lead to false positive and false negative results. Inaccuracy in predicting early pregnancy status of a cow leads to decrease profitability in the dairy and beef industry (Arbel *et al.* 2001). Therefore, development of non-invasive biomarkers that could be indicators of early pregnancy in bovine could be proposed as alternative tools for pregnancy diagnosis.

Recently, microRNAs (miRNAs); a subclass of small non-coding RNAs, 18-22 nucleotides long molecules emerged as key posttranscriptional regulators of gene expression through mRNA degradation and translational inhibition (Bartel 2004; Ying *et al.* 2006). MicroRNAs are involved in physiological conditions of bovine reproduction including follicular development (Salilew-Wondim *et al.* 2014; Gebremedhn *et al.* 2015), bovine granulosa cell proliferation and cell cycle transition (Gebremedhn *et al.* 2016) and granulosa cell differentiation (Andreas *et al.* 2016). In addition to the cellular miRNAs , matured circulating miRNAs are present in various body fluids including blood plasma (Chim *et al.* 2008; Noferesti *et al.* 2015), serum (Chen *et al.* 2008; Lawrie *et al.* 2008), follicular fluid (Sohel *et al.* 2013; Noferesti *et al.* 2015), saliva (Park *et al.* 2009), urine (Hanke *et al.* 2010) and breast-

milk (Kosaka *et al.* 2010). Unlike other RNA species like mRNA, rRNA and tRNA, the extracellular or circulating miRNAs are stable and withstand extreme conditions including extreme pH, higher temperature, repeated free-and-thaw procedures and RNAse digestion in the circulation (Valadi *et al.* 2007). Circulating miRNAs are protected from endogenous RNase activity in circulation through vesicle-mediated encapsulation in exosomes and microvesicles or through association with protein complexes like Ago2 (Cortez *et al.* 2011). The stable characteristics of circulatory miRNAs make them attractive to be used as predictive and diagnostic biomarkers in various pregnancy-related diseases and abnormalities like preeclampsia (Li *et al.* 2013), intrauterine growth restriction (Mouillet *et al.* 2010) and ectopic pregnancy (Miura *et al.* 2015). There are growing evidences indicating the presence of placenta-specific miRNAs in maternal circulation. The expression of the placenta-specific human chromosome 19 miRNA cluster (C19MC); hsa-miR-515-3p, hsa-miR-517a, hsa-miR-517c, hsa-miR-518b, and hsa-miR-526b increased in maternal circulation during the third trimester of pregnancy and declined after delivery (Kotlabova *et al.* 2011). The villous of trophoblast shade the C19MC miRNA cluster encapsulated in exosomes and could be the major source of placenta specific miRNAs in maternal circulation (Luo *et al.* 2009; Donker *et al.* 2012). Moreover, the human chromosome 14 miRNA cluster (C14MC) are also reported to be associated with pregnancy (Morales-Prieto *et al.* 2013). Another miRNAs like miR-141, miR-149, miR-299-5p, and miR-135, which are shown to be abundantly expressed in placenta were enriched in maternal plasma of pregnant women and their concentration decline after delivery (Chim *et al.* 2008). MicroRNAs in maternal serum exosomes derived from non-pregnant and day 30 and 90 pregnant ewes identified 25 miRNAs differentially regulated in maternal circulation according to pregnancy status (Cleys *et al.* 2014). Recently, circulatory miRNAs profiling in cow plasma samples derived from different stages of the estrous cycle and day 16 and 24 of pregnancy showed elevated miR-26a in pooled plasma samples of day 16 pregnant cows compared to the non-pregnant counterpart (Ioannidis and Donadeu 2016b). In this study, we profiled the expression pattern of circulatory miRNAs in maternal serum derived from pregnant and non-pregnant cows at day 19 and 24 post-insemination.

Materials and Methods

Study animals and sample collection

In a dairy farm in Bakersfield, CA, USA, a total of 154 lactating Holstein-Friesian cows at 50-80 days post-partum were estrous synchronized using standard procedures. Upon the onset

of the estrous, cows were inseminated with frozen semen and the day of insemination was regarded as Day 0. Blood samples were collected 19 and 24 days after insemination. Finally, the pregnancy status of each cow was assessed 35 days post-insemination using ultrasonography. Blood samples were retrospectively categorized as pregnant and non-pregnant. For the study, only cows in the 1st breeding of the lactation during the experiment period were considered. Moreover, cows with a blind or nonfunctional quarter in their teats were not considered. All experimental cows were kept in free stall penning, kept on the farm of study for at least 6 months and ear tagged with a unique identification number. Due to health reasons, 4 cows were removed post-enrollment and 2 cows with a blind quarter were removed post-enrollment.

Blood serum and cell separation

Blood samples were collected from all cows on consecutive study Days 19 through Day 24 post-insemination at roughly the same time each day from tail vein or in the event that the tail vein was not accessible, samples were taken from the jugular vein, using aseptic technique. Blood samples were taken using Vacutainer system employing 10.0 mL SST blood (serum) tubes. Within 1-2 hours of collection, samples were centrifuged on site and aliquots of serum were placed in a freezer (-4°C) and stored in -80°C until shipment.

RNA extraction and cDNA synthesis

Serum samples of 3 pooled replicates (4 animals/pool) from both day 19 and 24 pregnant and non-pregnant cows were subjected to RNA isolation using miRNeasy serum/plasma kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, serum samples were thawed at room temperature and 250 µl of the pooled serum samples were lysed and homogenized in 1250 µl of QIAzol lysis reagent (Qiagen, Germany) followed by addition of 250 µl chloroform. Samples were mixed vigorously and centrifuged at 12,000 xg for 15 minutes at 4°C. The upper aqueous phase was transferred into a new tube and thoroughly mixed with 1.5x of its volume of absolute ethanol. Samples were placed into RNeasy MiniElute spin column (Qiagen, Germany) and centrifuged at ≥ 8000 xg for 15 seconds at room temperature. After washing with RWT and RPE washing buffers, RNA was finally eluted in 20 µl of RNase-free water. RNA concentration was determined using NanoDrop 8000 spectrophotometer (Thermo scientific, USA) and frozen in -80°C until further use. Subsequently, a total of 100 ng of RNAwas used for cDNA synthesis using the miRCURY LNA™ Universal cDNA synthesis kit (Exiqon, Denmark) according to the manufacturer's

instructions. Briefly, 10 µl of RNA sample (with total concentration of 100 ng) was mixed with 8 µl of 5x reaction buffer, 4 µl of reverse transcriptase enzyme mix, 2 µl synthetic spike-in RNA and 16 µl of nuclease free water. The reactions were incubated at 42°C for 60 min followed by heat-inactivation at 95°C for 5 min and left at 4°C.

Expression profiling of extracellular miRNAs using PCR arrays

Before the genome-wide extracellular miRNA profiling, the impact of PCR inhibitors found in serum was assessed. For this, we measured the expression of miR-132 by synthesizing cDNA using different volumes of RNA input and the linearity and correlation was calculated. Due to the high miRNA sequence conservation among mammalian species, a commercially available microRNA Ready-to-Use PCR, Human panel I+II, V3.R (Exiqon, Denmark) was used to determine the expression of extracellular miRNAs in serum samples pregnant and non-pregnant cows. For this, a PCR master mix was prepared for every 384-well plate using 40 µl of cDNA samples, 2000 µl ExiLENT SYBR Green master mix (Exiqon, Denmark) and 1960 µl nuclease free water. After mixing briefly, 9 µl of PCR master mix was transferred to each well containing 1 µl of pre-aliquot miRNA-specific primer using Biomek FX robotic pipette (Beckman Coulter, USA). Assays were performed on the LightCycler 480 (Roche, Switzerland) with thermal cycling program of 95°C for 10 min followed by 45 amplification cycles (95°C for 10 s, 60°C for 1 min). Melting curve analysis was included at the end of the program to verify the specificity of the amplification reaction. At the end of PCR reactions, Data were exported following the analysis using the 2nd derivative method.

Quality control and PCR array data analysis

MicroRNAs with a quantification cycle (Cq) ≤ 40 in at least 75% of the samples within each treatment group were considered as detected. Stability in the expression of synthetic spike-in RNAs; UniSp6 and UniSp3 was assessed to monitor the effect of inhibitors during the cDNA synthesis reactions and PCR efficiency, respectively. Next, we performed hemolysis test using the "miR-ratio" method as described in Blondal *et al.* 2013). Principal component analysis (PCA) was also performed using PAST 2.07 software (http://folk.uio.no/ohammer/past). Raw Cq values were normalized using global mean expression, in which the geometric mean of all miRNAs detected in all samples is used as normalizer. Differential expressed of miRNAs were identified using web-based PCR array data analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php)

(Qiagen, Germany). MicroRNAs with fold changes fold change ≥ |2| and *P*-value ≤ 0.05 were considered as differentially expressed.

MicroRNA target gene prediction and pathway analysis

Target genes of differentially expressed miRNAs in serum samples of pregnant cows were computationally predicted using DIANA mirpath V. 3 (Vlachos *et al.* 2015b). For this, only experimentally validated target genes were identified using the tarbase 7.0 (Vlachos *et al.* 2015a), through the mirpath V. 3 interface. Significantly enriched pathways by predicted target genes were exported. The list of pathways and the associated target genes are indicated in Table 4.S1 and 4.S2.

Results

MicroRNAs are present in serum of pregnant and non-pregnant cows

Prior to the expression profiling of serum miRNAs, we assessed the inhibitory effect of enzymes present in serum (Al-Soud *et al.* 2000) using different volumes of RNA input for cDNA synthesis. Quantitative real-time PCR showed that different input volumes of RNA affect the expression of the tested miR-132 showing a correlation coefficient (r) of -0.96 (Figure 4.1A). The commercially available human miRNAs PCR array used for miRNA expression profiling contains primer sets for 748 matured human miRNAs, 6 sets of endogenous controls, 6 intra-plate calibrators and 8 empty negative control wells. Following that, the stability of spike-in RNAs which are indicators of efficiencies of cDNA synthesis (UniSp6) and qRT-PCR (UniSp3) was checked. It was shown that both spike-ins RNAs are stably expressed in serum samples of both pregnant and non-pregnant cows at day 19 and 24 post-insemination and no significant difference was observed among the treatment groups (Figure 4.1B). We then proof the level of hemolysis by checking the ratio of candidate miRNAs specific to serum/plasma and red blood cells. Briefly, the ratio between red blood cell enriched miR-451a and a miRNA stably expressed in circulation miR-23a was used to determine the level of hemolysis in serum and plasma. The miR-ratio analysis showed a ratio of 1.84 ± 0.25 (Figure 4.1C). Following this, miRNAs detected in pregnant and/or non-pregnant group were filtered by setting specific criteria. For this, a miRNA is considered as detected when it is amplified at least until the 40th cycle at least in two of the three biological replicates. Accordingly, a total of 302 and 316 miRNAs were detected in serum samples of day 19 pregnant and non-pregnant cows, respectively. Similarly, 356 and 325 miRNAs were detected in day 24 pregnant and non-pregnant cows, respectively (Figure 4.2A). Analysis of

the top 20 highly expressed miRNAs in all data sets showed that 16 miRNAs (miR-451a, miR-223-3p, miR-16-5p, miR-486-5p, miR-92a-3p, miR-145-5p, miR-23a-3p, miR-24-3p, miR-25-3p, miR-21-5p, miR-93-5p, miR-150-5p, miR-19b-3p, miR-192-5p, miR-320a, miR-191-5p) were commonly detected in all sample groups (Figure 4.2B). Interestingly, miRNAs known to be abundantly expressed in erythrocyte (miR-451a) and thrombocyte (miR-223-3p) appear to be the two most abundantly expressed miRNAs in serum samples of both pregnant and non-pregnant cows (Figure. 4.2B). In addition, PCA analysis on the pooled serum samples revealed clear separation between pregnant and non-pregnant cows both at day 19 and 24 post insemination (Figure 4.3A and 4.3B) and the first two principal components explain majority of the variation in the datasets. This signifies that the expression signature of extracellular miRNAs in serum can separate pregnant and non-pregnant cows effectively.

Differential expression of miRNAs in serum of pregnant cows

Comparative expression analysis of miRNAs between pregnant and non-pregnant cows at day 19 and 24 post-insemination revealed that 8 miRNAs to be differentially expressed in serum samples of pregnant cows at day 19 post-insemination. Among these, the expression level of miR-301b, miR-99a-3p, miR-433, and miR-638 were found to be elevated. Whereas, miR-324-5p, miR-188-5p, miR-615-3p and miR-491-5p were down-regulated in serum samples of day 19 pregnant cows compared to the non-pregnant counterparts (Figure 4.4A). Similarly, 23 miRNAs were differentially expressed in serum samples of pregnant cows at day 24 post-insemination and 16 of them were significantly up-regulated, while 7 miRNAs were down-regulated (Figure 4.4B). The list of miRNAs differentially expressed in serum samples of pregnant cows at day 19 and 24, the fold regulation, and the corresponding *p*-values are indicated in Table 4.1. We further looked into the chromosomal location of all differentially expressed miRNAs in serum samples of pregnant cows both at day 19 and day 24 post-insemination. It was revealed that 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p, and miR-323a-3p) and 1 miRNA (miR-433) differentially expressed in day 24 and day 19 pregnant cows, respectively, which are homologous to the pregnancy-associated human C14MC miRNAs and transcribed from chromosome 21 of bovine genome (Table 4.2).

Target gene and pathway analysis of differentially expressed miRNAs revealed that adherens junction and lysine degradation to be the top two pathways enriched by target genes of differentially expressed miRNAs in day 19 pregnant cows. Interestingly, ECM-receptor interaction and Fatty acid biosynthesis were the two highly enriched pathways by target genes of miRNAs which were differentially expressed in day 24 pregnant compared to non-

pregnant cows. List of all pathways significantly enriched by predicted target genes of differentially expressed miRNAs in day 19 and 24 pregnant cows are indicated in Table 4.S1 and Table 4.S2, respectively.

Discussion

The objective of the present study was to identify miRNAs in serum samples that could be associated with the early pregnancy status of dairy cows at day 19 and 24 post-insemination using miRNA PCR array technique. It was noted that higher number of miRNAs were detected in the circulation of day 24 pregnant cows. This could be associated with the differences in the number of exosome and export of exosome-associated miRNAs into the maternal circulation. On this regard, previous study showed that exosomes released into the circulation of pregnant women were higher than that of non-pregnant counterparts and positively correlated with the progression of the gestation period (Salomon *et al.* 2014). Among the top 20 miRNAs highly enriched in all experimental groups, 16 miRNAs were commonly detected in all data sets. Among these miRNAs, some are expressed in various types of blood cells, which can be indication of their cellular origin. Accordingly, miR-486, miR-92a, miR-16b are expressed in erythrocytes, miR-191 in platelets, miR-150, miR-27a, miR-23a in leukocytes and miR-223, miR-20a, miR-24 in thrombocytes (Pritchard *et al.* 2012). Similar results were also reported in Ioannidis and Donadeu (2016a) and Ioannidis and Donadeu (2016b). The presence of blood cells specific miRNAs in the circulation could be attributed to the active vesicle-mediated release of these miRNAs into the extracellular environment (Cortez *et al.* 2011; Haider *et al.* 2014). Since contamination of blood cells through hemolysis creates bias in miRNA-based screening tests (Kirschner *et al.* 2013), it is highly recommended to check the level hemolysis. In addition to the visual inspection of hemolysis, the miRNA-ratio test is one of the techniques widely used to check the level of hemolysis. A ratio between miR-451a and miR-23a, less than 7 is considered as absence of a measurable level of hemolysis (Blondal *et al.* 2013). In this experiment, the miRNAs-ratio test resulted in a mean value of 1.84 ± 0.25, which is below the threshold level and could indicate that the enrichment the blood cell miRNAs is not due to hemolysis and rather due to active shedding of vesicle-mediated miRNAs into the circulation (Haider *et al.* 2014; Shah *et al.* 2016).

In the present study, we found miR-149 and miR-29a among the miRNAs which were enriched in serum samples of day 24 pregnant cows. In another study, it was also reported that both miR-149 and miR-29a are placental origin miRNAs detected in maternal plasma

and their abundance was decreased after delivery showing higher clearance kinetics in the absence of pregnancy (Chim, Stephen S C *et al.* 2008). Similarly, higher expression of miR-29a in porcine endometrium was associated with pregnancy from healthy embryo and trophoblast cells (Wessels *et al.* 2013). Moreover, miR-301b was highly abundant in serum samples of pregnant cows at day 19 pregnant cows compare to the non-pregnant counterparts. Interestingly, Ioannidis and Donadeu (2016b) reported that miR-301b showed elevated expression in plasma of pregnant cows starting day 16 of the gestation period making it candidate with higher prediction potential of early pregnancy in bovine. Similarly, miR-99a which was significantly enriched in day 19 pregnant cows, was also reported to be abundantly expressed in plasma of pregnant woman (Kotlabova *et al.* 2013) and the same miRNA was among the miRNAs predominantly abundant in ovarian library derived from pregnant goat (Zhang *et al.* 2013).

Majority of the miRNAs reported to be associated with pregnancy are mainly transcribed from two chromosomal locations of the human genome: (C19MC) and chromosome 14 miRNA cluster (C14MC) (Morales-Prieto *et al.* 2013). Some miRNAs of the human C14MC cluster which corresponds to chromosome 21 of the bovine genome showed differential expression in serum of pregnant cows. For instance, miR-433 is enriched in serum of day 19 pregnant cows (Table 4.2). MiR-433 is pregnancy-associated miRNA which showed elevated abundance in the first trimester of pregnancy and decreases its abundance as the gestation proceeds to the third trimester (Miura *et al.* 2010). In addition, miR-487b was found to be elevated in serum of day 24 pregnant cows compared to the non-pregnant counterpart. Whereas, bta-miR-495, bta-miR-376b and bta-miR-323 were selectively enriched in serum of non-pregnant cows. During early pregnancy, trophoblast cell derived villous invade the maternal uterus for appropriate implantation. Therefore, improper and incomplete invasion of trohoblast cells leads to pregnancy complications (Pollheimer and Knofler 2012). MicroRNAs are reported to affect the implantation process by targeting important genes associated with trophoblast cell function (reviewed in (Doridot *et al.* 2013)). The expression of miR-34a was declined in day 24 pregnant cows compared to the non-pregnant counterparts. It was reported that miR-34a inhibits invasion of human trophoblast cells by directly targeting MYC (Sun *et al.* 2015) signifying the necessity to maintain the expression of miR-34a lower for proper early pregnancy implantation. During embryonic development, cell-to-cell communication is very crucial and the embryonic cells start to establish contact with extracellular matrix for proper cell invasion (Hynes 1992; Reddy, K V R and Mangale 2003). Target gene prediction and pathway analysis revealed that adherens junction and

ECM-receptor interaction to be the top pathways enriched by target genes of differentially expressed miRNAs in serum of day 19 and 24 pregnant cows, respectively (Table 4.S1 and 4.S2). In similar study comparative proteomic analysis of endometrial samples derived from pregnant and non-pregnant ewes during the peri-implantation period of pregnancy revealed that there was a dramatic increase in protein molecules involved in ECM-receptor interaction and focal adhesion in pregnant ewes (Zhao *et al.* 2015).

Conclusion

In conclusion, the expression signature of miRNAs in maternal circulation could be associated with the pregnancy status and be indicators of early pregnancy in bovine. Further validation work in an independent population with larger samples size could enhance the efforts towards the discovery of pregnancy diagnostic biomarker in bovine.

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Table 4.1: List of significantly differentially expressed serum circulatory miRNAs

MicroRNA ID	Comparison	Fold Change	*P*-Value
miR-301b	D19P Vs D19NP	25.6575	0.017962
miR-99a-3p	D19P Vs D19NP	21.8765	0.035381
miR-433	D19P Vs D19NP	7.523	0.012063
miR-638	D19P Vs D19NP	2.7094	0.033964
miR-324-5p	D19P Vs D19NP	-7.5966	0.039145
miR-188-5p	D19P Vs D19NP	-7.7922	0.029441
miR-615-3p	D19P Vs D19NP	-10.7681	0.000691
miR-491-5p	D19P Vs D19NP	-13.5669	0.039543
miR-214-5p	D24P Vs D24NP	21.5066	0.012231
miR-99b-3p	D24P Vs D24NP	21.0397	0.008245
let-7e-3p	D24P Vs D24NP	15.5629	0.003295
let-7f-1-3p	D24P Vs D24NP	15.527	0.024776
miR-338-3p	D24P Vs D24NP	13.0566	0.03714
miR-487b	D24P Vs D24NP	11.1197	0.006435
miR-29a-5p	D24P Vs D24NP	10.8531	0.016094
miR-125b-2-3p	D24P Vs D24NP	10.7409	0.003999
miR-502-3p	D24P Vs D24NP	10.4472	0.006895
miR-335-5p	D24P Vs D24NP	9.9524	0.007685
miR-149-5p	D24P Vs D24NP	8.1495	0.015961
miR-1538	D24P Vs D24NP	7.8628	0.02069
miR-342-5p	D24P Vs D24NP	7.701	0.002069
miR-664a-3p	D24P Vs D24NP	7.0455	0.000127
miR-25-5p	D24P Vs D24NP	6.5737	0.039168
miR-500a-5p	D24P Vs D24NP	5.5406	0.011349
miR-34a-5p	D24P Vs D24NP	-2.4339	0.008307
miR-495-3p	D24P Vs D24NP	-2.5996	0.042961
let-7i-5p	D24P Vs D24NP	-2.8745	0.023474
miR-422a	D24P Vs D24NP	-3.9999	0.011377
miR-376b-3p	D24P Vs D24NP	-6.5204	0.025471
miR-323a-3p	D24P Vs D24NP	-7.917	0.024205
miR-455-3p	D24P Vs D24NP	-18.6999	0.010084

Comparisons were done between pooled serum samples derived from day 19 pregnant (D19P) Vs day 19 non-pregnant (D19NP) and day 24 pregnant (D24P) Vs day 19 non-pregnant (D19NP)

Table 4.2: List of differentially expressed bovine miRNAs homologous to the pregnancy associated C14MC miRNA cluster

CM14C miRNA	Homologous bovine miRNA	Comparison	FC	p-Value	Chromosomal location in bovine genome
hsa-miR-487b	bta-miR-487b	D24P Vs D24NP	11.1197	0.006435	chr21: 67583558-67583641 [+]
hsa-miR-495-3p	bta-miR-495	D24P Vs D24NP	-2.5996	0.042961	chr21: 67573235-67573315 [+]
hsa-miR-376b-3p	bta-miR-376b	D24P Vs D24NP	-6.5204	0.025471	chr21: 67579062-67579154 [+]
hsa-miR-323a-3p	bta-miR-323	D24P Vs D24NP	-7.917	0.024205	chr21: 67565468-67565553 [+]
hsa-miR-433	bta-miR-433	D19P Vs D19NP	7.523	0.012063	chr21: 67428646-67428769 [+]

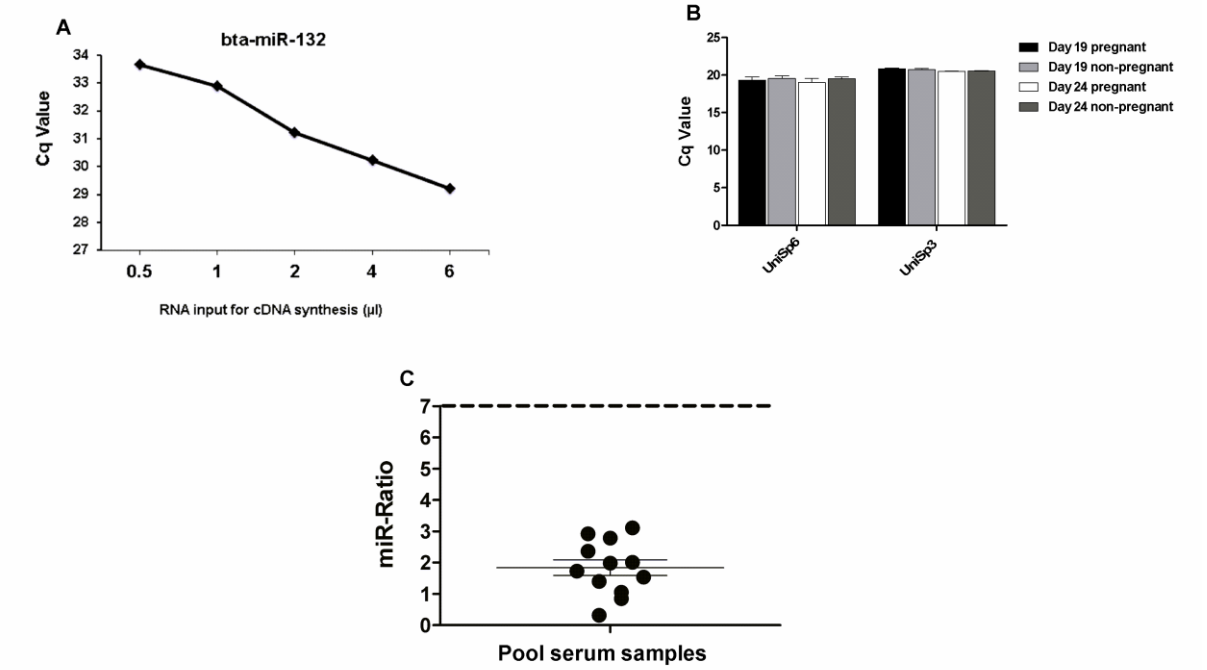

Figure 4.1: Quality control of serum miRNA profiling. Quantification of bta-miR-132 in serum sample using qRT-PCR using different RNA input volumes for cDNA synthesis in 10 µL reaction volume. The expression of miRNA and the RNA were negatively correlated (r= -0.96) (**A**). Stability of synthetic RNA spike-ins; UniSp6 and UniSp3 in serum samples of pregnant and non-pregnant cows 19 and 24 days post-insemination. No significant differences were observed indicating higher degrees of efficiencies in cDNA synthesis and qRT-PCR reactions (**B**). Determining the level of hemolysis in bovine serum samples using the miR-Ratio method (Cq bta-miR-451- Cq bta-miR-23a), showing a miR-Ratio value of 1.82 ± 0.25 indicating no detectable level of hemolysis (miR-Ratio < 7) (**C**).

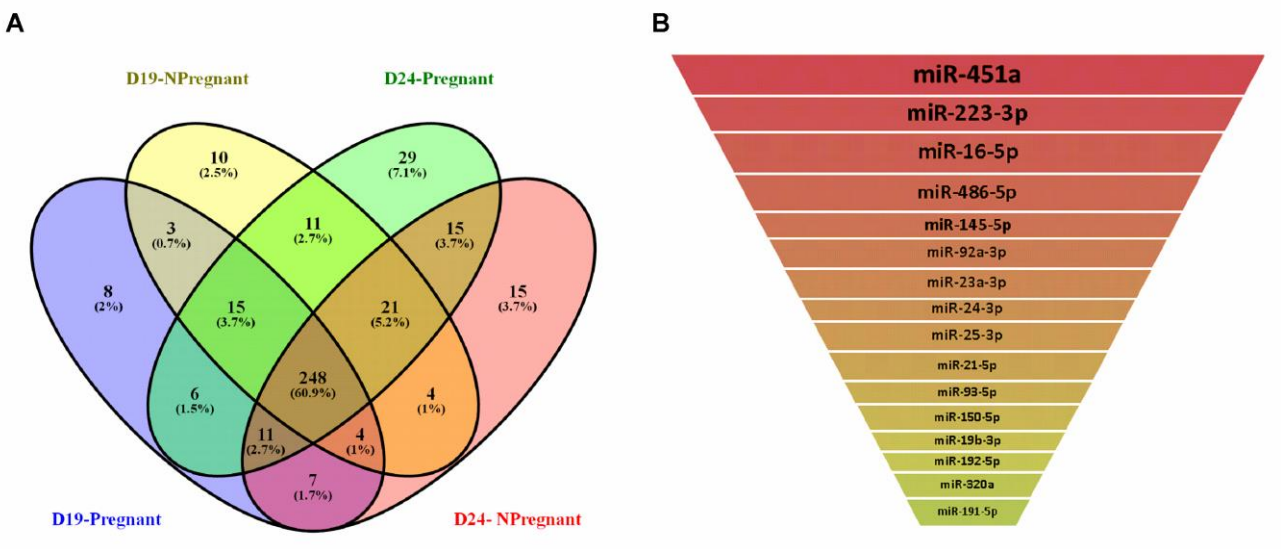

Figure 4.2: Abundance of miRNAs in bovine serum. The number and percentage of miRNAs detected in serum samples of pregnant and non-pregnant cows at day 19 and 24 post-insemination (**A**). Analysis of the top 16 miRNAs highly expressed in all treatment groups calculated as 2^{40-Cq} (**B**).

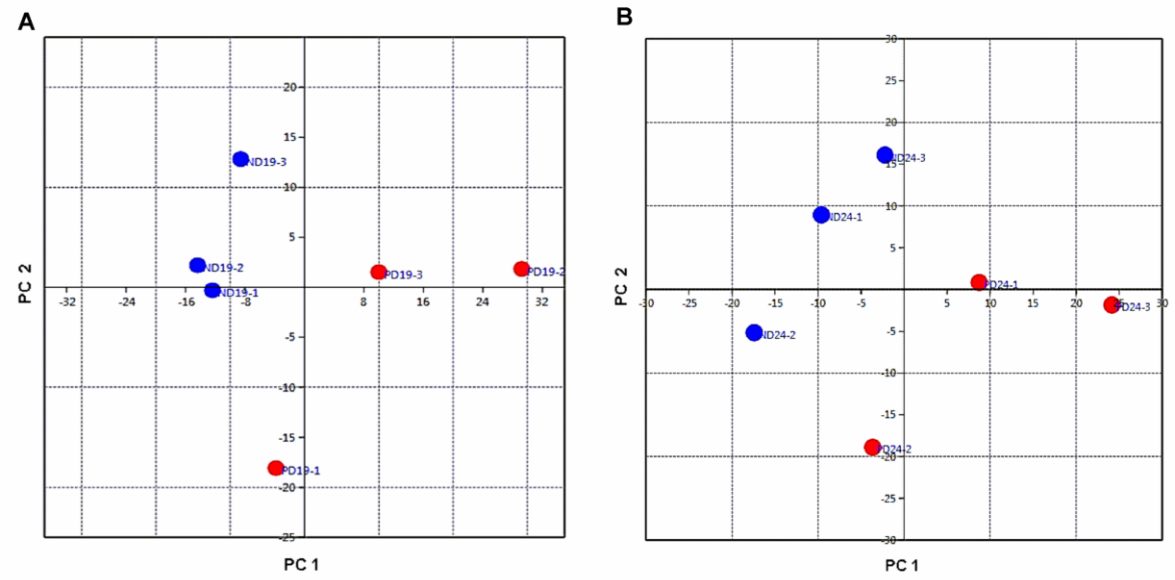

Figure 4.3: Principal component analysis using extracellular miRNAs in serum samples of pregnant and non-pregnant cows. PCA plot of day 19 pregnant and non-pregnant cows (**A**). PCA plot of day 24 pregnant and non-pregnant cows (**B**). Red dots represent biological replicates of pregnant cows while blue dots represent biological replicates of non-pregnant cows.

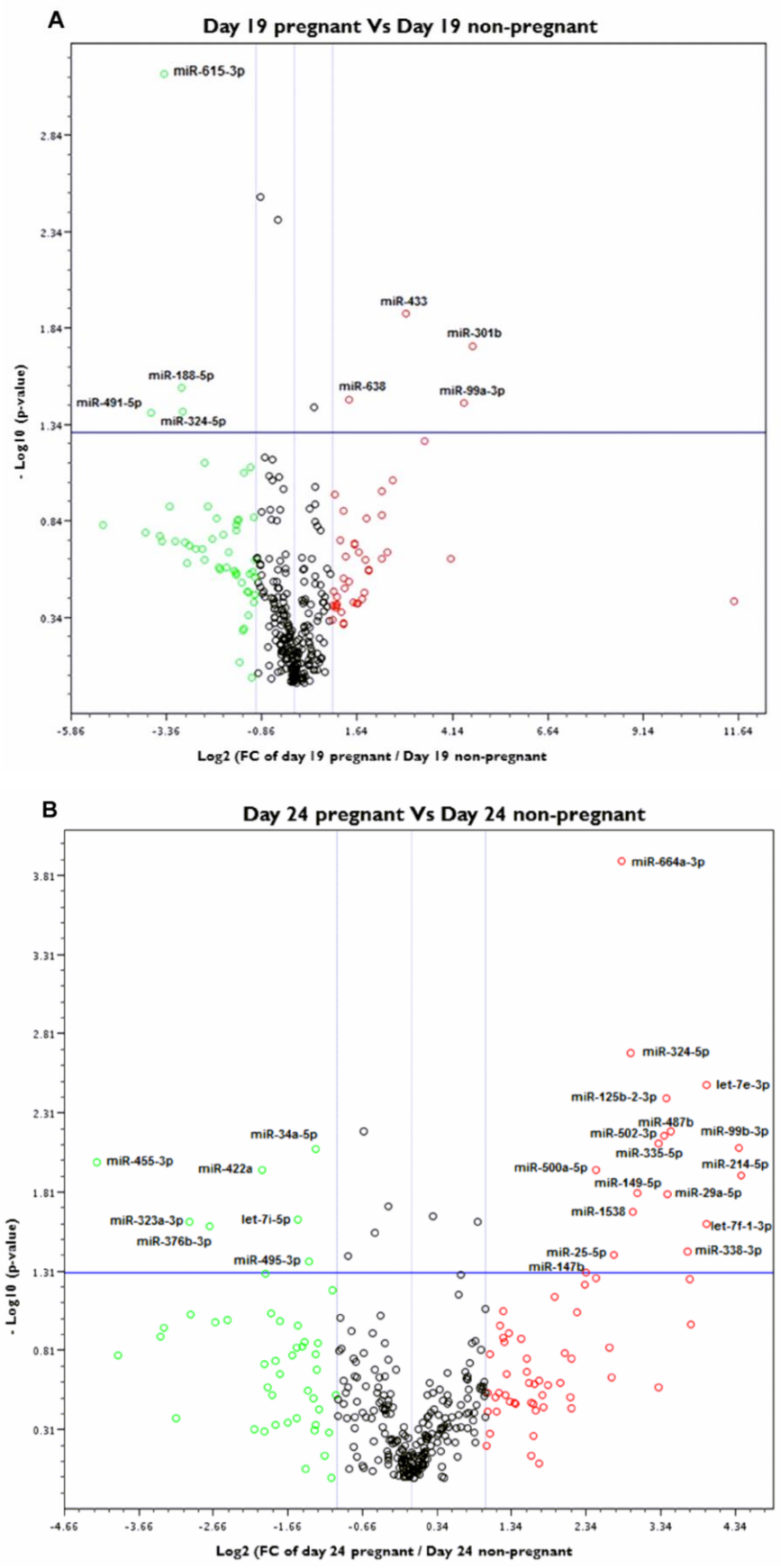

Figure 4.4: Differential expression analysis of extracellular miRNAs between pregnant and non-pregnant cows. Volcano plot of miRNAs differentially expressed in serum of day 19 pregnant cows (**A**) and in serum of day 24 pregnant cows (**B**). Red and

green dots represent up-regulated and down-regulated miRNAs, respectively. The horizontal blue line represents threshold of significant level (p=0.05) and the vertical grey lines represent threshold of fold regulations. The p-values are Log10 transformed while the fold regulation of each miRNAs is Log_2 transformed.

Chapter 5
General discussion

5.1 General discussion

The present PhD study aimed at determining the role of cellular miRNAs in bovine follicular development and assessed the potential use of extracellular miRNAs as potential indicators of early pregnancy in dairy cows. To attain the first objective, a genome-wide miRNA expression profile was performed in granulosa cells collected from preovulatory dominant and subordinate follicles derived from synchronized cows slaughtered at day 19 of the estrous cycle using next-generation sequencing technology. Moreover, the functional role of miR-183-96-182 cluster miRNAs in regulating the proliferation and cell cycle transition of granulosa cells was assessed using an in vitro granulosa cell culture model. In the last experiment, extracellular miRNAs were profiled in serum samples of pregnant and non-pregnant dairy cows 19 and 24 days post-insemination.

In the first manuscript, data demonstrating that miRNAs in granulosa cells of preovulatory dominant and subordinate follicles have distinct expression signature are presented. Based on the expression profile data, it was possible to clearly separate granulosa cells of preovulatory dominant from the subordinate follicles using PCA analysis as shown in Figure 5.1.

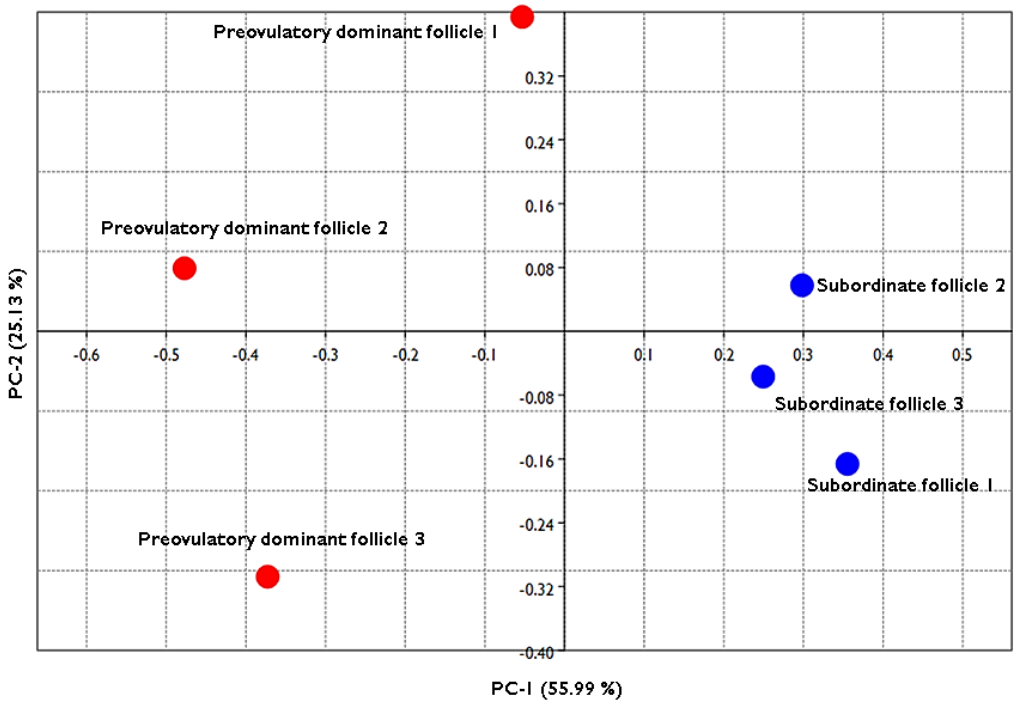

Figure 5.1: Principal component analysis of normalized miRNA expression data in granulosa cells of preovulatory and subordinate follicles. The first two principal components (PC) explain more than 80% of the variations between the two follicular categories.

Interestingly, granulosa cells of preovulatory dominant follicles were relatively more heterogeneous compared to the subordinate follicles (Figure 5.1). This is in agreement with previous report in which comparative transcriptome analysis of granulosa cells from healthy follicle and atretic follicles revealed higher variability among the healthy follicles (Hatzirodos *et al.* 2014a), in which the activation of the pro-apoptotic transcriptional factor TP53 and its downstream genes could further justify the high level of homogeneity among the atretic follicles. It was also reported that transcriptome variability increase with follicular size, which could be associated with the preferential activation of large sets of genes by LH surge in large antral follicles but not in smaller follicles (Hatzirodos *et al.* 2014b). Taken this together, the subordinate follicles which are destined to be atretic showed relatively homogeneous activity of transcriptional machinery both at mRNAs and miRNAs levels compared to the preovulatory dominant ones. The expression pattern of miRNAs in granulosa cells of preovulatory and subordinate follicles showed that majority of the miRNAs expressed in similar fashion in both preovulatory and subordinate follicles which signifies their housekeeping role during the follicular phase of the estrous cycle. MiR-26a, miR-10b and the let-7 family were among the abundantly expressed miRNAs both in preovulatory dominant and subordinate follicles of the follicular phase of the estrous cycle. In similar studies, miR-26a, miR-10b and the let-7 family were also abundantly expressed in both dominant and subordinate follicles during early luteal phase of bovine estrous cycle (Salilew-Wondim *et al.* 2014), in bovine ovaries (Hossain *et al.* 2009; Huang *et al.* 2011; Miles *et al.* 2012) suggesting their involvement in maintaining basal functions of ovarian cyclicity.

Comparative expression analysis between preovulatory dominant and subordinate follicles revealed that 64 miRNAs were differentially regulated and distinct miRNA clusters were preferentially enriched in either of the follicular categories. For instance, the miR-183-96-182, miR-132-212, miR-424- 450-542 cluster and miR-21were among the miRNAs which were found to be enriched in preovulatory dominant follicles (Table 2.4), while the miR-17-92 cluster miRNAs and miR-224 were among the miRNAs enriched in subordinate follicles (Table 2.5). The surge of LH during the follicular phase of the estrous cycle leads to the induction of massive miRNA expression change of the preovulatory follicle that could function as a survival factor to promote terminal differentiation of granulosa cells into progesterone secreting luteal cells (Quirk *et al.* 2004). Similar study in mouse reported selective enrichment of miR-132-212 cluster and miR-21 in granulosa cells following treatment with an ovulatory dose of LH (Fiedler *et al.* 2008; Carletti *et al.* 2010). The changes in the expression pattern of miRNAs observed in granulosa cells of the preovulatory

follicles during the follicular phase of the estrous cycle (i.e following the LH surge) is associated with the fact that the granulosa cells are the prime target for the miRNA-mediated changes in gene expression. Following the LH surge, the granulosa cells acquire luteal phenotypes and loss the follicular phenotype, which is accompanied by miRNA-mediated changes in gene expression (Carletti *et al.* 2010). Among the transcriptional factor involved in ovarian function, the pro-apoptotic FOXO1 was found to be enriched in granulosa cells of subordinate follicles compared to the preovulatory dominant follicles and validated to be target of the miRNA clusters enriched in preovulatory dominant follicles. Similarly, downregulation of FOXO1 gene through LH-induced luteinization was reported earlier (Liu *et al.* 2009). Taken together, selective enrichment and degradation of miRNAs in granulosa cells of preovulatory and subordinate follicle suggest their critical role in posttranscriptional regulation of genes associated with follicular fate determination.

Since the discovery of miRNAs, the number of miRNAs reported in the miRBase repository has drastically increased. Similarly, the number of miRNAs detected in mammalian ovaries, granulosa cells, and other follicular cells increased significantly (Fiedler *et al.* 2008; Hossain *et al.* 2009; McBride *et al.* 2012; Salilew-Wondim *et al.* 2014). However, only few studies have been conducted to decipher the functional role of these miRNAs in granulosa cells. The interaction between miRNA and target gene is complex in nature and this has hampered efforts of determining functions of individual miRNAs. Moreover, most miRNAs are co-expressed and act in clusters and functional study on single miRNAs become very challenging and attaining measurable biological changes become very difficult. Thus, functional study on cluster of co-expressed miRNAs could affect the function of granulosa cells under investigation (Maalouf *et al.* 2016). To get a good picture of the specific regulatory role of selected miRNAs in granulosa cells which were found to be differentially expressed in preovulatory dominant follicles, in the second experiment an in vitro granulosa cell culture model was used to perform loss-and-gain of function for the miR-183-96-182 cluster. The miR-183-96-182 cluster miRNAs were found to be highly enriched in preovulatory follicles and validated to target the FOXO1 transcription factor. Using loss-and-gain of function experiment, overexpression and inhibition of the miRNA cluster resulted in downregulation and enrichment of the protein and mRNA of FOXO1, respectively. We also showed that overexpression of the miRNA cluster enhance the rate of proliferation and cell cycle transition in FOXO1-dependent manner. Interestingly, the expression of FASL; a pro-apoptotic downstream transcript of FOXO1, was also affected in similar manner. In agreement with these findings, the expression of FOXO1 was reported to be altered by

several miRNAs in several mammalian cell types to induce cell proliferation (Wu *et al.* 2012; Yang *et al.* 2014), inhibit apoptosis of cells (Song *et al.* 2015). Similarly, inhibition of miRNAs known to target the FOXO1 gene; miR-27a, miR-96, and miR-182 in breast cancer cells increased the endogenous FOXO1 accumulation and resulted in decreased cell number and viability (Guttilla & White 2009). Taking these together, enrichment of the miR-183-96-182 cluster miRNA in preovulatory follicle could be involved in repressing the expression of transcription factor FOXO1 and its downstream apoptosis-related genes to prevent the granulosa cells in preovulatory follicle from follicular atresia and act as survival factors.

The successes of a dairy farming heavily depend on good reproductive management practices. Enhancing the reproductive performance of cows by the maintaining regular cyclicity and pregnancy is needed. Therefore, early detection of pregnancy within the first 3 weeks post-insemination plays paramount role in preventing elongated calving intervals. The pregnancy detection methods currently utilized have inefficiencies related to sensitivity, specificity, earliest time of detection and invasiveness, which result in false positive and negative results. As a consequence of the longer inter-calving intervals, profitability in the dairy industry declines significantly (Arbel *et al.* 2001). Thus, development of blood-based non-invasive biomarkers to detect early pregnancy could help improve the shortcomings of the currently used pregnancy detection tools. MicroRNAs in circulation of pregnant mothers are reported to indicate the pregnancy status and pregnancy-related complications (Morales-Prieto *et al.* 2013; Ioannidis & Donadeu 2016). In line with this, the third experiment was performed to detect extracellular miRNAs that could be used as indicators of early pregnancy in dairy cows. For this, serum samples of cows derived from 19 and 24 days after insemination were retrospectively categorized as pregnant and non-pregnant after ultrasonography examination 35 days post-insemination. Principal component analysis on the normalized expression pattern of 748 miRNAs showed a clear separation between pregnant and non-pregnant cows both at day 19 and 24 post-insemination. Among the miRNAs differentially expressed in serum of pregnant cows, bta-miR-487b, bta-miR-495, bta-miR-376b, bta-miR-323 and bta-miR-433 which are homologous to the C14MC human pregnancy-associated miRNAs were found to be differentially expressed in pregnant cows. The C14MC are among the placental-specific miRNA clusters associated with pregnancy implantation in human. In agreement with these finding, it was shown that the C14MC are differentially expressed in plasma of pregnant women compared to the non-pregnant counterparts (Miura *et al.* 2010). The results also showed that the adherens junction and ECM-receptor interaction were among the top pathways enriched by the predicted target

genes of the miRNAs preferentially expressed in serum of pregnant cows at day 19 and 24. Reports indicated that the adherens junction in the uterus of ovine is differentially regulated by pregnancy and progesterone, which helps the elongation and survival of conceptus by secreting trophic substances (Satterfield *et al.* 2007). Similarly, the ECM interaction is necessary during implantation and trophoblast cell invasion (MacIntyre *et al.* 2002; Zhao *et al.* 2015). These results suggested the potential use of extracellular miRNAs as indicators of early pregnancy in cattle.

5.2 Future prospects

The recent advancements in high-throughput sequencing technology have facilitated efforts in miRNAs profiling and increased the number of miRNAs detected in mammalian ovaries and follicular somatic cells. Moreover, the presence of extracellular miRNAs in almost all biological body fluids has provided a new opportunity in miRNA research and miRNA-based diagnostic biomarkers discovery. Profiling of both cellular and extracellular miRNAs has tremendous benefit to understand the spatio-temporal map of miRNAs and gives a clue with regards to the holistic functional role of miRNAs in the physiological conditions under scrutiny. In comparison with the number of miRNAs detected in mammalian ovaries, experiments on determining the functional role of individual and cluster miRNAs in follicular cells from various stages of follicular development is progressing at a slower pace. Thus, future efforts should focus on understanding the function of miRNAs in follicular cells by applying novel genetic tools including the CRISPR technology.

It is also necessary to further validate the extracellular miRNAs reported to be differentially regulated in serum of pregnant cows in independent herds with increased number of experimental cows. This could enhance the effort in search of diagnostic marker for early pregnancy detection. Moreover, functional studies on candidate extracellular miRNAs could further widen our understanding on the genetic regulation of maternal recognition of pregnancy and embryo implantation in greater depth.

Recently, shreds of evidence supporting the importance of functional polymorphisms associated with miRNA regulation started to emerge. MicroRNA-related single nucleotide polymorphisms (SNPs) in the miRNA loci or in their target gene could have regulatory role through modifications in miRNA biogenesis and/or ability to bind to the target genes (Sethupathy & Collins 2008). The density of SNPs and frequency of alleles within the pre-miRNA and the seed regions of mature miRNAs tend to be lower, and are under negative selection pressure (Saunders *et al.* 2007). Moreover, SNPs residing at the 3´-UTR of the

target genes that alter or create novel miRNA binding sites showed a higher degree of population differentiation (Richardson *et al.* 2011), implying the positive selection pressure that leads to differences among populations. Thus, the focus of future miRNA research should also include the miRNA-related SNPs both within the miRNA loci and at the 3´-UTR of target genes to explain the molecular mechanism of phenotypic variations in female reproduction related traits in cattle.

Chapter 6

Summary

Bovine follicular and embryonic development are among the highly orchestrated and dynamically regulated processes affected by multifarious hormones and intraovarian growth factors. All these factors are tightly regulated by genes, which are expressed in spatio-temporal manner. Transcriptional regulation of primordial follicle activation, recruitment of small antral follicles, selection of dominant follicles, ovulation of preovulatory dominant follicles, degradation of anovulatory subordinate follicles, early embryonic cleavages, activation of the embryonic genome, maternal recognition of pregnancy, embryo implantation in maternal endometrium and other processes beyond are evidenced to be regulated by the expression and interaction of multitude of transcripts in various follicular and embryonic cells. The emergence of miRNAs as posttranscriptional gene regulators added another layer of complexity into the regulation of follicular and embryonic development. Since their discovery, numerous studies have indicated the fundamental roles of miRNAs in almost all physiological conditions and diseases. Despite the growing number of miRNAs reported in several mammalian species and diverse tissue types, little is known about the expression signature and functional role of miRNAs in bovine follicular development and early pregnancy. The aim of this PhD thesis was to investigate the role of cellular miRNAs in bovine follicular development (chapter 2 and 3) and determining the potential use of extracellular miRNAs as indicators of early pregnancy in dairy cows (chapter 4).

In chapter 2, Simmental heifers (n=7) were estrous synchronized according to standard protocol and slaughtered 19 days after the onset of estrous. According to the surface diameter, ovarian follicles were categorized as preovulatory dominant (> 12 mm) and subordinate follicles (≤ 11 mm). Preovulatory dominant follicles were only found in 5 heifers while two heifers were excluded from the experiments as they had no a clear dominant follicle. Granulosa cells were aspirated from both follicular categories and miRNA enriched total RNA was isolated according to standard procedures. Total RNA samples were subjected to miRNA expression profiling using next-generation sequencing technology. Data analysis and expression profile of miRNAs was performed according to the workflow of miRDeep2. The expression of selected candidate miRNA clusters was determined in theca cells, COC and follicular fluid. Furthermore, the interaction of miR-183-96-182 cluster and its predicted target gene FOXO1 was validated using luciferase reporter assay. Results showed that the difference in the surface diameter of preovulatory dominant (15.4 ± 3.68 mm) and subordinate follicles (6.53 ± 0.99 mm) was statistically significant ($p < 0.001$). It was shown that 315 and 323 known mature miRNAs were detected in granulosa cells of

preovulatory dominant and subordinate follicles, respectively. Besides, 11 putative novel miRNAs were also predicted. Principal component analysis clearly separated both follicular categories according to the expression level of miRNAs in granulosa cells. Even though majorities of the detected miRNAs are equally expressed in both follicular categories, 64 miRNAs were found to be significantly differentially expressed in preovulatory dominant follicles. Among the 34 miRNAs, which were found to be enriched in preovulatory dominant follicles, the miR-183-96-182, miR-132-212 and miR-424-450-552 cluster miRNAs were co-expressed. Conversely, the miR-17-92 cluster miRNAs are among the 30 miRNA preferentially enriched in subordinate follicles. The expression of 9 randomly selected candidate miRNAs was validated using qRT-PCR and their expression was in agreement with the deep sequencing result. The relative abundance of miR-96, miR-182, miR-212, and miR-132 was assessed in theca cells, COC and follicular fluid using qRT-PCR. Accordingly, the relative abundance was higher in preovulatory dominant follicles as it was in the granulosa cells. In silico target gene prediction of one of the miRNA clusters enriched in preovulatory dominant follicles; the miR-183-96-182 cluster resulted in an array of genes known to be involved in pathways with important roles in follicular development. Luciferase reporter assay showed that the miR-183-96-182 coordinately suppress the expression of FOXO1. This was further supported by the lower level of FOXO1 in preovulatory dominant follicle compared with the subordinate follicles counterpart. Therefore, the preferential enrichment and degradation of miRNA in granulosa cells of preovulatory dominant and subordinate follicles during the later follicular stages of bovine estrous cycle supports the notion that miRNAs are involved in regulating genes and pathways important for follicular fate determination.

In chapter 3, granulosa cells aspirated from small growing antral follicles (surface diameter of 3-5 mm) were used for an in vitro cell culture system to determine the functional role of miR-183-96-182 in granulosa cells through the regulation of FOXO1 transcription factor and its downstream transcripts. Cells were cultured in F-12 medium supplemented with FBS. Loss-and-gain of function experiments were performed by transfecting cultured granulosa cells with LNA-based mimics, inhibitors of the miR-183-96-182 cluster miRNAs and the corresponding negative controls where lipofectamine was used as transfection reagent. After the modulation of the miRNAs, the mRNA and protein expression of the target gene was quantified followed by cell proliferation and cell cycle analyses. To further validate the results from the miRNA modulation experiments, FOXO1 was selectively downregulated using anti-FOXO1 siRNA followed by mRNAs and protein quantification, cell proliferation and cell

cycle assays. Results revealed that inhibition of a single miRNA from the cluster leads to a sponge effect. Whereas, overexpression of a single miRNAs selectively increase the expression of the overexpressed miRNA and no sponge effect was observed in the expression of other miRNAs within the cluster. Overexpression of the miR-183-96-182 cluster miRNAs leads to significant reduction in the mRNA and protein of FOXO1and the expression of FASL, a downstream target gene of FOXO1. Inhibition of the miRNA cluster increased the FOXO1 and FASL mRNA level, but the FOXO1 protein was not affected. Transfection of cultured granulosa cells with miR-183-96-182 cluster mimics increased the rate of cell proliferation. Whereas, inhibition of the miRNA cluster slowed down the rate at which cells proliferate. This was further supported by the changes in cell cycle profile, where overexpression of the miRNA cluster decreased the proportion of cells under G_0/G_1 arrest by increasing the proportion of cells under the S phase. There were no measurable changes in the cell cycle profile of granulosa cells upon inhibition the miRNA cluster. In agreement with the overexpression experiment, selective knockdown of the FOXO1 using siRNA increased the rate of granulosa cell proliferation, decreased the proportion of cells under G_0/G_1 arrest, and increased the proportion of cells in the S-phase of the cell cycle. Therefore, the miR-183-96-182 cluster miRNAs are important in promoting survival of granulosa cells by promoting cell proliferation and cell cycle transition by downregulating pro-apoptotic transcription factors and the associated downstream transcripts.

In chapter 4 of the thesis, the expression signature of extracellular miRNAs in serum samples derived from pregnant and non-pregnant cows was determined. For this, lactating dairy cows were synchronized 50-80 days post-partum according to standard procedures. Cows were inseminated with frozen semen and the day of insemination was considered as day 0. Serum samples were processed 19 and 24 days post-insemination and the pregnancy status of the cows was verified using ultrasonography 35 days post-insemination. Serum samples were retrospectively categorized as pregnant and non-pregnant and pooled serum samples (4 animals/pool) were subjected to RNA isolation. The expression of extracellular miRNAs in serum samples was performed using Ready-to-Use PCR array, Human panel I+II containing primers of 748 known mature miRNAs. After normalization and quality control procedures, a total of 302 and 316 miRNAs were detected in day 19 pregnant and non-pregnant cows, respectively. Similarly, 356 and 325 miRNAs were detected in day 24 pregnant and non-pregnant cows, respectively. Principal component analysis showed a clear separation between pregnant and non-pregnant cows both at day 19 and 24. It was also revealed that 8 and 23 miRNAs to be differentially expressed in day 19 and 24 the pregnant

group, respectively. Among the differentially expressed miRNAs, 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p, and miR-323a-3p) in day 24 pregnant cows and 1 miRNA (miR-433) in day 19 pregnant cows were found to be homologous to the known human pregnancy-associated C14MC miRNAs. Pathway analysis of the target genes of differentially expressed miRNAs in pregnant cows revealed adherens junction and ECM-interaction pathways to be important during pregnancy establishment.

In summary, the present study provides insights into the involvement of cellular miRNAs in granulosa cells and their regulatory role during bovine follicular development. Distinct miRNA clusters preferentially co-expressed in granulosa cells of preovulatory dominant follicles support the notion that miRNAs are involved in regulating genes important for follicular fate determination. Moreover, the unique expression signatures of extracellular miRNAs in serum samples of pregnant and non-pregnant cows signify their potential to be used as indicators of early pregnancy in dairy cows.

Die bovine follikuläre und embryonale Entwicklung gehören zu den dynamisch regulierten Prozessen, die von vielfältigen Signalwegen und intra-ovariellen Wachstumsfaktoren beeinflusst werden. Diese Faktoren werden durch Gene streng räumlich und zeitlich begrenzt reguliert. Die Transkriptionsregulierung der Primordialfollikel, deren Aktivierung, die Rekrutierung von kleinen Antralfollikeln, die Auswahl der dominanten Follikeln, die Ovulation vom dominanten Follikel, der Abbau von anovulatorischen subordinaten Follikeln, die frühe embryonale Teilung, die Aktivierung des embryonalen Genoms, die mütterliche Erkennung der Trächtigkeit, die Einnistung des Embryos ins mütterliche Endometrium werden durch die Expression und die Interaktion einer Vielzahl von Transkripten in verschiedenen follikulären und embryonalen Zellen beeinflusst. Die Einbeziehung von miRNAs als posttranskriptionelle Genregulatoren ist eine weitere komplexe Ebene in der Regulation der follikulären und embryonalen Entwicklung. Seit ihrer Entdeckung haben zahlreiche Studien die grundlegenden Rollen von miRNAs in fast allen physiologischen Zuständen und Krankheiten untersucht. Trotz der wachsenden Zahl an Publikationen von miRNAs in verschiedenen Tierarten und Gewebetypen ist nur wenig über die Expressionsignatur und funktionelle Rolle von miRNAs in der Follikelreifung und frühen Trächtigkeit beim Rind bekannt. Das Ziel dieser Doktorarbeit war es, die Rolle der zellulären miRNAs während der Follikelreifung (Kapitel 2 und 3) und die Bestimmung der möglichen Verwendung von extrazellulären miRNAs als Indikatoren der frühen Trächtigkeit bei Milchkühen (Kapitel 4) zu untersuchen.

In Kapitel 2 wurden Fleckvieh Färsen (n = 7) nach Standard-Protokoll synchronisiert und die Tiere 19 Tage nach dem Beginn des Östrus geschlachtet. Die Ovarialfollikel wurden in präovulatorische dominante (> 12 mm) und subordinante Follikel (≤ 11 mm) klassifiziert. Präovulatorische dominante Follikel wurden nur in 5 Färsen gefunden, während zwei Färsen von den Experimenten ausgeschlossen wurden, da sie keine klaren dominanten Follikel hatten. Granulosazellen wurden von beiden Follikelkategorien aspiriert, miRNA-angereicherte Gesamt-RNA wurde nach Standardverfahren isoliert. Die Gesamt-RNA-Proben wurden für die Erstellung eines miRNA-Expressionsprofils unter Verwendung der „Next-Generation Sequenztechnologie“ verwendet. Die Datenanalyse und das Expressionsprofil von miRNAs wurde mit dem Arbeitspaket miRDeep2 durchgeführt. Die Expression von ausgewählten Kandidaten-miRNA-Clustern wurde in Theka-Zellen, Kumuls-Oocyten-Komplexen (COC) und Follikelflüssigkeit bestimmt. Weiterhin wurde die Interaktion des miR-183-96-182-Clusters mit seinem Zielgen FOXO1 unter Verwendung eines Luciferase-Reporter-Assays validiert. Die Ergebnisse zeigten, dass der Unterschied im

Durchmesser von präovulatorisch dominanten (15,4 ± 3,68 mm) zu subordinanten Follikeln (6,53 ± 0,99 mm) statistisch signifikant (p <0,001) war. Es wurden 315 und 323 bekannte miRNAs in Granulosazellen von präovulatorisch dominanten und subordinanten Follikeln nachgewiesen. Außerdem wurden 11 neue miRNAs identifiziert. Eine Hauptkomponentenanalyse trennte deutlich die beiden Follikelkategorien nach dem Expressionsniveau der miRNAs in den Granulosazellen. Obwohl die Mehrheit der detektierten miRNAs in beiden Follikelklassen gleichermaßen exprimiert waren, wurden 64 signifikant differentiell exprimierte miRNAs in präovulatorisch dominanten Follikeln identifiziert. Unter den 34 miRNAs, die in präovulatorisch dominanten Follikel hoch reguliert waren, waren die miR-183-96-182, miR-132-212 und miR-424-450-552 Cluster co-exprimiert. Umgekehrt gehören die miR-17-92-Cluster-miRNAs zu den 30 miRNAs, die vorzugsweise in subordinaten Follikeln angereichert sind. Die Expression von 9 zufällig ausgewählten Kandidaten-miRNAs wurde mittels qRT-PCR validiert. Die relative Expression von miR-96, miR-182, miR-212 und miR-132 wurde in Theka-Zellen, COC und Follikelflüssigkeit untersucht, sie war in präovulatorisch dominanten Follikeln höher als in den Granulosazellen.

Eine In-Silico-Zielgen-Analyse zeigte, dass eine Vielzahl von Genen des miR-183-96-182-Clusters in Signalwegen, die eine wichtige Rolle in der Follikelentwicklung spielen, involviert sind. Der Luciferase-Reporter-Assay ergab, dass miR-183-96-182 die Expression von FOXO1 unterdrückt. Dies wurde durch die niedrigere Expression von FOXO1 in präovulatorisch dominanten Follikeln im Vergleich zu den subordinaten Follikeln unterstützt. Daher bestätigt die Anreicherung und der Abbau von miRNA in Granulosazellen von präovulatorisch dominanten und subordinaten Follikeln während der späteren Follikelstadien des Rinder-Östruszyklus die Annahme, dass miRNAs an der Regulierung von Genen und Signalwegen beteiligt sind, die für die follikuläre Entwicklung wichtig sind.

In Kapitel 3 wurden Granulosazellen, die von kleinen wachsenden Antrum-Follikeln (Oberflächendurchmesser von 3-5 mm) aspiriert wurden, für das In Vitro-Zellkultursystem verwendet, um die funktionelle Rolle von miR-183-96-182 in Granulosazellen durch die Regulation von FOXO1 Transkriptionsfaktoren und seine nachgeschalteten Transkripte zu bestimmen. Die Zellen wurden in F-12-Medium kultiviert, das mit fötalem Rinderserum versetzt wurde. „Loss-and-gain of function“ Experimente wurden durch Transfektion von kultivierten Granulosazellen mit LNA-basierten Stimulatoren und Inhibitoren der miR-183-96-182-Cluster-miRNAs und den entsprechenden Negativkontrollen, bei denen Lipofectamin als Transfektionsreagens verwendet wurde, durchgeführt. Nach der Modulation der miRNAs

wurden die mRNA- und Proteinexpression des Zielgens quantifiziert, gefolgt von Zellproliferations- und Zellzyklusanalysen. Zur Validierung der Ergebnisse wurde FOXO1 selektiv unter Verwendung von anti-FOXO1-siRNA herunterreguliert, gefolgt von mRNAs und Protein-Quantifizierung, Zellproliferations- und Zellzyklusanalyse. Die Ergebnisse zeigten, dass die Hemmung einer einzelnen miRNA aus dem Cluster zu einem Schwamm-Effekt führt. Die Überexpression der miR-183-96-182-Cluster-miRNAs führt zu einer signifikanten Reduktion der mRNA- und Proteinexpression von FOXO1 und der Expression von FASL, einem nachgeschalteten Zielgen von FOXO1. Die Inhibition des miRNA-Clusters erhöhte die FOXO1- und FASL-mRNA Expression, aber das FOXO1-Proteinniveau wurde nicht beeinflusst. Die Transfektion von kultivierten Granulosazellen mit miR-183-96-182-Clusterstimulatoren erhöhte die Zellproliferation, während die Inhibition des miRNA-Clusters die Geschwindigkeit der Zellproliferation verlangsamte. Dies zeigte auch die Veränderungen des Zellzyklusprofils. Die Überexpression des miRNA-Clusters verringerte den Anteil der Zellen während des G_0 / G_1-Arrest, der Anteil der Zellen während der S-Phase wurde erhöht. Es gab keine messbaren Veränderungen im Zellzyklusprofil von Granulosazellen nach Inhibierung des miRNA-Clusters. In Übereinstimmung mit diesem Experiment erhöhte der selektive Knockdown von FOXO1 die Rate der Granulosazellenproliferation, verringerte den Anteil der Zellen während des G_0 / G_1-Arrest und erhöhte den Anteil der Zellen in der S-Phase des Zellzyklus. Daher sind die miR-183-96-182-Cluster-miRNAs wichtig, um das Überleben von Granulosazellen zu fördern und die Zellproliferation und den Zellzyklusübergang zu verbessern, indem sie pro-apoptotische Transkriptionsfaktoren und die zugehörigen nachgeschalteten Transkripte herabregulieren.

In Kapitel 4 der Arbeit wurde die Signatur von extrazellulären miRNAs in Serumproben von trächtigen und nicht trächtigen Kühen bestimmt. Hierzu wurden laktierende Milchkühe 50-80 Tage postpartum nach Standardverfahren synchronisiert. Die Kühe wurden mit gefrorenem Samen besamt und der Tag der Besamung wurde als Tag 0 betrachtet. Es wurden Serumproben 19 und 24 Tage nach der Insemination gewonnen und der Trächtigkeitsstatus der Kühe mittels Ultrasonographie 35 Tage nach der Insemination verifiziert. Die Serumproben wurden retrospektiv als trächtig und nicht-trächtig sortiert und gepoolt (4 Tiere / Pool) einer RNA-Isolierung unterzogen. Messungen der Expression extrazellulärer miRNAs in Serumproben erfolgte unter Verwendung eines gebrauchsfertigen PCR-Arrays, Human Panel I + II, das Primer von 748 bekannten miRNAs enthielt. Nach der Normalisierung und der Qualitätskontrolle wurden insgesamt 302 und 316 miRNAs am Tag 19 bei trächtigen bzw. nicht trächtigen Kühen nachgewiesen. Ähnlich, konnten 356 und 325

miRNAs an Tag 24 bei trächtigen bzw. nicht-trächtigen Kühen nachgewiesen werden. Die Hauptkomponentenanalyse zeigte eine klare Trennung sowohl zwischen trächtigen und nicht trächtigen Kühen als auch am Tag 19 und 24. Weiterhin zeigten 8 und 23 miRNAs eine differentielle Expression sowohl am Tag 19 als auch 24 der trächtigen Gruppe. Unter den unterschiedlich exprimierten miRNAs waren 4 miRNAs (miR-487b, miR-495-3p, miR-376b-3p und miR-323a-3p) am Tag 24 der trächtigen Kühe und eine miRNA (miR-433) am Tag 19 der trächtigen Kühe homolog zu den bekannten humanen schwangerschaftsassoziierten C14MC-miRNAs. Die Signalweg-Analyse der Zielgene von differentiell exprimierten miRNAs bei trächtigen Kühen ergab, dass die Signalwege der Adhäsionsverbindung und der ECM-Interaktion während der Trächtigkeit von Bedeutung sind.

Zusammengefasst bietet die vorliegende Studie einen Einblick in die Beteiligung zellulärer miRNAs in Granulosazellen und ihrer regulatorischen Rolle während der Entwicklung der Ovarfollikel. Verschiedene miRNA-Cluster, die bevorzugt in Granulosazellen von präovulatorisch dominanten Follikeln exprimiert werden, unterstützen die Annahme, dass miRNAs an der Regulation von Genen beteiligt sind, die für die Entwicklung des follikulären Wachstums wichtig sind. Darüber hinaus zeigen die einzigartigen Expressionssignaturen extrazellulärer miRNAs in Serumproben von trächtigen und nicht trächtigen Kühen ihr Potenzial, als Indikatoren für die Erkennung der Frühträchtigkeit bei Milchkühen Verwendung zu finden.

Chapter 7
References

References of chapter 1

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Chapter 8
List of appendices

Appendix 1: Supplementary materials of chapter 2

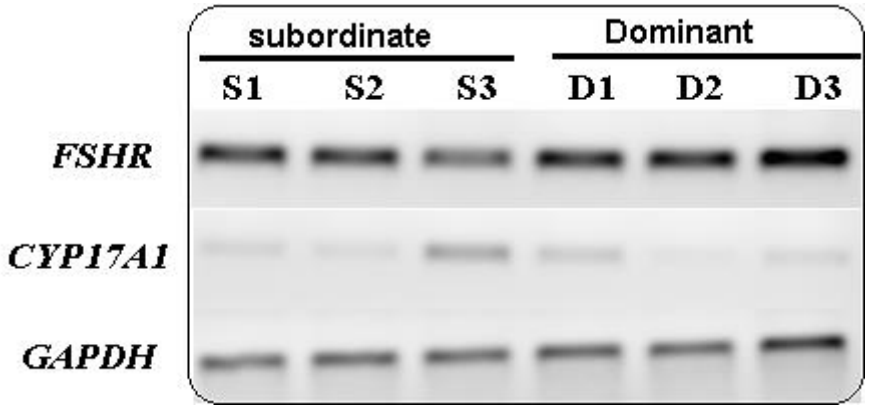

Figure 2.S1: Purity of granulosa cells check-up using granulosa cells-specific gene markers. Granulosa cell-specific marker gene (FSHR) was detected in both dominant and subordinate follicles at higher level as indicated by strong bands, while theca cell-specific marker gene (CYP17A1) had weaker band. Efficiency of cDNA synthesis was confirmed using housekeeping GAPDH gene. Legend: S1, S2, S3 and D1, D2, D3 represent granulosa cell samples derived from subordinate and preovulatory dominant follicles of day 19 of the estrous cycle, respectively.

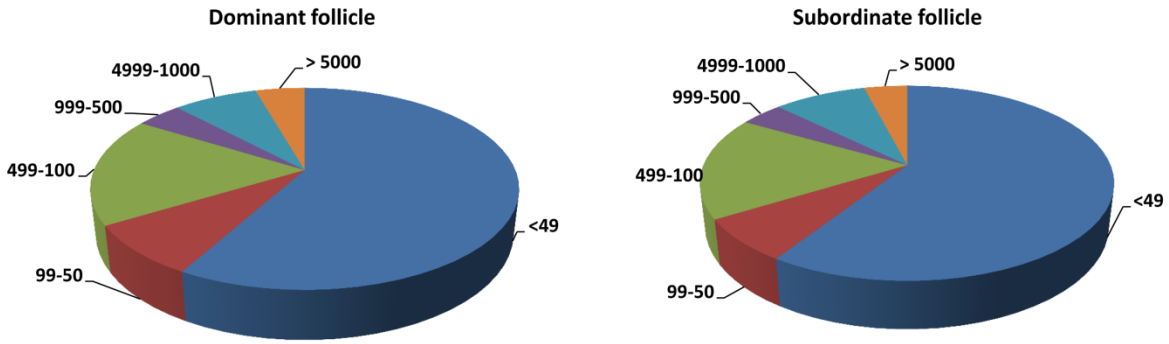

Figure 2.S2: Read count distribution of detected miRNAs in libraries of preovulatory dominant and subordinate follicles

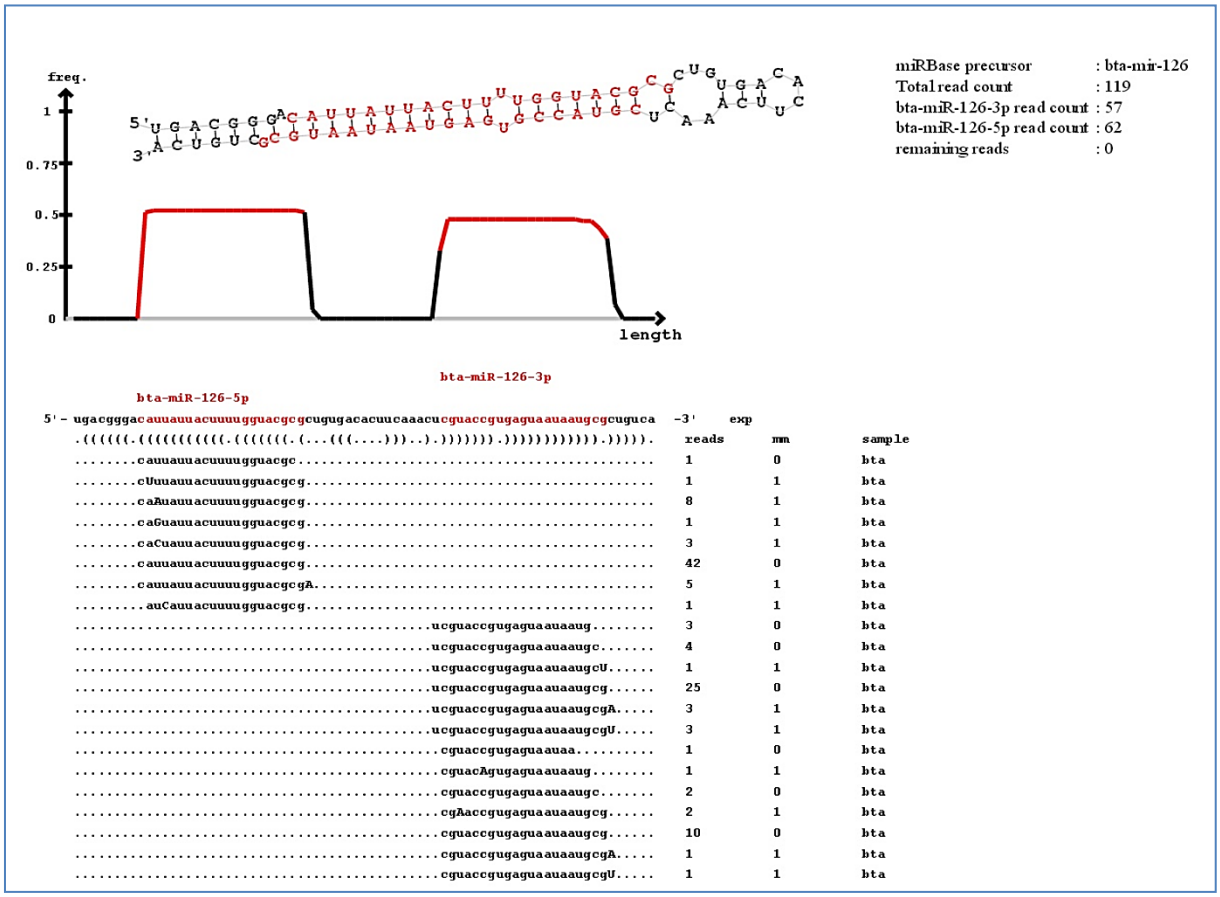

Figure 2.S3: Graphic illustration of a representative miRNA precursor (bta-mir-126) with functional 5p and 3p arms

Table 2.S1: List of primers and adaptors used during library construction, PCR amplification and luciferase assay

List of primers for semi quantitative and quantitative PCR

Gene Name		Sequence (5´-3´)	Tm (°C)	Accession no.
GAPDH	For.	AATGGAGCCATCACCATC	55	NM_001034034
	Rev.	GTGGTTCACGCCCATCACA		
FSHR	For.	GCAGTCGAACTGAGGTTTGT	55	NM_174061
	Rev.	AGATATCGGAGGTTGGGAAG		
CYP17A1	For.	CTACTTGCCCTTTGGAGCAG	57	NM_174304
	Rev.	GGAGTCATGAGGTGCTACCC		
FOXO1	For.	AAGAGCGTGCCCTACTTCAA	57	XM_583090
	Rev.	CTCTTCTCCTGGGGGATTTC		

List of adapters and primers used for library construction

Adapter/Primer	Sequence (5´-3´)
RNA 5´ adapter	GTTCAGAGTTCTACAGTCCGACGATC
RNA 5´ adapter (RC)	GATCGTCGGACTGTAGAACTCGAAC
RNA 3´ adapter	TGGAATTCTCGGGTGCCAAGG
RNA 3´ adapter (RC)	CCTTGGCACCCGAGAATTCCA
RNA PCR primer	AATGATACGGCGACCACCGAGATCTACACGT TCAGAGTTCTCCAGTCCGA
RNA PCR primer (RC)	TCGGACTGTAGAACTCTGAACGTGTAGATCTC GGTGGTCGCCGTATCATT
RNA Reverse transcription primer	GCCTTGGCACCCGAGAATTCCA
RNA Reverse transcription primer (RC)	TGGAATTCTCGGGTGCCAAGGC

RC: reverse complement For: forward primer Rev: reverse primer

List of primers used for luciferase assay

Oligo name	Sequence (5´-3´)	Tm ^{o}C)
Wild-type FOXO1-3´-UTR-Forward	CGTGGAGCTCTCCTTTCGTCAGACTTGGCA	70
Wild-type FOXO1-3´-UTR-Reverse	GACTCTCGAGGCACACCAGGATCCAAAAGTC	70
Mutant FOXO1-3´-UTR-Forward	CGTGGAGCTCCAGATAAGGACTTCAGTCGTGGAAATTCATC TCGAGGACT	75
Mutant FOXO1-3´-UTR-Reverse	AGTCCTCGAGATGAATTTCCACGACTGAAGTCCTTATCTGG AGCTCCACG	75

Table 2.S2: List of all detected miRNAs in granulosa cells of preovulatory dominant and subordinate follicles

MicroRNA ID	Subordinate 1	Subordinate 2	Subordinate 3	Preovulatory 1	Preovulatory 2	Preovulatory 3
bta-miR-26a	58078.42433	84347.45043	66642.01629	40653.2587	50130.92778	53623.51194
bta-miR-10b	44809.68549	64938.17255	59179.46108	27546.39404	26579.40053	28537.10593
bta-miR-202	3157.880899	4109.63532	3608.541618	11998.63893	14128.67348	10841.65644
bta-let-7a-5p	9269.830485	10117.43518	10265.28587	10809.39165	10327.01037	10744.06249
bta-miR-21-5p	6335.901344	1618.894355	4419.53131	8373.157605	7611.736979	9255.556379
bta-miR-22-3p	1719.111693	1414.678032	1678.945454	8220.427336	10434.94845	7823.384989
bta-let-7i	9496.198988	8396.432664	8073.645783	8185.969898	9365.38025	8423.230734
bta-miR-27b	11121.0576	10221.28878	14569.84929	8045.346297	10085.90195	7613.915046
bta-let-7f	10089.91282	10892.40995	11425.32221	8023.926808	8300.017432	8268.508617
bta-miR-143	5182.308118	8893.882682	8875.313755	6678.224134	4600.685224	4125.129671
bta-miR-191	8332.133198	7455.641228	7046.185024	6533.875404	8884.565189	7733.725506
bta-miR-186	4992.99638	5318.351591	4690.896954	5812.131756	6920.65294	5965.132692
bta-miR-30a-5p	3551.004847	3958.654876	4349.100532	5368.841463	6013.692752	5821.51883
bta-miR-125a	5991.917889	6870.919277	8483.80149	5326.933767	6996.349772	4094.978694
bta-miR-182	42.69583868	36.65421182	66.28779091	5237.530683	3547.938544	4204.474346
bta-miR-99b	7794.810096	6952.954894	19763.08341	4870.605525	4555.827842	2383.514047
bta-miR-30d	4082.688876	3990.945491	3490.46649	4713.218846	5420.734235	4346.501315

bta-miR-148a	5511.791099	6596.885408	6111.941471	4424.521386	4367.987556	4510.744793
bta-miR-98	1704.61122	2044.781388	1788.734608	3391.729507	2579.299455	2368.438559
bta-miR-92a	11390.92753	15143.4258	9806.450068	2949.370497	3783.439799	2419.219151
bta-miR-486	350.4281099	360.4330829	208.1850933	2852.517155	1968.117628	230.8930052
bta-miR-183	14.50047351	12.21807061	23.82217486	2629.009445	2003.162457	1868.567104
bta-let-7g	2264.490614	2295.251835	2419.504368	2234.145823	2681.630357	2014.561307
bta-miR-132	48.33491172	12.21807061	46.60860298	1952.898621	1508.329464	2980.979453
bta-miR-204	465.6263162	456.4322091	382.1905445	1802.962198	1900.831555	1505.961938
bta-let-7c	1263.957941	1297.733642	1508.047243	1544.997049	1661.124921	1200.484938
bta-let-7b	1176.149518	1202.607235	1129.999686	1544.997049	1926.063832	1551.98185
bta-miR-16b	1843.976882	1937.43691	1846.736425	1424.861655	1366.748353	1698.769499
bta-miR-26b	1263.957941	1379.769259	1308.148124	1407.167294	1868.590312	1406.781094
bta-miR-92b	1325.987745	1904.273576	1194.215983	1385.747805	1912.0459	1624.978951
bta-miR-151-5p	1672.387945	1429.514261	2278.642813	1299.138567	1741.027132	1394.879393
bta-miR-151-3p	2151.709153	1973.218403	1686.195681	1260.956	1682.151819	1441.692751
bta-miR-30e-5p	2436.07955	2513.431667	2459.898491	1233.948818	1358.337594	1808.265151
bta-miR-181a	1073.03504	937.3005593	1629.229611	1172.484198	1881.20645	1573.404912
bta-miR-195	777.3864968	1035.917844	694.9860578	1147.339581	1448.052357	1053.697289
bta-miR-125b	1107.67506	1285.515572	2489.935146	1026.272904	1101.809441	752.1875223
bta-miR-31	2020.39931	2101.508144	2242.391677	1025.341622	1663.928507	1374.249777
bta-let-7e	854.7223555	905.8826635	939.4222868	1022.547776	985.4606071	803.7615612
bta-miR-450b	378.6234751	303.7063265	498.1941786	984.3652084	738.745007	1138.596091

bta-miR-25	1885.867139	2070.962968	1661.33776	962.9457195	1372.355525	938.6475093
bta-miR-148b	999.7270906	837.8105558	673.2353764	838.1539144	1013.496471	963.2443587
bta-miR-99a-5p	1139.092753	999.2636317	3506.002691	769.239037	672.8607274	599.8457456
bta-miR-23b-3p	601.7696509	637.9578295	649.4132016	713.3621094	707.905557	580.8030235
bta-miR-103	476.0988804	532.3587907	471.2647635	671.4544137	754.164732	645.0722105
bta-miR-30c	1167.288118	1299.479081	866.9200155	642.5846677	727.5306615	618.0950209
bta-miR-30b-5p	456.7649157	484.3592276	467.1217766	618.3713324	811.6382525	594.2916183
bta-miR-423-5p	990.0601083	856.1376617	1069.926375	614.6462039	698.0930047	638.7246365
bta-miR-451	61.22422151	50.61772108	29.00090852	597.8831256	521.4670638	30.15097663
bta-miR-660	1007.782909	1178.171094	936.3150466	556.906712	786.4059752	713.3086313
bta-miR-192	800.7483707	775.8474835	565.5177162	520.5867091	569.128032	514.1534962
bta-miR-140	800.7483707	1027.19065	901.0996577	500.0985023	628.0033456	606.9867664
bta-miR-24-3p	396.3462761	341.2332576	438.120868	440.4964461	562.119066	360.2248261
bta-miR-29a	257.7861958	201.598165	233.0430149	428.3897785	576.1369979	382.4413352
bta-miR-424-5p	53.16840289	73.30842363	114.9678874	411.6267002	403.7164365	514.946943
bta-miR-21-3p	150.6438082	41.89052779	129.4683416	407.9015717	342.0375364	407.8316313
bta-miR-16a	337.5388001	382.2510661	315.9027536	388.344647	385.4931251	362.6051663
bta-miR-215	43.50142054	42.76324712	52.82308338	374.3754151	800.423907	308.6507871
bta-miR-342	340.7611276	383.9965048	341.7964219	361.3374653	466.7971297	304.6835533
bta-miR-6119-5p	994.8935995	978.3183678	712.5937523	361.3374653	469.600716	366.5724001
bta-miR-423-3p	712.1343659	759.2658162	687.7358307	352.9559262	463.9935433	360.2248261
bta-let-7d	475.2932985	479.1229116	408.0842128	340.8492585	438.761266	270.5653429

bta-miR-155	412.4579133	120.4352674	294.1520722	339.9179764	288.7693955	460.9925637
bta-miR-574	495.4328451	603.0490563	556.1959956	338.0554122	541.0921683	269.7718962
bta-miR-873	45.11258427	47.12684377	32.10814872	291.4913058	236.9030478	375.3003144
bta-miR-107	203.8122111	194.6164104	219.5783074	291.4913058	346.242916	327.6935092
bta-miR-130a	468.8486436	633.5942328	764.3810889	289.6287415	309.7962933	581.5964703
bta-miR-221	100.6977327	58.47219504	94.2529527	285.903613	229.8940819	242.0012598
bta-miR-93	483.3491172	525.377036	490.9439514	278.453356	375.6805728	301.5097663
bta-miR-339a	371.3732383	351.7058896	304.5095395	274.7282275	409.3236092	257.0767481
bta-miR-339b	371.3732383	351.7058896	304.5095395	274.7282275	409.3236092	257.0767481
bta-miR-142-5p	114.3926244	94.25368753	124.289608	258.8964313	165.4115955	49.98714547
bta-miR-145	155.4772994	298.4700105	278.6158712	258.8964313	225.6887023	115.843226
bta-miR-199a-3p	240.8689767	267.924834	422.584667	254.2400207	318.2070524	184.0796468
bta-miR-199c	240.8689767	267.924834	422.584667	254.2400207	318.2070524	184.0796468
bta-miR-30f	111.1702969	137.0169347	130.5040884	252.3774565	280.3586364	161.8631377
bta-miR-127	174.811264	221.6707096	487.8367112	250.5148922	302.7873273	245.1750468
bta-miR-2284x	167.5610273	177.1620238	187.4701587	245.8584816	333.6267774	229.3061117
bta-miR-2284y	167.5610273	177.1620238	187.4701587	245.8584816	333.6267774	229.3061117
bta-miR-497	245.7024679	289.7428172	254.7936963	243.0646352	328.0196046	209.4699429
bta-miR-421	114.3926244	115.1989514	109.7891537	238.4082246	206.0635978	238.034026
bta-miR-10a	263.4252688	353.4513282	355.2611294	234.683096	185.0367	188.8403273
bta-miR-1839	194.9508106	196.361849	225.7927878	234.683096	318.2070524	256.2833014
bta-miR-128	308.5378531	312.4335198	237.1860018	212.332325	262.1353251	145.2007559

bta-miR-6123	174.811264	158.8349179	161.5764903	204.882068	250.9209796	209.4699429
bta-miR-15b	343.983455	309.8153618	354.2253827	197.431811	271.9478773	262.6308754
bta-miR-532	256.1750321	317.6698358	279.6516179	196.5005288	260.7335319	231.686452
bta-miR-2313-3p	1.611163724	7.854473961	3.107240199	194.6379646	183.6349069	11.9017013
bta-miR-197	219.1182664	278.3974659	177.1126913	188.1189897	211.6707705	107.1153117
bta-miR-374b	115.1982063	143.12597	152.2547697	179.7374505	274.7514637	162.6565845
bta-miR-146b	306.9266894	342.9786963	291.044832	172.2871935	239.7066342	272.1522364
bta-miR-652	129.6986798	120.4352674	125.3253547	162.9743723	304.1891205	157.8959039
bta-miR-100	292.4262159	260.07036	1699.660389	159.2492437	161.2062159	140.4400754
bta-miR-450a	37.86234751	28.79973786	51.78733665	158.3179616	124.7595932	114.2563325
bta-miR-1246	379.429057	307.1972038	3860.228074	152.7302689	273.3496705	2027.256455
bta-miR-425-5p	155.4772994	117.8171094	105.6461668	148.0738582	145.7864909	181.6993065
bta-miR-320a	204.6177929	236.5069382	207.1493466	142.4861655	218.6797364	104.7349715
bta-miR-199a-5p	139.3656621	159.7076372	449.5140821	139.6923191	155.5990432	84.89880262
bta-miR-212	0.805581862	1.745438658	11.39321406	136.8984727	86.91117729	111.0825455
bta-miR-6529	114.3926244	109.9626355	78.71675171	131.3107799	164.0098023	86.48569612
bta-miR-361	318.2048355	336.869661	442.263855	119.2041123	176.625941	169.7976052
bta-miR-27a-3p	147.4214807	123.0534254	132.5755818	115.4789838	157.0008364	128.5383741
bta-miR-374a	112.7814607	85.52649424	77.68100497	110.8225731	98.12552275	153.1352234
bta-miR-708	15.30605538	6.109035303	15.53620099	107.0974446	218.6797364	108.7022052
bta-miR-484	169.9777729	189.3800944	132.5755818	97.78462335	107.938075	74.58399482
bta-miR-222	58.00189406	32.29061517	56.96607031	96.85334122	89.71476366	64.26918703

bta-miR-19b	174.811264	182.3983398	117.0393808	94.05949484	110.7416614	75.37744158
bta-miR-7	157.0884631	92.50824887	80.78824517	89.40308421	140.1793182	118.2235663
bta-miR-15a	83.78051364	79.41745894	64.21629744	88.47180208	123.3578	84.89880262
bta-miR-129	1.611163724	2.618157987	4.142986932	84.74667357	120.5542137	56.33471949
bta-miR-129-5p	1.611163724	2.618157987	4.142986932	84.74667357	120.5542137	56.33471949
bta-miR-181b	104.725642	142.2532506	161.5764903	81.02154506	144.3846978	119.017013
bta-miR-32	14.50047351	20.9452639	21.75068139	80.09026294	75.69683184	76.17088833
bta-miR-2478	157.0884631	77.67202028	47.64434972	80.09026294	130.3667659	27.77063637
bta-miR-146a	112.7814607	77.67202028	110.8249004	79.15898081	51.86634774	58.71505975
bta-miR-331	79.75260433	82.03561692	95.28869943	79.15898081	71.49145229	48.40025196
bta-miR-23a	56.39073033	49.74500175	89.07421903	78.22769868	100.9291091	83.31190911
bta-miR-378	697.6338924	663.26669	487.8367112	76.36513443	190.6438728	196.7747949
bta-miR-769	331.0941452	302.8336072	188.5059054	75.4338523	82.70579775	63.47574027
bta-miR-210	41.89025682	48.87228242	31.07240199	72.64000592	85.50938411	80.1381221
bta-miR-28	45.91816613	38.39965048	34.17964219	72.64000592	126.1613864	90.45292989
bta-miR-129-3p	1.611163724	0.872719329	0	71.70872379	78.5004182	37.29199741
bta-miR-101	99.08656902	90.76281021	96.32444617	67.05231316	53.26814092	69.02986755
bta-miR-874	52.36282102	31.41789584	55.93032358	67.05231316	74.29503865	55.54127274
bta-miR-1468	79.75260433	89.89009089	175.0411979	67.05231316	102.3309023	65.06263378
bta-miR-193b	54.77956661	79.41745894	61.10905724	63.32718465	100.9291091	45.22646495
bta-miR-152	29.00094703	16.58166725	26.92941506	62.39590252	67.28607274	37.29199741
bta-miR-1343-3p	146.6158989	157.0894792	179.1841848	62.39590252	113.5452478	50.78059222

bta-miR-424-3p	10.4725642	13.96350926	7.250227131	57.73949188	78.5004182	70.61676106
bta-miR-1271	136.9489165	123.0534254	733.3086869	55.87692763	53.26814092	42.05267793
bta-miR-17-5p	141.7824077	163.1985145	111.8606472	48.42667061	68.68786593	38.08544416
bta-miR-542-5p	7.250236757	4.363596645	13.46470753	45.63282423	40.65200228	43.63957144
bta-miR-2483-5p	18.52838282	13.09078993	13.46470753	40.97641359	35.04482955	27.77063637
bta-miR-126-5p	56.39073033	54.1085984	94.2529527	40.97641359	36.44662274	7.934467534
bta-miR-505	56.39073033	60.2176337	48.68009645	40.04513147	51.86634774	42.05267793
bta-miR-301a	23.361874	19.19982524	22.78642813	39.11384934	39.2502091	30.15097663
bta-miR-106b	77.33585874	68.07210766	55.93032358	39.11384934	64.48248638	56.33471949
bta-miR-126-3p	40.2790931	49.74500175	64.21629744	36.32000296	23.8304841	10.31480779
bta-miR-20a	136.1433347	129.1624607	88.0384723	35.38872083	43.45558865	31.73787014
bta-miR-2285f	45.91816613	31.41789584	27.96516179	35.38872083	43.45558865	72.99710132
bta-miR-2284z	27.38978331	27.92701853	24.85792159	34.4574387	23.8304841	40.46578442
bta-miR-224	75.72469502	62.83579169	87.00272557	34.4574387	44.85738183	30.94442338
bta-miR-150	24.16745586	14.83622859	34.17964219	32.59487445	30.83945001	12.69514805
bta-miR-96	0	0	1.035746733	31.66359232	26.63407046	19.04272208
bta-miR-345-3p	12.88930979	13.09078993	15.53620099	31.66359232	36.44662274	23.00995585
bta-miR-181c	53.16840289	36.65421182	43.50136278	30.7323102	26.63407046	34.1182104
bta-miR-1260b	70.89120385	48.87228242	33.14389545	29.80102807	71.49145229	21.42306234
bta-miR-335	443.0700241	592.5764244	314.8670068	26.07589956	15.419725	23.8034026
bta-miR-744	41.89025682	54.98131773	58.00181705	26.07589956	29.43765683	12.69514805
bta-miR-194	14.50047351	14.83622859	15.53620099	26.07589956	50.46455456	15.86893507

bta-miR-324	19.33396469	13.09078993	14.50045426	25.14461743	25.23227728	26.18374286
bta-miR-502b	44.30700241	42.76324712	27.96516179	25.14461743	32.24124319	27.77063637
bta-let-7a-3p	36.25118379	37.52693115	15.53620099	25.14461743	36.44662274	23.00995585
bta-miR-328	13.69489165	13.09078993	17.60769446	22.35077105	21.02689773	8.727914288
bta-miR-29b	4.02790931	5.236315974	4.142986932	21.41948892	16.82151819	15.86893507
bta-miR-1388-5p	28.19536517	28.79973786	19.67918793	20.4882068	15.419725	15.07548832
bta-miR-2285k	66.05771268	51.49044041	166.755224	20.4882068	21.02689773	15.07548832
bta-miR-185	12.88930979	9.599912619	12.4289608	17.69436042	12.61613864	14.28204156
bta-miR-296	143.3935714	133.5260573	100.4674331	17.69436042	29.43765683	23.00995585
bta-miR-19a	48.33491172	29.67245719	19.67918793	16.76307829	14.01793182	10.31480779
bta-miR-454	18.52838282	13.09078993	19.67918793	16.76307829	23.8304841	20.62961559
bta-miR-6526	119.2261156	112.5807934	187.4701587	14.90051403	26.63407046	27.77063637
bta-miR-190b	9.666982343	12.21807061	10.35746733	13.96923191	18.22331137	23.00995585
bta-miR-34a	6.444654895	7.854473961	6.214480398	13.96923191	26.63407046	7.934467534
bta-miR-1307	30.61211075	34.90877316	26.92941506	13.96923191	30.83945001	26.18374286
bta-miR-22-5p	4.02790931	0.872719329	1.035746733	13.96923191	33.64303637	16.66238182
bta-miR-3431	78.14144061	71.56298498	50.75158992	13.03794978	53.26814092	26.18374286
bta-miR-2387	10.4725642	11.34535128	5.178733665	12.10666765	9.812552275	4.760680521
bta-miR-504	4.02790931	6.981754632	3.107240199	12.10666765	9.812552275	4.760680521
bta-miR-34c	4.02790931	1.745438658	2.071493466	12.10666765	14.01793182	9.521361041
bta-miR-214	28.19536517	26.18157987	45.57285625	12.10666765	19.62510455	11.10825455
bta-miR-592	20.13954655	26.18157987	34.17964219	12.10666765	23.8304841	23.8034026

bta-miR-365-3p	19.33396469	37.52693115	22.78642813	11.17538553	11.21434546	9.521361041
bta-miR-29c	1.611163724	6.109035303	4.142986932	11.17538553	12.61613864	7.141020781
bta-miR-876	0.805581862	4.363596645	1.035746733	10.2441034	4.205379547	10.31480779
bta-miR-1296	59.61305778	53.23587907	34.17964219	10.2441034	5.607172729	5.554127274
bta-miR-2484	13.69489165	22.69070255	33.14389545	10.2441034	9.812552275	19.04272208
bta-miR-199b	11.27814607	12.21807061	58.00181705	10.2441034	11.21434546	5.554127274
bta-miR-99a-3p	6.444654895	15.70894792	14.50045426	10.2441034	16.82151819	10.31480779
bta-miR-6120-3p	24.97303772	19.19982524	12.4289608	9.312821272	2.803586364	5.554127274
bta-miR-2285g	12.88930979	6.981754632	10.35746733	9.312821272	5.607172729	7.934467534
bta-miR-2313-5p	0	0	1.035746733	9.312821272	5.607172729	0.793446753
bta-miR-500	8.861400481	6.981754632	8.285973864	9.312821272	5.607172729	4.760680521
bta-miR-2285q	20.94512841	13.09078993	12.4289608	9.312821272	11.21434546	9.521361041
bta-miR-410	1.611163724	6.109035303	12.4289608	9.312821272	15.419725	9.521361041
bta-miR-6518	10.4725642	5.236315974	9.321720597	8.381539144	2.803586364	4.760680521
bta-miR-130b	17.72280096	18.32710591	25.89366832	8.381539144	15.419725	15.86893507
bta-miR-149-5p	11.27814607	14.83622859	16.57194773	8.381539144	18.22331137	8.727914288
bta-miR-760-3p	18.52838282	6.981754632	8.285973864	7.450257017	2.803586364	7.141020781
bta-miR-181d	7.250236757	8.72719329	5.178733665	7.450257017	5.607172729	4.760680521
bta-miR-2284aa-1	4.02790931	3.490877316	3.107240199	7.450257017	5.607172729	15.86893507
bta-miR-2284aa-2	4.02790931	3.490877316	3.107240199	7.450257017	5.607172729	15.86893507
bta-miR-2284aa-3	4.02790931	3.490877316	3.107240199	7.450257017	5.607172729	15.86893507
bta-miR-2284aa-4	4.02790931	3.490877316	3.107240199	7.450257017	5.607172729	15.86893507

bta-miR-677	37.86234751	27.0542992	77.68100497	7.450257017	8.410759093	46.81335845
bta-miR-18a	7.250236757	13.09078993	9.321720597	7.450257017	9.812552275	8.727914288
bta-miR-362-5p	12.08372793	10.47263195	17.60769446	7.450257017	11.21434546	13.48859481
bta-miR-378b	53.97398475	46.25412444	46.60860298	7.450257017	14.01793182	19.04272208
bta-miR-1	0.805581862	1.745438658	3.107240199	6.51897489	1.401793182	1.586893507
bta-miR-193a-3p	3.222327448	6.981754632	0	6.51897489	2.803586364	3.967233767
bta-miR-3613	3.222327448	2.618157987	3.107240199	6.51897489	8.410759093	7.141020781
bta-miR-375	0	0.872719329	0	6.51897489	8.410759093	7.141020781
bta-miR-2419-5p	17.72280096	6.109035303	14.50045426	6.51897489	11.21434546	24.59684936
bta-miR-17-3p	14.50047351	13.09078993	13.46470753	6.51897489	12.61613864	19.04272208
bta-miR-491	4.02790931	1.745438658	7.250227131	6.51897489	19.62510455	5.554127274
bta-miR-6524	1.611163724	0.872719329	1.035746733	5.587692763	2.803586364	0.793446753
bta-miR-1306	8.055818619	8.72719329	7.250227131	5.587692763	5.607172729	9.521361041
bta-miR-425-3p	8.861400481	10.47263195	6.214480398	5.587692763	7.008965911	12.69514805
bta-miR-345-5p	1.611163724	4.363596645	2.071493466	5.587692763	8.410759093	3.967233767
bta-miR-503-3p	6.444654895	3.490877316	1.035746733	5.587692763	16.82151819	5.554127274
bta-miR-6119-3p	18.52838282	21.81798322	13.46470753	5.587692763	18.22331137	2.38034026
bta-miR-2308	0	0.872719329	1.035746733	4.656410636	0	0
bta-miR-2440	12.88930979	10.47263195	12.4289608	4.656410636	1.401793182	3.967233767
bta-miR-2443	1.611163724	1.745438658	3.107240199	4.656410636	1.401793182	0
bta-miR-144	0.805581862	1.745438658	0	4.656410636	2.803586364	0
bta-miR-2299-5p	7.250236757	6.109035303	6.214480398	4.656410636	2.803586364	5.554127274

bta-miR-2403	0.805581862	1.745438658	1.035746733	4.656410636	2.803586364	0
bta-miR-2285c	8.055818619	3.490877316	9.321720597	4.656410636	4.205379547	4.760680521
bta-miR-383	4.02790931	6.109035303	4.142986932	4.656410636	4.205379547	5.554127274
bta-miR-2483-3p	9.666982343	9.599912619	8.285973864	4.656410636	5.607172729	9.521361041
bta-miR-1291	3.222327448	5.236315974	10.35746733	4.656410636	7.008965911	7.934467534
bta-miR-455-3p	6.444654895	7.854473961	6.214480398	4.656410636	7.008965911	3.967233767
bta-miR-2885	12.08372793	7.854473961	39.35837585	3.725128509	0	40.46578442
bta-miR-122	0	0	1.035746733	3.725128509	1.401793182	2.38034026
bta-miR-1940	2.416745586	1.745438658	3.107240199	3.725128509	1.401793182	6.347574027
bta-miR-432	0.805581862	0	3.107240199	3.725128509	2.803586364	0.793446753
bta-miR-493	0	2.618157987	5.178733665	3.725128509	4.205379547	3.173787014
bta-miR-502a	2.416745586	4.363596645	0	3.725128509	5.607172729	3.967233767
bta-miR-877	5.639073033	4.363596645	5.178733665	3.725128509	5.607172729	7.934467534
bta-miR-378c	17.72280096	20.9452639	15.53620099	3.725128509	9.812552275	13.48859481
bta-miR-3432	8.055818619	4.363596645	6.214480398	3.725128509	14.01793182	5.554127274
bta-miR-133a	2.416745586	2.618157987	3.107240199	2.793846381	0	1.586893507
bta-miR-190a	12.08372793	3.490877316	1.035746733	2.793846381	0	0.793446753
bta-miR-2332	8.861400481	12.21807061	35.21538892	2.793846381	0	14.28204156
bta-miR-2411-3p	16.11163724	27.92701853	82.85973864	2.793846381	0	60.30195326
bta-miR-499	3.222327448	0.872719329	3.107240199	2.793846381	0	1.586893507
bta-miR-2285b	4.833491172	0.872719329	5.178733665	2.793846381	1.401793182	0.793446753
bta-miR-2285e	0	1.745438658	0	2.793846381	1.401793182	0

bta-miR-2285u	4.833491172	0.872719329	5.178733665	2.793846381	1.401793182	0.793446753
bta-miR-411a	0.805581862	4.363596645	8.285973864	2.793846381	1.401793182	5.554127274
bta-miR-2284v	9.666982343	6.981754632	3.107240199	2.793846381	2.803586364	3.173787014
bta-miR-2336	3.222327448	9.599912619	6.214480398	2.793846381	2.803586364	2.38034026
bta-miR-2425-5p	4.02790931	3.490877316	1.035746733	2.793846381	2.803586364	0.793446753
bta-miR-665	2.416745586	2.618157987	6.214480398	2.793846381	2.803586364	6.347574027
bta-miR-2284w	6.444654895	2.618157987	3.107240199	2.793846381	4.205379547	0
bta-miR-2366	0	0	1.035746733	2.793846381	4.205379547	3.173787014
bta-miR-326	3.222327448	0.872719329	0	2.793846381	4.205379547	0
bta-miR-1814c	6.444654895	2.618157987	14.50045426	2.793846381	5.607172729	1.586893507
bta-miR-381	1.611163724	2.618157987	13.46470753	2.793846381	5.607172729	4.760680521
bta-miR-2310	6.444654895	2.618157987	14.50045426	2.793846381	7.008965911	1.586893507
bta-miR-452	8.861400481	10.47263195	5.178733665	2.793846381	7.008965911	3.173787014
bta-miR-137	0	0	1.035746733	1.862564254	0	1.586893507
bta-miR-2284b	0	1.745438658	0	1.862564254	0	1.586893507
bta-miR-449a	41.89025682	6.981754632	19.67918793	1.862564254	0	2.38034026
bta-miR-495	0	0.872719329	0	1.862564254	0	0.793446753
bta-miR-223	1.611163724	0.872719329	5.178733665	1.862564254	1.401793182	1.586893507
bta-miR-2328-5p	0.805581862	0	0	1.862564254	1.401793182	1.586893507
bta-miR-2487	0.805581862	1.745438658	12.4289608	1.862564254	1.401793182	38.87889092
bta-miR-488	2.416745586	0.872719329	1.035746733	1.862564254	1.401793182	0.793446753
bta-miR-2346	1.611163724	1.745438658	2.071493466	1.862564254	2.803586364	0

bta-miR-2320-3p	0.805581862	1.745438658	1.035746733	1.862564254	4.205379547	0
bta-miR-2424	5.639073033	16.58166725	44.53710952	1.862564254	4.205379547	15.07548832
bta-miR-362-3p	0.805581862	0	3.107240199	1.862564254	4.205379547	0.793446753
bta-miR-628	2.416745586	6.109035303	3.107240199	1.862564254	4.205379547	1.586893507
bta-miR-6517	9.666982343	10.47263195	5.178733665	1.862564254	4.205379547	4.760680521
bta-miR-671	5.639073033	3.490877316	4.142986932	1.862564254	4.205379547	3.173787014
bta-miR-483	0.805581862	2.618157987	4.142986932	1.862564254	5.607172729	4.760680521
bta-miR-503-5p	4.02790931	0.872719329	5.178733665	1.862564254	5.607172729	7.934467534
bta-miR-379	3.222327448	0.872719329	5.178733665	1.862564254	7.008965911	1.586893507
bta-miR-211	0	0	0	0.931282127	0	1.586893507
bta-miR-2284t-5p	1.611163724	1.745438658	4.142986932	0.931282127	0	0.793446753
bta-miR-2285j	1.611163724	0	1.035746733	0.931282127	0	0
bta-miR-2285l	3.222327448	0	1.035746733	0.931282127	0	1.586893507
bta-miR-2312	0	0	0	0.931282127	0	0.793446753
bta-miR-2376	0	0	0	0.931282127	0	0.793446753
bta-miR-2431-3p	1.611163724	0.872719329	1.035746733	0.931282127	0	0
bta-miR-2461-3p	0.805581862	0	0	0.931282127	0	2.38034026
bta-miR-2465	0.805581862	0	0	0.931282127	0	1.586893507
bta-miR-301b	0.805581862	1.745438658	0	0.931282127	0	0.793446753
bta-miR-369-3p	0	3.490877316	0	0.931282127	0	0.793446753
bta-miR-487b	0	0.872719329	1.035746733	0.931282127	0	0
bta-miR-541	0.805581862	1.745438658	0	0.931282127	0	1.586893507

bta-miR-6520	0.805581862	0.872719329	1.035746733	0.931282127	0	0
bta-miR-6523	19.33396469	4.363596645	6.214480398	0.931282127	0	15.07548832
bta-miR-6531	0	2.618157987	1.035746733	0.931282127	0	0
bta-miR-9-5p	2.416745586	2.618157987	0	0.931282127	0	0.793446753
bta-miR-106a	6.444654895	0.872719329	1.035746733	0.931282127	1.401793182	0
bta-miR-1249	0	0.872719329	0	0.931282127	1.401793182	0
bta-miR-135a	0.805581862	0	0	0.931282127	1.401793182	0
bta-miR-139	0	0	1.035746733	0.931282127	1.401793182	0
bta-miR-188	0.805581862	0.872719329	1.035746733	0.931282127	1.401793182	1.586893507
bta-miR-2284h-5p	0	0	0	0.931282127	1.401793182	1.586893507
bta-miR-2320-5p	1.611163724	0.872719329	4.142986932	0.931282127	1.401793182	2.38034026
bta-miR-2340	0	2.618157987	1.035746733	0.931282127	1.401793182	0.793446753
bta-miR-2447	0	0	0	0.931282127	1.401793182	0
bta-miR-29d	0	0.872719329	0	0.931282127	1.401793182	0
bta-miR-340	2.416745586	4.363596645	4.142986932	0.931282127	1.401793182	2.38034026
bta-miR-4286	8.055818619	3.490877316	3.107240199	0.931282127	1.401793182	3.173787014
bta-miR-545-3p	0.805581862	0.872719329	1.035746733	0.931282127	1.401793182	0.793446753
bta-miR-545-5p	0.805581862	0.872719329	1.035746733	0.931282127	1.401793182	0.793446753
bta-miR-582	0.805581862	0.872719329	0	0.931282127	1.401793182	0
bta-miR-1224	0	0	0	0.931282127	2.803586364	0.793446753
bta-miR-193a-5p	1.611163724	2.618157987	2.071493466	0.931282127	2.803586364	2.38034026
bta-miR-218	6.444654895	4.363596645	2.071493466	0.931282127	2.803586364	2.38034026

bta-miR-2285s	0	0	0	0.931282127	2.803586364	0
bta-miR-380-3p	2.416745586	0.872719329	4.142986932	0.931282127	2.803586364	1.586893507
bta-miR-6525	1.611163724	1.745438658	0	0.931282127	2.803586364	0.793446753
bta-miR-411c-3p	0.805581862	0.872719329	2.071493466	0.931282127	4.205379547	2.38034026
bta-miR-141	4.02790931	0	0	0.931282127	5.607172729	0.793446753
bta-miR-2284t-3p	14.50047351	12.21807061	10.35746733	0.931282127	5.607172729	8.727914288
bta-miR-1248	0	0.872719329	4.142986932	0	0	0.793446753
bta-miR-138	0.805581862	0.872719329	1.035746733	0	0	0
bta-miR-1434-5p	2.416745586	1.745438658	4.142986932	0	0	0
bta-miR-153	4.02790931	0.872719329	0	0	0	0.793446753
bta-miR-1777a	0.805581862	0	1.035746733	0	0	0
bta-miR-196a	4.02790931	4.363596645	0	0	0	0
bta-miR-20b	5.639073033	5.236315974	2.071493466	0	0	0.793446753
bta-miR-2285m	0.805581862	0	2.071493466	0	0	0
bta-miR-2285n	1.611163724	0	1.035746733	0	0	0.793446753
bta-miR-2285r	1.611163724	0.872719329	2.071493466	0	0	0.793446753
bta-miR-2314	1.611163724	0	2.071493466	0	0	0
bta-miR-2350	0	0.872719329	1.035746733	0	0	0
bta-miR-2397-3p	1.611163724	0	2.071493466	0	0	0
bta-miR-2404	1.611163724	1.745438658	30.03665526	0	0	19.04272208
bta-miR-2416	1.611163724	0.872719329	0	0	0	0
bta-miR-2435	2.416745586	1.745438658	0	0	0	0.793446753

bta-miR-2904	8.055818619	1.745438658	11.39321406	0	0	25.39029611
bta-miR-33a	0.805581862	0	1.035746733	0	0	0
bta-miR-409a	2.416745586	2.618157987	4.142986932	0	0	0
bta-miR-449b	1.611163724	0.872719329	3.107240199	0	0	0
bta-miR-455-5p	0	2.618157987	2.071493466	0	0	2.38034026
bta-miR-543	0.805581862	0.872719329	2.071493466	0	0	0
bta-miR-9-3p	0.805581862	0.872719329	0	0	0	0
bta-miR-1388-3p	0.805581862	2.618157987	4.142986932	0	1.401793182	1.586893507
bta-miR-154c	3.222327448	0	1.035746733	0	1.401793182	0
bta-miR-2284f	0	0	0	0	1.401793182	0.793446753
bta-miR-2284k	1.611163724	0	1.035746733	0	1.401793182	0.793446753
bta-miR-2285o	0	0	0	0	1.401793182	0.793446753
bta-miR-2323	0.805581862	0.872719329	3.107240199	0	1.401793182	0
bta-miR-2330-5p	0.805581862	0.872719329	1.035746733	0	1.401793182	0.793446753
bta-miR-2339	2.416745586	3.490877316	2.071493466	0	1.401793182	4.760680521
bta-miR-2382-3p	0.805581862	0	1.035746733	0	1.401793182	0
bta-miR-2446	2.416745586	0	0	0	1.401793182	3.967233767
bta-miR-330	0.805581862	0	1.035746733	0	1.401793182	2.38034026
bta-miR-338	2.416745586	0.872719329	2.071493466	0	1.401793182	0.793446753
bta-miR-411c-5p	1.611163724	0	2.071493466	0	1.401793182	0.793446753
bta-miR-6522	4.833491172	4.363596645	3.107240199	0	1.401793182	0.793446753
bta-miR-2344	12.08372793	6.981754632	5.178733665	0	2.803586364	0

bta-miR-2415-3p	2.416745586	1.745438658	1.035746733	0	2.803586364	0
bta-miR-2887	6.444654895	7.854473961	16.57194773	0	2.803586364	11.9017013
bta-miR-33b	0.805581862	0	1.035746733	0	2.803586364	0
bta-miR-449c	16.11163724	5.236315974	8.285973864	0	2.803586364	3.173787014

Numbers represents the normalized read count of each miRNAs in biological triplicates of both preovulatory and subordinate follicles

Table 2.S3: Top 20 gene ontology terms enriched by predicted target genes of differentially expressed miRNAs in preovulatory dominant follicles

Target genes of up regulated miRNAs		Target genes of down regulated miRNAs	
GO term	**Enrichment P-value**	**GO term**	**Enrichment P-Value**
GO:0042127~ regulation of cell proliferation	1.37E-12	GO:0042127~ regulation of cell proliferation	3.48E-19
GO:0010604~ positive regulation of macromolecule metabolic process	3.06E-10	GO:0010604~ positive regulation of macromolecule metabolic process	3.86E-15
GO:0043067~ regulation of programmed cell death	1.06E-09	GO:0010557~ positive regulation of macromolecule biosynthetic process	2.54E-12
GO:0010941~ regulation of cell death	1.16E-09	GO:0051254~ positive regulation of RNA metabolic process	4.44E-12
GO:0042981~ regulation of apoptosis	3.11E-09	GO:0031328~ positive regulation of cellular biosynthetic process	6.56E-12
GO:0043069~ negative regulation of programmed cell death	3.95E-09	GO:0009891~ positive regulation of biosynthetic process	8.81E-12
GO:0060548~ negative regulation of cell death	4.16E-09	GO:0045893~ positive regulation of transcription, DNA-dependent	3.51E-11
GO:0008284~ positive regulation of cell proliferation	1.10E-08	GO:0045935~ positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6.35E-11
GO:0043066~ negative regulation of apoptosis	1.62E-08	GO:0051173~ positive regulation of nitrogen compound metabolic process	1.14E-10
GO:0048534~ hemopoietic or lymphoid organ development	1.74E-08	GO:0045944~ positive regulation of transcription from RNA polymerase II promoter	4.92E-10
GO:0030097~ hemopoiesis	2.46E-08	GO:0045941~ positive regulation of transcription	6.06E-10
GO:0002520~ immune system development	4.36E-08	GO:0030182~ neuron differentiation	6.62E-10
GO:0001501~ skeletal system development	7.56E-08	GO:0007423~ sensory organ development	9.05E-10
GO:0042325~ regulation of phosphorylation	9.72E-08	GO:0010628~ positive regulation of gene expression	9.96E-10
GO:0045597~ positive regulation of cell	9.88E-08	GO:0006357~ regulation of transcription from RNA	1.05E-09

differentiation		polymerase II promoter	
GO:0019220~ regulation of phosphate metabolic process	1.99E-07	GO:0042325~ regulation of phosphorylation	1.70E-09
GO:0051174~ regulation of phosphorus metabolic process	1.99E-07	GO:0043066~ negative regulation of apoptosis	2.34E-09
GO:0045596~ negative regulation of cell differentiation	2.73E-07	GO:0008284~ positive regulation of cell proliferation	2.41E-09
GO:0007423~ sensory organ development	5.78E-07	GO:0043069~ negative regulation of programmed cell death	2.83E-09
GO:0031328~ positive regulation of cellular biosynthetic process	6.53E-07	GO:0060548~ negative regulation of cell death	2.94E-09

Table 2.S4: Representative list of pathways related to follicular development enriched by target genes of differentially expressed miRNAs

Pathway	Predicted target genes involved in pathway ¥	List of DE miRNAs involved in pathway	*p*-value*
Axon guidance	DPYSL2, CFL2, SEMA5A, SEMA4A, SEMA3, SEMA4A, SEMA6A, RAC1, RAC2, PPP3CC, PPP3R1, PAK1, PAK2, PAK6, MET, PLXNA2	**bta-miR-132, bta-miR-152, bta-miR-182, bta-miR-183, bta-miR-204, bta-miR-212, bta-miR-221, bta-miR-29b, bta-miR-339b, bta-miR-375, bta-miR-424-5p, bta-miR-96,** *bta-miR-1271, bta-miR-17-5p, bta-miR-19a, bta-miR-20a, bta-miR-214, bta-miR-296, bta-miR-409a, bta-miR-449a, bta-miR-769*	0.037 to 3.58E+12
Wnt signaling pathway	FZD4, FZD1, FZD6, WNT9A, WNT3A, FZD7, WNT7A, LRP6, FZD2, WNT8B, SMAD4, WNT1, WNT11, TBL1XR1, TCF7L1	**bta-miR-129-5p, bta-miR-132, bta-miR-152, bta-miR-182, bta-miR-183, bta-miR-204, bta-miR-212, bta-miR-421, bta-miR-424-5p, bta-miR-96,** *bta-miR-10b, bta-miR-1271, bta-miR-17-5p, bta-miR-20a, bta-miR-214, bta-miR-296, bta-miR-409a, bta-miR-449a*	0.041 to 7.07E+09
MAPK signaling pathway	MAP4K3, MAP3K14, MAP3K3, MAP4K4, IKBKB, TRAF2, TGFBR1, TNFRSF1A, BDNF, FGF1, FGF2, CACNB3, CACNB4, PDGFRA, GRB2, NRAS, MRAS, KRAS, RRAS. RASA1	**bta-miR-182, bta-miR-204, bta-miR-22-3p, bta-miR-424-5p, bta-miR-873, -bta-miR-96,** *bta-miR-1271, bta-miR-1296, bta-miR-17-5p, bta-miR-20a, bta-miR-2240, bta-miR-296, bta-miR-365-3p*	0.046 to 1.15E+12
Progesterone-mediated oocyte maturation	IGF1, GNAI1, GNAI2, PIK3R1, PRKACA, PRKACB, PIK3R3, RPS6KA3, CDC23, CPEB1, MAPK3	**bta-miR-182, bta-miR-424-5p, bta-miR-96**, *bta-miR-19a, bta-miR-296, bta-miR-365-3p, bta-miR-99a, bta-miR-99b*	0,028 to 1.40E+12
Calcium signaling pathway	ADCY1, ADCY7, PRKACB, CACNA1H, CACNA1I, CACNA1A, ITPR1, ATP2A3, GNAQ, RYR2	**bta-miR-129-5p, bta-miR-182, bta-miR-204, bta-miR-96,** *bta-miR-19a, bta-miR-296*	0.045 to 0.002

Oocyte meiosis	IGF1, ADCY1, ADCY7, IGF1R, PRKACA, SMC1A, PPP2R5C, ADCY6, ADCY2, CPEB1, MAP2K1, PPP2R5E	**bta-miR-132, bta-miR-182, bta-miR-424-5p, bta-miR-96,** *bta-miR-19a*	0.033 to 0.001
GnRH signaling pathway	FSHB, GNAS, ITPR2, PLCB2, GNAQ	**bta-miR-182, bta-miR-96,** *bta-miR-1271, bta-miR-19a, bta-miR-296*	0,024 to 4.88E+12
TGF-beta signaling pathway	BMP8B, TGFBR1, BMPR2, GDF6, SMAD6, SMAD2, RBL1, TGFBR2, SMAD7, NOG, E2F5, BMP2, SKP1	**bta-miR-152, bta-miR-183**, *bta-miR-17-5p, bta-miR-20a, bta-miR-214*	0,042 to 1.59E+12
ErbB signaling pathway	PIK3R1, STAT5B, SHC1, ERBB4, SHC4, GRB2	**bta-miR-129-5p, bta-miR-221, bta-miR-339b, bta-miR-96**	0.045 to 4.53E+11
Cell cycle	GADD45A, CCND1, RBL1, RBL2, RB1, WEE1, CCND3, E2F3, CCND2	**bta-miR-152, bta-miR-424-5p, bta-miR-450b,** *bta-miR-20a*	0.049 to 0.010
Apoptosis	AKT3, CASP9, BCL2, XIAP, TNFRSF1A, CASP8, CASP7, IRAK4	*bta-miR-1271, bta-miR-17-5p, bta-miR-365-3p*	0.041 to 0.,014
p53 signaling pathway	CDK6, CDKN1A, CHEK1, BAI1, CCND3, PTEN, SHISA5	**bta-miR-202, bta-miR-424-5p**, *bta-miR-20a*	0.042 to 0.021
RNA Degradation	PAPOLB, PAPOLA, CNOT6L, PAPOLG, CNOT7, XRN1, CNOT6, CNOT4, PATL1, DCP2, DDX6	*bta-miR-17-5p, bta-miR-19a*	0.027-0.025
Hedgehog signaling pathway	WNT4, GSK3B, PTCH1	*bta-miR-769*	0.018
VEGF signaling pathway	MAPK1, KRAS, NFAT5, PPP3CC, PXN	**bta-miR-132**	0.078

Jak-STAT signaling pathway	IL2RB, SOCS2, STAM2, CBL, LIFR, SOCS4, SOCS5, STAT1, IL10, PTPN11 LIF, SPRY1, NTF, CLCF1, SPRED2 IL2RG, IL12B, PIK3R3, AKT3, GHR	**bta-miR-421**	0.0061

List of all miRNAs predicted to be involved in each pathway are indicated. MiRNAs highlighted in bold and italic are up and down regulated in preovulatory dominant follicles, respectively.

¥: Only representative predicted genes are presented

*: Ranges of p-values for genes involved in specific pathway

Appendix 2: Supplementary materials of chapter 3

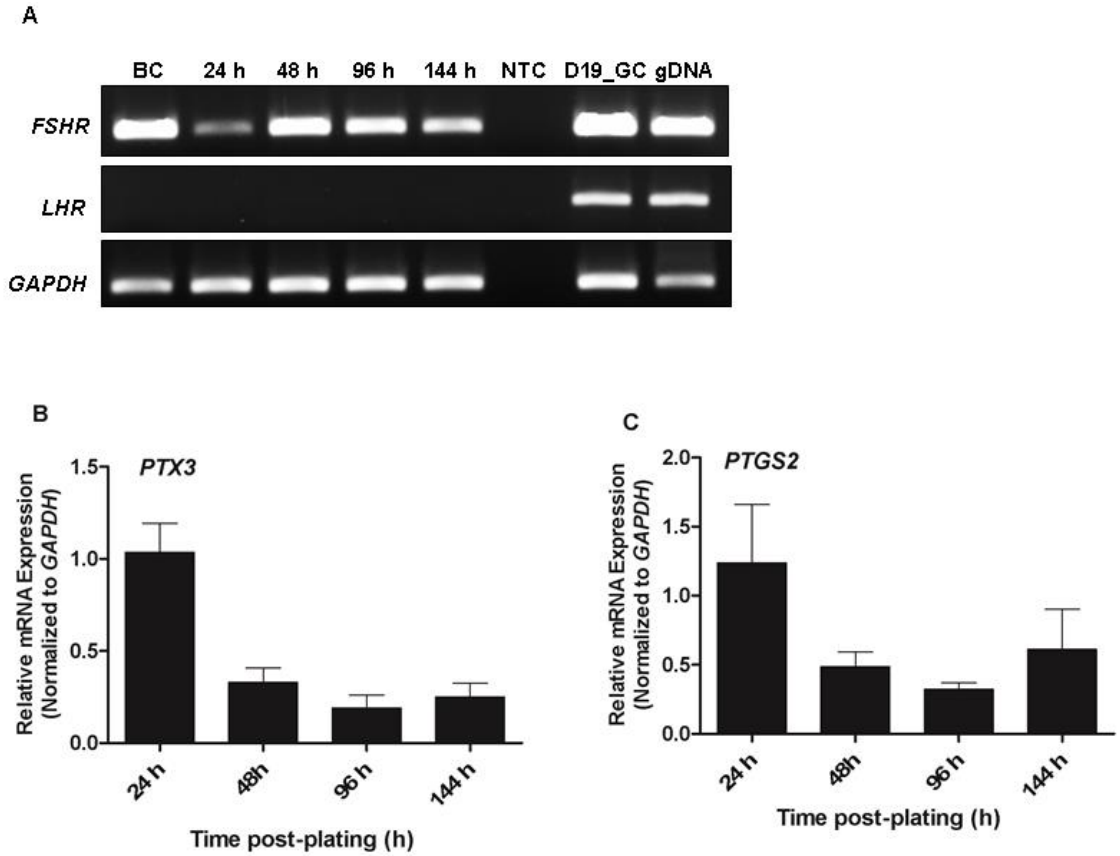

Figure 3.S1: Detection of Granulosa and luteinizing cell specific marker genes. Identity of granulosa cells and possible transformation into luteal cells were assessed by semi-quantitative PCR. Granulosa cell-specific marker gene *FSHR* was detected in granulosa cells both before and after plating. The Luteinizing cell marker gene (*LHR*) was not detected in the cells both before and after culture. RNA sample isolated from luteinizing granulosa cells derived from preovulatory dominant follicle on day 19 of estrous cycle was used as positive control. Genomic DNA (gDNA) was used as PCR positive control (A). *In vitro* culture decrease the expression of LH induced ovulatory genes (*PTX3*) and (*PTGS2*) signifying the absence luteinization (B, C).

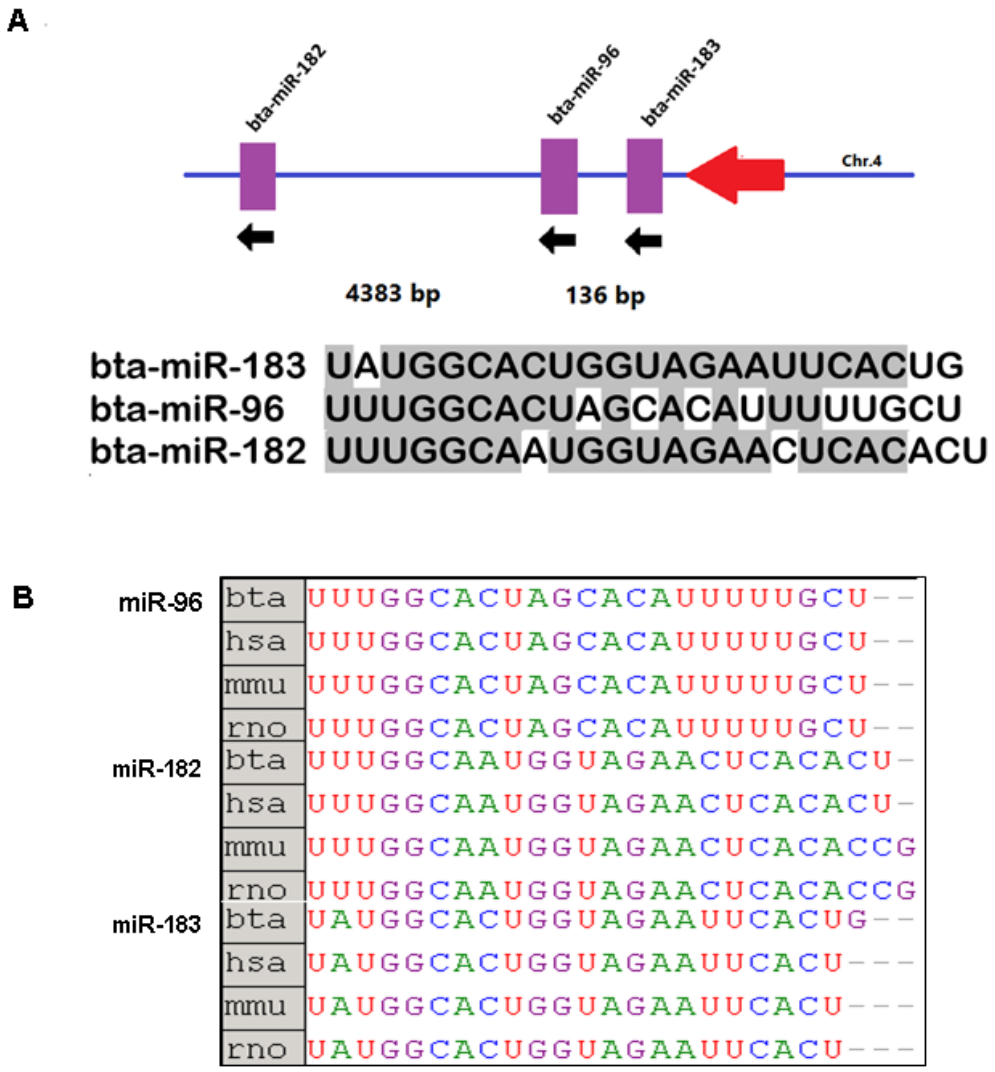

Figure 3.S2: Genomic organization and sequence conservation of bovine miR-183-96-182 cluster miRNAs. MiR-183-96-182 cluster is transcribed from intergenic region of chromosome 4 of bovine genome. Members of the miRNA cluster have higher sequence homology with similar seed region (A). Members of the miRNAs cluster are evolutionarily conserved across different mammalian species. b*ta, Bos taurus; hsa, Homo sapiens; mmu, Mus musculus; rno, Rattus norvegicus* (B).

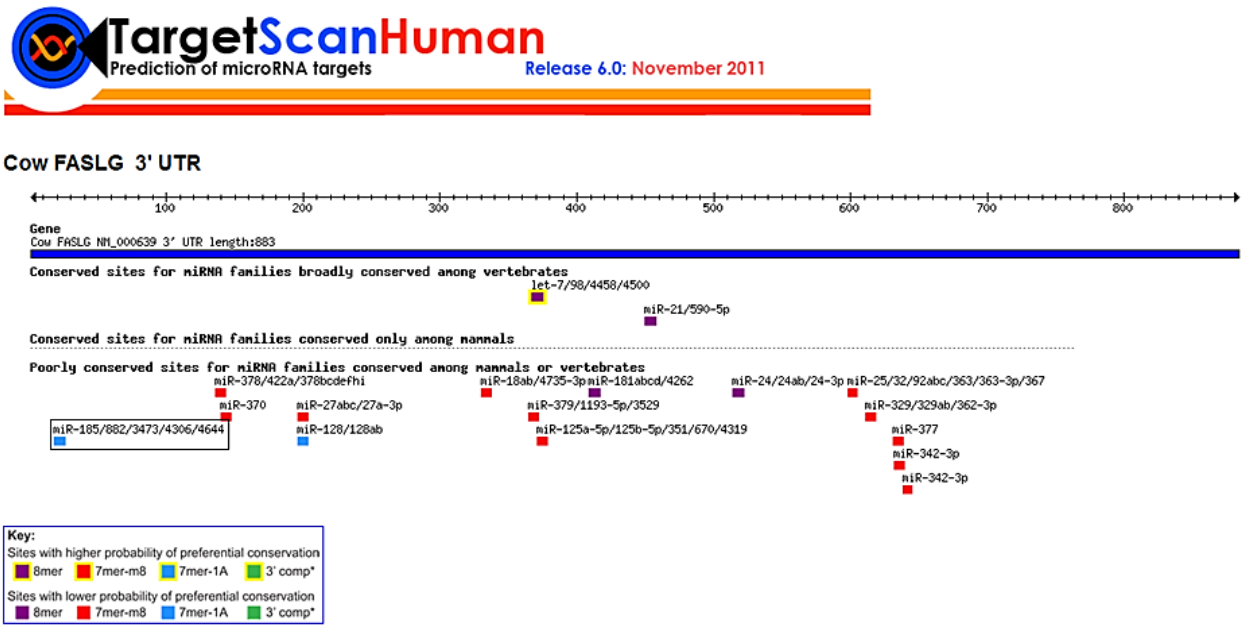

Figure 3.S3: FASL is not a predicted target gene of miR-183-96-182 cluster miRNAs. MiRNA target prediction using target scan 6.0 (Lewis *et al.* ; Grimson *et al.* 2007; Friedman *et al.* 2009) showed that the 3´-UTR of bovine *FASL* harbors the binding sites of several miRNAs. However, there are no conserved binding sites for the miR-183-96-182 cluster miRNAs.

Table 3.S1: SiRNA constructs used

Product	Sequence (5´-3´)
FOXO1-siRNA	5´-GCC AAG TCT GAC GAA A-3´
	5´-CCG CAC GAC TTG AAA A-3´
NC-siRNA	5´-AAC ACG TCT ATA CGC-3´

Table 3.S2: Primers used in this study

Gene	Primer sequence (5´-3´)	Tm(°C)	Usage	Accession No.
FOXO1	F: AAGAGCGTGCCCTACTTCAA R: CTCTTCTCCTGGGGGATTTC	57	qRT-PCR	XM_583090
FasL	F: AAGAGGAGGGACCACAACA R: TGGGGTGACCTATTTGCTTC	55	qRT-PCR	NM_001098859
GAPDH	F: AATGGAGCCATCACCATC R: GTGGTTCACGCCCATCACA	55	qRT-PCR	NM_001034034

Table 3.S3: Cell cycle distribution of granulosa cells transfected with miR-183-96-182 Cluster miRNAs inhibitors

Product	G_0/G_1 (%)	S (%)	G_2/M (%)
miR-183-Inh	73.92 ±0.52	12.14 ±0.77	13.94 ±1.28
miR-96-Inh	72.20 ±0.78	13.56 ±0.36	14.23 ±1.15
miR-182-Inh	72.91 ±0.26	12.97 ±0.52	14.11 ±0.78
Pool-Inh	72.56 ±0.86	13.27 ±1.08	14.17 ±0.28
NC-Inh	73.74 ±0.99	11.89 ±0.63	14.38 ±1.47

Appendix 3: Supplementary materials of chapter 4

Table 4.S1: Pathways analysis of predicted target genes of miRNAs differentially expressed in day 19 pregnant cows

KEGG pathway	Adjusted p-value	Number target genes	Number of miRNAs	Experimentally validated target Genes
Adherens junction	2.01E-07	14	3	ACTB, SMAD2, TCF7L2, VCL, FYN, CDH1, WASF2, ACTN4, BAIAP2,IQGAP1, TJP1, RAC1, INSR, SRC
Lysine degradation	2.60E-05	9	3	SETD1B, NSD1, KMT2D, DOT1L, SUV420H2, KMT2B, KMT2D, ACAT2, KMT2C,
Viral carcinogenesis	6.33E-05	30	3	YWHAH, CCND2, PKM, HIST4H4, CDK6,STAT5B, DDX3X, CHD4, YWHAQ, TP53, HDAC4, PMAIP1, IRF3, DDB1, RBL1, HIST1H2BG, YWHAZ, HDAC2, RAC1, SRC, CDC20, ACTN4, IL6ST, TBP, RELA, SRF, APKAPK2, JAK1, CCND3, UBR4,
Central carbon metabolism in cancer	0.000154642	12	3	PKM, NTRK3, SLC7A5, TP53, AKT2, HIF1A, AKT1, LDHA, PGAM1, HK1, PTEN, SLC1A5
Measles	0.00047783	26	3	IL2RG, GSK3B, OAS3, HSPA1A, IL2RB, CCND2, TAB2, CDK6, FYN, STAT5B, MSN, IFNAR2, TP53, IFNAR1, AKT2, MAVS, AKT1, CD46, IRF3, TNFRSF10B, EIF2S1, GNB2L1, HSPA1B, RELA, JAK1, CCND3
Transcriptional misregulation in cancer	0.004114175	26	3	CCNT2, BAIAP3, HMGA2, IL2RB, CCND2, HOXA9, HIST2H3D, PBX1, HIST2H3C, TP53, PTK2, HIST1H3D, HDAC2, H3F3A, DOT1L, PPARG, BCL2L1, WHSC1, H3F3B, TFE3, RELA, DDIT3, HIST1H3J, HIST2H3A, GOLPH3, PAX3,
Huntington's disease	0.006595323	18	3	POLR2B, REST, AP2B1, HTT, COX5A, ATP5B, TP53, SDHC, APAF1, DLG4, POLR2A, DNAL1, NDUFS1, HDAC2, ATP5H, PPARG, NDUFA13, TBP
Hippo signaling	0.008457064	18	3	ACTB, GSK3B, DVL3, YWHAH, SMAD2, TCF7L2,

pathway				CCND2, YWHAQ, CDH1, CSNK1D, DLG4, YWHAZ, TEAD2, FBXW11, SERPINE1, CCND3, AJUBA, BIRC2
Steroid biosynthesis	0.009284639	2	2	DHCR24, CYP51A1
Protein processing in endoplasmic reticulum	0.012870894	21	3	HSPA1A, UGGT1, HSP90AA1, UBE2E2, XBP1, GANAB, DNAJB1, SEC24C, BLOC1S5-TXNDC5, SEC24A, SEC61B, EIF2S1, SSR3, UBE2D1, UBE2D2, RPN2, CALR, HSPA1B, RRBP1, DDIT3, SYVN1
Bacterial invasion of epithelial cells	0.012870894	13	3	ACTB, ITGB1, SEPT8, VCL, CD2AP, PTK2, CDH1, ARPC2, WASF2, RAC1, SRC, SEPT9, RHOG
RNA transport	0.014281428	25	3	SAP18, NUP85, TACC3, DDX20, NUP160, EEF1A2, RPP30, UBE2I, PNN, EIF4A2, EIF4G1, POM121C, GEMIN5, EIF2S1, SUMO3, EIF4EBP2, ACIN1, XPO1, NUP188, KPNB1, EIF1, EIF4A1, EIF4G2, SRRM1, EIF3I
Shigellosis	0.015983739	12	3	PFN1, ACTB, ITGB1, VCL, ARPC2, WASF2, RAC1, SRC, UBE2D2, RELA, FBXW11, RHOG
Pathogenic Escherichia coli infection	0.036572299	11	3	ACTB, ITGB1, TUBA1B, TUBB, FYN, YWHAQ, TUBA1A, CDH1, TUBA1C, ARPC2, YWHAZ
Ubiquitin mediated proteolysis	0.039469388	21	3	FBXW7, HUWE1, UBE2E2, FZR1, MAP3K1, UBE2I, TRIP12, UBA1, DDB1, CDC20, PIAS4, UBE2D1, UBE2M, UBE2D2, UBE2S
Cell cycle	0.041305505	18	3	GSK3B, YWHAH, SMAD2, CCND2, FZR1, MCM5, WEE1, CDK6, YWHAQ, TP53, RBL1, YWHAZ, HDAC2, CDC20, TFDP1, PRKDC, ANAPC13, CCND3
p53 signaling pathway	0.041305505	13	3	CCND2, CDK6, TP53, APAF1, PMAIP1, SHISA5, TNFRSF10B, RRM2, PTEN, SERPINE1, CCNG2, PPM1D, CCND3
Pancreatic cancer	0.041305505	9	3	SMAD2, CDK6, TP53, AKT2, AKT1, RAC1, BCL2L1, RELA, JAK1

Table 4.S2: Pathways analysis of predicted target genes of miRNAs differentially expressed in day 24 pregnant cows

KEGG pathway	Adjusted p-value	Number target genes	Number of miRNAs	Experimentally validated target Genes
ECM-receptor interaction	0	38	5	ITGB8, LAMB1, COL6A5, CD36, LAMA5, THBS1, COL4A5, ITGB6, VTN, ITGB4, COL6A6, ITGA1, COL3A1, ITGB5, HSPG2, COL6A1, LAMC3, ITGAV, ITGA11, COL4A2, COL5A1, COL1A1, ITGA2, ITGA10, COL1A2, LAMC1, COL11A1, THBS3, COL6A3, GP9, FN1, TNC, SDC4, TNN, COL5A2, SPP1, SEPT5, COL4A1
Fatty acid biosynthesis	0	5	3	FASN, ACSL4, ACSL1, ACSL6, ACACA
Prion diseases	0	4	2	PRNP, PRKX, MAPK1, PRKACB
Fatty acid metabolism	2.44E-12	15	3	FASN, SCD5, ACOX1, ACOX3, ACAA2, PTPLA, CPT2, ACADVL, SCD, ACSL4, ACSL1, HSD17B12, ACSL6, MECR, ACACA
Adherens junction	7.56E-11	50	6	ACTB, CSNK2A2, TGFBR1, MET, WASL, RAC2, CTNND1, BAIAP2, PVRL2, TCF7L2, ACTG1, TCF7L1, IQGAP1, PTPRM, IGF1R, EGFR, VCL. PTPRF, SNAI1, TJP1, SORBS1, FYN, MLLT4, NLK, CDH1, PTPN6, SMAD4, CTNNB1, CTNNA1, ACTN1, WASF2, CSNK2A1, ARP2, PTPRJ, RAC1, INSR, SRC, MAPK3, SSX2IP, EP300, LEF1, PARD3, Smad2, PVRL3, TCF7, FGFR1, MAPK1, MAP3K7, TGFBR2, PVRL1
Transcriptional misregulation in cancer	4.05E-09	81	4	BMI1, GOLPH3L, TCF3, MEN1, CDKN2C, SSX1, NFKB1, MET, SPI1, FUS, CXCL8, BAIAP3, ETV6, ELK4, HIST3H3, RUNX1, HMGA2, HDAC1, AFF1, IL2RB, DUSP6, CCND2, HIST2H3D, PBX1, CD40, SLC45A3, HIST2H3C, MLLT3, IGF1R, ZEB1, PAX8, TP53, SSX2, ETV5, CCNT1, ATM, CEBPB, MLLT1, MYC, HIST1H3D, MMP9, KLF3, BMP2K, CD86, NCOR1, JMJD1C, HDAC2, H3F3A, ATF1, KDM6A,

				RARA, HIST1H3H, SP1, BCL6, IGF1, PPARG, WHSC1, H3F3B, IGFBP3, PINT1, KMT2A, MYCN, CDKN1A, BIRC3, PER2, TFE3, MEF2C, FUT8, EWSR1, SIX4, IL6, PLAU, HOXA10, HIST1H3J, HIST2H3A, GOLPH3, TGFBR2, SIN3A, RXRA, TAF15, Mycn
Cell cycle	1.25E-06	71	5	ESPL1, CDC6, RBL2, CDKN2C, E2F1, SMC1A, CCNB1, SFN, CDK4, E2F2, YWHAE, CCNA2, CDC25C, ORC2, HDAC1, MCM6, YWHAG, CDK2, CCND2, ORC1, MCM4, CUL1, FZR1, MCM5, STAG2, CDKN2B, STAG1, WEE1, CDK1, CDKN2A, CDK6, TGFB1, MCM7, TP53, ATM, MCM2, CDKN1C, ANAPC4, CCND1, SMAD4, CCNE2, E2F5, SKP2, E2F3, PKMYT1, MYC, RBL1, TTK, RB1, MAD2L2, YWHAZ, HDAC2, CDC7, CDC20, MAD2L1, CDC23, ANAPC5, EP300, CDC27, CDKN1A, PRKDC, ORC6, E2F4, PLK1, ANAPC13, MDM2, MCM3, CCND3, CDC25A, TGFB3, TFDP2
Proteoglycans in cancer	1.77E-06	92	2	PPP1CA, CAMK2D, BRAF, ACTB, STAT3, PDCD4, MET, CBL, SDC1, NRAS, MAP2K2, THBS1, WNT1, PTCH1, PIK3R2, ARHGEF12, PPP1CC, ACTG1, PXN, FRS2, RDX, IQGAP1, RAF1, IGF1R, EGFR, TLR4, PPP1R12B, TGFB1, FZD3, MSN, ITGB5, MAPK13, ARAF, RPS6KB2, TP53, HSPG2, HPSE, CTTN, VAV2, AKT2, PTK2, ITGAV, GPC3, ANK3, SLC9A1, PPP1R12A, DROSHA, PLCG1, CASP3, PTPN6, CCND1, GPC1, CTNNB1, HIF1A, MYC, MMP9, IGF2, CBLC, PRKACA, FLNA, FZD2, PTPN11, PRKX, RAC1, SRC, PLAUR, MAPK3, PPP1R12C, FZD1, PRKCB, FAS, IGF1, GAB1, NANOG, PIK3CA, HOXD10, FN1, CDKN1A, MAP2K1, ITPR3, VEGFA, FGFR1, MAPK1, ITPR2, PLAU, GRB2, MDM2, ELK1, WNT9A, RRAS, CD44, PRKACB

Hippo signaling pathway	7.07E-05	39	4	PPP1CA, ACTB, TGFBR1, YWHAH, YAP1, YWHAE, BTRC, PPP2CA, TCF7L2, YWHAG, CCND2, BMP5, ACTG1, GLI2, AMOT, FZD3, LIMD1, CCND1, CTNNA1, TP73, MYC, PPP2R1A, LLGL1, FZD2, TEAD1, YWHAZ, BMP2, PPP2CB, BMPR1A, PARD3, Smad2, SMAD7, MOB1A, LATS1, FBXW11, TGFBR2, WNT9A, BMPR2, BIRC2
Hepatitis B	0.001464018	73	2	STAT3, E2F1, TGFBR1, NFKB1, CDK4, CXCL8, ATF2, E2F2, NRAS, MAP2K2, CCNA2, ATF6B, CREB5, PIK3R2, CDK2, IFNB1, RAF1, CHUK, BCL2, BIRC5, MAP3K1, TLR4, CDK6, DDX3X, TGFB1, CREB3, TP53, HSPG2, CREB1, APAF1, AKT2, MAVS, CASP3, JUN, CCND1, SMAD4, CCNE2, E2F3, MAPK8, TIRAP, MYC, MMP9, IRF3, NFKBIA, RB1, YWHAZ, TBK1, SRC, MAPK3, CASP9, PRKCB, FAS, EP300, CREB3L2, PIK3CA, CREB3L1, CDKN1A, MAP2K1, STAT1, CYCS, MAP2K4, PTEN, NFATC3, IL6, MAPK1, NFATC1, GRB2, BAD, JAK1, ELK1, ATF4, TGFB3, IKBKE
RNA transport	0.001906104	32	2	SAP18, CYFIP2, EIF3D, TACC3, NUP98, EIF4EBP1, FMR1, EEF1A2, ALYREF, EIF5, EIF2S3, NUP214, SENP2, PABPC3, RNPS1, NUP107, EIF3A, THOC5, EIF4B, EIF4G1, EIF1B, PABPC1, FXR1, EEF1A1, DDX39B, RAN, ACIN1, XPO1, KPNB1, EIF2B2, EIF3C, EIF3I
Other types of O-glycan biosynthesis	0.003847068	15	2	ST3GAL3, OGT, POMT2, B3GALT4, RFNG, PLOD3, GXYLT1, B4GALT1, ST6GAL1, POMGNT1, EOGT, B4GALT3, POFUT1, MGAT5B, COLGALT1

Pathways in cancer	0.004107947	157	2	BRAF, STAT3, PDGFRA, E2F1, TGFBR1, NFKB1, MET, ADCY1, SPI1, FIGF, GNA12, CDK4, CXCL8, RAC2, CBL, E2F2, ADCY7, NRAS, FGFR3, CRKL, STK4, MAP2K2, BID, GNAS, LAMA5, CRK, RUNX1, WNT1, PTCH1, RAD51, CUL2, TPR, HDAC1, TCF7L2, TGFA, PIK3R2, ARHGEF12, CDK2, GNA13, GLI2, TCF7L1, BDKRB2, ETS1, RAF1, CHUK, BCL2, BIRC5, CDKN2B, IGF1R, EGFR, GNB1, CDKN2A, CDK6, TGFB1, FZD3, PAX8, EPAS1, MLH1, ARAF, TP53, PLCB3, AKT2, PTK2, LAMC3, GNA11, ITGAV, FH, CDH1, ARNT2, PLCG1, CASP3, JUN, CCND1, SMAD4, CTNNB1, LPAR1, MSH6, CTNNA1, CCNE2, AXIN2, SKP2, COL4A2, HIF1A, E2F3, MAPK8, MYC, MMP9, PTGER2, CBLC, PDGFB, PRKACA, EGLN3, NFKBIA, KIT, VEGFC, PTGS2, FZD2, RB1, FGF9, HSP90B1, HDAC2, PRKX, RAC1, MAPK3, RARA, CASP9, FGF18, BMP2, FZD1, EGLN2, PRKCB, FAS, LAMC1, IGF1, EP300, PPARG, GNAQ, GNAI2, PIK3CA, RALGDS, LEF1, FN1, CDKN1A, MAP2K1, BIRC3, RALB, SLC2A1, GNG4, STAT1, CYCS, SUFU, VEGFB, FGFR2, ITGA6, VEGFA, PTEN, FGFR1, FOXO1, IL6, MAPK1, FGF7, GNG5, GRB2, ADCY9, TGFBR2, TFG, BAD, JAK1, MDM2, WNT9A, PLCB4, PDGFRB, PRKACB, COL4A1, TGFB3, BIRC2, RXRA
Lysine degradation	0.005996849	15	2	WHSC1L1, SETD1B, HADH, PLOD2, ASH1L, KMT2D, SUV420H1, DOT1L, SUV420H2, KMT2A, SUV39H2, KMT2E, SETD1A, EHMT1, KMT2B
Thyroid hormone signaling pathway	0.006568914	62	3	ACTB, MED13L, NRAS, MAP2K2, RCAN1, MED14, MED12, PLCD3, HDAC1, PIK3R2, ACTG1, RAF1, MED13, SLC16A10, THRA, NOTCH2, RHEB, NCOA3, TP53, GATA4, PLCB3, MED4, AKT2, MED16, ITGAV, SLC9A1, NOTCH1, PLCG1, TSC2, CCND1, CTNNB1, ATP1B3, HIF1A, MYC, PRKACA, NCOR1, HDAC2, PRKX, KAT2A, SRC, MAPK3, CASP9, PRKCB, KAT2B, EP300, NOTCH3, PIK3CA, MAP2K1,

				SLC2A1, PFKFB2, ATP2A2, STAT1, ATP1A1, FOXO1, MAPK1, BAD, MDM2, PLCB4, NCOA1, SIN3A, PRKACB, RXRA
Viral carcinogenesis	0.008089296	95	2	RBL2, STAT3, NFKB1, GTF2E2, CDK4, ATF2, NRAS, RASA2, YWHAE, CCNA2. GTF2H1, ATF6B, HDAC7, CREB5, HDAC1, DLG1, LTBR, PIK3R2, YWHAG, HIST1H2BC, CDK2, CCND2, PXN, HIST1H4C, CDKN2B, CDK1, HIST4H4, CDKN2A, GTF2B, CDK6, DDX3X, CREB3, CHD4, HIST1H2BD, HIST1H2BA, TP53 ,HNRNPK, CREB1, GSN, PMAIP1, CASP3, JUN, CCND1, CCNE2, SKP2, CCR5, PRKACA, IRF3, DNAJA3, NFKBIA, RBL1, HIST1H2BG, HIST1H4H, RB1, JAK3, YWHAZ, HDAC2, PRKX, KAT2A, RAC1, SRC, MAPK3, CDC20, RBPJ, HIST1H2BL, HIST1H4J, KAT2B, EP300, CREB3L2, PIK3CA, CREB3L1, CDKN1A, HIST2H2BE, HDAC8, MAPK1, TBPL1, GRB2, BAD, JAK1, MDM2, ATF4, CCND3, PRKACB, UBR4, SCRIB, HIST1H4J, HIST1H2BO, HIST1H4E, HIST1H4B, HIST1H2BM, HIST1H4K, HIST1H2BJ, HIST3H2BB, HIST1H4D, HIST1H4A

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Publication and contributions

Published peer reviewed articles

1. **Gebremedhn S**, Salilew-Wondim D, Ahmad I, Sahadevan S, Hossain MM, Hoelker M, et al. (2015) MicroRNA expression profile in bovine granulosa cells of preovulatory dominant and subordinate follicles during the late follicular phase of the estrous cycle. PLoS ONE 10(5): e0125912.doi:10.1371/journal.pone.0125912

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5. Dessie Salilew-Wondim†, Sally Ibrahim†, **Samuel Gebremedhn**, Dawit Tesfaye, Maike Heppelmann, Heinrich Bollwein, Christiane Pfarrer, Ernst Tholen, Christiane Neuhoff, Karl Schellander and Michael Hoelker (2016). Clinical and subclinical endometritis

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6. Dawit Tesfaye, Dessie Salilew-Wondim, **Samuel Gebremedhn**, Md Mahmodul Hasan Sohel, Hari Om Pandey, Michael Hoelker and Karl Schellander. Potential role of microRNAs in mammalian female fertility. Reprod Fertil Dev, 2017, 29, 1–16 http://dx.doi.org/10.1071/RD16266

7. Gabriella Mamede Andrade†; Juliano Coelho da Silveira†; Claudia Perrini; Maite Del Collado; **Samuel Gebremedhn**; Dawit Tesfaye; Flávio Vieira Meirelles*; Felipe Perecin* (2017) The Role of PI3K-Akt Signaling Pathway in Bovine Oocyte Developmental Competence. PLos One (under review)

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Conference abstract/poster

1. **Gebremedhn, S**., Salilew-Wondim, D., Hoelker, M., Rings, F., Neuhoff, C., Tholen, E., Looft, C., Schellander, K., and Tesfaye, D. (2015). MicroRNA-183~96~182 CLUSTER PROMOTE BOVINE GRANULOSA CELL PROLIFERATION THROUGH COORDINATED REGULATION OF *FOXO1*. *Reprod Fertil and Dev* **28**, 194–195. (Abstr). 42th Annual Conference of the International Embryo Transfer Society (IETS), 23.-26.1.2016, Louisville, KN, USA,

2. **Gebremedhn S**, Ahmad I, Salilew-Wondim D, Sahadevan S, Hoelker M, Rings F, Uddin M J, Tholen E, Looft C, Schellander K, Tesfaye D (2014): Expression profiling of noncoding microRNAs in bovine granulosa cells of preovulatory dominant follicle using deep sequencing. *Reprod Fertil and Dev* **26**, 1: 113 (Abstr). 40th Annual Conference of the International Embryo Transfer Society (IETS), 11.-14.1.2014, Reno NV, USA,

3. Salilew-Wondim D, Ahmad I, **Gebremedhn S**, Sahadevan S, Hoelker M, Rings F, Uddin M J, Tholen E, Looft C, Schellander K, Tesfaye D (2014): Regulatory microRNA enrichment and degradation in granulosa cells during bovine follicular recruitment and dominance. *Reproduction Fertility and Development* **26**, 1: 113 (Abstr). 40th Annual

Conference of the International Embryo Transfer Society (IETS), 11.-14.1.2014, Reno NV, USA

4. Ahmad I, Salilew-Wondim D, Sahadevan S, **Etay S G**, Hoelker M, Rings F, Uddin M J, Tholen E, Looft C, Tesfaye D, Schellander K (2013): Investigation of miRNAs enrichment and degradation in bovine granulosa cells during follicular development using next-generation sequencing. DGfZ/GfT-Gemeinschaftstagung, 4./5.9.2013, Göttingen, Deutschland, Tagungsband: (Proc)

5. **S. Gebremedhn**, D. Salilew-Wondim, M. Hoelker, E. Held, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2015) Identification and Functional Analysis of Regulatory MicroRNAs in Bovine Granulosa Cells. Vortragstagung der DGfZ und GfT am 16./18. September 2015 in Berlin: (Proc)

6. **S. Gebremedhn**, D. Salilew-Wondim, M. Hoelker, E. Held, C. Neuhoff, E. Tholen, K. Schellander, D. Tesfaye (2016) Circulatory microRNAs in maternal serum as potential indicators of pregnancy in bovine Vortragstagung der DGfZ und GfT am 20./21. September 2016 in Hannover: (Proc)

Computer and software skills

- Good command on Microsoft office packages
- Graphpad Prism
- Serial cloner (Virtual cloning)
- Flex array microarray data analysis tool

Trainings

1. Hands-On" workshop NGS Genomics data analysis" using CLC genomics workbench. Qiagen November 3rd-4th, 2015 in DZIF, Gießen, Germany
2. Applied bioinformatics: Theodor Brinkmann Graduate School. university of Bonn
3. Preparing conference posters for international conferences, July 28-2016. Bonn graduate center (BGC), University of Bonn
4. Multi-scale imaging for the study of animal reproduction (Summer school) , 21-24 June 2016, INRA, Tours, France