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Investigation of miRNAs enrichment and degradation in bovine granulosa cells during follicular development

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Dedicated to my Sweet Mom and Dad, my Wife and my Loving Son

Investigation of miRNAs enrichment and degradation in bovine granulosa cells during follicular development

The granulosa cells in the mammalian ovarian follicle respond to gonadotropin signalling and are involved in the processes of folliculogenesis and oocyte maturation. Although, several studies have been done on spatio temporal expression of genes during follicular development, little is known about the post-transcriptional regulation of those genes. This study unravelled the basic knowledge on bovine miRNA prevalence and expression pattern during the early luteal phase of the bovine estrous cycle. For this, miRNAs enriched total RNA isolated from granulosa cells of subordinate follicles (SF) and dominant follicles (DF) obtained from heifers slaughtered at day 3 and day 7 of the estrous cycle and were subjected for miRNAs deep sequencing. The data analysis revealed that 291 and 318 mature miRNAs were detected in granulosa cells of SF and DF, respectively at day 3 of estrous cycle, while 314 and 316 were detected in granulosa cells of SF and DF, respectively, at day 7 of estrous cycle. A total of 244 detected miRNAs were common to all follicle groups, of which 15 miRNAs including bta-miR-10b, bta-miR-26a, let-7 families, bta-miR-92a, bta-miR-191, bta-miR-125a, bta-miR-148 and bta-miR-30a-5p, were highly abundant ($\geq$3000 reads) in both SF and DF at both days of the estrous cycle. At day 3 of the estrous cycle, 16 miRNAs including bta-miR-449a, bta-miR-449c, bta-miR-212, bta-miR-21-3p, bta-miR-183 and bta-mir-34c were differentially expressed (DE) in granulosa cell of subordinate follicle groups. Similarly, at day 7 of the estrous cycle, a total of 108 miRNAs including bta-mir-409a, bta-miR-2446, and bta-mir-383 were altered in granulosa cells of SF compared to DF. Nine miRNAs including bta-miR-21-3p, bta-miR-708, and bta-miR-335 were commonly DE between SF and DF at day 3 and day 7 of the estrous cycle. In addition to known miRNAs, a total of 21 novel miRNAs were identified and detected in granulosa cells of SF and/or DF at day 3 and day 7 of the estrous cycle. The majority of the DE miRNAs were found to be involved in regulation of programmed cell death and regulation of cell proliferation. In addition, the DE miRNAs were found to be involved in Wnt signaling, TGF-beta signaling, oocyte meiosis, MAPK signaling, focal adhesion, axon guidance and gap junction. Therefore, our findings suggest that temporal variation in the abundance of mature miRNAs during bovine follicular development in SF and DF of granulosa cells, which may be associated with recruitment, selection and development of bovine follicles.

Untersuchung der miRNA Anreicherung und Abbau in bovine Granulosazellen während der Follikelreifung

Die Granulosazellen aus ovarialen Säugertierfollikeln reagieren auf Gonadotropin-Signale und sind an den Prozessen der Follikulogenese und Eizellenreifung beteiligt. Obwohl bereits mehrere Studien über die spatio-temporale Genexpression während der follikulären Entwicklung erfolgten, ist bisher wenig über die post-transkriptionelle Regulierung dieser Gene bekannt. Daher befasst sich diese Studie mit der Untersuchung von der bovine miRNA Prävalenz und ihrer Expressionsmuster während der frühen Lutealphase des bovinen Östruszyklus. Dafür wurde miRNA angereicherte Gesamt-RNA aus Granulosazellen von untergeordneten Follikeln (SF) und dominanten Follikeln (DF) am Tag 3 und Tag 7 des Östruszyklus von geschlachteten Färsen isoliert und mittels Deep Sequenzierung analysiert. Durch die Datenanalyse konnten jeweils 291 und 317 miRNAs in Granulosazellen von SF und DF am Tag 3 des Östruszyklus ermittelt werden. Für Tag 7 des Östruszyklus gewonnene Granulosazellen konnten 314 und 316 miRNAs identifizierten werden. In allen Follikelgruppen wurden insgesamt 244 miRNAs detektiert, wobei 15 miRNAs einschließlich bta-miR-10b, bta-miR-26a, let-7 families, bta-miR-92a, bta-miR-191, bta-miR-125a, bta-miR-148 und bta-miR-30a-5p in beiden SF und DF und auch an beiden Tagen des Östruszyklus hoch reguliert ($\geq$3000 reads) waren. Am Tag 3 des Östruszyklus waren 16 miRNAs einschließlich bta-miR-449a, bta-miR-449c, bta-miR-212, bta-miR-21-3p, bta-miR-183 und bta-mir-34c unterschiedlich in Granulosazellen der untergeordneten Follikelgruppe exprimiert (DE). Genauso zeigten am Tag 7 des Östruszyklus insgesamt 108 miRNAs einschließlich bta-mir-409a, bta-miR-2446, und bta-mir-383 in SF Granulosazellen im Vergleich zu DF eine unterschiedliche Expression. Neun miRNAs, u.a. bta-miR-21-3p, bta-miR-708 und bta-miR-335 waren sowohl am Tag 3 als auch am Tag 7 DE zwischen SF und DF des Östruszyklus. Insgesamt wurden 21 neue miRNAs zusätzlich zu den bekannten miRNAs in den Granulosazellen von SF und/oder DF am Tag 3 und 7 des Östruszyklus identifiziert und detektiert. Die Mehrheit der DE miRNAs sind an der Regulierung des programmierten Zelltods und der Regulierung der Zellproliferation beteiligt. Gleichwohl waren diese DE miRNAs auch an den Signalwegen Wnt, TGF-beta, Meiose der Eizelle, MAPK, fokal Adhäsion, Axon Guidance und Gap Junction involviert. Deshalb lassen unsere Ergebnisse darauf schließen, dass temporale Variationen in der Anreicherung von miRNAs während der bovinen Follikelentwicklung in SF und DF aus Granulosazellen, welche mit der Rekrutierung, Selektion und Entwicklung boviner Follikel assoziiert werden, vorkommen können.

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List of abbreviations

A	Adenine
Ago	Argonaute protein
AI	Artificial insemination
AMH	Anti-Müllerian hormone
aRNA	Amplified ribonucleic acid
ATP	Adenosine tri phosphate
BLAST	Basic local alignment search
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Bta	*Bos taurus*
C	Cytosine
cDNA	Complementary deoxy ribonucleic acid
cKO	Conditional knockout
CL	Corpus luteum
CMF	Calcium magnesium free
COC	Cumulus oocyte complex
cRNA	Complementary ribonucleic acid
Cx	Connexin
ddH2O	Demineralised millipore water
DE	Differentially expressed
DEPC	Diethylpyrocarbonate
DESeq	Differntial expressed sequencing
DF	Dominant follicle
DGCR8	DiGeorge syndrome critical region gene 8
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
E2	Estradiol
EBV	Epstein-Barr virus
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

ER	Endoplasmic reticulum
ES	Embryonic stem cell
FC	Fold change
FDR	False discovery rate
FF	Follicular fluid
FGF	Fibroblast growth factor
Fig	Figure
For	Forward primer
FSH	Follicle stimulating hormone
G	Guanine
G6PDH	Glucose 6 phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GEO	Gene expression omnibus
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GVBD	Germinal vesicle breakdown
HCC	Hepatocellular carcinoma
hCG	Human chorionic gonadotropin
HPG	Hypothalamic–pituitary–gonadal
hr	Hour
IPA	Ingenuity pathway analysis
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
IVT	In vitro transcription
kDa	Kilo dalton
KGF	Keratinocyte growth factor
KL	Kit ligand
KO	Knock out
LH	Luteinizing hormone

LNA	Locked nucleic acid
MAPK	Mitogen-activated protein kinase
MCGF	Mast cell growth factor
MeOH	Methanol
mg	Milligrams
MI	Metaphase I
MII	Metaphase II
min	Minute
miRISCs	miRNA-induced silencing complexes
miRNA	Micro RNA
mi-RNPs	Micro-ribonucleoprotein
MPs	Micro particles
mRNA	Messenger ribonucleic acid
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National center for biotechnological information
NGS	Next generation sequencing
nt	Nucleotides
P4	Progesterone
P-bodies	Processing bodies
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGCs	Primordial germ cells
PGF2α	Prostaglandin F2α
POF	Premature ovarian failure
POF	Premature ovarian failure
Pre-miRNA	Precursor micro RNA
Pri-miRNA	Primary micro RNA
PVA	Polyvinyl alcohol
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative Real Time PC

RC	Reverse complement
RefSeq	Reference sequence
Rev	Reverse primer
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RNP	Ribonucleoprotein
rpm	Revolution per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse-trancription
SCNT	Somatic cell nuclear transfer
sec	Second
SF	Subordinate follicle
siRNA	Small interfering RNA
T	Testosterone
TC	Theca cell
TCM	Tissue culture media
TE	Trophectoderm
TGF-ß	Transforming growth factor beta
tRNA	Transfer ribonucleic acid
U	Uracil
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WC	Watson-Crick
ZP	Zona pelluciada
°C	Degree centigrade

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1 Introduction

One or multiple oocytes ovulate per reproductive cycle in mammalian species. The commencement of follicular growth is alike across species, with numerous follicles growing to the antral stage. From the pool of these antral follicles, a cluster of follicles (cohort) are recruited which continue growth toward ovulation. In mono-ovulatory species (e.g., cattle, horses, humans), single follicle is selected from the cohort and continues its growth and attains the ovulatory capacity, whereas, the fate of other follicles is atresia (Ginther et al. 2000b, Ginther et al. 2001, Hodgen 1982, Zeleznik 2001). Two major functions of the mammalian ovary are the production of germ cells (oocytes), which allow continuation of the species, and hormone production, primarily steroids (mainly estrogens and progestins) and peptide growth factors, which are critical for ovarian function, regulate the hypothalamic-pituitary-ovarian axis, and development of secondary sex characteristics (Edson et al. 2009). Within ovary follicular granulosa cells border and foster oocytes, and yield sex steroid hormones. It is understood that during growth, the ovarian surface epithelial cells enter into the ovary and develop into granulosa cells when correlating with oogonia to form follicles (Hummitzsch et al. 2013).

In the reproductive lifespan of mammals, a continuously gentle stream of primordial follicles is released from dormancy and move in the growing follicle pool. As soon as growth is initiated, the follicle boards on a complex path of development during which the oocyte progresses through a series of highly orchestrated phases of development which are essential for its fruitful ovulation and fertilization. This process begins as soon as the pool of primordial follicles is established in the ovary and continues until the pool is devastated and folliculogenesis ends (Hutt and Albertini 2007).

Follicular development is the outcome of multifarious hormonal and biochemical interactions that could be triggered or deactivated within the follicular environment in a spatiotemporal fashion. Mammalian ovaries consist of follicles as elementary functional units, and each follicle comprises of an oocyte enclosed by one or multiple layers of somatic granulosa cells and theca cells (Tu et al. 2014). The layer and number of granulosa cells may differ reliant on the size and phase of follicular development. For instance, in primordial follicle, the small, non-growing functionally immature oocyte is

surrounded by a single layer of squamous granulosa cells (Aerts and Bols 2010, Buccione et al. 1990). Generally in domesticated animal species the stock of primordial follicles begins during fetal life. The transition from non-growing to growing follicles is a steady process, which originates shortly after the formation of the primordial follicles and continues throughout reproductive life (Fortune et al. 1998). The primordial follicles then advance to the primary follicles by commencing follicle growth and transforming the single layer of granulosa from flattened to a cuboidal morphology and there by undergoes proliferation and differentiation of granulosa cells which results in an increase in the number of granulosa cells and accompanied by enlargement of the oocyte volume which may gradually become Graafian follicle (Aerts and Bols 2010). This involuntary and compound transit of the primordial follicle into large sized antral follicles is primarily initiated by morphological conversion and functional differentiation of the granulosa cells. Therefore, when the follicles start to develop from state of resting pool, the oocytes continue to grow and the granulosa cells proliferate until the stage of preantral follicle (Thomas and Vanderhyden 2006). The pre-antral phase of folliculogenesis is characterized by zona pellucida formation, granulosa cell proliferation, the recruitment of thecal cells to the follicular basal lamina and a vivid increase in oocyte volume (Pedersen 1969). In consequence, during these acute periods, oocyte development is governed by paracrine interactions between the oocyte and the granulosa cells by which the oocyte controls the growth and development of granulosa cells and vice-versa (Buccione et al. 1990). Therefore for symbiotic survival of both cell types, the granulosa and oocyte undergo a bidirectional communication by establishing a gap junction mediated syncytium (Aerts and Bols 2010, Buccione et al. 1990). The bidirectional crosstalk between the oocyte and the somatic cell type (granulosa and theca cells) affects the hormonal production and the expression of genes associated with follicular development (Palma et al. 2012). Hence, the growth, development and notable functional differentiation of the granulosa cells are one of the significant events that are required for follicle maturation (Yada et al. 1999). The granulosa cells are indeed specialised in production of estradiol hormone, inhibin and activin (Hatzirodos et al. 2014a). Therefore, the fate of follicular growth and development is believed to be mainly determined by the growth and development of the granulosa cells (Clement et al. 1997).

In bovine, follicular development is characterized by recruitment of a group of follicles in 2 or 3 follicular waves though high rate of growth is initiated only in one of the follicles which later on succeeded to be dominant over the others and becomes ovulatory (Ahmad et al. 1997). However, the dominant follicle could not be ovulated when the follicular development is happening through the luteal phase of estrous cycle. Here the main query is to recognize the mechanism how the follicular atmosphere assists one follicle to develop dominant and thereby shadow the development of other follicles and they become atretic. This may lead to the hypothesis that the follicular microenvironment could have distinctive molecular signals which would affect differently the bidirectional crosstalk between the oocytes and the companion somatic cells in the subordinate and dominant follicles. This may perhaps clue to the hypothesis that the follicular atmosphere could have distinctive molecular indications which would mark contrarily the bidirectional crosstalk between the oocytes and the surrounding somatic cells in the subordinate and dominant follicles.

Two key developments during the last decade have dramatically increased knowledge of the molecular control of follicular development. First is the advent and application of genomic technologies to study ovarian function leading to the identification of hundreds of new genes putatively involved in ovarian processes in different species. Second a major development has been the discovery and characterization of animal microRNAs (miRNAs); 19-22 nucleotides non-coding RNAs which function as key regulators of gene expression post-transcriptionally. miRNAs works by interact with the 3´-UTR of the target mRNA. For instance, it has been reported that, a total of 13162 genes were detected in germinal vesicle (GV) oocytes and their companion CCs, of those 1516 and 2727 are exclusively expressed in oocytes and CCs, respectively, while 8919 are expressed in both. Moreover, a total of 265 transcripts are differentially expressed between oocytes cultured with (OO+CCs) and without (OO-CCs) CCs, of which 217 and 48 are over expressed in the former and the later groups, respectively. The presence or absence of oocyte and CC factors during bovine oocyte maturation can have a profound effect on transcript abundance of each cell types, thereby showing the prevailing molecular cross-talk between oocytes and their corresponding CCs (Regassa et al. 2011). Using a heterologous approach Tesfaye and co-scientists revealed a set of 59 differentially expressed miRNAs, of which 31 and 28 miRNAs were found to be

preferentially expressed in immature and matured oocytes, respectively (Tesfaye et al. 2009). In another study, a total of 47 and 51 miRNAs were highly abundant in immature and matured oocytes, respectively, compared with their surrounding cumulus cells. Furthermore, expression analysis of six miRNAs enriched in oocyte miR-205, miR-150, miR-122, miR-96, miR-146a and miR-146b-5p at different maturation times showed a dramatic decrease in abundance from 0 h to 22 h of maturation (Abd El Naby et al. 2013). These results confirmed the presence of distinct sets of miRNAs in oocytes or cumulus cells and the presence of their dynamic degradation during bovine oocyte maturation which indicating the important potential role of miRNAs during the dynamic stage of follicular development. The significance and involvement of miRNAs in the ovarian function, and follicular development has also been described by several authors (Donadeu et al. 2012, Hossain et al. 2009, Toloubeydokhti et al. 2008, Tripurani et al. 2010), but their existence, richness and sequential expression in the subordinate and dominant follicles during the bovine luteal phase of the estrous cycle needs to be revealed.

Several signaling molecules including the TGF-beta superfamily members, follicle stimulating hormone receptor, luteinizing hormone receptor, cytochrome 450s (CYP11A1, CYP17A1, CYP19A1), GDF9, IGF-1, IGF-II, IGFBP2 and several of genes have been found to be altered in granulosa and/or theca cells depending on the size and stage of follicular development (Bao and Garverick 1998a, Hayashi et al. 2010, Sisco et al. 2003, Spicer et al. 2011, Vitt et al. 2000a). Abnormal expression of those developmentally related genes and gene products in the oocytes and supporting cells could then lead to cellular communication dysfunction and dysregulation of normal follicle recruitment and development (Toloubeydokhti et al. 2008). However, the posttranscriptional regulatory mechanism of genes and gene products associated with follicular recruitment, selection and dominance during the luteal phase of the estrous cycle is still poorly understood.

In the recent years, the fast progress of next generation sequencing (NGS) technologies haas enormously boosted the profiling of miRNA expression levels as well as identification of novel miRNA genes. High-throughput miRNA profile analysis from different ovarian somatic cell types appears rare among the publications so far. Based

on that, we formulate an aim of the current study was to fill the gap in information regarding the miRNA profile in bovine follicular granulosa cells from subordinate and dominant follicles at different time points of estrous cycle. The study was performed using next-generation sequencing to determine annotated as well as novel miRNAs. We aimed to examine the degree of difference between granulosa cells from subordinate and dominant follicles obtained at day 3 and day 7 of estrous cycle and regarding their miRNA profile, then to predict the potential targets of differential expressed miRNAs in either cell type or at each time points. Therefore, the objectives of this study were to understand the availability and abundance of miRNAs in bovine granulosa cells derived from subordinate and dominant follicles during bovine follicular development across the estrous cycle.

2 Literature review

2.1 Ovary and folliculogenesis

The two key functions of the ovary are gametogenesis (the production of female gametes) and steroidgenesis (the production of steroid hormones), which play dynamic roles to control the reproductive tract for fertilization and the establishment of pregnancy (Knight and Glister 2006). After the formation of zygote by the unification of an oocyte and a spermatozoon, all cells up to the eight-cell stage of embryogenesis appear to have analogous totipotency, because of the fact of same morphological appearance for all these cells. However, with the formation of a 16-cell morula, the cells begin the process of differentiation with cells being allocated to either the inside or outside of the embryo. This process is inflated further at the blastocyst stage by defining the lineages: trophectoderm (future placenta), epiblast (future embryo), and primitive endoderm (future yolk sac). Cells with in the epibalst eventually form the precursors of the primordial germ cells (PGCs), claimed to be the initial cells of the ovary by the result of implantation and further differentiation. By the arrival of PGS in to the indifferent gonad, eventually the formation of ovary occurs and allow the PGCs to differentiate in to oocytes, which enter meiosis and subsequently arrest; this differentiation stage and entry into meiosis propose that the last of the oocyte "stem cells" (*i.e*., the PGCs) likely disappear at this stage of fetal life. The meiotically arrested oocytes eventually become surrounded by pre-granulosa cells and form individual primordial follicles, the resting pool of oocytes that have the potential to be recruited into the growing follicle pool in the postpubertal mammal. The meiotically arrested oocytes ultimately become enclosed by pre-granulosa cells and form distinct primordial follicles, while the resting pool of oocytes that have the potential to be recruited into the growing follicle pool in the post pubertal mammal, to be fertilized, and to contribute to the next generation (Edson et al. 2009).

Folliculogenesis is the outcome of a follicle which is recruited, matured and sequentially ovulated following the growth and differentiation of the oocyte and its surrounding somatic cells (Gougeon 1996, Knight and Glister 2001). The major steps in ovarian folliculogenesis (Figure 2.1) include the formation of the inactive pool of primordial

follicles, the recruitment and selection of primordial follicles for growth and development resulting in the development of follicles through the primary, secondary, antral, and preovulatory stages and ovulation, which ends up with the establishment of a corpus luteum (CL) from remaining somatic cells of the follicle (Edson et al. 2009). The cataloging of the follicle is based on the size of oocyte, the morphology of granulosa cells and the number of granulosa cell layers surrounding the oocyte (Braw-Tal and Yossefi 1997, Lussier et al. 1987). It is recognized that folliculogenesis in bovine species occur in the course of fetal development. Once oocytes attainments to diplotene stage of prophase I, it's surrounded by a single layer of squamous pre-granulosa cells inactive on a basement membrane and forming the non-growing or primordial follicle (Picton et al. 1998). The bulky population of non-growing primordial follicles serves as the pool of developing follicles and oocytes until the end of a female's reproductive life. Primordial follicles are transformed into primary follicles by changing the flattened granulosa cells to cuboidal form by allowing the oocyte to attain its extensive growth phase. (Eppig 2001, van Wezel and Rodgers 1996). Expansion to the secondary follicle stage also known as preantral follicle or growing follicle is characterized by the presence of a second layer of granulosa cells, increased oocyte diameter (Driancourt et al. 1991), zona pellucida and cortical granule formation, development of gap junction between oocyte and granulosa cell which may serve both to maintain meiotic arrest and oocyte growth (Braw-Tal and Yossefi 1997, Fair et al. 1997b), first measurable signs of oocyte RNA synthesis (Fair et al. 1997a) and gonadotrophin responsiveness where the follicular stimulating hormone (FSH) receptor mRNA expression has been detected in follicles with one to two layers of granulosa cells in cattle (Bao and Garverick 1998, Fair 2003, Xu et al. 1995). Development beyond the early antral follicle stage is clearly dependent upon gonadotrophins, antral follicles are sometimes called tertiary follicles and well developed antral follicles are often stated to as Graafian follicle (Eppig 2001). The transition to antral follicle is characterized by formation of antrum which is fluid filled cavity surrounded by multiple layer of granulosa cells, this fluid contain important regulatory substance to be delivered to oocyte (Braw-Tal and Yossefi 1997, Eppig 2001, Lussier et al. 1987). After antrum formation, granulosa cells are divided into two distinct subtypes, the cumulus granulosa surrounding and in intimate metabolic contact with the oocyte forming a structure called cumulus oocyte complex (COC) and the

mural granulosa lining the follicle wall and adjacent to the basal lamina, the mural granulosa cells nearest the antrum are called periantral granulosa cells (Eppig 2001).
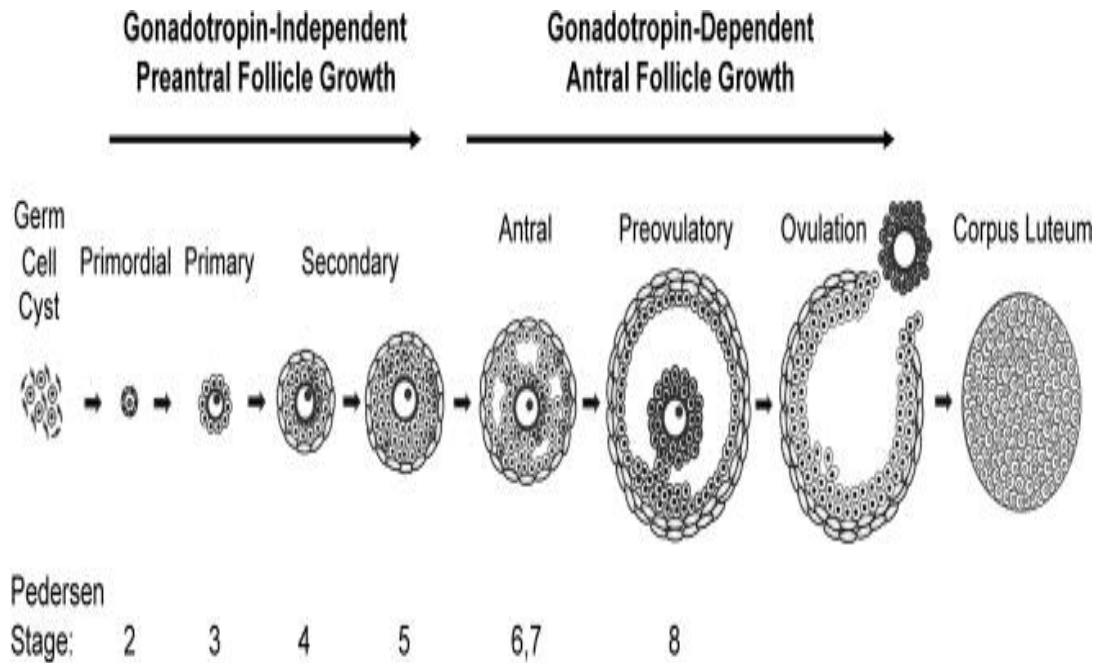
Figure 2.1: Schematic presentation of the major stages of mammalian folliculogeneis.

These stages are divided into two separate groups. (i) Intraovarian regulation of folliculogenesis (primordial, primary, and secondary stages). (ii) Gonadotropin-dependent regulation of folliculogenesis (antral, preovulatory, and ovulation of the follicle) (Edson et al. 2009).

Ideally a fully grown follicle known as graafian follicle is a three-dimensional structure with an antrum in the center and surrounded by a diverse set of cell types. The main discrete histologic components in graafian follicle comprise (Figure 2.2) of theca interna, mural granulosa cells, cumulus granulosa cells, oocyte antrum filled with follicular fluid (Aerts and Bols 2010).
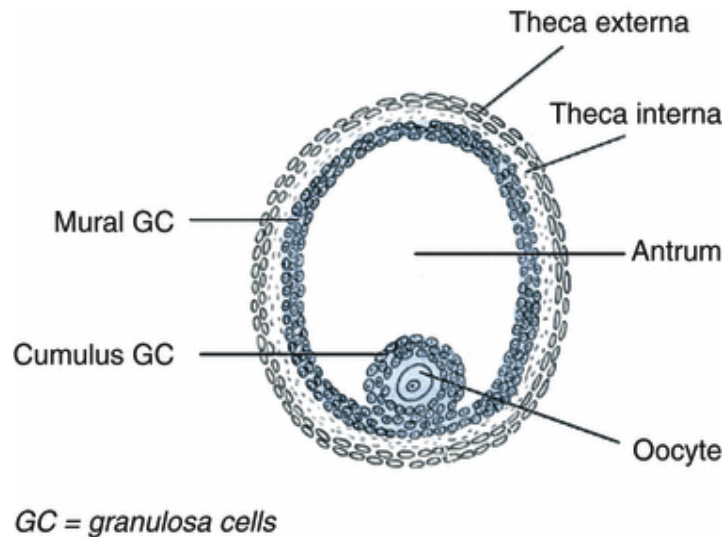
Figure 2.2: Morphology of a graffian follicle (Aerts and Bols 2010).

In mammals, follicular growth and development is commenced and organized by a multifarious interaction between physiological, molecular, and genetic elements. During the course of follicular development many cellular changes occur that regulate cell differentiation and are prerequisite for the provision of oocyte competency. These changes are intervened in a self-motivated approach by autocrine, paracrine and endocrine factors including growth factors and hormones acting to control cell signalling, cell-cell adhesion, and cell-extracellular matrix interactions (Albertini et al. 2001). Follicular growth is furthermost clearly apparent by the growth of the pre-antral follicles comprising primordial, primary and secondary stage follicles. This growth is categorized by the expansion of the follicular basal lamina which is associated by a proliferation in oocyte size and the number of granulosa cells. Once this process is initiated, the thecal cells are also signalled to begin differentiation in preparation for oocyte release and possible fertilization (Albertini et al. 2001). A better thoughtful of the many factors contributing to follicular maturation is vital to understanding ovarian function.

2.2 Regulation of folliculogenesis by paracrine and hormonal factors

The growth, development and maturation of ovarian follicles across the life span of an animal are indispensable courses for efficient reproduction. Germ cell numbers are established during fetal development. Soon afterwards, primordial follicles form and

initiate to change through primordial, primary and preantral stages of development. Folliculogenesis can be categorized into 2 main phases: 1) the gonadotrophin-independent phase and 2) the gonadotrophin dependant phase. The first phase is allied with primordial to preantral follicles and involves growth and differentiation of the oocyte and granulosa cells; pituitary gonadotrophins are not necessary for this growth phase, although they may impact growth. The second phase is associated with antral to ovulatory follicles and hinge largely on the manifestation of circulating levels of follicle stimulating hormone (FSH) and luteinising hormone (LH). This stage marks follicle receptiveness to FSH and LH, comprises the growth and development of granulosa cells and the recruitment of theca cells. The factors that rule oocyte and primordial follicle development during the gonadotrophin-independent phase stay unclear however increasing evidence has advised that growth factors such as TGF-β1 and activins play main roles during these processes (Knight 1996, Rosairo et al. 2008, Webb et al. 2004, Woodruff and Mather 1995).

2.2.1 Gonadotropin-independent phase

Paracrine signalling between the oocyte and its surrounding companion cells is indispensable to the processes of oogenesis and folliculogenesis in mammals. The key paracrine factors which regulate preantral follicle growth include kit ligand (KL), anti-müllerian hormone (AMH), growth differentiation factor-9 (GDF-9) and activins.

2.2.1.1 Kit Ligand and c-Kit in the ovary

Recently, the role and importance of growth factors in ovarian folliculogenesis has been broadly studied in various species. Particularly kit ligand (KL), which was one of the first growth factors identified in the ovarian follicle, plays a crucial role in mammalian oogenesis and folliculogenesis (Thomas and Vanderhyden 2006). Since its identification in 1990, *in vivo* and *in vitro* studies have shown that the functions of this system (Figure 2.3) in the mammalian ovary include the establishment of primordial germ cells (PGCs), activation of primordial follicles, oocyte survival and growth, proliferation of granulosa cells, theca cell recruitment and maintenance of meiotic competence (Hutt et al. 2006, Thomas et al. 2008).
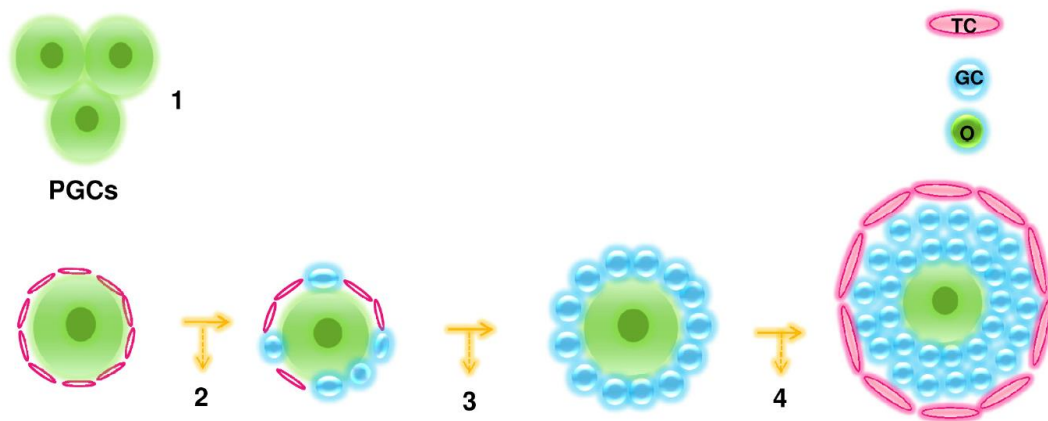
Figure 2.3: Several functions of the KL/c-Kit system in the ovary: 1) Establishment of primordial germ cells; 2) Activation of primordial follicles; 3) Oocyte survival and growth; 4) Proliferation of granulosa cells and recruitment of theca cells. PGCs: primordial germ cells; TC : theca cells; GC: granulosa cells; O: oocyte (Celestino J.J 2009).

Kit Ligand also named as stem cell factor, steel factor, or mast cell growth factor (MCGF), is a locally produced factor that has numerous characters in ovarian function from embryogenesis onward (Driancourt et al. 2000, Yoshida et al. 1997). In follicles, the expression of the mRNA for KL has been studied in the granulosa cells of several species like rat by (Ismail et al. 1996), ovine by (Tisdall et al. 1999), mouse by (Doneda et al. 2002), human by (Hoyer et al. 2005) and caprine by (Silva et al. 2006).

KL affects target cells through binding to its receptor c-Kit, a member of the tyrosine kinase receptor family. During postnatal ovarian development, c-Kit mRNA and protein are present in oocytes of all stages of follicular development. In addition, c-Kit is expressed in interstitial and thecal cells of antral follicles in rodent (Motro and Bernstein 1993), ovine (Clark et al. 1996) and caprine (Silva et al. 2006). KL as the first granulosa cell-derived growth factor can directly stimulate theca cell growth and androstenedione production in the absence of gonadotropins (Parrott and Skinner 1997).

Further i*n vivo* and *in vitro* studies support the knowledge that KIT-KITL signaling is essential for early follicular growth. For example, when newborn mice are injected with

ACK2 (an antibody to KIT, which blocks its interaction with KITL), follicular growth is blocked resulting in an ovary populated with only primordial stage follicles. On the other hand, when neonatal rat ovaries were treated in whole organ culture with recombinant KITL, there is an acceleration of the primordial to primary follicle transition, resulting in an increased number of growing follicles (Parrott and Skinner 1999). Jointly, these studies indicate the KL is crucial for the transition from primordial to primary follicles and the initiation of follicle development (Edson et al. 2009).

2.2.1.2 Anti-Mullerian Hormone

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a glycoprotein hormone belongs to the transforming growth factor-β family of growth and differentiation factors and involved in regulation of folliculogenesis. AMH is note worthy for its properties during sex differentiation. Initially, AMH was identified because of its role in male sex differentiation. AMH is synthesized in the sertoli cells of the fetal testis and induces degeneration of the Müllerian ducts, the anlagen of the female internal reproductive structure, which in the female differentiate into the oviducts, the uterus and the upper part of vagina (Josso et al. 1993, Lee and Donahoe 1993).

 At the time of sex differentiation, no ovarian AMH activity was expressed in the ovary, but few days later after birth AMH mRNA expression has been noticed in the ovarian granulosa cells of rodent, agreeing with the initiation of primary follicle growth (Beppu et al. 2000, Lawson et al. 1999).

Some studies of immunohistochemistry and mRNA *in situ* hybridization in sheep (Bezard et al. 1987) and rodents (Baarends et al. 1995) have exposed the definite expression of AMH in granulosa cells of initially growing, preantral and small antral follicles, while the signs for AMH expression were lost in non-atretic big antral follicles as well as in all atretic follicles. Similarly prior to 36 weeks of gestation no AMH expression in the fetal and neonatal tissues of human was detected (Rajpert-De Meyts et al. 1999). There are also some evidences by (Durlinger et al. 1999) that AMH knockout (AMHKO) mice specify that AMH displays an inhibitory effect on initial follicle

recruitment. Instantly after birth, a normal-sized primordial follicle reservoir has been fashioned in AMHKO animals, but depletion of the primordial follicle pool was enhanced, subsequent in premature termination of the estrus cycle. Additionally, ovarian follicles of AMH null mice treated *in vivo* with exogenous FSH look to be more delicate to FSH compared with follicles from wild type mice, causing in an enlarged number of follicles reaching the ovulatory stage compared with wild type mice (Durlinger et al. 1999, Durlinger et al. 2001) by the involvement of AMH in mouse primordial follicle selection and growing follicle cyclic recruitment. The in vitro explanations are obviously reliable with the concept of AMH as a negative feedback signal of the number of growing follicles that are present in the ovary (Figure 2.4).
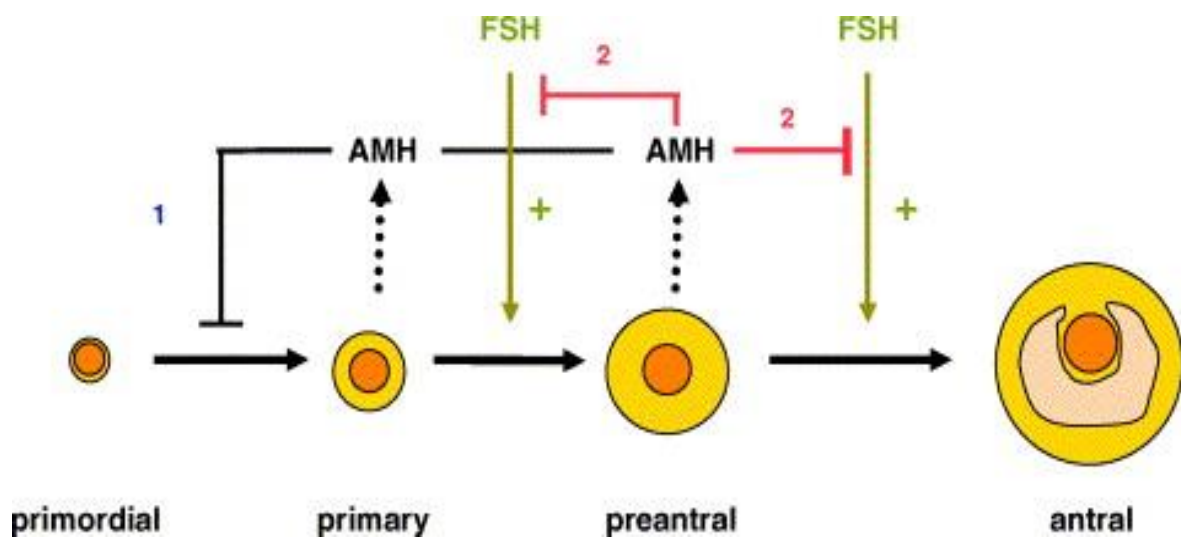
Figure 2.4: Action of AMH in the postnatal mouse ovary. AMH produced by the small growing (primary and preantral) follicles in the postnatal ovary has two sites of action in the postnatal ovary. It inhibits recruitment (1), while it also inhibits the stimulatory effect of FSH on the growth of preantral and small antral follicles (2) (Visser and Themmen 2005).

2.2.1.3 Growth differentiation factor 9

Growth differentiation factor 9 (GDF-9) also known as GDF-9B, is another major factor belonging to the TGF-β family has been shown to be selectively expressed by oocytes throughout ovulation commencing from initial -stage follicles – primary

follicles in rodents and primordial follicles in cattle and sheep (Bodensteiner et al. 1999, Elvin et al. 2000, Jaatinen et al. 1999, McGrath et al. 1995).

GDF-9 has also shown their vital role in mice granulosa cell development, as somatic cells fail to develop beyond the primary stage with a null mutation in the GDF-9 gene thus by causing infertility and viewing its obligation during early ovarian folliculogenesis (Dong et al. 1996), their by designating that oocyte-derived GDF-9 is necessary for endorsing follicle progression. This conclusion is strengthened by the result that GDF-9 treatment *in vivo* (Vitt et al. 2000) or *in vitro* (Nilsson and Skinner 2002) boosts the development of initial to late-stage primary follicles in the rat.

Mice with null mutations in the GDF -9 gene have also shown reduced proliferation and failings in differentiation in granulosa cells, as well as nonappearance of theca layer development (Dong et al. 1996, Elvin et al. 1999) .

Additionallly, lesser granulosa cells experience apoptosis, and KL*,* as well as the peptide inhibin, are melodramatically increased in GDF-9 null mice granulosa cells compared to controls, signifying that GDF-9 inhibits granulosa cell assembly of these growth factors (Elvin et al. 1999).

2.2.1.4 Activins

Activins are growth and differentiation factors belong TGF-β superfamily, govern and arrange multiple physiological processes and are indispensable for the development, growth and functional dependability of most tissues, including the pituitary. Activins produced by pituitary cells roles in union with central, peripheral, and other local factors to affect the function of gonadotropes and to accomplish normal reproductive axis (reviewed in (Welt et al. 2002). Activins, comprising of dimers of two inhibin-β subunits (βA and/or βB subunits) linked by disulfide bonds. Activins, generally formed by granulosa cells in the ovary, are requisite for the ovarian development and for reproductive functions, as mice with genetic deletions of activin constituents are infertile (Pangas et al. 2007). Mammalian ovaries express both types of activin receptors (ALK4 and ActRIIA/B) and in the rat, both βA and βB subunits are highly expressed in

granulosa cells of developing follicles, while theca cells express tiny or no β subunit mRNAs (Drummond et al. 1996, Drummond et al. 2002, Meunier et al. 1988, Roberts et al. 1993). In divergence, the oocyte does not perform to express either subunit exhibits both type I and type II receptors (Sidis et al. 1998). During folliculogenesis, the differentiation status of granulosa cells determines the response to activins (Findlay 1993). Activin is produced by granulosa cells of primary to tertiary follicles of the ovary (Rabinovici et al. 1992, Zhao et al. 2001). Activin has been found to promote the release of FSH from the anterior pituitary (Katayama et al. 1990). In the ovary, it displays an atmosphere in endorsing aromatase activity, antral cavity formation and granulosa cell proliferation (Findlay 1993, Mizunuma et al. 1999, Zhao et al. 2001). Furthermore, activin is provoked by follistatin and by inhibin binding to its receptors (Lewis et al. 2000).
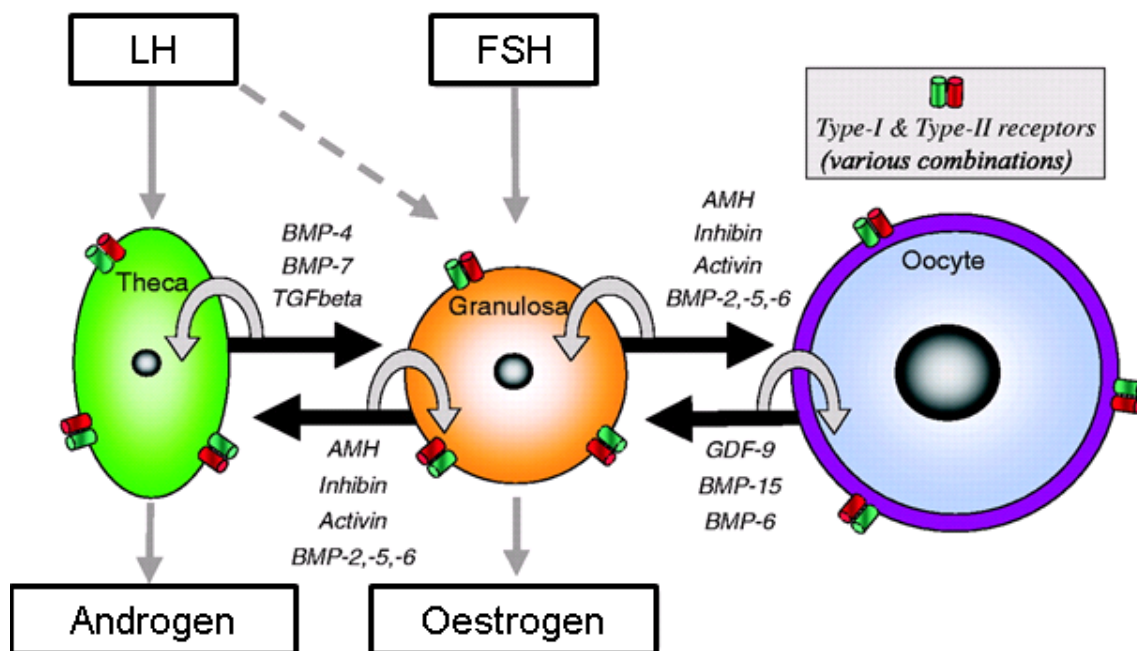
Figure 2.5: Members of the TGF-β superfamily feature prominently amongst the growing list of extracellular ligands implicated in the bi-directional communication between theca and granulosa cells, and granulosa cells and oocyte. Both autocrine (thick grey arrows) and paracrine (thick black arrows) signalling events are likely, depending on the expression of appropriate combinations of type-I and type-II receptors on the cell surface (Knight and Glister 2006).

2.2.2 Gonadotropin-dependent phase

During each estrous cycle in cattle the advance stages of antral follicle development are characterized by two or three follicular waves. These follicular waves seem to be essential and have been predicted prior to puberty and through other stages of anestrus. A cluster of follicles are recruited in each wave of growth in cattle and continue to grow to approximately 6 to 8 mm in diameter. In mono-ovulatory species, such as cattle the fate of mostly follicles is atresia only one follicle from the cohort of identical follicles is selected for continued growth and becomes dominant (Adams 1999, Ireland et al. 2000). Antral follicle is under gonadotropic control when it attains growth atleast 2 mm in diameter (Campbell et al. 1995) as verified by treatment of hypogonadotropic cattle with bovine follicular fluid and estradiol (Figure 2.6).
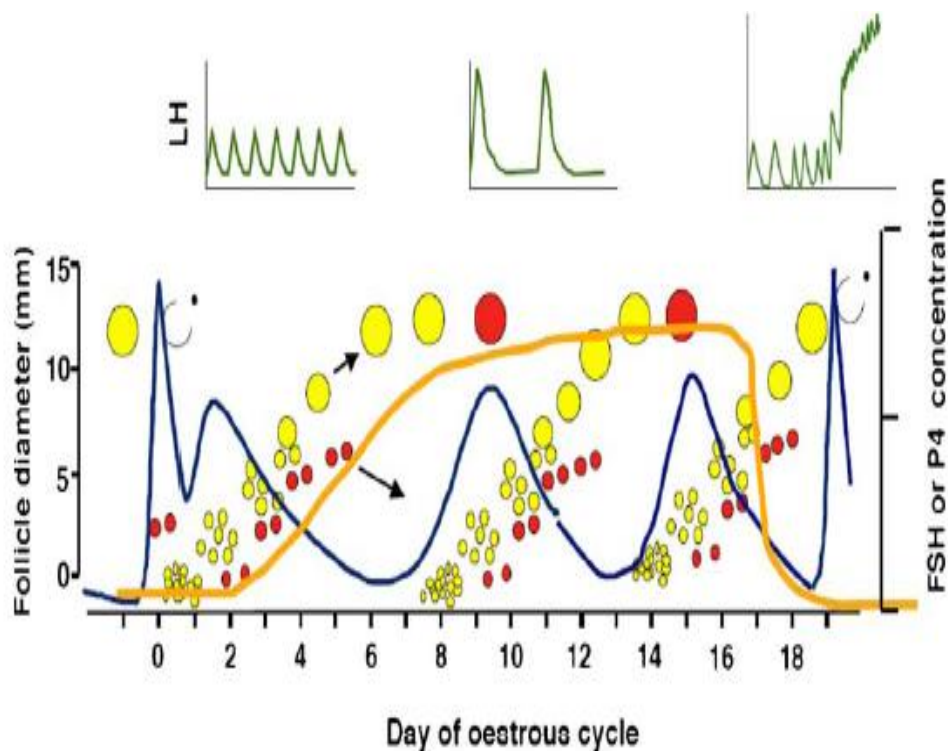
Figure 2.6: Schematic depiction of the pattern of secretion of follicle-stimulating hormone (FSH; blue line), luteinizing hormone (LH; green lines), and progesterone (P4; orange line); and the pattern of growth of ovarian follicles during the estrous cycle in cattle. Healthy growing follicles are shaded in yellow, atretic follicles are shaded red. A surge in LH and FSH concentrations occurs at the onset of estrus and induces ovulation. The pattern of secretion of LH pulses

during an 8-h window early in the luteal phase (greater frequency, lesser amplitude), the mid-luteal phase (lesser frequency, lesser amplitude) and the follicular phase (high frequency, building to the surge) is indicated in the inserts in the top panel (Campbell et al. 2003).

2.2.3 Gonadotropin regulation of follicular maturation during the estrous cycle

Cattle are polyestrous animals. Estrous cycle is the period from one estrus to the next estrus. For the cow and heifer, this period averages 21 days, with a typical range of 18 to 24 days in length. The estrous cycle is regulated by the hormones of the hypothalamus (gonadotropin-releasing hormone; GnRH), the anterior pituitary (follicle-stimulating hormone; FSH and luteinizing hormone; LH), the ovaries (progesterone; P4, estradiol; E2 and inhibins) and the uterus (prostaglandin F2α; PGF). By mechanisms of positive and negative feedback these hormones govern the estrous cycle of cattle (Roche 1996).

The gonadotropin dependent regulation of folliculogenesis is initiated by gonadotropin-releasing hormone (GnRH), which is a 10 amino acid peptide hormone secreted from neurons in the hypothalamus (Reece 2004). Isolation and chemical characterization of gonadotropin-releasing hormone were first discovered in 1971 from the hypothalamus of pigs. Later the translation from basic discovery to clinical usefulness with numerous signs was swift in human medicine (Conn and Crowley 1991). Synthesis occurs in neurons found in the ventral portion of the hypothalamus, specifically in the arcuate nucleus, as well as in the preoptic nucleus of the anterior portion of the hypothalamus. Many neurotransmitters and neuropeptides are involved in the regulation of GnRH secretion (Hadley 1999). After its release, GnRH is transported from the hypothalamus to the pituitary gland via the hypophyseal portal blood system and binds to its G-protein coupled receptor on the cell surface of the gonadotroph cells (Kakar et al. 1993, Moenter et al. 1992).

This binding release intracellular calcium which activates intermediaries in the mitogen activated protein kinase (MAPK) signaling pathway concluding in the release of FSH and LH from storage sections in the cytoplasm. The release of these gonadotropins is

differentially regulated by GnRH and is hooked on the area of the hypothalamus that is stimulated by neural depolarization to release GnRH. Upon stimulation of the arcuate nucleus, the hypothalamus mediates tonic or basal secretion of LH. When the preoptic nucleus is stimulated, there is a surge release of both FSH and LH. Finally, if there is a surge release of GnRH prior to ovulation, this mediates a secretory surge of LH, with less FSH (Reece 2004).

In the course of follicular development, selected follicles experience an irreversible progression of oocyte growth, granulosa cell proliferation, and theca cell differentiation. These resulting follicles become immensely responsive to gonadotropins secreted from the anterior pituitary gland with the consequence of high levels of FSH signal and thus by endorsing the development to the pre-ovulatory stage. This period is characterized by an increased capacity to synthesize estradiol, formation of the large fluid filled antrum, and the expression of the LH receptor. These phenotypic changes permit for an increased level of circulating estradiol. When estradiol attains the peak, it acts on the hypothalamic-pituitary axis leading to an increased release of LH, known as the LH surge (Couse et al. 2005). Rupturing of follicle and ovulation occurs by the expression of LH receptor in healthy pre-ovulatory follicles. After the occurrence of this, the granulosa cells then experience terminal differentiation forming the corpus luteum. During the course of follicular development the gonadotropin dependent mechanisms occur over the signalling cascades prompting from FSH and LH expression. In demands for these endocrine hormones to complete their obligatory functions, the genes encoding their receptors must also be expressed (Couse et al. 2005).

2.2.4 Gonadotropin regulation of final maturation of the preovulatory follicle and selection

The gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) are protein hormones (Figure 2.7) secreted by the pituitary gland, comprising of a common α-subunit and a hormone-specific   β-subunit. Transcriptional regulation of gonadotropin subunits involves basal gene expression as well. Further, synthesis and secretion of FSH and LH are both positively and negatively regulated by steroids and gonadal peptides reviewed by (Brown and McNeilly 1999).

For each wave of growth comprise emergence, selection and dominance followed by either atresia or ovulation of the DF. Both FSH and LH have a noticeable role in ovarian follicle development. Given that follicles are involved in the positive and negative feedback mechanisms of the hypothalamic–pituitary–gonadal (HPG) axis (estradiol and inhibins), these hormones have a leading role in the regulation of the estrous cycle of cattle. The commencement of gonadotropin dependent follicle development is characterized by the emergence of a follicle cohort normally consisting of 5–20 follicles ≥5mm and is associated with a transient increase in FSH concentration (Adams et al. 1992, Sunderland et al. 1994). This results the beginning of dependency of follicle growth on FSH (Adams et al. 1992) with FSH receptors (FSH-R) localized within the granulosa cells of the follicles by day 3 of the follicle wave (Camp et al. 1991, Evans and Fortune 1997). This permits FSH to perform its required down stream signalling effects including endorsing cellular growth and proliferation (Richards 1994, Richards et al. 1998).Transient increase in FSH concentrations further encourages an increase in aromatase enzyme activity (P450arom; CYP19), in the granulosa cells of ovarian follicles, which transforms androgen to estrogen (Hillier 1994). The diameter of the dominant follicle increases by its selection from the cohort of follicles and is accepted as the largest healthy follicle in the cohort (Gougeon and Lefevre 1983). Follicular fluid, estradiol and inhibin concentartions are elevated by the increase of dominant follicles diameter (Hillier 1994).
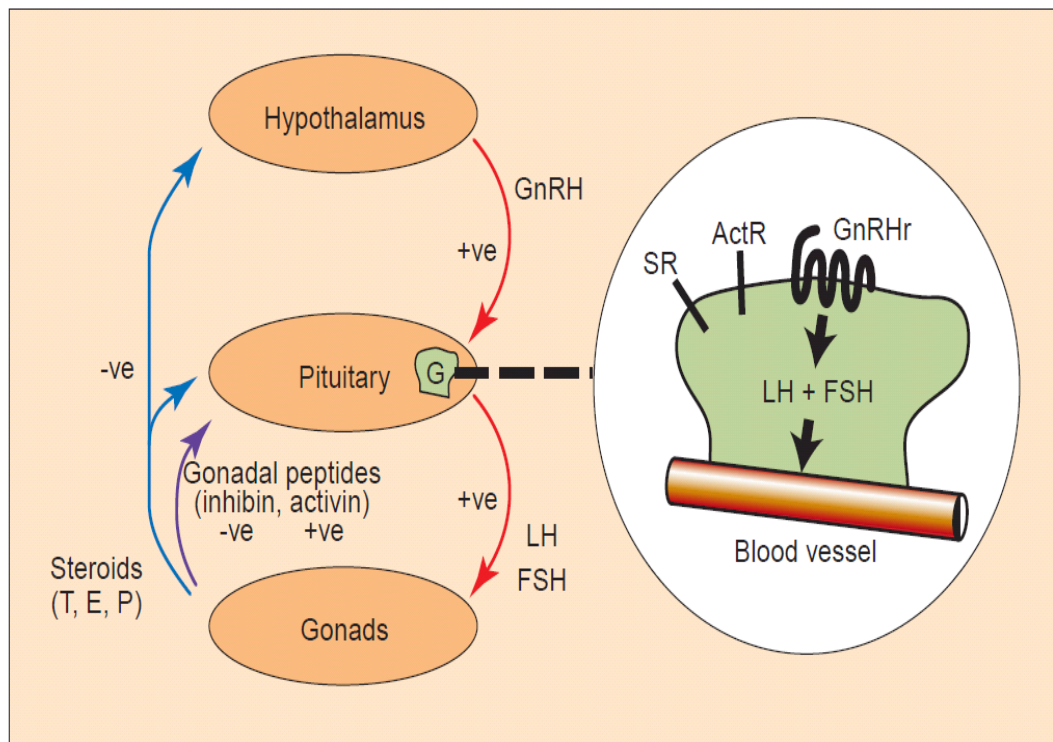
Figure 2.7: Schematic presentation of the hypothalamic–pituitary–gonadal axis showing positive and negative regulators of gonadotrophin hormone gene expression. Gonadotrophin-releasing hormone (GnRH) synthesized in and released from the hypothalamus binds to GnRH receptor (GnRHr), a seven transmembrane G-protein-coupled receptor located on the surface of the gonadotroph. The binding of GnRH to the GnRHr triggers the synthesis, and ultimately the secretion, of LH and FSH into the vascular system. A stylized steroid receptor (SR) is also indicated on the gonadotroph cell, this represents androgen, oestrogen and progesterone receptor. Testosterone (T), oestrogen (E) and progesterone (P) negatively regulate gonadotrophin synthesis directly at the pituitary and via downregulation of hypothalamic GnRH secretion. The gonadal peptides, inhibin and activin, have opposing roles in regulating gonadotrophin synthesis and seem to regulate production of FSH. Activin transactivates its own receptor (activin receptor; ActR) but it is yet to be determined whether inhibin signals through the same or an unidentified receptor (Brown and McNeilly 1999).

Suppression of FSH happens by the appearance of dominance, when the dominat follicle attains the diameter of 9mm, there by stopping further follicle wave emergence

till the dominant follicle either experiences atresia or ovulated. The upsurge in estradiol concentrations in performance with inhibin are the main endocrine signals that suppress FSH concentrations from the anterior pituitary gland via negative feedback reducing FSH to basal concentrations (Ginther et al. 2000b, Ginther et al. 2000a, Sunderland et al. 1994). The selected dominant follicles shows progressively response to LH (Ginther et al. 2000a) and continues growth in the aspect of decreasing FSH concentrations. Regardless of the stage of the estrous cycle through which follicles developed, the shift from FSH (Adams et al. 1992) to LH dependency (Kulick et al. 1999) is spread through the presence of LH receptors (LH-R) on the granulosa cells (Xu et al. 1995). LH-R is confined to the theca and granulosa cells of healthy follicles, at different stages of follicle development (Camp et al. 1991).

As the follicle accomplishes the growth, the theca cell LH-R increases and LH-R is attained by the granulosa cells of the follicle undergoing selection to become the dominant follicle (Bao et al. 1997, Braw-Tal and Roth 2005, Xu et al. 1995). In addition, evidence suggests transient increases in circulating LH concentrations that occur at or around the time of follicle selection (Ginther et al. 2003), permits the dominant follicle to continue E2 production and grows in the face of declining FSH concentrations (Ireland and Roche 1983). During the early luteal phase of the follicular development slighter amplitude and greater frequency (20–30 pulses/24 h) LH pulses occur, while in the mid-luteal period LH pulses are of larger amplitude and lesser frequency (6–8 pulses/24 h) both of which are of inadequate amplitude and frequency for final maturation and successive ovulation of the dominant follicle (Rahe et al. 1980).

Dominant follicles are produced during the luteal phase of the estrous cycle go through atresia, E2 and inhibin production decreases, and removes this negative feedback block to the hypothalamus/pituitary, FSH secretion can raise and a new follicle wave emerges. The production of high concentrations of estradiol is a defining characteristic of the dominant follicle (Ireland and Roche 1982, Ireland and Roche 1983) and prior to visible differences in follicle diameter; the supposed dominant follicle has larger follicular fluid concentrations of estradiol compared with other follicles in its cohort (Fortune 1994, Mihm et al. 2000, Sunderland et al. 1994). The synthesis of estradiol is reliant on the production of androgens in the theca cells and subsequent aromatization of these

androgens to estrogens in the granulosa cells known as the two cell/two gondatropin model. (Fortune and Quirk 1988). Production of estradiol from growing follicles is dependent on sufficient LH pulse frequency (Crowe et al. 2001a, Crowe et al. 2001b). The binding of LH to its receptors in the theca cells drives the conversion of cholesterol to testosterone through a series of catalytic reactions. Testosterone, once produced in the theca cells, diffuses out into the granulosa cells where it is converted to estrogens by the aromatase enzyme (Dorrington et al. 1975). At the follicular phase of the estrous cycle, when the progesterone concentrations are at basal level, the vast concentration of estradiol produced by the pre-ovulatory dominant follicles induces a GnRH surge from the hypothalamus. The subsequent LH surge is of adequate amplitude and frequency to stimulate final maturation and ovulation of the dominant follicle (Sunderland et al. 1994).

2.3 Genetic regulation of folliculogenesis

Recently two developmental keys have preciously increased understanding on the molecular control of follicular and luteal development. One is the arrival and submission of genomic technologies to study ovarian function leading to the identification of hundreds of novel genes   putatively elaborate in ovarian processes in different species (Agca et al. 2006, Bogan et al. 2009, Espey and Richards 2002, Evans et al. 2004, Hamel et al. 2008). The second foremost development has been the discovery and classification of animal microRNAs (Ambros 2004).

During the course of follicular development, a compact, coordinated transcriptional activity is involved. Dynamic progresses have been made in understanding transcriptional gene regulation during follicular and luteal development (Liu et al. 2009, Patel et al. 2009, Pisarska et al. 2011, Wu and Wiltbank 2002). Follicular development entails the appropriate expression of multiple genes at different follicular developmental stages. Several studies in oocyte biology on natural altered in sheep (Fabre et al. 2006) and on mutant mouse models revealed that the expression of diverse oocyte-specific genes is crucial during early folliculogenesis in a stage specific expression pattern (Choi and Rajkovic 2006). Genes such as *Figla*, *Nobox*, *Pten*, *Foxo3A* and nerve growth factor are convoluted in the development of primordial follicles, whereas genes such as βFGF,

GDF-9 and BMP 4 are involved in the transformation from primordial to primary follicles. Some other genes like BMP15 are not uttered in the oocyte until the primary follicle stage and are involved in the transition from primary to secondary follicles, as presented in sheep reviewed by (Monget et al. 2012). Studies on large scale expression have been directed in multiple species. Two studies in specific identified the explicit transcriptome of human oocytes in the quiescent state. These studies tinted enhanced functions such as transcription, RNA post transcriptional modifications and molecular mechanisms in human oocytes (Bonnet et al. 2011, Grondahl et al. 2013, Markholt et al. 2012).

However, little is known about the post-transcriptional mechanisms involvement. Assuming the dynamic nature of the mammalian ovary, miRNAs are predicted to play crucial roles in the regulation of ovarian function. Spatial and temporal information on miRNA expression patterns would assist the identification of the regulatory miRNA networks.

2.4 MicroRNAs

MicroRNAs (miRNAs) represent a thrilling discovery in the field of biology in recent years. MicroRNAs (miRNAs) control gene expression in animals, plants, and unicellular eukaryotes by endorsing degradation or inhibiting translation of target mRNAs. miRNA expression is often tissue specific and developmentally regulated both at transcriptional and posttranscriptional level (Siomi and Siomi 2010). MicroRNAs (miRNAs) short , non-coding RNAs,19-25 nucleotides in length, are key regulators of gene expression that have significant roles in a wide range of biological processes, including cell division, cell proliferation and differentiation, apoptosis and metabolism while some robust evidence suggests the involvement of miRNAs pathogenesis in human diseases such as cancer and metabolic disorders (Bartel 2009, Carthew and Sontheimer 2009, Krol et al. 2010). Functionally significant small non-coding RNAs (lin-4) was firstly identified in nematodes in 1993 (Lee et al. 1993, Wightman et al. 1993) and was consider as worm specific. Later on in 2001, the researchers took keen interest and by recognizing the function of this family of RNA that includes miRNA and

identified that their worth was not limited to lower-order organisms (Lau et al. 2001, Lee and Ambros 2001).

2.5 Function of microRNAs

To accomplish the regulatory functions, miRNAs are incorporated into ribonucleoprotein (RNP) complexes known as miRNA-induced silencing complexes (miRISCs) or micro-ribonucleoprotein (mi-RNPs). RISC and miRNP complexes are also linked functionally. The mammalian let-7 and other miRNPs can function as RISC nucleases, capable to cleave RNAs that bear sequences perfectly complementary to miRNAs. Argonaute family proteins are the major machineries of these complexes. AGO1 to AGO4 are the four AGO proteins in mammals (Hutvagner and Zamore 2002, Liu et al. 2004, Meister et al. 2004, Pillai et al. 2004). MiRNAs guide the Argonaute (AGO) proteins to the target mRNAs by their perfect or near-perfect complementary sequence to the 3'UTR of the mRNA, resulting in silencing of the gene expression reviewed by (Bushati and Cohen 2007).

In plant, miRNAs varying from animal miRNAs on the basis of their biogenesis and method of target regulation. Mostly plant miRNAs bind to their mRNA targets with perfect or nearly perfect complementary sequence, and trigger the endonucleolytic mRNA cleavage through the RNAi-like mechanism (Vaucheret 2006). While In animals, miRNAs usually bind base pair with their target genes imperfectly. The seed region (position 2 to 8) of miRNAs is most important for target recognition and silencing (Doench et al. 2003, Lewis et al. 2005). It has been suggested that adjacent and perfect base pairing in the 5' proximal seed region of the miRNAs and the 3'UTR of its target mRNAs is strictly required for the interaction (Eulalio et al. 2008, Filipowicz et al. 2008). Some research show that both the 3'UTR and 5'UTR (Doench et al. 2003, Zeng et al. 2002) and even the mRNA-coding regions (Forman et al. 2008). A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence) may comprise the potential binding sites of miRNAs. Usually, the number of miRNA binding site imitates the degree of translation repression. It is normally believed that miRNAs regulate gene expression by

translational repression or cause deadenylation and degradation of mRNA (Eulalio et al. 2008, Filipowicz et al. 2008, Nilsen 2007).

miRNA target prediction is a lively arena with increasing thoughtfulness on advance of new analysis tools (Peterson et al. 2014). Computational prediction and validation of miRNA targets are essential for revealing the comprehensive functions of miRNAs in gene regulation. Computational prediction of miRNA targets is much more puzzling in animals than in plants, because animal miRNAs mostly shows imperfect base-pairing with their target sites, which stance a trail for the precise computational identification of animal miRNA targets (Brennecke et al. 2005, Lai 2004, Lewis et al. 2005). By divergence, plants miRNAs bind their targets by entirely or nearly entirely complementarity, so target identification is forthright (Jones-Rhoades and Bartel 2004).

First computational miRNA target prediction programs concentrated on the fruit fly (Enright et al. 2003, Rajewsky and Socci 2004, Stark et al. 2003) and mammalian (Kiriakidou et al. 2004, Lewis et al. 2003) genome was independently developed by several research groups. Significant advances have been made in computational target prediction (Sethupathy et al. 2006) and many diverse algorithms have been developed for the prediction of miRNA targets such as TargetScan (Grimson et al. 2007) , PicTar (Krek et al. 2005), miRanda (Enright et al. 2003), PITA (Kertesz et al. 2007), DIANA-microT (Kiriakidou et al. 2004), RNAhybrid (Rehmsmeier et al. 2004), microInspector (Rusinov et al. 2005), MovingTargets (Burgler and Macdonald 2005), rna22 (Miranda et al. 2006), NBmiRTar (Yousef et al. 2007) and Nucleus (Rajewsky and Socci 2004). These mentioned algorithms make predictions mainly based on various significant features of miRNA-target nucleotide sequence interaction. Although different algorithms utilize different sets of features, a few important features including seed match, free energy, sequence conservation and site accessibility are the four common features for miRNA target prediction tools and are briefly described as:

2.5.1 Seed Match

The first 2–8 position nucleotides starting at the 5′ end and totaling toward the 3′ end is termed as seed sequence of a miRNA (Lewis et al. 2003). Mostly for tools, a seed match

is a Watson-Crick (WC) match between a miRNA and its target in the seed sequence. A WC match between a miRNA and mRNA nucleotide occurs when adenosine (A) pairs with uracil (U) and guanine (G) pairs with cytosine (C). A perfect seed match between the miRNA and the mRNA target has no gaps in alignment within the WC matching. 6mer, 7mer-m8, 7mer-A1 and 8mer are the main types of seed matches which could be considered depending on the algorithm (Brennecke et al. 2005, Krek et al. 2005, Lewis et al. 2003, Lewis et al. 2005).

2.5.2 Conservation

Conservation mentions to the upkeep of a sequence across species. Conservation analysis may emphasis on regions in the 3′ UTR, the 5′ UTR and the miRNA seed region, or any combination of the three. Generally, there is higher conservation in the miRNA seed region than in the non-seed region (Lewis et al. 2003). In a small fraction of miRNA:mRNA target interactions, there is conserved pairing at the 3′ end of the miRNA which can pay compensation for seed mismatches, and these sites are called 3′ compensatory sites (Friedman et al. 2009). In the milieu of predicting miRNA targets in 3′ UTRs, conservation analysis may provide evidence that a predicted miRNA target is functional because it is being selected for and furthermore there is accumulative interest in conservation analysis of the genomic regions flanking the miRNA gene and miRNA target genes. As examples, conservation analysis has been functional to the promoter regions of miRNAs and their target genes (Fujiwara and Yada 2013), and to the co-localization of independently transcribed miRNAs and flanking protein coding genes (Ohler et al. 2004).

2.5.3 Free Energy

To measure the stability of a biological system the term free energy or Gibs free energy can be used. Free energy states to the minimum free energy and shows how strong the binding of a miRNA:mRNA is. Free energy is usually considered as a negative real value for which kcal/mol unit is used. True binding could be considered by the stable binding structure in the result of low free energy (Yue et al. 2009).

2.5.4 Site Accessibility

Site accessibility is a degree of the comfort with which a miRNA can pinpoint and hybridize with an mRNA target. In the result of transcription, mRNA adopts a secondary structure which can hinder with a miRNA's ability to bind to a target site (Mahen et al. 2010). Two step development is involved in the hybridization of miRNA:mRNA, firstly miRNA binds to a short reachable region of the mRNA and secondly miRNA completes binding to a target when the mRNA secondary structure unfolds (Long et al. 2007). Consequently, the possibility that mRNA is the target of a miRNA, the projected quantity of energy to make a site accessible to a miRNA can be estimated.

Inorder to avoid some limitations in the existing algorithms a new tool named as miREcords for animal miRNA:mRNA interactions, was established consisting of two components the validated target component and the predicted target component. The predicted targets component of miRecords is an integration of predicted miRNA targets formed by 11 conventional miRNA target prediction databases. As the maximum complete integration of predicted miRNA targets, it is predictable to provide significant help to researchers exploring new miRNA targets (Xiao et al. 2009).

Studies conducted on the miRNA target predictions and microyrray put forward that an individual miRNA could regulate multiple genes post-transcriptionally (Lim et al. 2005). Furthermore, an messenger RNA can be regulated by a set of miRNAs, in a cooperative manner, in which the translational repression of an individual target gene may only be operative by interaction of different miRNAs (Krek et al. 2005).

A large set of diverse miRNAs are communicated in different types of cells and tissues, thus by making a multifarious regulatory network between miRNAs and their target genes to regulate the functions of the specific cells and tissues. Indeed, miRNAs play their role in various biological processes including cell proliferation, development, apoptosis, organogenesis, tumorigenesis, haematopoiesis (Ambros 2004, Baehrecke 2003, Hwang and Mendell 2007). The following sections give a brief review of the literature on function of miRNAs in reproduction.

2.6 MiRNAs in cell cycle regulation

Gametogenesis and development comprises proliferation of the germ cells and their accompanying somatic cells. miRNAs are involved in the regulation of various biological processes, such as cell proliferation (Dong et al. 2010), apoptosis (Chan et al. 2005) and differentiation (Goljanek-Whysall et al. 2012). Du and coworkers (Du et al. 2014) reported that miR-545 inhibited cell proliferation *in vitro* and *in vivo* and also invented that miR-545 caused cell cycle arrest at the G0/G1 phase and induced cell apoptosis in lung cancer cells by targeting cyclin D1 and CDK4 genes. Results showed that miR-503 blocked the proliferation of hepatocellular carcinoma (HCC) cells by introduction of G1 phase arrest through Rb-E2F signaling pathways. (Xiao et al. 2013). Recent study verified that miR-107 is downregulated in breast cancer samples related with normal tissue and may perform a crucial role in breast cancer progression. Abundance of miR-107 expression causes cellular growth inhibition, migration and G1 phase arrest by targeting CDK8 (Li et al. 2014).

First time the role of miRNAs in apoptosis was stated in 2003 when miR-14 and bantam were exposed to regulate cell death in Drosophilia (Brennecke et al. 2003, Xu et al. 2003). Bantam, formerly recognized as a gene that causes overgrowth of wing and eye tissue (Hipfner et al. 2002), was validated to be a miRNA and was exposed to endorse proliferation and prevent apoptosis by targeting the proapoptotic gene hid1(Brennecke et al. 2003). On the other hand miR-14, recognized in a presentation for genes that modify cell death in the eye of Drosophila, was revealed to inhibit apoptosis by regulating the effector caspase Drice (Xu et al. 2003).

Another study identified targets and biological processes generated by the transfection of human cells with miRNAs. Cellular growth and cell cycle progression was negatively regulated by mir-16 family miRNAs, thus by showing that over-expression of mir-16 family miRNAs induced G0/G1 capture in cultured human tumour cells, and that domination of many identified targets of mir-16 family miRNAs induced G0/G1 accumulation. CDK6, one of the identified targets and was a cell cycle kinase could be triggered by binding to D-type cyclins during early G1 phase (Linsley et al. 2007). Cyclin-dependent kinase inhibitor p21/CDKN1A being a direct target of miR-106b and

it shut up shows a major role in miR-106b-triggered cell cycle phenotypes (Ivanovska et al. 2008).

2.7 MiRNAs in development

MicroRNA expression may contribute to developmental robustness because of the fact that microRNAs are the main post-transcriptional regulators of developmental gene expression and its importance to alter the expression of several target genes (Karp et al. 2011). Role of miRNAs in the regulation of development are evidenced by the study that some of the miRNAs in the embryonic stem cells appear in the cell specific pattern by inhibiting as ES cells differentiate into embryoid bodies and are down regulated in adult mouse organs (Houbaviy et al. 2003). Another study showed that miRNAs can play key role in a spatial temporal expression pattern in zebra fish embryonic development. The results showed that the majority of the miRNAs what were used in the study were down regulated in early development while highly expressed during segmentation and later stages (Wienholds et al. 2005). Similarly, mutation in the gene dicer1 in mice caused the depleted stem cells in embryos thus by causing death early in development (Bernstein et al. 2003). With the advancement of development, a diverse set of miRNAs were involved from oocyte stage to tadpole stage in *Xenopus laevis*, thus showing the stage-specific temporal expression of miRNAs during development (Watanabe et al. 2005).

2.8 MiRNAs in female reproduction

For fruitful reproduction, appropriate development and function of the female reproductive tract are necessary stages. Directives of the distinguished functions of the organs that constitute the female reproductive tract are well documented to happen at various levels including transcription, translation, and posttranslational modifications. Posttranscriptional gene regulation mediated by miRNA has ascended as a central mechanism governing normal tissue development and function. MicroRNA in mammalian cells are vital post-transcriptional regulators and function by modulating translation or degradation of their target mRNA (Nothnick 2012). In order to explore the role of miRNAs in reproductive tissues, widespread miRNA expression profiling has

been measured indicating the presence of large numbers of miRNA within reproductive tissues and cells. Thousands of miRNA have now been recognized in a variety of organisms. (Fiedler et al. 2008, Hassan et al. 2010, Lin et al. 2013, Nagaraja et al. 2008, Tesfaye et al. 2009, Yao et al. 2009). In this part the discussion is being made on the role of miRNAs in folliculogenesis.

2.9 MiRNAs in ovary

Recently it has begun to discover the regulatory role of small RNA molecules in ovarian cells, recognizing their impact on gonadal development, proliferation, apoptosis, steroidogenesis, ovulation and corpus luteum progression. This emergent area of research has pushed and reformed our understanding on how ovarian function is regulated. MiRNAs are expressed during different stages of ovarian advancement and are expected to play significant roles during gonadal development and folliculogenesis. Here, we discuss the current intimation and role of miRNA involvement in ovarian development and function.

Folliculogenesis is the outcome of series of complex and synchronized processes, which comprise morphological and functional alterations in different types of follicular cells and their communications. Sequential recruitment of growing follicles, atresia, ovulation, and luteal tissue development and regression are dynamically regulated events that recapped on a cyclical basis within the ovary. These processes are under control of closely harmonized endocrine and paracrine factors to grow a number of ovulatory follicles that are species and breed dependent (Hunter et al. 2004). These actions comprise intense changes in cellular growth, angiogenesis, steroidogenesis, cell cycle status, and apoptosis and are tightly regulated at the endocrine and tissue level. These steady state cyclic alterations are organized by tightly regulated expression and interaction of a assembly of genes in different compartments of the ovary (oocyte, mural granulosa, cumulus granulosa cells and theca cells) to enable oocyte development (Bonnet et al. 2008). Defects in regulatory control can lead to ovarian failure such as premature ovarian failure (POF) due to disruption of folliculogenesis, block of ovulation, and corpus luteum (CL) insufficiency (Carletti and Christenson 2009).

It is apparent that germ cells of the ovary and the supporting granulosa cells of the follicles, tickle a discourse that is important for granulosa cells growth and differentiation and oocyte growth, maturation and ovulation. Inside the ovary, granulosa cells are considered as an important location for the production of estrogen which is utilized for local use as well as providing endocrine signaling to other tissues (Nelson and Bulun 2001).

The luteinizing hormone (LH) surge prompts multiple firmly connected ovarian processes, including steroidegenesis, maturation of oocyte and ovulation (Jamnongjit et al. 2005). Like wise many roles of miRNA in folliculogenesis, current studies have provided demonstration for the important function of Dicer, which is an RNase III enzyme accountable for miRNA processing obligatory for oogenesis regulation. The importance of small RNA in the ovary is cleared by the proof of *Dicer1* conditional knockout (cKO) mouse ovarian tissue , in which *Dicer1* is removed specifically in follicular granulosa cells which led to an enlarged primordial follicle pool bestowed , enhanced early follicle recruitment and extra degenerate follicles in the cKO ovaries. This study showed that dicer 1 plays a crucial role in follicular cell development through the differential regulation of mRNA expression (Lei et al. 2010).

In a study by deleting Dicer in growing oocytes in mouse showed incomplete meiosis, which illustrates that oocytes lacking Dicer arrest in meiosis I with numerous disarrayed spindles and chromosomal congression defects. Additionally, they proposed that miRNAs might be elaborate in the degradation of many maternal transcripts essential to completion of meiotic maturation (Murchison et al. 2007). Matching result attained from mouse injected with Dicer siRNA showed significant reduction in maturation rate, increased uncharacteristic chromosomal and spindle organization, and reduced transcripts of Dicer miRNAs, spindle formation proteins and spindle check points genes, thus, specified that Dicer and miRNA appear to have an important role during oogenesis and meiosis accomplishment (Liu et al. 2010).

In a study of differentially expressed plasma microRNAs in premature ovarian failure patients they found regulatory effects on proliferation and apoptosis of granulosa cells by effecting different signaling pathways including the AKT signaling pathway, steroid

hormone receptor signaling pathways. They concluded that mir-23a may show an important role in regulating apoptosis via decreasing the levels of X-linked inhibitor of apoptosis protein (XIAP) and increasing caspase-3 cleavage (Yang et al. 2012).

3 Material and methods

3.1 Materials

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

3.1.1 Chemicals, kits, biological and other materials

Chemicals, kits, biological and other materials	Manufacturer/Supplier
10x PCR buffer	Genaxxon bioscience GmbH, Germany
5x First-Strand buffer	Invitrogen Life Technologies, Karlsruhe, Germany
Acetic acid	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
AllPrep® DNA/RNA Micro Kit	QIAGEN, Hilden, Germany
Ammonium acetate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Calcium chloride dihydrate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Chloroform	Roth , Karlsruhe, Germany
Citric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe, Germany
dNTPs	Roth , Karlsruhe, Germany
Dithiothreitol (DTT)	Invitrogen Life Technologies,

	Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylenediaminetetra acetic acid (EDTA)	Roth , Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Gentamycin sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hemi-calcium lactate	Promega, WI, USA
Hyaluronidase	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hydrochloric acid	Roth, Karlsruhe, Germany
Hydroxylamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Igepal	Roth, Karlsruhe, Germany
Ionomycin	Sigma-Aldrich, Chemie GmbH, Munich, Germany
Isopropyl -D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
L-Glutamine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
MicroRNA LNA^{TM} PCR primer set	Exiqon, Vedbaek, Denmark
MiRNeasy® mini kit	QIAGEN, Hilden, Germany
Nuclease free water	Exiqon, Vedbaek, Denmark
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
QIAquick PCR Purification Kit	QIAGEN, Hilden, Germany
Random primer	Promega, WI, USA

Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA
RNA later	Sigma-Aldrich, MI, USA
Ribo-nuclease inhibitor (Rnasin)	Promega, WI, USA
RNA 6000 Nano LabChip® kit	Agilent Technologies Inc, CA, USA
Roti PAGE gradient (4-20%)	Carl Roth GmbH, Karlsruhe, Germany
Rotiphorese Gel 30 (37, 5:1)	Carl Roth GmbH, Karlsruhe, Germany
RQ1 Rnase-free Dnase	Promega, WI, USA
Sample Loading Solution (SLS)	Beckman Coulter, Krefeld, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium bicarbonate	Sigma-Aldrich chemie, Steinheim, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Superscript II reverse transcriptase	Invitrogen, CA, USA
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific, Rockford, U.S.A.
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
Universal cDNA synthesis kit II	Exiqon, Vedbaek, Denmark
ExiLENT SYBR®GREEN master mix	Exiqon, Vedbaek, Denmark

3.1.2 Reagents and media preparation

During this experiment, the following reagents and media formulation were used. All solutions used in this study were prepared with deionised and demineralised millipore water (ddH2O) and when necessary, the pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl). In addition, the solutions or buffers were

subsequently filtered through 0.2 µl filter and autoclaved at 120°C for 20 min when it was necessary.

Name of the Buffer/media	Constituents	Volume / amount
Physiological saline solution (NaCl 9%)	Sodium chloride	9.0 g
	added to water	1000.0 ml
	Water up to	1000.0 ml
70% Ethanol	Ethanol (100%)	700 ml
	Water	300 ml
10x CMF (Calcium magnesium free PBS)	Sodium chloride (NaCl)	8 g
	Potasium chloride (KCl)	0.2 g
	Sodium hydrogen phosphate (Na_2HPO_4)	1.15
	Potassium hydrogen phosphate (KH_2PO_4)	0.2 g
	Water up to	1000 ml
10x PBS	NaCl	8.77 g
	Na2HPO4	1.50 g
	NaH2PO4	2.04 g
	Water up to	1000.0 ml
1x PBS	10x PBS	100 ml
	DEPC-treated water upto	1000 ml
3% BSA in PBS	BSA	30g
	10x PBS : added to	1,000.0 ml
3M Sodium acetate, pH 5.2	Sodium acetate	123.1 g
	ddH_2O added to	500 ml
	ddH_2O added to	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml

dNTP solution	dATP (100 mM)	10 μl
	dGTP (100 mM)	10 μl
	dTTP (100 mM)	10 μl
	ddH$_2$O added to	400 μl
EDTA 100 Mm (pH 2.0)	EDTA	37.224 g
	Water up to	1000.0 ml
4% paraformaldehyde	Paraformaldehyde	4.0 g
	1X PBS	100.0 ml
Lysis buffer	Igepal (0.8%)	0.8 μl
	40U RNasin	5.0 μl
	DTT	5.0 μl
	ddH2O added to	100.0 μl

3.1.3 Equipment

Equipment	Manufacturer
Agilent 2100 Bioanalyzer	Agilent Technologies , CA, USA
Electrofusion machine CFA 400	Kruess, Munich, Germany
Electrophoresis unit	BioRad, Munich, Germany
CEQ^{TM} 8000 Genetc Analysis	Beckman Coulter, Krefeld, Germany
Illumina Hiseq 2000	GATC Bio Tech AG Konstanc, Germany
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Millipore apparatus	Millipore Corporation, USA
MyCycler Thermal Cycler	Bio-Rad Laboratories, CA, USA
Stereomicroscope SMZ 645	Nikon, Japan
StepOnePlus real-time PCR system	Applied Bio Systems
Ultra-low freezer (-80°C)	Labotect GmbH, Göttingen, Germany
Universal centrifuge 233MK	Hermel, Wehingen, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.4   List of software programs and statistical packages

Programs (softwares) and statistical packages	Source
BLAST program	http://www.ncbi.nlm.nih.gov/Blast/
Bos taurus genome	ftp://ftp.ensembl.org/pub/release-72/fasta/bos_taurus/dna/
Bowtie 1.3	http://sourceforge.net/projects/bowtie-bio/files/bowtie/old/0.11.3/
Cutadapt	https://code.google.com/p/cutadapt/
DAVID Bioinformatic Resource 6.7	https://david.abcc.ncifcrf.gov/
EndNote X1	Thomson
ENSEMBL genome browser	http://www.ensembl.org/
Entrez Gene	http://www.ncbi.nlm.nih.gov/gene/
FASTQC version 0,10,0	http://www.bioinformatics.babraham.ac.uk/projects/fastqc
GraphPad prism v.5	GraphPad software, Inc.
KEGG pathway	http://www.genome.jp/kegg/pathway.html
MiRBase v.19	http://www.mibase.org/
miRDB	http://www.mirdb.org/
MicroRNA.org-Target and Expression	http://www.microrna.org/
miRecords	http://mirecords.biolead.org/
PicTar	http://www.pictar.org/
Primer Express® software v.2.0	Applied Biosystems, Foster City, CA, USA
RT^2 PCR Array Data Analysis Version 3.5	http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/

Prism for windows (ver.5.0)	GraphPad software, Inc.
Seqtk tools	https://github.com/lh3/seqtk
TargetScan 5.1	http://www.targetscan.org/
Weight to molar quantity (for nucleic acids) converter	http://www.molbiol.ru/eng/scripts/01_07.html

3.2   Methods

3.2.1   Experimental layout
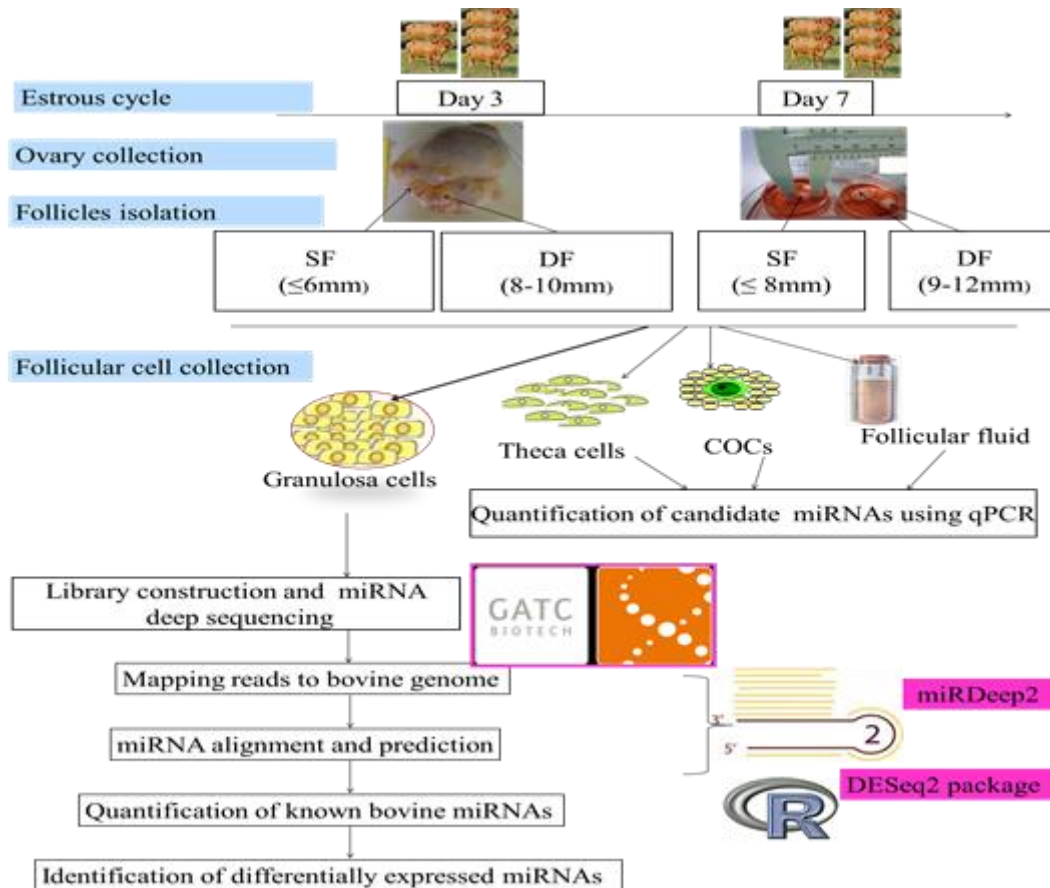
Figure 3.1 Brief overview of the present study

SF= Subordinate follicles (DF) = Dominant follicles

3.2.2 Animals and treatment

Handling of experimental animals was in accordance with the 1972 rules and regulations of German law of animal protection and the experiment was licensed by the animal protection office of the University of Bonn. Healthy Simmental heifers (n =13) aged between 15 to 20 months and weighing between 380 and 500 kg were used in this study. All experimental heifers received chief characteristics and they were kept under the same farm and housing conditions. Prior to collection of experimental samples, all heifers used for the experiment were estrous synchronized as previously described (Gad

et al. 2011, Ghanem et al. 2011, Hoelker et al. 2012, Salilew-Wondim et al. 2010). Briefly, the heifers received intra muscular administration of 500 mg of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue cloprostenol (Estrumate; Munich, Germany) twice within 11 days interval. Two days after each of the $PGF_{2\alpha}$ treatments, animals received 0.02 mg GnRH-analogue buserelin (Receptal) (Intervet, Boxmeer, the Netherlands). Common signs of estrus were monitored by visual observation and careful rectal palpation 3 days after the last $PGF_{2\alpha}$ injection. Following this, a total of 6 and 7 heifers were slaughtered at day 3 and day 7 of the estrous cycle, respectively at nearby slaughter house. The ovaries were then collected from each experimental animal and transported to laboratory in a thermo-flask containing warm phosphate buffer saline solution (PBS) at a temperature of $38^{o}C$.

3.2.3 Follicle isolation and categorization

Immediately up on arrival in the laboratory within 1 hour after collection, the ovaries were washed   three times with 0.9% NaCl solution. The ovaries were then rinsed in 70% alcohol for 30 sec followed by washing in 0.9% NaCl solution three times. Each ovary was checked for the presence of cystic ovaries and corpus luteum. The ovaries from 3 cows (1 cow at day 3 and 2 cows at day 7 of the estrous cycle) were discarded from the experimental group. The ipsilateral or contralateral part of the ovary was noted based on the previous corpus luteum and the follicles were then isolated from ipsilateral side of ovaries by blunt-dissection using scissors and forceps. The diameter of each follicle was measured using a caliper and then classified as subordinate and dominant follicles depending on their diameter as recommended by (Ireland et al. 1980) with minor modifications. Briefly, at day 3 of the estrous cycle, follicles with follicular diameter of ≤ 6 mm (n = 43) were categorized as subordinated follicle (SF) and follicles with a diameter of 8-10 mm (n = 9) were considered as dominant follicle (DF). On the other hand, at day 7 of the estrous cycle, follicles with a diameter of ≤ 8 mm (n = 58) were considered as SF and those with 9-13 mm diameter (n = 3) were categorized as DF.

3.2.4 Collection of follicular fluid and follicular cells (granulosa cells, theca cells,
      cumulus oocyte complex)

Following classification of follicles as SF and DF at days 3 and 7 of estrous cycle, each
of the follicles were cut at its top edge by the help of sharp scalpel and the follicular
contents were released into the 35 mm sterile petri dish. The follicular fluid containing
granulosa cells remained in the plate after cumulus oocyte complex recovery was
pooled according to the size of their corresponding follicles in a sterile microcentrifuge
tube and centrifuged at 300g for 10 min. While follicular fluid was collected as
supernatant, the granulosa cells were collected as pellet from the bottom of the tube.
Follicular fluid was snap frozen and stored at -80°C for further use. The granulosa cell
pellets were then washed twice with DPBS (Gibco, Life Technologies, UK) containing
0.025% polyvinyl alcohol (PVA) without Ca^{2+} and Mg^{2+} and stored at -80°C for
downstream experiment. Theca cells were isolated after gently scraping the follicular
wall with a blind edge of forceps and washed with Ca^{2+} and Mg^{2+} free DPBS to remove
any granulosa cell contamination. Then the theca cells (theca interna and theca externa)
were pealed carefully and scraped off with forceps. After consecutive washings in order
to remove granulosa cells, the theca cells were transferred into 0.65 ml falcon tube
containing RNA later (Sigma) and stored at $-20^{o}C$ for further use.

3.2.5 Total RNA extraction

Total RNA enriched with small RNA was extracted from granulosa cells, theca cells,
follicular fluid and cumulous oocyte complexes using the miRNeasy® mini kit (Qiagen
GmbH, Hilden, Germany), according to the manufacturer's instructions with minor
modifications.

3.2.5.1 Total RNA isolation from surrounding follicular cells (granulosa cells and theca
cells)

The total RNA enriched with miRNAs was extracted from the follicular cells (granulosa
cells and theca cells) samples using RNeasy mini kit (Qiagen, Hilden, Germany) with
slight modifications from the manufacturer's instructions. The cells were disrupted by

adding 700 µl QIAzol Lysis Reagent and homogenized by vortexing for 1 min followed by incubation at 15-25 ^{o}C temperature for 5 min. After this 140 µl chloroform was added to the homogenate, the tube shacked vigorously for 15 sec and centrifuged for 15 min at 12000 xg at 4°C in refrigerated universal centrifuge 233MK (Hermle Labortechnik, Wehingen, Germany). The upper aqueous phase was transferred carefully to a new 2 ml collection tube and 1.5 volumes (525 µl) of 100% ethanol was added and mixed thoroughly by pipetting up and down several times. Without any delay after mixing, 700 µl of the sample was pipetted onto RNeasy Mini spin column and centrifuged at 11000 rpm for 15 sec at room temperature. The flow-through was discarded and the previous step was repeated with the left over samples. Following this, 350 µl of RWT buffer was added to the spin column and centrifuged at 11000 rpm for 15 sec. Total RNA samples were freed from any Genomic DNA contamination by performing on-column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). A total of 80 µl (10 µl DNase + 70 µl buffer RDD) of diluted DNase I solution was pipetted directly onto the RNeasy mini spin column membrane and were placed on the bench top at 20-30°C for 15 min. Following incubation, 350 µl RWT buffer was added to the spin column and centrifuged for 15 sec at 11000 rpm at room temperature. After discarding the flow-through, 500 µl RPE buffer was added to the spin column followed by 15 sec centrifugation 11000 rpm. This step was repeated for the second time and centrifuged at the same speed for 2 min. The spin column was then transferred to a new 2 ml collection tube and centrifuged at 14000 rpm for 1 min to eradicate any possible leftover of RPE buffer and residual flow-through remained on the spin column. To elute total RNA, the RNeasy spin column was transferred into new 1.5 ml collection tube to which 30 µl of RNase-free water was added to the centre of the RNeasy spin column membrane and centrifuged at speed of 11000 rpm for 1 min at room temperature. Isolated total RNA was stored in -80°C until the further step.

3.2.5.2 Total RNA isolation from follicular fluid

Total RNA was isolated from follicular fluid samples using miRNeasy$^{®}$ mini kit (Qiagen, Hilden, Germany) according to the protocol adopted for serum or plasma with minor modification. Briefly, 5 volume of Qiazol lysis buffer was added to the follicular fluid, vortexed for 3-4 min and incubated for 8 min at room temperature. Following

incubation, 200 µl of chloroform was added to each sample, vigorously shacked for 1 min and incubated for 5 min. After incubation the samples were centrifuged at 12000 xg for 15 min at 4°C.The upper aqueous phase was transferred carefully to a new 2 ml collection tube and 1.5 volumes (525 µl) of 100% ethanol was added and mixed thoroughly by pipetting up and down several times. Directly after mixing, 700 µl of the sample was pipetted onto RNeasy Mini spin column and centrifuged at 11000 rpm for 30 sec at room temperature. The flow-through was discarded and the previous step was repeated with the left over samples. Following this, 700 µl of buffer RPE was added to the spin column and centrifuged at 11000 rpm for 1 min at room temperature. After this spin column were washed three times by adding 500 µl RPE buffer and centrifuged for 1 min at 11000 rpm at room temperature. After discarding the flow-through, the spin column was then transferred to a new 2 ml collection tube and centrifuged at 14000 rpm for 2 min at room temperature. The tube was left over open for 1 min that allowed the evaporation of residual ethanol portion from the spin column. To elute total RNA, the RNeasy spin column was transferred into new 1.5 ml collection tube to which 30 µl of RNase-free water was added to the centre of the RNeasy spin column membrane and centrifuged at speed of 11000 rpm for 1 min at room temperature. Isolated total RNA was stored in -80°C until the further step.

3.2.5.3 Purification and isolation of total RNA containing small RNAs from cumulous oocyte complexes

Total RNA was purified and isolated from the cumulous oocyte complexes samples by using AllPrep DNA/RNA Micro Kit followed by RNeasy mini kit (Qiagen, Hilden, Germany) by the manufacturer's instructions. The cells were disrupted by adding 75 µl of buffer RLT plus and homogenized by pipetting and transferred to the top of a Qiashredder column placed in a 2 ml collection tube. The sample tube was then rinsed with 275 µl of buffer RLT Plus and the entire volume was pipetted over the sample in a Qiashredder column and centrifuge for 2 min at 14000 rpm at room temperature. The homogenized lysate was transferred to an AllPrep DNA spin column placed in a 2 ml collection tube by closing the lid gently, and centrifuged for 30 sec at 10000 rpm at room temperature. After these 1.5 volumes (usually 525 µl) of 100% ethanol was added to the flow through from the AllPrep DNA spin column, and mixed well by pipetting.

700 µl of the sample was transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied) by closing the lid gently, and centrifuged for 15 s at 11,000 rpm) followed by discarding the flow through. Last step was repeated until the whole sample passed through the membrane with discarding the flow-through each time. The collection tube was used to next step by adding 500 µl Buffer RPE to the RNeasy MinElute spin column, closing the lid gently, and centrifuged for 15 sec at 11,000 rpm. The flow-through was discarded. Reuse The collection tube was to next step by adding 500 µl Buffer RPE to the RNeasy MinElute spin column by closing the lid gently, and centrifuged for 2 min at 11,000 rpm. The collection tube with the flow-through was discarded. After this the RNeasy MinElute spin column was placed in a new 2 ml collection tube (supplied) by opened lid of the spin column, and centrifuged at full speed for 5 min. The collection tube with the flow-through was discarded. The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube by adding 14 µl RNase-free water directly to the center of the spin column membrane. The lid was closed gently, and centrifuged for 1 min at full speed at room temperature. Isolated total RNA was stored in -80°C until the further step.

3.2.5.4 Quantity and quality control of isolated RNA

The quantity and purity of each RNA sample was assessed with spectrophotometer NanoDrop ND-800 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was analyzed using the RNA 6000 Nano Kit and Small RNA Kit with the 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). All samples were of high quality with the absorbance wavelength ratio (A260/A280) of $\geq$ 1.9 and the RNA Integrity Number of $\geq$ 8. RNA samples stored at -80ºC pooled accordingly and send to the company for deep sequencing of miRNAs. On average, 100 ng of RNAwas used as starting material for small RNA library preparation, miRNA sequencing library construction and sequencing. A total of 12 granulosa samples (three biological replicated of granulosa cells from SF or DF at day 3 or day 7 of the estrous cycle) were used for miRNA deep sequencing. However, the total RNA samples from COCs, follicular fluid and theca cells were used for measuring the expression of candidate miRNAs generated from granulosa cells.

3.2.5.5 Purity of isolated granulosa cells

Purity of isolated granulosa cells and conceivable contamination of theca cells were confirmed by the presence and absence of granulosa cell and theca cell specific markers genes namely; follicle-stimulating hormone receptor (FSHR) and Cytochrome P450 17A1 (CYP17A1), respectively. The program primer3web version 4.0.4 (http://bioinfo.ut.ee/primer3/) was used to design sequence-specific primers of bovine GAPDH, FSHR and CYP17A1 genes (Table 3.1). For this, cDNA (20 µl) was synthesized using First Strand cDNA Synthesis Kit (Thermo scientific, MA, USA) according to manufacturer´s instruction. Briefly; total RNA samples isolated from granulosa cells were diluted to obtain 1 µg of RNA per 20 µl of reaction volume. One µl of oligo (dt) 18 primer was added to total RNA samples and were incubated at 65°C for 5 min. 9 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 min. Finally, reactions were ended by heating at 75°C for 5 min. Efficiency of cDNA synthesis was confirmed by polymerase chain reaction (PCR) amplification of known housekeeping gene (GAPDH) primers. PCR was performed using the following cycling conditions: Pre20 incubation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C (FSHR and GAPDH) and 57°C (CYP17A1) for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR product was loaded into 2% agarose gel electrophoresis 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 specific bands.

Table 3.1: Details of primers design for gene markers of follicular cells

Gene		Sequence (5´-3´)	Annealing temperature (^{O}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		

For: forward primer Rev: reverse primer

3.2.6   Library preparation and sequencing

The miRNA enriched total RNA samples from granulosa cells from subordinate and dominant follicles at day 3 and day 7 of estrous cycle were subjected to construction of tagged miRNA sequencing libraries (22 to 30 nt) by a commercial company GATC BioTech AG (Konstanz, Germany) according to Illumina Small RNA Sample Preparation protocol. Briefly, specific 3´and 5´ RNA adapters were ligated to each end of the RNA molecule followed by purification of 1st and 2nd adapter ligation products. The 3´ RNA adapter is modified to capture miRNAs and other small RNA species in the sample. Single stranded cDNA was synthesized by reverse transcription using RT primers. Complementary DNA samples were PCR amplified using specific primers (Table 3.2). PCR amplified cDNA constructs were gel purified and band fraction size range of 140-160 nucleotides were excised using 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, filtering of the data and index sorting were performed by the CASAVA Pipeline version 1.8.0. Raw FASTQ sequence reads of 50 nucleotides length were obtained.

Table 3.2: List of adapters and primers used for library construction and PCR amplification

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	AATGATACGGCGACCACCGAGATCT
	ACACGTTCAGAGTTCTCCAGTCCGA
RNA PCR primer (RC)	TCGGACTGTAGAACTCTGAACGTGT
	AGATCTCGGTGGTCGCCGTATCATT
RNA Reverse transcription primer	GCCTTGGCACCCGAGAATTCCA
RNA Reverse transcription primer (RC)	TGGAATTCTCGGGTGCCAAGGC

RC: reverse complement

3.2.7   Sequence Quality control and pre-processing

All Fastq files were subjected to a preliminary sequence quality control procedure using FASTQC version 0,10,1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Based on input reads, FASTQC provide plots for quality control measures such as per base GC content, per sequence quality scores, per base sequence quality scores and over represented sequences. The 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, sequence reads with Phred score lower than 20 were removed at first. In the second step, sequence reads shorter than 18 bp after trimming were also removed from all the data sets. These sequences trimmings were performed using a combination of Cutadapt (Martin 2012) (https://code.google.com/p/cutadapt/) and Seqtk tools (https://github.com/lh3/seqtk).

 The *Bos taurus* genome release 72 was downloaded from ensemble genome browser (ftp://ftp.ensembl.org/pub/release-72/fasta/bos_taurus/dna/) was used as the reference

genome build to align the reads after quality control. The genome was indexed with Bowtie 1.3 (Langmead et al. 2009) (http://sourceforge.net/projects/bowtie-bio/files/bowtie/old/0.11.3/). Moreover, the Fasta file of all matured bovine miRNA, precursor bovine miRNAs and mature miRNAs of model species such as human, mouse and rat were downloaded from mirBase database (release 20: June 2013) (http://mirbase.org/ftp.shtml). The downloaded Fasta files contained 755 bovine mature miRNAs, 766 bovine precursor miRNAs, 2042 human mature miRNAs, 1281 mouse mature miRNAs and 723 rat mature miRNAs respectively. The results presented here are based on miRNA prediction algorithm miRDeep2 and associated workflow.

The first step of the workflow, "Mapper" maps Fasta formatted input reads to the given reference genome index (Friedlander et al. 2012). This read to reference genome mapping is used in the miRDeep2 algorithm for the identification of known and novel microRNAs. The second step of the workflow, "Quantifier" takes as input Fasta format converted reads and sequences of known mature and precursor miRNAs (Friedlander et al. 2012). The number of reads mapped to each known mature and precursor miRNAs were quantified to generate the miRNA expression data.

The raw FASTQ files and processed CSV files containing expression data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE56002.

3.2.8   Identification of known and novel miRNAs

Novel miRNAs were predicted from mature, star and loop sequence according to the RNAfold algorithm using miRDeep2 (Friedlander et al. 2012, Hofacker 2003). RNAfold was used for prediction of RNA secondary structures. In addition, we also calculated the estimated probability that the sequence to be true a positive novel miRNA candidate. Apart from this, the sequence should not be mapped to the rRNA or tRNA. A sequence was considered as a novel miRNA when it was not matched to any of the known miRNA in the mirBase.

3.2.9   Data normalization and analysis of differential expression of miRNAs

Following detection and identification of miRNAs, the expression level of those miRNAs was compared between the granulosa samples of SF and DF to understand whether accumulation or degradation of miRNA is associated with follicular selection, recruitment and dominance. For this, four comparisons were performed:

(1) To understand whether accumulation or degradation of miRNAs is associated with follicular selection and recruitment in early luteal phase, the expression pattern of miRNAs were compared between the granulosa cells of SF and DF at day 3 of the estrous cycle.

(2) To investigate whether the miRNA expression is associated with follicular selection and dominance during the early luteal phase, the expression pattern of miRNAs was compared between granulosa cells of SF and DF at day 7 of the estrous cycle.

(3) To understand the temporal miRNAs expression dynamics in DF during the luteal phase of the bovine estrous cycle, the miRNA expression pattern of granulosa cells in DF samples collected at day 7 was compared to the DF samples collected at day 3 of the estrous cycle.

(4) The miRNA expression pattern of the granulosa cells of SF was also compared between day 3 and day 7 of the estrous cycle to identify the miRNAs that could be changing in SF follicles during the early luteal phase of the estrous cycle.

Differential expression of miRNAs were analyzed using the R statistical software (RDC Team 2006) and DESeq2 package (Anders and Huber 2010). The expression data from second step of the workflow ("Quantifier") was used. To normalize the read count differences in each sample, DESeq2 normalization step generated a fictitious sample, calculated from the geometric mean of read count of all samples. The read count for each gene in each sample is divided by this geometric mean to obtain the normalized counts. For each miRNA in each granulosa sample, the observed count is the ratio of raw count for each miRNA to the geometric mean across the samples. DESeq2 employs a negative binomial distribution based method to identify differentially expressed genes or miRNAs. The Benjamini–Hochberg procedure of false discovery rate adjustment (Benjamini and Hochberg 1995) was used to correct the p values for multiple testing. At

the end of analysis, miRNAs with $\log_2$ fold change difference ≥ 1.0, with p value ≤ 0.05 and false discovery rate (FDR) $\leq$ 10% were considered as differentially expressed miRNAs. The heatmaps and clustering analysis of the differentially expressed miRNAs were generated using PermutMatrix (Caraux and Pinloche 2005).

3.2.10 MiRNA target gene prediction and functional annotation (Insilico Analysis)

The functional annotation of differentially expressed miRNAs was analyzed based on the functional annotation of their potential target genes. The predicted target genes were identified using miRecords http://mirecords.biolead.org/, (Xiao et al. 2009), an online animal miRNA-target interaction tool which integrates 12 miRNA-target prediction tools, including DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, PicTar, PITA, and TargetScan. The target genes which were predicted by at least four prediction tools were selected and submitted to DAVID Functional Annotation Bioinformatics Microarray Analysis tool (Da Wei Huang and Lempicki 2008) and the potential KEGG, Panther and Reactome pathways significantly enriched by target genes of each miRNAs were identified.

3.2.11 Validation of selected differentially expressed miRNAs using qPCR

The expression levels of 12 differentially expressed miRNAs were further validated using qPCR. For that, 88 ng miRNA enriched total RNA was reverse transcribed using universal cDNA synthesis kit (Exiqon) following the manufactures protocol. Briefly, a 20 μl reaction mix consisting of total RNA, reaction buffer, enzyme mixes and nuclease-free water was incubated at 42°C for 60 min, followed by 5 min incubation at 95°C. At the end of reaction period, the resulting cDNA was diluted 40x in RNAse free water. The qPCR was then performed in 20 μl reaction volume containing cDNA of each sample mixed with reverse and forward primer of each miRNA, PCR master mix (SYBR Green, thermo stable DNA polymerase) and nuclease-free water in the StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA). The real time PCR thermocycler parameter was set to 95°C of denaturation for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. At the end of 40 cycles, the melting curve was analyzed at 95°C for 15 s, 60°C for 1 min and the temperature

was increased at the rate of 0.3/sec until it reached to 95°C and the reaction was incubate at 95°C for 15 sec. At the end of the reaction, the qPCR data with acceptable dissociation curve and the amplification plot was used for further quantification analysis. The data was analyzed using the delta thresh hold cycle (delta Ct) method. The delta Ct values were generated after the expression of each miRNA was normalized against the arithmetic mean expression value of U6 and 5S. The within similarities of biological replicates and differences between samples of the qPCR data was then compared with the data generated by next generation sequencing.

3.2.12 Characterization of the expression of candidate miRNAs in follicular cells (theca cells, COCs and follicular fluid)

Following identification of differentially expressed miRNAs between the granulosa cells of SF and DFat days 3 and 7 of estrous cycle, we decided to understand whether the expression observed in granulosa cell could follow a similar pattern in theca cells, follicular fluid and COCs. For this, selected differentially expressed miRNA both at day 3 and day 7 of the estrous cycle were analyzed in theca cells, follicular fluid and COCs (Table 3.3). For this, the miRNA enriched total RNA was isolated from theca cells, follicular fluid and COCs of SF and DF whose granulosa cells were used for the next generation sequencing was subjected to cDNA synthesis and qPCR analysis. The qPCR reaction, the thermo cycler parameters and the data analysis was performed exactly as indicated above.

Table 3.3: List of selected differentially expressed miRNAs for their characterization in follicular cells (theca cells, COCs and follicular fluid) at day 3 and 7 of estrous cycle

Day3 SF vs DF	Day7 SF vs DF	Day3&7 DF vs DF
bta-mir-222	bta-miR-409a	bta-mir-155
bta-miR-21-3p	bta-miR-335	bta-miR-221
bta-mir-21-5p	bta-miR-195	bta-mir-222
bta-mir-155	bta-miR-214	bta-miR-21-3p
bta-miR-221	bta-mir-21-5p	bta-miR-708
bta-miR-708	bta-miR-21-3p	bta-mir-21-5p

bta-miR-214	bta-mir-222	bta-miR-365-5p
bta-miR-335	bta-mir-155	bta-miR-214
bta-miR-34c	bta-miR-708	bta-mir-195
	bta-miR-221	bta-miR-335
	bta-miR-2487	bta-mir-409a
	bta-mir-365-5p	

SF**:** Subordinate follicle, DF: Dominant follicle

3.2.13 Statistical analysis

Expression data of selected candidate miRNAs in granulosa cells, theca cells, cumulus oocyte-complex and follicular fluid derived from SF and DF at day 3 and day 7 of estrous cycle was done using comparative threshold cycle (Ct) method (Livak and Schmittgen 2001). Expression data was normalized against the geometric mean of the expression of 2 endogenous reference miRNAs; 5s Ribosomal RNA (5s rRNA) and *U6* small non-coding small nuclear RNA (U6 snRNA). Two-tailed student´s *t*-test was used to discover statistical differences in the mean expression value between treatment groups (SF vs DF at day 3 of estrous cycle), (SF vs DF at day 7 of estrous cycle), (SF vs SF at day 3 and day 7 of estrous cycle), (DF vs DF at day 3 and day 7 of estrous cycle), 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. All experiments were performed in three biological replicates and relative expression values are presented as mean ± SD of normalized Ct values.

4 Results

Simmental heifers were estrous synchronized, at day 3 of the estrous cycle, follicles with follicular diameter of ≤ 6 mm (number of follicles = 43) were categorized as subordinate follicle (SF) and follicles with a diameter of 8-10 mm (number of follicles = 9) were considered as dominant follicle (DF). On the other hand, at day 7 of the estrous cycle, follicles with a diameter of ≤ 8 mm (number of follicles = 58) were considered as SF and those with 9-13 mm diameter (number of follicles = 3) were categorized as DF.

4.1 Isolation efficiency from bovine follicles

The purity of granulosa cells isolated from follicles was checked using *FSHR* as granulosa cell specific CYP17A1 as theca cell specific markers. As indiacted in figure 4.1 below all granulosa cells showed a specific band for *FSHR,* while negligible amount of bands were detected using CYP17A1 gene in the SF and DF group at day 7 of estrous cycle. On the other hand in the SF and DF group at day 3 of estrous cycle samples showed some bands of CYP17A1 gene in some samples.
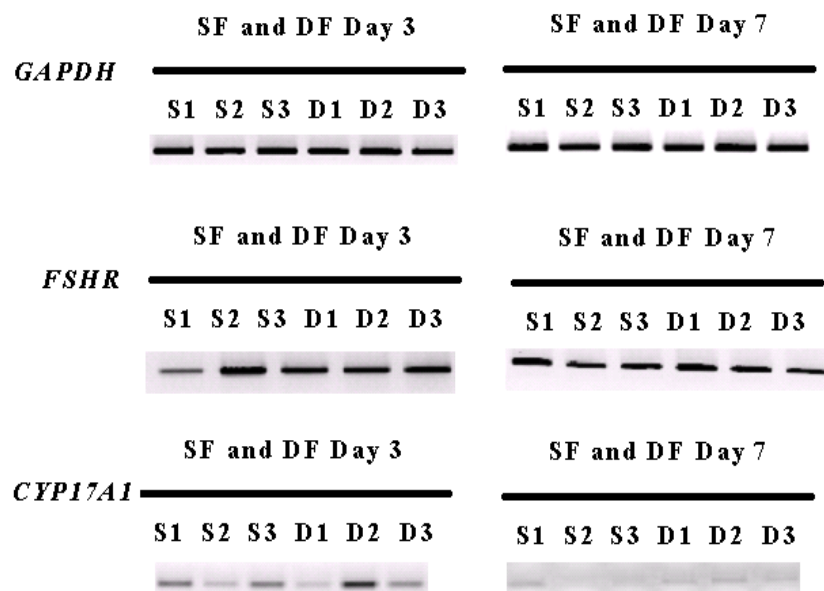
Figure 4.1: Granulosa cell-specific marker gene (FSHR) was detected in subordinate and dominant follicles at higher level as indicated by strong bands, while theca cell-specific marker gene (CYP17A1) had poor band.

4.2 Identification of known miRNAs in granulosa cells of subordinate and dominant follicles at day 3 and day 7 of the estrous cycle

MiRNAs libraries of 12 (three biological replicates for each sample) granulosa samples from SF and DF on day 3 and day 7 of post estrous were sequenced using Illumina HiSeq 2000 small RNA deep sequencing technology and clusters of sequence reads with maximum 50 base-pair (bp) length were obtained. At day 3 of the estrous cycle, the raw sequence data within the biological replicates of SF groups ranges from 3.9 to 7.7 million reads, whereas in DF, the read count ranges from 8.7 to 9.5 million reads. Similarly, at day 7 of the estrous cycle, raw sequence data in granulosa cells of SF was between 6.7 and 8.1 million reads whereas in DF groups the raw read counts was between 7.5 and 9.2 million. However, after filtering adapters, PCR primers, non informative sequences and sequences shorter than 18 bp, the mean read counts of SF and DF samples at day 3 of the estrous cycle were 1.5 ± 0.32 and 1.9 ± 0.18 million reads, respectively. Similarly, at day 7 of the estrous cycle, the mean read count was 1.9 ± 0.18 and 1.6 ± 0.15 in granulosa samples of SF and DF, respectively. After quality filtering the total number of reads obtained per replicate from SF and DF at day 3 and day 7 of estrous cycle ranged from 1,057,154 to 2,116,142 of which 57% to 69% were aligned to the reference genome (Table 4.1).

Table 4.1: Summary of sequence read alignments to reference genome

Group	Biological Replicates	Total number of reads	Unmapped reads	Mapped reads	Percentage of unmapped reads	Percentage of mapped reads
Subordinate	1	1691367	964766	726601	0.43	0.57
follicles	2	1083734	642467	441267	0.407	0.593
day 3	3	1594922	989857	605065	0.379	0.621
Dominant	1	1973220	1274237	698983	0.354	0.646
follicles	2	2031526	1219346	812180	0.4	0.6
day 3	3	1694104	1149755	544349	0.321	0.679
Subordinate	1	1779229	1120328	658901	0.37	0.63
follicles	2	1786030	1045365	740665	0.415	0.585
day 7	3	2116142	1222335	893807	0.422	0.578
Dominant	1	1701710	1159412	542298	0.319	0.681
follicles	2	1057154	732656	324498	0.307	0.693
day 7	3	1970550	1294502	676048	0.343	0.657

Following this, miRDeep2.pl of mirDeep2 script was used to detect known and novel miRNAs. Consequently, miRNAs with at least 1 raw read count in at least 2 of the three biological replicates were measured as detected miRNAs. Thus, 43025 and 4400226 reads of SF and DF, respectively at day 3 of the estrous cycle and 411736 and 358128 sequence reads of SF and DF, respectively at day 7 of the estrous cycle were matched to the known bovine miRNAs available in miRbase. Based on this analysis, the results revealed that a total of 291 and 318 known miRNAs were detected in granulosa cells of SF and DF, respectively at day 3 of the estrous cycle and 274 miRNAs were commonly expressed in both granulosa cell groups. On the other hand, 314 and 316 known miRNAs were detected in granulosa cells of SF and DF, respectively at day 7 of the estrous cycle of which 279 were detected in both granulosa cell groups. In addition, taking all data together, a total of 244 miRNAs were commonly detected in all granulosa cells in both follicles sizes at both days of the estrous cycle (Figure 4.2), of which 15 miRNAs named bta-miR-10b, bta-miR-26a, bta-miR-99b, bta-miR-27b, let-7 families (bta-let-7f, bta-let-7a-5p and bta-let-7i), bta-miR-92a, bta-miR-191, bta-miR-125a, bta-miR-148a, bta-miR-186, bta-miR-143, bta-miR-30d and bta-miR-30a-5p, were dominantly abundantly by > 3000 reads (Table 4.2). On the basis of this the results

exposed that majority of the detected miRNAs are co-expressed between granulosa cells of SF versus SF and DF versus DF at day 3 and day 7 of estrous cycle.
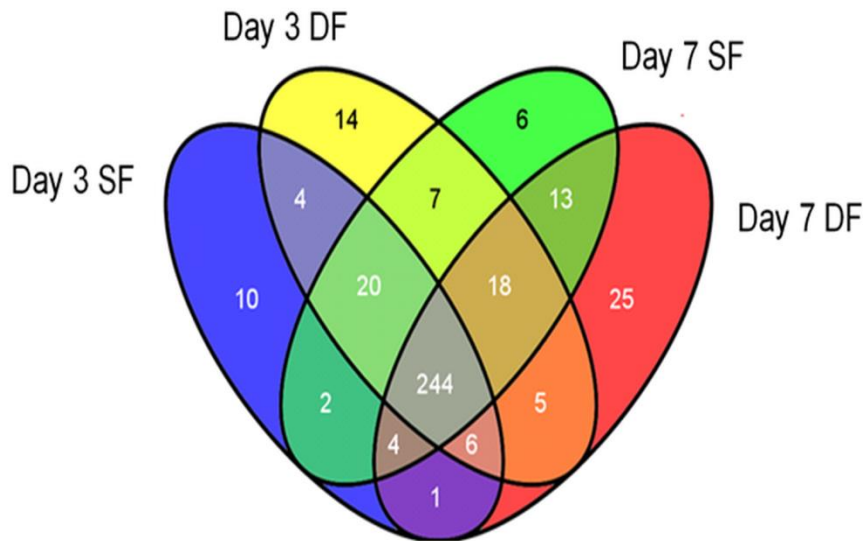
Figure 4.2: Venn diagram showing the number of known miRNAs detected uniquely or commonly in SF and DF granulosa samples at day 3 and day 7 of the estrous cycle. SF Day 3 and DF Day 3 indicate the subordinate and dominant follicles, respectively at day 3, while SF Day 7 and DF Day 7 indicate the subordinate and dominant follicles, respectively at day 7 of the estrous cycle.

Table 4.2: The top most abundant miRNAs with > 3000 read counts in granulosa samples of SF or DF at day 3 and/or day 7 of the estrous cycle

miRNAs	Day 3 SF No of reads	Day 3 DF No of reads	Day 7 SF No of reads	Day 7 DF No of reads
bta-miR-10b	37494	46559	63323	14742
bta-miR-26a	37300	53636	61103	42278
bta-miR-99b	18612	31817	15768	7583
bta-miR-27b	12922	15894	11789	20250
bta-let-7f	11065	14697	10439	7936
bta-let-7a-5p	10134	14314	10027	10415
bta-let-7i	8944	13802	11203	7270
bta-miR-92a	8853	7929	11115	4348
bta-miR-191	8073	15492	9996	5147
bta-miR-125a	7425	10142	8046	4713
bta-miR-148a	5055	6336	6100	6254

bta-miR-186	3795	5778	5052	5272
bta-miR-143	3737	3802	3906	10194
bta-miR-30d	3534	5499	4400	2993
bta-miR-30a-5p	3453	4314	3460	4188

No of reads describe the average number of read counts aligned to each miRNA

4.3 Identification of differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 3 of estrous cycle

To understand whether miRNAs expression is altered during follicular recruitment and selection, during the early luteal phase, we investigated the expression pattern of miRNAs at day 3 of the estrous. Therefore, following detection of the known miRNAs, their expression difference between the granulosa cells derived from SF and DF was analysed using the DESeq2 by comparing the $\log_2$ transformed read counts of granulosa cells of SF and DF. The expression pattern of 280 miRNAs was found to be altered by $\geq$ 1.5 fold changes compared to the granulosa cells of the DF of which 57% were activated while the rest 43% were repressed in the SF groups. However, when the selection parameters were limited to $\log_2$ fold change $\geq$ 1.0 (absolute fold change $\geq$ 2), p value < 0.05 and FDR $< 10\%$, only 16 known miRNAs were found to be significantly differentially expressed between the granulosa cells of SF and DF groups. Among these, the expression level of 14 miRNAs including bta-miR-449a, bta-miR-449c, bta-miR-212, bta-miR-222, bta-miR-21-3p and bta-miR-155 were increased while the expression level of 2 miRNAs (bta-miR-183, bta-miR-34c) was decreased in SF groups. The hierarchical clustering, the average expression, the p value and the FDR values of the differentially expressed miRNAs are described in figure 4.3.

SF	DF		Log 2 FC	P value	FDR
		bta-miR-449a	4.0	0.000	0.000
		bta-miR-449c	3.4	0.001	0.024
		bta-miR-222	2.7	0.000	0.000
		bta-miR-212	2.8	0.005	0.067
		bta-miR-21-3p	2.3	0.000	0.000
		bta-miR-155	2.2	0.000	0.000
		bta-miR-21-5p	1.9	0.000	0.000
		bta-miR-221	1.8	0.000	0.000
		bta-miR-214	1.3	0.006	0.078
		bta-miR-708	1.7	0.008	0.097
		bta-miR-677	1.5	0.001	0.010
		bta-miR-199a-5p	1.2	0.000	0.000
		bta-miR-335	1.2	0.000	0.000
		bta-miR-1246	1.4	0.009	0.098
		bta-miR-183	-1.3	0.002	0.026
		bta-miR-34c	-2.5	0.005	0.067

Figure 4.3: The hierarchical clustering of differentially expressed miRNAs between the granulosa cells of SF and DF at day 3 of the estrous cycle along with their average expression difference (FC=$\log_2$ fold change), *p* value, and false discovery rate (FDR). Positive and negative FC values indicate up and downregulation of miRNAs, respectively in SF compared to DF granulosa cells. The red and green colours designate high and low expression of miRNAs, respectively.

4.4 Identification of differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 7 of estrous cycle

To uncover whether the degradation or enrichment of miRNAs is associated with follicular selection and dominance during the early luteal phase, the expression profiles of miRNAs were then compared between granulosa cells of SF and DF at day 7 of the estrous cycle using the DESeq2 package. The results revealed that a total of 272 known bovine miRNAs exhibited ≥ 1.5 fold change differences between the two granulosa cell groups of which 49% of those miRNAs were enriched while the rest 51% of them were reduced in the granulosa cells of SF. However, when the miRNAs were filtered based on the criteria of $\log_2$ fold change ≥ 1.0, *p* value ≤ 0.05, FDR ≤ 10%, a total of 108

known miRNAs were differentially expressed between the granulosa cells of SF and DF, of which the expression level of 51 miRNAs were increased while the expression level of 57 miRNAs were reduced in the granulosa cell of SF. Among these, bta-miR-2332, bta-miR-409a, bta-miR-2446, bta-miR-383, bta-miR-2404 and bta-miR-335 were not detected in granulosa cells of DF. On the other hand, from 57 downregulated miRNAs, bta-miR-184, bta-miR-365-5p, bta-miR-2487 and bta-miR-2389 were not absent in granulosa cells of SF. In addition, the expression of 12 miRNA families including miR-130 (*a, b*), bta-miR-181 (*a, b, c, d*), bta-miR-199 (*a-3p, a-5p, b, c*), bta-miR-2285 (*k, t*), bta-miR-2411(*-3p, -5p*), bta-miR-2483 (*-3p, -5p*), bta-miR-29 (*a, b*), bta-miR-339 (*a, b*), bta-miR-365 (*-3p, -5p*), bta-miR-455 (*-3p, -5p*), bta-miR-92, and bta-miR-99 (*a, -5p, b*) were differentially expressed between the granulosa cells of SF and DF (Table 4.3). The expression and hierarchical clustering of all differentially expressed miRNAs and the top 36 ones are described in figure 4.4A and 4.4B, respectively. Moreover, the list of 108 differentially expressed miRNAs with their corresponding fold change, *p* values and FDR is provided in appendix 1.
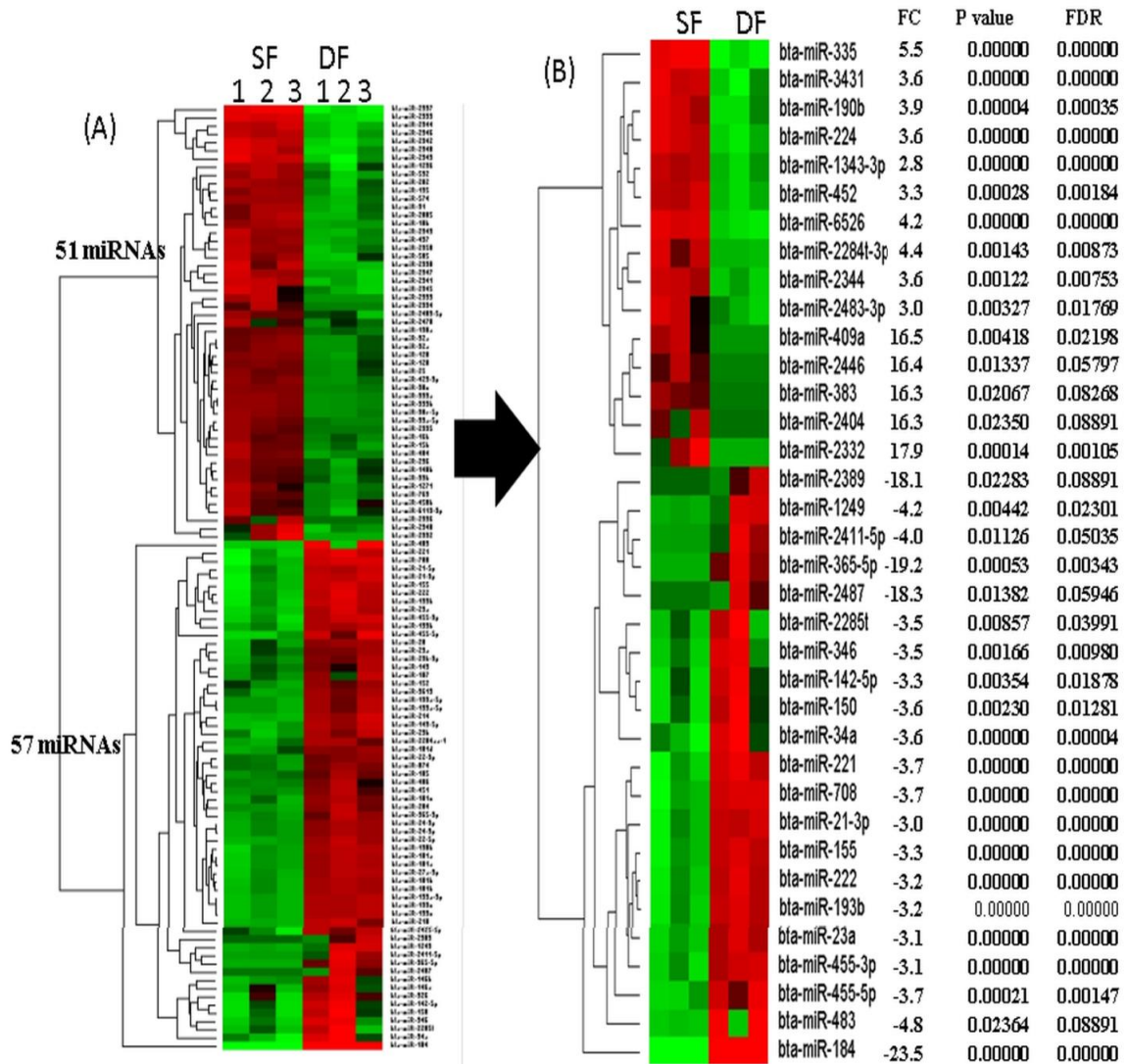
Figure 4.4: Differentially expressed miRNAs between granulosa cells of SF and DF at day 7 of the estrous cycle. (A) The expression patterns and hierarchical clustering of 108 differentially expressed miRNAs between granulosa cells of SF and DF. The numbers 1, 2, 3 under SF and DF indicate the biological replicates. (B) The expression patterns and hierarchical clustering of top 36 differentially expressed miRNAs along with their average expression difference (FC=$\log_2$ fold change), p values and false discovery rate (FDR). The red and green colours indicate high and low expression, respectively. Positive and negative FC values indicate up and downregulation of miRNA, respectively in SF compared to the DF granulosa cells.

Table 4.3: MiRNA families co-expressed or co-repressed in granulosa cells of SF compared to DF at day 7 of the estrous cycle

miRNAs	FC	*p* value
bta-miR-130a	1	0.0001
bta-miR-130b	-2.5	< 0.0001
bta-miR-181a	-2.4	< 0.0001
bta-miR-181b	-2.3	< 0.0001
bta-miR-181c	-1.8	< 0.0001
bta-miR-181d	-1.5	0.074
bta-miR-199a-3p	-2.5	< 0.0001
bta-miR-199a-5p	-2.1	< 0.0001
bta-miR-199b	-2.4	< 0.0001
bta-miR-199c	-2.9	< 0.0001
bta-miR-2285k	2.6	< 0.0001
bta-miR-2285t	-3.5	0.0085
bta-miR-2411-3p	2.4	0.0055
bta-miR-2411-5p	-4	0.0112
bta-miR-2483-3p	3	0.0032
bta-miR-2483-5p	1.2	0.0212
bta-miR-29a	-1.4	0.0001
bta-miR-29b	-2.2	0.0018
bta-miR-339a	1.6	< 0.0001
bta-miR-339b	1.6	< 0.0001
bta-miR-365-3p	-1.9	< 0.0001
bta-miR-365-5p	-19.2	0.0005
bta-miR-455-3p	-3.1	< 0.0001
bta-miR-455-5p	-3.8	0.0002
bta-miR-92a	1.4	< 0.0001
bta-miR-92b	2.2	< 0.0001
bta-miR-99a-5p	1.3	< 0.0001
bta-miR-99b	1.1	0.002

FC=Fold change in $\log_2$ scale. Positive FC values indicate upregulation and negative FC values indicate downregulation of miRNAs in SF compared to DF.

4.5 Commonly differentially expressed miRNAs between the granulosa cells of subordinate and dominant follicles at day 3 and day 7 of estrous cycle

Compared to day 3, the number of differentially expressed miRNAs between the granulosa cells of SF and DF was higher at day 7 of the estrous cycle (16 vs 108 miRNAs). However, 9 miRNAs were commonly differentially expressed between granulosa cells of SF and DF both at day 3 and 7 of the estrous cycle. Interestingly, 8 miRNAs namely (bta-miR-199a-5p, bta-miR-214, bta-miR-708, bta-miR-221, bta-miR-21-5p, bta-miR-155, bta-miR-21-3p and bta-miR-222) were found to be enriched in SF at day 3 of the estrous cycle but at day of 7 the estrous cycle those miRNAs were repressed in SF compared to the DF groups (Table 4.4).

Table 4.4: The list of commonly differentially expressed miRNAs at day 3 and day 7 of estrous cycle between granulosa cells of subordinate and dominant follicles

miRNAs	Day 3 FC	p value	Day 7 FC	p value
bta-miR-199a-5p	1.154918723	5.66E-09	-2.05261	5.15E-09
bta-miR-335	1.183533497	7.48E-08	5.512602	5.64E-31
bta-miR-214	1.279954695	0.006387	-2.67598	8.52E-11
bta-miR-708	1.696020455	0.008131	-3.69984	3.68E-13
bta-miR-221	1.840344086	7.95E-08	-3.7374	3.23E-11
bta-miR-21-5p	1.918453892	3.4E-32	-2.79013	1.26E-09
bta-miR-155	2.173146128	3.48E-13	-3.25402	1.44E-11
bta-miR-21-3p	2.312488135	1.73E-10	-3.01701	4.68E-10
bta-miR-222	2.729778837	1.29E-08	-3.17048	5.02E-11

Positive fold change means up regulation and negative fold change means down regulation (FC=Log_2 fold), P value.

4.6 Temporal enrichement or degradation of miRNAs in granulosa cells of DF during the early luteal phase of the estrous cycle

We determined the temporal miRNA expression patterns in granulosa cells of DF between day 3 and day 7 of the estrous cycle to uncover the miRNAs expression dynamics in dominant follicles during the luteal phase of the bovine estrous cycle. Thus, after normalization of the read counts, the number of detected miRNAs were quite similar in both granulosa cells of DF at day 3 (n= 318) and day 7 of the estrous cycle (n=316) of which 262 miRNAs were commonly detected at both time points. The scatter plot of all those miRNAs is indicated in figure 4.5 to depict the overall expression difference. In addition to this, we also analysed the miRNAs whose expression is significantly different in DF between the two time points. Accordingly, a total of 131 miRNAs were differentially expressed between day 3 and day 7 of the estrous cycle of which 51 and 80 miRNAs were increased at day 3 and at day 7 of the estrous cycle, respectively (Figure 4.6). Among miRNAs increased at day 7 of the estrous cycle, bta-miR-2389, bta-miR-29d, bta-miR-363, bta-miR-1249, bta-miR-338, bta-miR-129-3p, bta-miR-129-5p, bta-miR-129, bta-miR-142-3p, bta-miR-449b, bta-miR-2285t and bta-miR-346 were not detected at day 3 (Figure 4.6A). On the other hand, among the miRNAs that were repressed at day 7 of the estrous cycle bta-miR-409a, bta-miR-2332 and bta-miR-196a were detected at day 3 but disappeared at day 7 of the estrous cycle (Figure 4.6A). Others namely, bta-miR-143, bta-miR-99b, bta-miR-191, bta-miR-16b, bta-miR-99a-5p and bta-miR-30e-5p were expressed by > 500 reads counts both at day 3 and day 7 of the estrous cycle. However, the expression pattern of these miRNAs was significantly decreased at day 7 compared to day 3 of the estrous cycle. The expression pattern of miRNAs expressed both at day 3 and day 7 but downregulated at later groups is shown in figure 4.6B and those upregulated are indicated in figure 4.6C. Moreover, the expression of 12 miRNA families including bta-miR-29 (a, b, c, d), bta-miR-449 (a, b, c), bta-miR-181 (a, b, c, d), bta- miR-455 (-3p, -5p), bta-miR-99 (b, a-5p) and bta-miR-2483 (-5p, -3p) were co-overexpressed or co-repressed at day 7 compared to day 3 (Table 4.5). The list of all differentially expressed miRNAs with their corresponding fold change and *p*-value in granulosa cells of DF during the early luteal phase of the estrous cycle is described in appendix 2.
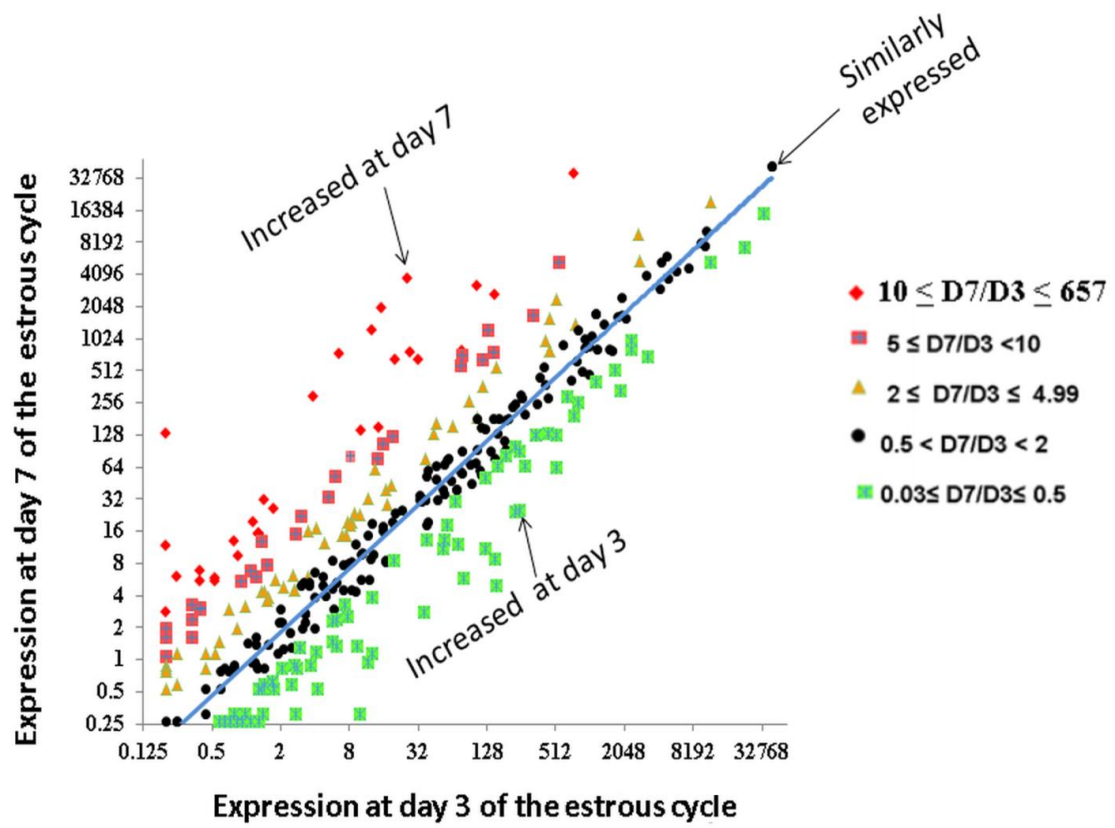
Figure 4.5: Scatter plot showing the read count ratio of 357 miRNAs between day 7 and day 3 in the granulosa cells of DF.
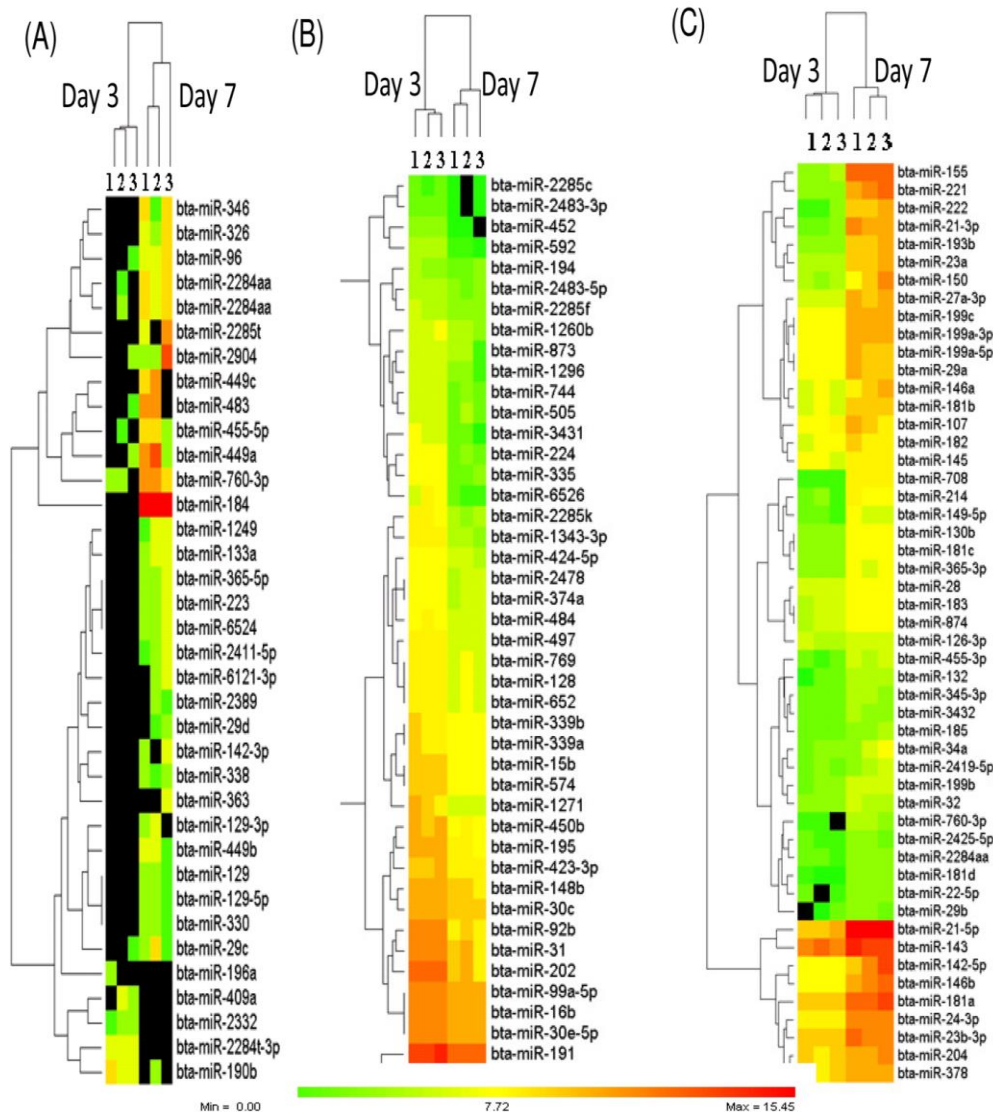
Figure 4.6: The heatmaps and the hierarchical clustering depicting the expression patterns of differentially expressed miRNAs in granulosa cells of DF between day 3 and day 7 of the estrous cycle. (A) The expression patterns of miRNAs detected only at day 3 (top) or at day 7 (bottom) of the estrous cycle in granulosa cells of DF. (B) The expression patterns of miRNAs detected both at day 3 and at day 7 of the estrous cycle but significantly increased in the former group. (C) The expression patterns of miRNAs expressed both at day 3 and at day 7 of the estrous cycle but significantly increased in the later group. The colour scale shows the $\log_2$ transformed expression values. Zero colour scale indicates miRNAs with ≤ 1 average read count. Numbers, 1, 2 and 3 on the heatmaps describe the number of biological replicates used in each sample group. Day 3 and Day 7 indicate the stages of the estrous cycle.

Table 4.5: MiRNA families co-expressed or co-repressed in granulosa cells of DF at day 7 compared to day 3 of the estrous cycle

miRNAs	FC	P value
bta-miR-99b	-1.7	< 0.0001
bta-miR-99a-5p	-1	< 0.0001
bta-miR-455-5p	3.2	0.0003
bta-miR-455-3p	3.1	< 0.0001
bta-miR-449c	4.1	0.00092
bta-miR-449b	16.7	0.0007
bta-miR-449a	4	0.00011
bta-miR-365-5p	4.5	0.00209
bta-miR-365-3p	2.7	< 0.0001
bta-miR-29d	16.4	0.01292
bta-miR-29c	2.7	0.0166
bta-miR-29b	2.3	0.00317
bta-miR-29a	2.4	< 0.0001
bta-miR-2483-5p	-1.6	0.0005
bta-miR-2483-3p	-2.8	0.00188
bta-miR-2285t	18	0.0002
bta-miR-2285k	-3.3	< 0.0001
bta-miR-2285f	-1.1	0.0093
bta-miR-199c	3.2	< 0.0001
bta-miR-199b	2.2	< 0.0001
bta-miR-199a-5p	2.5	< 0.0001
bta-miR-199a-3p	3.2	< 0.0001
bta-miR-181d	2.5	0.00013
bta-miR-181c	2.6	< 0.0001
bta-miR-181b	2.9	< 0.0001
bta-miR-181a	3.3	< 0.0001
bta-miR-146b	4.1	< 0.0001
bta-miR-146a	3.2	< 0.0001
bta-miR-142-5p	4.9	< 0.0001
bta-miR-142-3p	16.6	0.002
bta-miR-129-5p	16.6	0.002
bta-miR-129-3p	17.8	0.002

FC=Fold change in $\log_2$ scale. Positive FC values indicate upregulation and negative FC   values describe downregulation of miRNAs at day 7 compared to day 3.

4.7 Temporal accumulation or degradation of miRNAs in granulosa cells of SF during the early luteal phase of the estrous cycle

The temporal miRNA expression difference in the granulosa cells of SF between day 3 and 7 of the estrous cycle was investigated to uncover the miRNAs expression dynamics occurring in subordinate follicles during the early luteal phase of the bovine estrous cycle. Results showed that only 5 miRNAs were differentially expressed between the two time points. From these, the expression level of 4 miRNAs, namely bta-miR-1271, bta-miR-100, bta-miR-424-5p and bta-miR-2285k was downregulated while the expression level of only one miRNA, bta-miR-155, was significantly upregulated in granulosa cells of SF at day 7 compared to day 3 (Table 4.6). Moreover, 4 of these 5 miRNAs, namely, bta-miR-1271, bta-miR-424-5p, bta-miR-155 and bta-miR-2285k were also differentially expressed in granulosa cells of DF between day 3 and day 7 of the estrous cycle. Therefore, 127 miRNAs were uniquely differentially expressed in DF while only one miRNA was differentially expressed in SF between day 3 and day 7 of the estrous cycle. Thus, the temporal miRNA expression dynamics in the DF was more pronounced than the SF groups between the two time points.

Table 4.6: List of differentially expressed miRNAs between granulosa cells of SF during the early luteal phase of the estrous cycle

miRNAs	FC	$p$value	FDR
bta-miR-1271	1.708093486	1.00E-05	0.001839181
bta-miR-424-5p	1.058624784	0.000369811	0.025924203
bta-miR-2285k	1.058150829	0.00053725	0.030412265
bta-miR-100	1.036116917	4.20E-06	0.001839181
bta-miR-155	-1.780583915	7.87E-06	0.001839181

Positive fold change means up regulation and negative fold change means down regulation (FC=Log_2 fold), P value and false discovery rate (FDR).

4.8 Target prediction and functional annotation for differentially expressed miRNAs across the estrous cycle

To understand further physiological functions and biological processes of the differentially expressed miRNAs between granulosa cells of the subordinate and dominant follicles in the follicular development, *in silico* analysis was performed in order to identify the potential target genes. Due to the reason that number of experimentally validated miRNA targets is limited, we used miRecords at http://mirecords.biolead.org/, which contain animal miRNA potential targets according to combinations of the broadly used target prediction programs DIANA, Micro Inspector, miRanda, Mir Target2, miTarget, Pictar, PITA, RNA hybrid and miRDB. For considering a gene as a potential target of a specific miRNA we set the criteria of at least 4 target prediction programs. GO annotation and KEGG, PANTHER and REACTOME pathway analysis were performed to identify functional modules regulated by these differentially miRNAs in two groups (SF and DF) of follicles at different time points (day 3 and day 7) of estrous cycle.

4.8.1 Target prediction, functional annotation and canonical pathways identified for differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 3 of estrous cycle

Target genes for the 16 differentially expressed miRNAs between granulosa cells of SF and DF at day 3 of estrous cycle were predicted *in silico*. A total of 3319 target genes were identified (Fig 4.7). Among these 3053 genes were identified for up regulated miRNAs and 607 were identified for down regulated miRNAs between granulosa cells of SF and DF. Among them 395 including ACVR2A, ACVR2B, FOXA1, FOXN2, FOXO1, FOXP1, SOX6 genes were uniquely present in the downregulated list, while 2841 genes including ACAD9, ACBD3, ACCN1, ACLY, ACTA1, ACTN1, ACTR2, ACTR8, ACVR1B, ACVR1C, HOXA1, WNT1,WNT9A genes   were uniquely identified in the upregulated list. Moreover, 212 genes including ACVR2A, ACVR2B, FOXA1, FOXN2, FOXO1, FOXP1, SOX6 genes were commonly identified between the two groups.

Figure 4.7: Venn diagram showing the targeted genes predicted to be regulated by differentially expressed miRNAs at day 3 of estrous cycle between granulosa cells of subordinate and dominant follicles.

Other than the differentially expressed miRNAs between granulosa cell of SF and DF follicles at day 3 of estrous cycle, also the interest was developed in the signalling pathways and biological functions that could be potentially targeted by these differentially expressed miRNAs. The biological processes in gene ontology were carried on in the analysis by revealing genes targeted by differentially expressed miRNAs. Twelve main GO terms were enriched with a p-value < 0.05 (Table 4.7). Mostly the genes targeted by DE miRNAs were associated with transcriptional categories, regulation of RNA metabolic process, metabolic process, regulation of programmed cell death, axonogenesis, biological adhesion, cell-cell adhesion, regulation of cell death and cell fate commitment. To uncover, the role of those differentially expressed miRNAs, their target genes were predicted using miRecords tool and significantly enriched pathway ($P \leq 0.05$) by those genes was analysed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). Accordingly, the pathway analysis results at day 3 of estrous cycle showed that cadherin signaling, apoptosis, ErbB signaling, MAPK signaling, Ras, signaling by BMP, 5HT1 type receptor mediated signaling and adipocytokine signaling pathways were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of SF, whereas the focal adhesion, gap junction, histamine H1 receptor mediated signaling pathway and angiotensin II-stimulated signaling through G proteins and beta arrestin were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of DF.

Interestingly, Wnt signaling pathway, TGF-beta signaling pathway, signaling by NGF, oxytocin receptor mediated signalin pathway, notch signaling pathway, axon guidance and adherens junction were enriched by genes targeted by miRNAs either enriched or repressed in granulosa cells of SF (Table 4.8). Despite of the fact of false-positive predictions which could occur every time, we expect that these targets have greater chance of being regulated by miRNAs in granulosa cells of SF and DF during the follicular development.

Table 4.7: Gene ontology analysis of potential target genes of miRNAs differentially expressed between granulosa cells of subordinate and dominant at day 3 of estrous cycle

miRNAs ID	GO ID & Terms	List of some genes
bta-miR-335↑,bta-miR-21-3p↑,bta-miR-449a↑,bta-miR-21-5p↑,bta-miR-214↑,bta-miR-221↑,bta-miR-222↑,bta-miR-199-5p↑,bta-miR-212↑,bta-miR-183↓	GO:0045449~regulation of transcription	CCNT2, HLF, SNIP1, ONECUT2,MEF2A, CNOT8, ZNF630, E2F7, E2F3, SLC2A4RG, IL16, ZBTB39, ZNF200, TLR4, ZNF395, MLL2, L3MBTL4, PIM1,CNOT2, CTCF, ZEB2, RORB,ENY2, BMI1, MEF2C
bta-miR-335↑,bta-miR-449a↑,bta-miR-1246↑,bta-miR-221↑,bta-miR-222↑,bta-miR-21-5p↑,bta-miR-21-3p↑,bta-miR-199-5p↑,bta-miR-212↑,bta-miR-183↓	GO:0006350~transcription	CCNT2, HLF, ONECUT2, MAX,E2F3, SLC2A4RG, ARID4A, BACH1, HLF, ZNF518B,MORF4L1, BMI1,ZNF83, ZEB2, EIF2C2, ELF2, RCBTB1, PCGF5, CDCA7,ZNF84, HLF,NFE2L1, ZFPM2, ING3, NFYC
bta-miR-21-3p↑,bta-miR-199-5p↑,bta-miR-221↑,bta-miR-	GO:0051252~regulation of RNA metabolic process	MEF2A, CNOT8, ZNF630, E2F7, PASK,ZNF84, HLF, ZNF614,CNOT2, ZEB2,

449a↑,bta-miR-222↑,bta-miR-335↑,bta-miR-212↑		RORB,E2F3, SLC2A4RG, ARID4A,ZNF83, ZEB2, CTCF,HLF, SNIP1, ONECUT2, LASS5, NPAS2, RNF141, HAND1,HIRA,
bta-miR-21-3p↑,bta-miR-199-5p↑,bta-miR-221↑,bta-miR-335↑,bta-miR-449a↑,bta-miR-222↑,bta-miR-212↑, bta-miR183↓,bta-miR-335↑,bta-miR-21-5p↑,bta-miR-21-3p↑,bta-miR-199-5p↑,bta-miR-212↑,	GO:0006355~regulation of transcription, DNA-dependent	MEF2A, CNOT8, ZNF630,ZNF84, HLF, ZNF614, ZNF468, ZNF81, ZNF558, CREM,CNOT2, ZEB2, RORB,HLF, ONECUT2, SNIP1, E2F3, SLC2A4RG,ZNF83, ZEB2, CTCF, HIRA, ZEB2, FOXO3
	GO:0010604~positive regulation of macromolecule metabolic process	ENY2, MEF2C, NFYC, FOXO1, FKBP1A, SOX6,SATB2, ONECUT2, TEAD1, CDC23,CNBP, GLIS2,IL6ST, NAA15, GJA1, TBP, FOXO3,CAV1, ACVRL1, BTRC, ONECUT2,ATXN1, PSMA2, MAPK1
bta-miR-222↑,bta-miR-21-5p↑,bta-miR-449a↑,bta-miR-335↑,bta-miR-21-3p↑,bta-miR-199-5p↑,bta-miR-212↑,bta-miR-183↓	GO:0051254~positive regulation of RNA metabolic process	MLL, KLF12, NTF3, TEAD1, NFYB,KLF6, BMP3, CNBP, NTF3, SOX2, SOX5,TBL1XR1, BMP3, SATB2, NPAS2, SATB2, HAND1,MAF, EPAS1, TBX5, EPAS1,EGR1, ENY2, MEF2C, BCL10, FOXO3,
bta-miR-212↑,bta-miR-21-5p↑,bta-miR-214↑,bta-miR-183↓	GO:0043067~regulation of programmed cell death	RTN4, PTGS2, ZAK, DEDD, GDF5, SOX4, FOXO3,APH1B, TLR4, GCLM, TIMP3, ACVR1C,TRAF1, YWHAZ, CADM1, ADORA2A, AKAP13,MEF2C, ING3, CDK5R1, SNCB, PTGS2, FOXO1, NR3C1,
bta-miR-214↑,bta-miR-21-3p↑,bta-	GO:0007409~axonogenesis	SLITRK1, NRP1, PVRL1, NRXN3, DRD2, ROBO2, TNN, UNC5C,

miR-449c↑,bta-miR155↑,bta-miR-449a↑bta-miR-34c↓,bta-miR-183↓		GAP43, CXCL12, CHL1, PARD3, NUMBL, ALCAM, SEMA4F, PRKCA, NRP2
bta-miR-1246↑,bta-miR-21-3p↑,bta-miR-221↑	GO:0022610~biological adhesion	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, FNDC3A, THBS2, DLG1, ITGB8, FAT2, PCDHA10, CD4
bta-miR-1246↑,bta-miR-21-3p↑,bta-miR-221↑	GO:0016337~cell-cell adhesion	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, FNDC3A, THBS2, DLG1, ITGB8, FAT2, PCDHA10, CD4
bta-miR-21-3p↑,bta-miR-21-5p↑	GO:0010941~regulation of cell death	ALS2, C7, TSPO, DNM1L, PTGER3, MSH2, APH1B, RFFL, ARHGEF12,MEF2A, LITAF, MMD, GJA1, TBP, PPT1, FOXO3, SLC11A2
bta-miR-21-5p↑,bta-miR-212↑,bta-miR-183↓	GO:0045165~cell fate commitment	SPRY2, SPRY1, NTF3, TBX2, SOX2, SOX5,GATA2, SPRY1, SALL1, FOXA1,ITGB1

Note: Top most terms are listed here

Table 4.8: The most enriched pathways of target genes for differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 3 of estrous cycle

miRNAs ID	Term	Genes
mir-214↑,mir-1246↑,mir-214↑,mir-212↑,mir-449a↑,mir-222↑,mir-221↑,mir-183↓	Wnt signaling pathway	FZD4, FZD7, ACVR1B, PLCB4, HOXA4, SMARCC1, HOXC4, SMARCD1, PPP2CB, WNT9A,PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHA2, PCDHA3, PCDHA4, PCDHA5, SENP2, TBL1XR1, PLCB4, VANGL1, VANGL2, PPP2CB, WNT9A, FZD4, FZD7,EP300, NLK, PPP2R5C, PRICKLE2, NFAT5, PPP3CC,
mir-21-5p↑,mir-199a-5p↑, mir-212↑,mir-214↑,mir-183↓	TGF-beta signaling pathway	SMAD7, BMPR2, SMURF2, ACVR1C, BMP8A, PITX2, ACVR2A, BMP3, ACVR1B, ACVR2B, ACVRL1, KRAS, RAB10, MAPK1, ACVR2B, EP300, FOXA1, GDF5, FOXE1, FOXO3, CITED2, FKBP2
mir-222↑,mir-221↑,mir-21-3p↑,mir-214↑,mir-183↓	Signalling by NGF	CDKN1B, RALA, FRS2, BCL2L11, PIK3R1, ITPR2, MAPK1, PDPK1, PRDM4, ADORA2A, SQSTM1, PPP2CB, MEF2C, RPS6KA3, PPP2CA, PSEN2, PPP2CB, NTRK2, RALA, FOXO1, FRS2, IRS1
mir-449a↑,mir-183↓,mir-34c↓	Oxytocin receptor mediated signaling pathway	PRKCQ, PLCG1, CACNB1, CACNB3, VAMP2, PLCB1, CACNA1C, PRKCA, PLCB4, GNB1, CACNB4, GNG4, GNG5 GNAQ, PRKCI, CACNB3, VAMP3, VAMP2
mir-449a↑,mir-34c↓	Notch signaling pathway	NOTCH2, DLL1, JAG1,NOTCH1, APH1A, DLL1, JAG1, NUMBL

mir-199a-5p↑,mir-449a↑, mir-222↑,mir-214↑,mir-221↑,mir-212↑,mir-183↓	Axon guidance	EPHA7, KRAS, PLXNA2, GSK3B, NTNG1, SRGAP2, ABLIM1, MAPK1, NRP1, GNAI2, LIMK2, SEMA6D, PLXNA2, ABLIM3, SRGAP3, DPYSL5, ARHGEF12,PAK7, GNAI3, GNAI2, SEMA6D, EFNB2, PPP3R1, SEMA3C, PAK1
mir-212↑,mir-214↑,mir-34c↓	Adherens junction	MAPK1, TJP1, EP300, NLK, TCF7L2, TCF7L1,PVRL4, FGFR1,ACVR1B, SORBS1, PVRL1, TGFBR1, ACTN1, SSX2IP,PARD3, WASF1, LEF1, VCL
mir-221↑	5HT1 type receptor mediated signaling pathway	PRKY, SNAP29, GNAI3, GNAI2, GNB3, SNAP23
mir-449c↑	Adipocytokine signaling pathway	IRS4, ACSL1, ACSL4
mir-222↑,mir-221↑,mir-212↑	Apoptosis	BBC3, PPP3R1, APAF1, BMF, KPNA1, BCL2L11
mir-222↑,mir-221↑ mir-1246↑	Cadherin signaling pathway	PSMA2, TJP1, PSMD12, DYNLL2, CASP7, PCDHA6, PCDHA7, PCDHA8, PCDHA2, PCDHA3, PCDH11Y, PCDHA4, PCDH11X, PCDHA5, PCDHA1, PCDHAC2, PCDHAC1,
mir-199a-5p↑,mir-199a-5p↑,mir-199a-5p↑	ErbB signaling pathway	CDKN1B, KRAS, GSK3B, CBL, PIK3CD,PAK7, CDKN1B, ERBB4, PLCG1, CBL, MAPK10, PAK1,
mir-199a-5p↑	MAPK signaling pathway	FGF18, NTF3, MAP3K1, RASGRP1, SOS2, IL1B, MAPK10, FGF12, DUSP8, STK3, ACVR1C
mir-199a-5p↑	Ras Pathway	RPS6KA6, TIAM1, RPS6KA2, MAP3K1, MAP2K3, SOS2, MAP2K4, RHOB

mir-199a-5p↑	Signaling by BMP	UBE2D3, SMAD7, BMPR2, SKI, SMURF2, UBE2D1
mir-34c↓	Angiotensin II-stimulated signaling through G proteins and beta-arrestin	MAPK1, MAP2K1, GNAQ, PRKCI, GRK6
mir-34c↓	Focal adhesion	MAPK1, MAP2K1, GSK3B, PDGFRA, FIGF, COL4A6, VCL
mir-34c↓	Gap junction	MAPK1, MAP2K1, GNAQ, PDGFRA, HTR2C
mir-183↓	Histamine H1 receptor mediated signaling pathway	PRKCA, PLCB4, GNB1, GNG4, GNG5

(P value < 0.05)

4.8.2: Target prediction, functional annotation and canonical pathways identified for differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 7 of estrous cycle

A total of 19710 target genes were identified by differentially expressed miRNAs between granulosa cells of SF and DF at day 7 of estrous cycle (Fig 4.8). Out of total 5004 genes including the families of ABCA, CASP, CCDC, COX, MAPK, NOTCH and WNT genes were uniquely identified for up regulated miRNAs and 2688 genes including the family of FOX, HOX, LOC, and ZNF genes were uniquely predicted for down regulated miRNAs between granulosa cells of SF and DF at day 7 of estrous cycle. 6009 genes including the family of E2F, EIF2C, FOX, FZD, SOX, and SMARCA genes were commonly identified between the two groups.
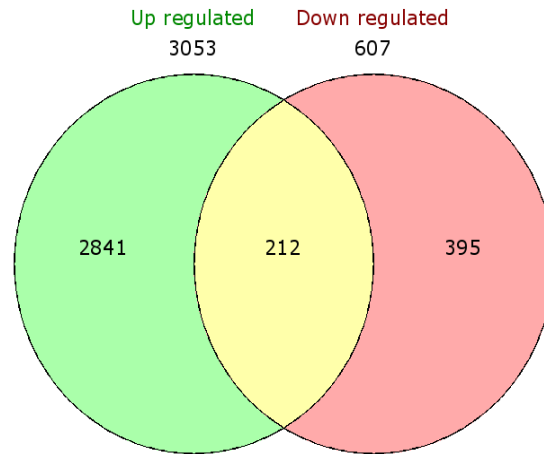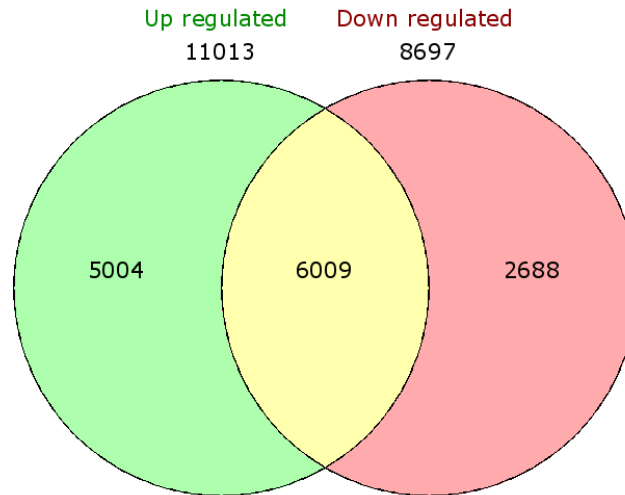
Figure 4.8: Venn diagram showing the targeted genes predicted to be regulated by differentially expressed miRNAs at day 7 of estrous cycle between granulosa cells of subordinate and dominant follicles.

The gene ontology annotation and pathway analysis were performed to identify functional modules of the differentially expressed miRNAs at day 7 of the estrous cycle between granulosa cells of SF and DF. The GO annotation enrichment results showed that regulation of transcription, regulation of RNA metabolic process, regulation of gene expression, cell adhesion, regulation of cell proliferation, cell adhesion, cell motion, cell morphogenesis, apoptosis, regulation of cell growth, and ovarian follicle development are the main significantly GO terms (Fig 4.9).

The predicted target genes of each miRNAs were uploaded to the DAVID Bioinformatics Resources version 6.7 tool and significantly ($P \leq 0.05$) enriched molecular pathways were identified. The results showed that among 108 miRNAs, the targets of 84 miRNAs were found to be involved in metabolic and signaling molecular pathways. Metabolic pathways (panthothenate and CoA biosynthesis, O-glycan and N-glycan biosynthesis, metabolism of vitamins and cofactors, D-glutamine and glutamate metabolism, biosynthesis of unsaturated fatty acids), Jak-STAT and cell cycle pathways were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of SF, whereas the Ras signaling pathway, lipids, lipoproteins, cysteine, methionine and carbohydrates metabolic pathways were enriched by genes potentially targeted by miRNAs repressed in granulosa cells of SF. Interestingly, axon guidance, Wnt signaling

pathway, GnRH signaling pathway, MAPK-signaling, oocyte meiosis, TGF-beta signaling pathway and focal adhesion were enriched by genes targeted by miRNAs either enriched or repressed in granulosa cells of SF (Figure 4.10, Figure 4.11). The top most enriched pathways of target genes for differentially expressed miRNAs between granulosa cells of SF and DF at day 7 of estrous cycle are shown in appendix 3.
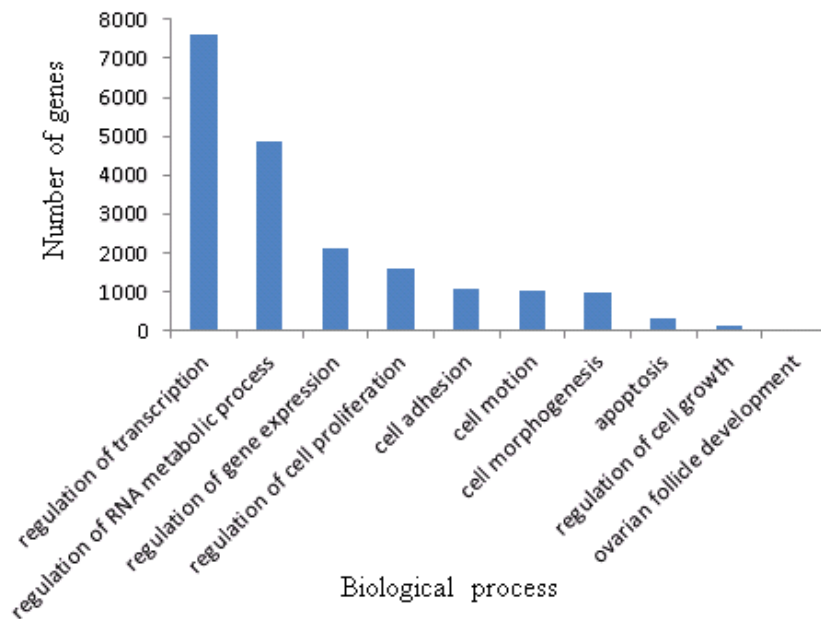
Figure 4.9: Partial gene ontology (GO) classification annotated for predicted target genes in biological process of miRNAs differentially expressed between granulosa cells of SF and DF at day 7 of estrous cycle.
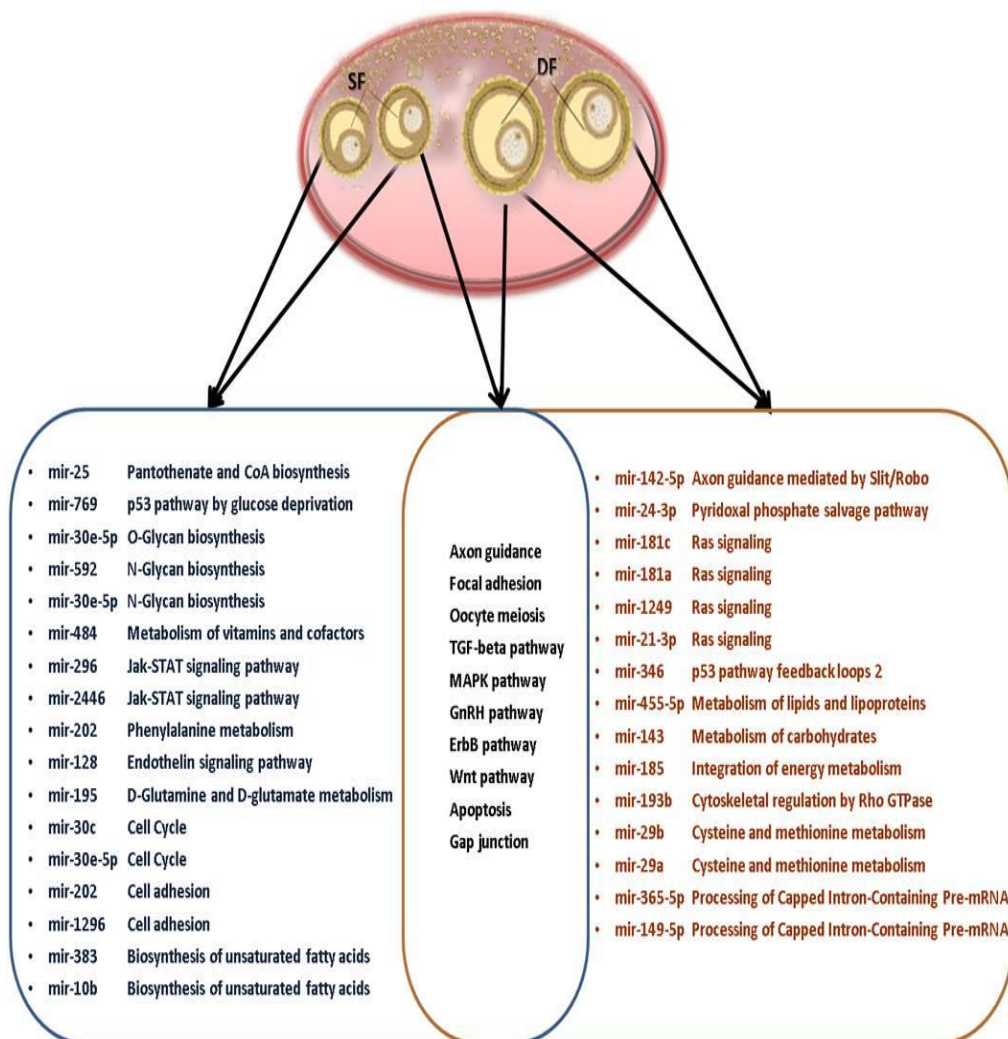
Figure 4.10: Significant molecular pathways (P ≤ 0.05) enriched by genes targeted by differentially expressed miRNAs between the granulosa cells of SF and DF at day 7 of the estrous cycle. Pathways enriched by genes potentially targeted only by miRNAs increased in SF are indicated in the left box, pathways enriched by genes potentially targeted by both miRNAs repressed and activated in SF are shown in the middle box while pathways enriched by genes potentially targeted only by miRNAs upregulated in DF but repressed in SF are described in the right box.
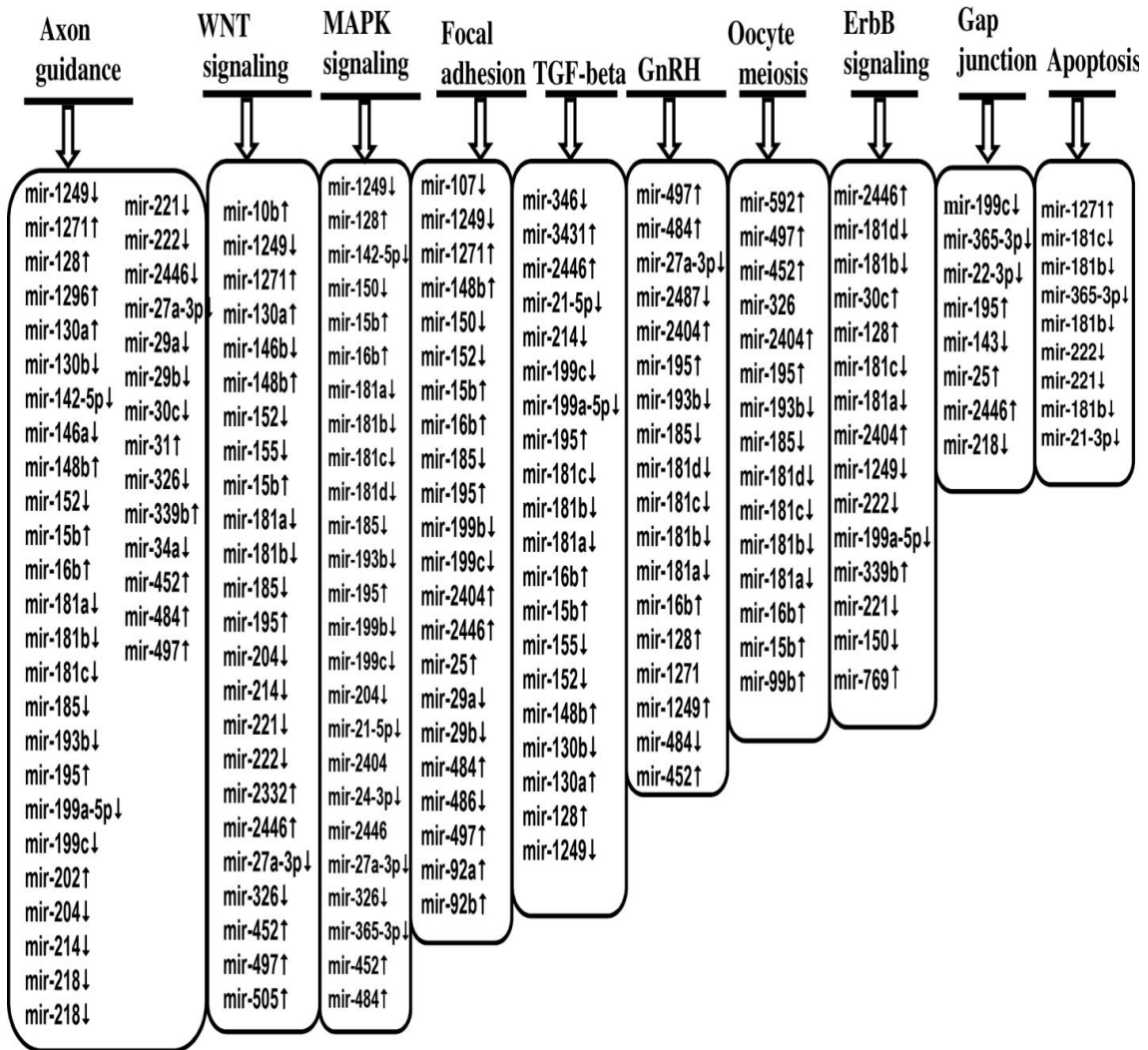
Figure 4.11: The list of differentially expressed miRNAs whose target genes are enriched (p ≤ 0.05) in Wnt signaling, GnRH signaling, MAPK, signaling, oocyte meiosis, TGF-beta signaling, focal, adhesion, ErbB, gap junction, axon guidance and apoptosis. (↑) indicates increased expression while (↓) depicts the reduction of miRNA expression in granulosa cells of SF compared to DF groups at day 7 of the estrous cycle.

4.8.3   Target prediction, functional annotation and canonical pathways identified for commonly differentially expressed miRNAs between the granulosa cells of subordinate and dominant follicles at day 3 and day 7 of estrous cycle

The software was allowed to predict miRNA target genes, performed the enrichment of KEGG, PANTHER and REACTOME pathways from the predicted targets. *Insilco* analysis of the function of the miRNAs via their target genes indicated that these miRNAs were found to be involved in 2 or more pathways. For instance, miR-221 and mir-222 were found to be involved in Wnt and cadherin signaling pathways by targeting protocadherin alpha family of genes (*PCDHA1,-2,-3,-4, -5,-6,-7,-8* etc.). In addition, miR-221, is also involved in apoptosis and 5HT1 type receptor mediated signaling by targeting genes involved in these pathways. Similarly, mir-21-5p was found to be involved in TGF-beta signaling pathway, regulation of actin cytoskeleton and MAPK signaling pathway and mir-199a-5p was found to be involved in TGF-beta signaling pathway, epidermal growth factor receptor (EGFR) signaling, membrane trafficking and insulin signaling pathway (Figure 4.12).

miRNA	Day 3	Day 7	Pathways	Target genes
miR-708	↑	↓	PI3 kinase pathway	NRAS, PTGER3, FOXO1, FOXJ3
			Interleukin signaling pathway	NRAS, FOXO1, IFT81, FOXJ3, IKBKB
miR-222	↑	↓	PI3 kinase pathway	YWHAG, FOXL1, GNAI3, GNAI2, FOXN2, FOXJ3, PIK3R1
			Wnt signaling pathway	PCDHA6,-7,-8, -2,-11Y,-3, -11X,-4, -5,-1, -2,-1, -10, -12,-13
			Cadherin signaling pathway	PCDHA6, -7, -8,-3,-11Y,-4,-11X,-5,-1,-2, -1, -10,-12,-13
miR-221	↑	↓	Wnt signaling pathway	PCDHA6,-7,-8,-9,-2,-3,-4,-5,-1,-2,-C1, -10,-12,-13
			Cadherin signaling pathway	PCDHA6,-7,-8,-9,-2,-11Y,-3,-11X,-4, -10,-A5, -1,-C2,-C1, -10, -12,-13
			Apoptosis	BBC3, STK24, PPP3R1, APAF1, BMF, KPNA1, BCL2L11
			5HT1 type receptor mediated signaling	PRKY, SNAP29, GNAI3, GNAI2, GNB3, SNAP23
miR-21-5p	↑	↓	TGF-beta signaling pathway	SMAD7, BMPR2, SMURF2, ACVR1C, BMP8A, PITX2
			Regulation of actin cytoskeleton	FGF18, TIAM1, ARHGEF7, ITGB8, CFL2, PIKFYVE, SOS2, FGF12, VCL
			MAPK signaling pathway	FGF18, NTF3, MAP3K1, RASGRP1, SOS2, IL1B, MAPK10, FGF12
			Wnt signaling pathway	SENP2, TBL1XR1, PLCB4, VANGL1, VANGL2, PPP2CB, WNT9A, FZD4
miR-214	↑	↓	TGF-beta signaling pathway	MAPK1, ACVR1B, ACVR2B, ACVRL1, TGFBR1, PPP2CB, BMP8B, RBX1
			Phosphatidylinositol signaling system	CDS2, CDIPT, PLCB4, DGKG, DGKZ, ITPR3, PTEN
			Insulin signaling pathway	MAPK1, PDPK1, CRKL, SOCS2, SORBS1, FLOT2, CBL, IKBKB, TRIP10
			Adherens junction	PVRL4, FGFR1, MAPK1, ACVR1B, SORBS1, PVRL1, TGFBR1, ACTN1
			Ubiquitin mediated proteolysis	MAP3K1, UBR5, TRIM32, UBE4B, UBA3, BIRC3, UBE2L3, CDC27
miR-21-3p	↑	↓	MAP kinase cascade	RPS6KA6, RPS6KA2, MAP2K3, SOS2, MAP2K4
			Ubiquitin mediated proteolysis	MAP3K1, UBR5, TRIM32, UBE4B, UBA3, BIRC3, UBE2L3, CDC27, UBE2Q1
			Apoptosis signaling pathway	MAP4K3, BAG4, MAP3K1, CASP7, MAP2K3, MAP2K4, TP53, HSPA14
miR-199a-5p	↑	↓	Signaling by EGFR	KRAS, CBL, CLTC, PXN
			TGF-beta signaling pathway	ACVR2A, BMP3, ACVR1B, ACVR2B, ACVRL1, KRAS, RAB10, JUNB
			Membrane Trafficking	STX4, AP1G1, ARCN1, CLTC
			Insulin signaling pathway	KRAS, GSK3B, CBL, PIK3CD, HK2, IKBKB, PPARGC1A, PCK1
miR-155	↑	↓	Wnt signaling pathway	PCDHA1,-6, -8,-9,-2, -3, -4,-5, -9, -10, -12, SMAD2, SMAD1
			TGF-beta signaling pathway	SP1, GDF6, SMAD2, RPS6KB1, SMAD1, ACVR1C

Figure 4.12: Uniquely and commonly differentially expressed miRNAs between the granulosa cells of SF and DF at day 3 and day 7 of the estrous cycle and their pathways enriched by their potential target genes. (↑) shows upregulation while (↓) indicate downregulation of commonly differentially expressed miRNAs in the granulosa cells of SF compared to DF at day 3 or day 7 of the estrous cycle.

4.8.4 Target prediction and functional annotation of differentially expressed miRNAs in granulosa cells of dominant follicles between day 3 and day 7 of estrous cycle

The gene ontology annotation and pathway analysis were performed to identify functional modules of the differentially expressed miRNAs in granulosa cells of DF between day 3 and day 7 of estrous cycle. A total of 19897 target genes were identified by differentially expressed miRNAs between granulosa cells of DF at day 3 and 7 of estrous cycle. Out of total 3235 genes were uniquely identified for up regulated miRNAs and 4296 were uniquely predicted for down regulated miRNAs, while 6183 genes were commonly identified in granulosa cells of DF between day 3 and day 7 of estrous cycle. The pathway analysis results showed that including transcription, the neuroactive ligand-receptor interaction, apoptosis signaling pathway, natural killer cell mediated cytotoxicity, Fc gamma R-mediated phagocytosis, tight junction, metabolism of carbohydrates, notch signaling pathway, axon guidance mediated by Slit/Robo and axon guidance mediated by netrin were enriched by genes targeted by miRNAs elevated at day 7, whereas the vitamin D and Phenylalanine metabolism, O-glycan and N-glycan biosynthesis, mRNA metabolic process, cell cycle and biosynthesis of unsaturated fatty acids were enriched by genes targeted by miRNAs downregulated at day 7 the estrous cycle. On the other hand, Wnt signaling, vascular endothelial growth factor (VEGF) signaling, TGF-beta signaling, epidermal growth factor receptor (EGFR) signaling, oocyte meiosis, MAPK signaling, GnRH signaling, focal adhesion, axon guidance, angiogenesis, calcium signaling and ubiquitin mediated proteolysis were enriched by genes potentially targeted by both down and upregulated miRNAs (Figure 4.13). The top most enriched pathways of target genes for differentially expressed miRNAs in granulosa cells of dominant follicles between day 3 and day 7 of estrous cycle are described in appendix 4.
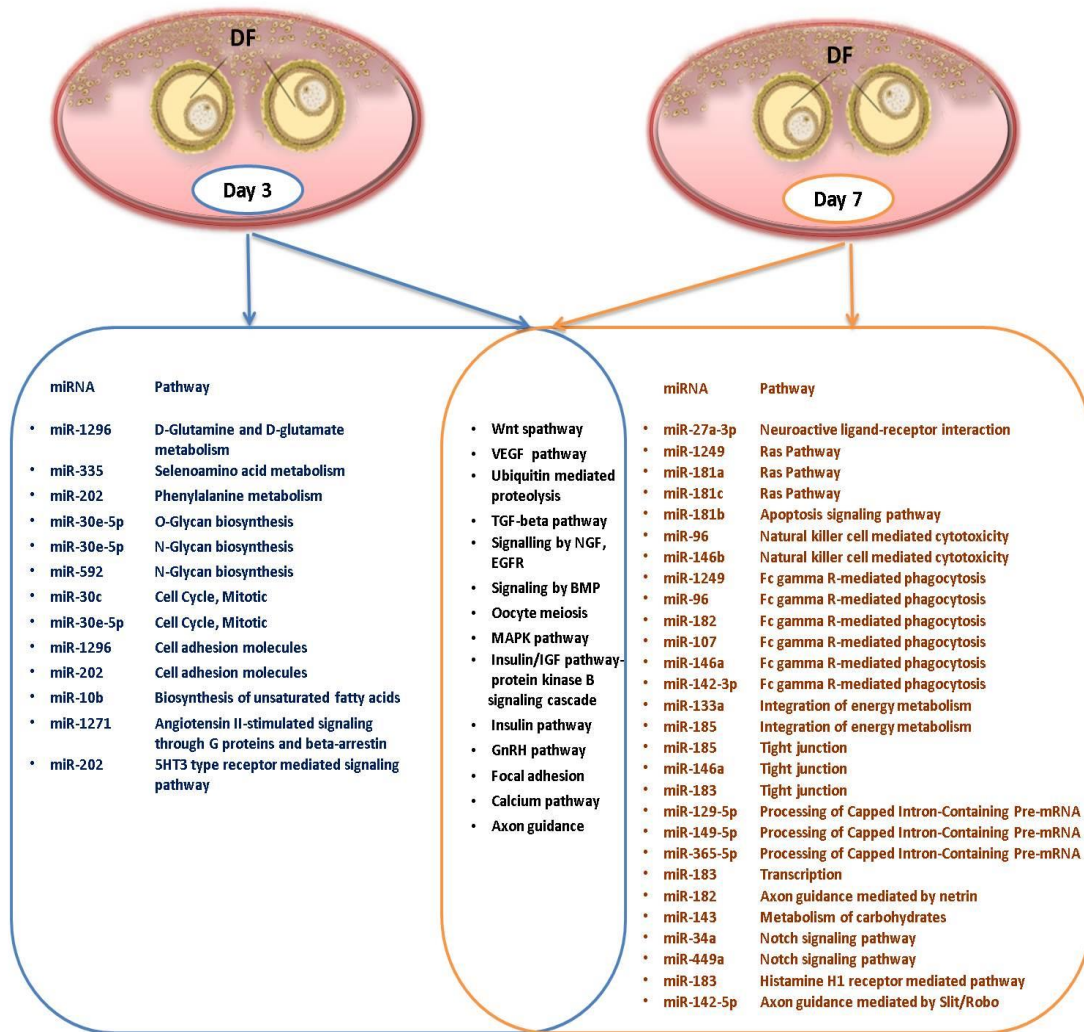
Figure 4.13: Graphical illustration of DF follicles and molecular pathways enriched by genes targeted by differentially expressed miRNAs between day 3 and day 7 of the estrous in granulosa cells of DF. Pathways significantly (P ≤ 0.05) enriched by genes potentially targeted only by miRNAs enriched at day 3 are indicated in the left box. Pathways enriched by genes potentially targeted by miRNAs increased at day 3 and day 7 of the estrous cycle are listed in the middle box and pathways enriched by genes potentially targeted only by miRNAs increased at day 7 of the estrous cycle are described in the right box. DF, dominant follicle, Day 3 and Day 7 indicate the stages of the estrous cycle post estrus.

4.8.5 Target prediction and functional annotation of differentially expressed miRNAs in granulosa cells of subordinate follicles between day 3 and day 7 of estrous cycle

The gene ontology annotation and pathway analysis were performed to identify functional modules of the differentially expressed miRNAs in granulosa cells of SF between day 3 and day 7 of estrous cycle. A total of 3326 target genes were identified by differentially expressed miRNAs in granulosa cells of SF at day 3 and 7 of estrous cycle. Out of total 478 genes were uniquely identified for up regulated miRNAs and 2713 were uniquely predicted for down regulated miRNAs in granulosa cells of SF between day 3 and day 7 of estrous cycle. Moreover, 135 genes were commonly identified in granulosa cells of SF between day 3 and day 7 of estrous cycle. Similarly, the pathway analysis revealed top pathways that were enriched in differentially expressed miRNAs. MAPK signaling pathway, Wnt signaling pathway, signalling by NGF, cadherin signaling pathway, integrin signalling pathway, focal adhesion, axon guidance, GnRH signaling pathway, apoptosis, oxytocin receptor mediated signaling pathway, insulin signaling pathway and progesterone-mediated oocyte maturation ranked among the most enriched pathways targeted by both up regulated and down regulated miRNAs (Table 4.9).

Table 4.9: The most enriched pathways of target genes for differentially expressed miRNAs between granulosa cells of subordinate follicles at day 3 and day 7 of estrous cycle

miRNAs Id	Term	Genes count	Genes
mir-1271↓,mir-424-5p↓	MAPK signaling pathway	62	MAP2K7, RASA1, PRKCA, MAP2K1, NF1, MAPK11, CACNA2D2, MAP4K4, RPS6KA3, MAP3K14, CACNA1C, PLA2G5, DUSP6, FGFR1, MAP2K1, GNA12, PPM1A, PTPRR
mir-1271↓,mir-424-	Wnt signaling	59	RAC1, NFAT5, FRAT2, NFATC4,

5p↓,mir-155↑	pathway		CHP, FBXW11, PLCB2, WNT8B, FZD4, WNT2B, FZD6,PRICKLE2, SFRP4, LRP6, TBL1X, TBL1XR1, DVL3, WNT3A, BTRC, SMAD3
mir-1271↓,mir-424-5p↓	Signalling by NGF	52	TRIB3, FOXO1, FOXO3, FOXO4,ATF1, PRKAR2B, LINGO1, KRAS, CASP9, MAPKAP1,CASP2, FRS2, PIK3R1, PPP2R1A, MAP2K1, PRKCI, MAPK11,
mir-1271↓,mir-155↑	Cadherin signaling pathway	51	PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHAC2, PCDHGA2, PCDHAC1, CDH22, PCDHA10, PCDHA11, PCDHA12, PCDH17, PCDHGB4, WNT2B, FZD6, FYN,
mir-1271↓	Integrin signalling pathway	41	ARFGAP1, ENAH, CAV1, GRB2, COL3A1, MAP3K3, RAC2, ITGB8, ITGAV, RAC1, RHOB, COL6A1, SHC1, MAP2K7, PIK3R1, MAP2K1,MAPK11, NAV3, COL4A6, ITGA6,
mir-1271↓	Focal adhesion	31	CAV1, XIAP, GRB2, COL3A1, DOCK1, PAK2, RAC2, ITGB8, ITGAV, RAC1, , COL4A4, PRKCA, COL4A2, VAV3, MAP2K1, MYLK2, ITGA2, COL4A6, CCND1, ITGA6,
mir-1271↓	Axon guidance	25	ABLIM1, ABLIM2, GNAI3, EFNA1, EFNA3, PPP3R1, KRAS, PAK2, RAC2, RAC1, NFAT5, NTN4, DPYSL5, ARHGEF12, NTN1, SEMA6A, SEMA6B,
mir-1271↓	GnRH	20	ADCY7, MAP2K1, ADCY6,

	signaling pathway		MAPK11, MMP2, ITPR2, PLCB4, MAP3K4, KRAS,
mir-1271↓	Apoptosis	16	TRAF2, XIAP,PPP3R1, ENDOD1, ATM, CASP9, RIPK1, PRKAR1A, CHP, MAP3K14,
mir-1271↓	Oxytocin receptor mediated signaling pathway	14	PRKCA, PRKCI, CACNB1, CACNB2, CACNB4, GNG12, PRKCE, PLCB4, PLCD3, GNB4, VAMP3, CACNA1C, PLCB2, GNG7
mir-424-5p↓	Insulin signaling pathway	14	MAP2K1, PRKAB2, PHKA1, PDE3B, MKNK1, IRS1, PRKAR2A, TSC1, SOS2,
mir-424-5p↓	Progesterone-mediated oocyte maturation	10	RPS6KA6, RPS6KA3, GNAI3, MAP2K1, ADCY5, PDE3B, CDC23, AKT3, CDC25A,

(↑) shows upregulation while (↓) indicate downregulation of differentially expressed miRNAs in the granulosa cells of subordinate follicles at day 3 and day 7 of estrous cycle, (P value < 0.05).

4.9 Novel miRNAs detected in granulosa cells of subordinate and dominant follicles at days 3 and 7 of estrous cycle

In addition to known miRNAs, novel miRNAs were identified from the sequenced data. For this, miRDeep2 novel miRNAs prediction tool was used and a sequence was considered as novel miRNA when it was not aligned to known miRNA in mirBase database. Once the sequence is confirmed as novel miRNA, it was considered as a detected when the average read counts in at least two of the biological replicates within the same sample group was $\geq$ 10 read counts. Based on this analysis, a total of 21 candidate novel miRNAs with base pair length of 17-22 were identified and detected in granulosa cells of SF or DF at day 3 or day 7 of the estrous cycle. Out of 21 candidate novel miRNAs, 13 candidates were found to be localized in the intergenic region while 8 of them were found in to be located the intronic part of CEP20,VCPIP1, EIFF31, ERRCC3, GNB1L, TEX14 or RPL12 genes (Table 4.10).

Table 4.10: Novel candidate miRNAs detected in granulosa cells of SF or/and DF at day 3 or/and day 7 of the estrous cycle

Ids. no. miRNAs	Sequence of novel miRNAs	Ch	Precursor coordinate	DNA strand	Day 3 SF	Day 3 DF	Day 7 SF	Day 7 DF	Genomic location
X_itw_0276	ccccgggcccgcgguuc	X	62078855..62078921	minus	11858	·	14018	·	Intergenic
13_itw_0295	cccggggagcccggcggu	13	38543580..38543652	minus	1190	1691	734	1096	Intergenic
X_itw_0140	auuggcaugucuuggaaugaag	X	30338368..30338426	minus	70	81	33	·	Intronic
7_itw_0345	ccgccgguccccgcccc	7	31762590..31762667	plus	57	·	·	·	Intronic (*CEP20*)
X_itw_0247	ugauugguacuucuuagagugga	X	29130215..29130265	plus	20	22	17	·	Intergenic
X_itw_0078	ugauaauacaaccugauaagu	X	82023929..82023981	minus	18	27	18	16	Intergenic
17_itw_0191	aaaaccggaaugaauuuuuuga	17	44700968..44701028	plus	14	12	20	·	Intergenic
X_itw_0207	ugauuggcauuucuuagagugga	X	29133927..29133985	plus	14	12	12	·	Intergenic
14_itw_0201	cggcggcggcggcgacu	14	32908534..32908603	minus	13	13	·	·	Intronic (*VCPIP1*)
5_itw_0362	aucccacuccugacacca	5	110183750..110183789	minus	12	44	30	13	Intronic (*EIF3l*)
X_itw_0284	uacugugccucgaauggguaug	X	30309767..30309823	plus	·	24	10	·	Intergenic
24_itw_0112	auaaccggaaugaacuuuuuga	24	6436128..6436188	plus	·	19	·	·	Intergenic
25_itw_0146	uccaaguaauucaggauagu	25	41470615..41470665	plus	·	17	17	·	Intergenic
2_itw_0270	caaaaaguucguccagauuuuu	2	5205393..5205455	plus	·	28	16	·	Intronic (*ERCC3*)
17_itw_0056	ggccaggggcgugucgggcucu	17	74856138..74856194	minus	·	14	·	·	Intronic (*GNB1L*)
13_itw_0186	agaaaaguuuguuugggguuuuu	13	73541941..73542001	minus	·	·	·	23	Intergenic
11_itw_0182	gccgaaagcaugggaacaggc	11	98192709..98192784	minus	·	·	·	34	Intronic (*RPL12*)

The numbers under SF and DF columns indicate the average read counts for each novel candidate miRNAs, Ch = chromosome number

4.10 Validation of deep sequencing data for their expression pattern between the granulosa cells of subordinate and dominant follicles at day 3 and day 7 of estrous cycle by using qRT-PCR

Although the results obtained by sequencing are believed to be more realistic than other methods, we opted to measure the expression level of selected candidate miRNAs using the qPCR by assuming that if the results obtained by the higher sensitive method are validated by relatively lower sensitive technique, the results obtained could be more reliable. Accordingly, the results of the qPCR data showed a similar trend of miRNA expression patterns as that of the deep sequencing result. Differences and similarities within and between biological replicates of the qPCR data along with the deep sequencing data is described in figure 4.14.
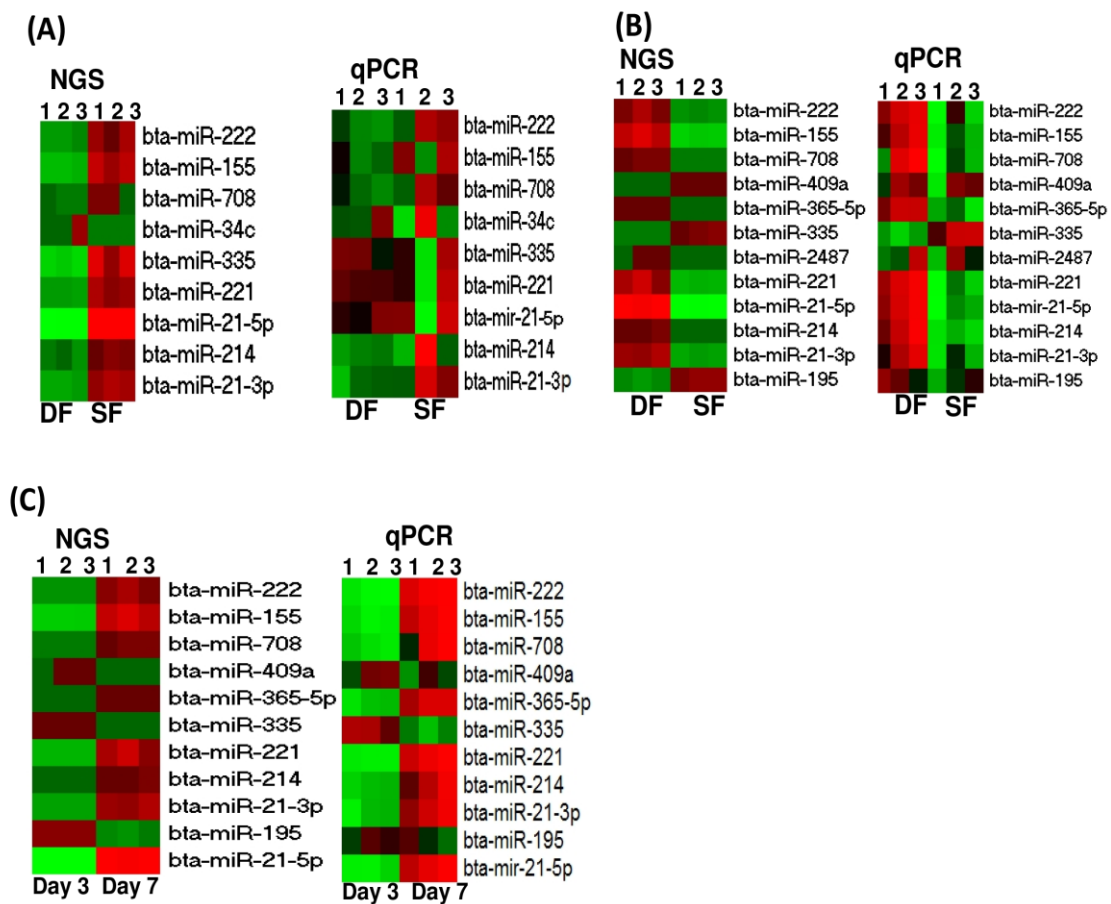
Figure 4.14: The heatmap showing the qPCR data along with the deep sequencing data for randomly selected differentially expressed miRNAs. (A) The expression pattern of candidate miRNAs in granulosa cells of SF and DF at day 3 of the

estrous cycle. (B) The expression pattern of candidate miRNAs in granulosa cells of SF and DF at day 7 of the estrous cycle. (C) The expression pattern of candidate miRNAs in granulosa cells of DF at day 3 and day 7 of the estrous cycle. The red and green colours indicate high and low expression, respectively. NGS and qPCR indicate the results obtained from next generation deep sequencing and quantitative real time qPCR, respectively. Numbers, 1, 2 and 3 on the heatmaps indicate the number of biological replicates used in each sample group.

4.11 Expression of differentially expressed miRNAs in companion follicular cells (granulosa cells, theca cells, COCs and follicular fluid) of subordinate and dominant follicles at day 3 of estrous cycle

The relative abundance of selected candidate miRNAs was determined in theca cells, cumulus-oocyte-complex and follicular fluid derived from corresponding SF and DF from which the granulosa cells were isolated. Results showed that the (bta-miR-21-3p and bta-miR-155) was higher in granulosa cells (GC), theca cells (TC), cumulus oocyte complex (COC) and follicular fluid (FF) of the SF compared with the DF counterparts (Figure 4.3). Similarly, bta-miR-214, bta-miR-221 and bta-miR21-5p were higher in follicular fluid, granulosa cells and theca cells while it was downregulated in cumulus-oocyte-complex of the SF compared to the DF. Moreover, bta-miR-708 and bta-miR-222 was higher in granulosa cells and theca cells while lower in the follicular fluid and COCs of SF compared to the dominant follicles. Among these bta-miR-34c was lower in all follicular cells except theca cells where as bta-miR-335 was higher in granulosa cells while lower in theca and COCs of the subordinate group at day 3 of estrous cycle (Figure 4.15).
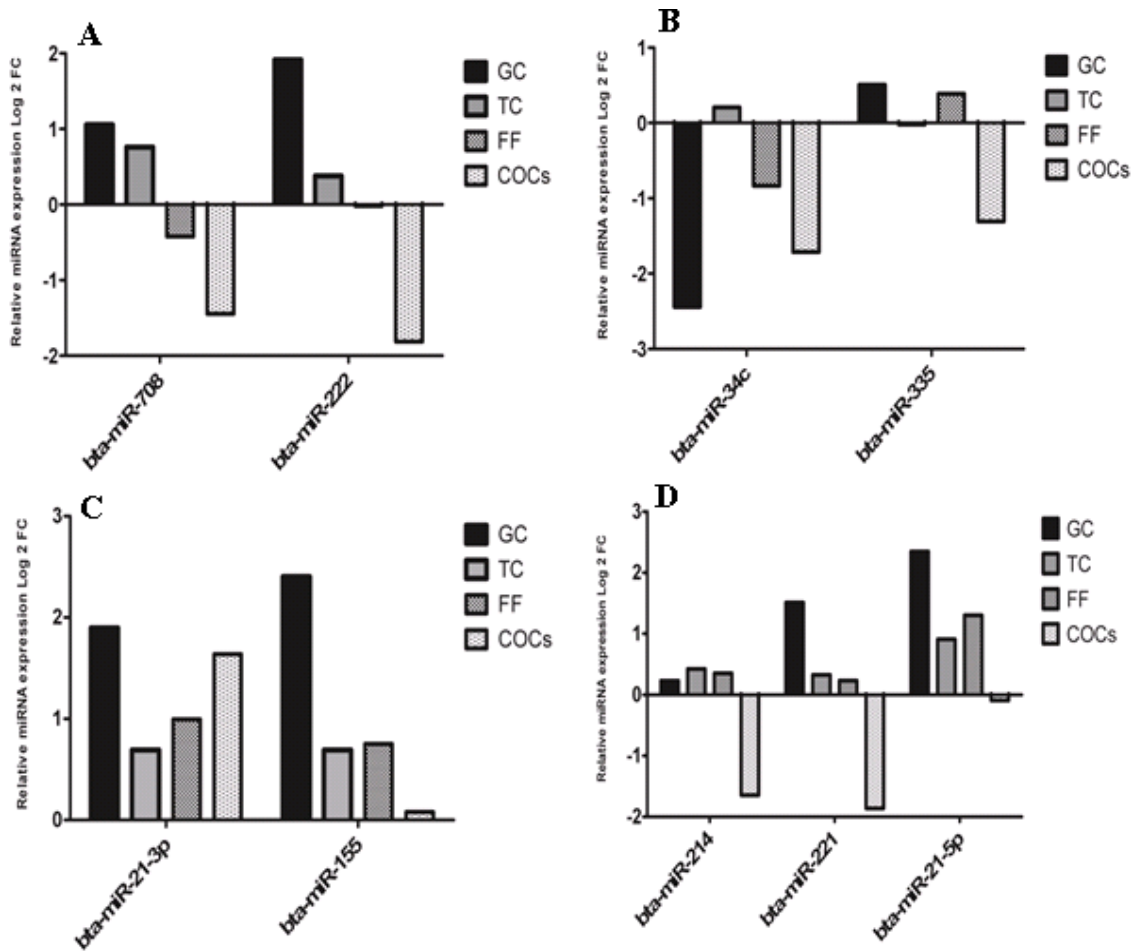
Figure 4.15: Expression pattern of miRNAs in different follicular cells at day 3 of estrous cycle (A) bta-miR-21-3p and bta-miR-155 (B) bta-miR-214, bta-miR-221 and bta-miR21-5p (C) bta-miR-708 and bta-miR-222 (D) bta-miR-34c and bta-miR-335   in companion follicular cells of both subordinate and dominant follicles using qPCR. The mean expression value of target miRNA was normalized against U6 snRNA and 5s rRNAs as an endogenous control. Relative expression values were calculated using ΔΔCT method.

4.12 Expression of differentially expressed miRNAs in granulosa and theca cells of subordinate and dominant follicles at day 7 of estrous cycle

The relative abundance of selected candidate miRNAs was determined in granulosa cells and theca cells from subordinate and dominant follicles. Results showed that ten selected miRNAs (bta-miR-409a, bta-miR-195, bta-miR-214, bta-miR-21-5p, bta-miR-21-3p, bta-miR-222, bta-miR-155, bta-miR-708, bta-miR-221 and bta-miR-365-5p) were higher in granulosa and theca cells of SF group with the counter part of DF (Figure 4.16). While bta-miR-335 was enriched in granulosa cells and lower in theca of the SF compared to the DF. Moreover, bta-miR-2487 was lower in granulosa cells and higher in theca cells of SF compared to the DF at day 7 of estrous cycle.
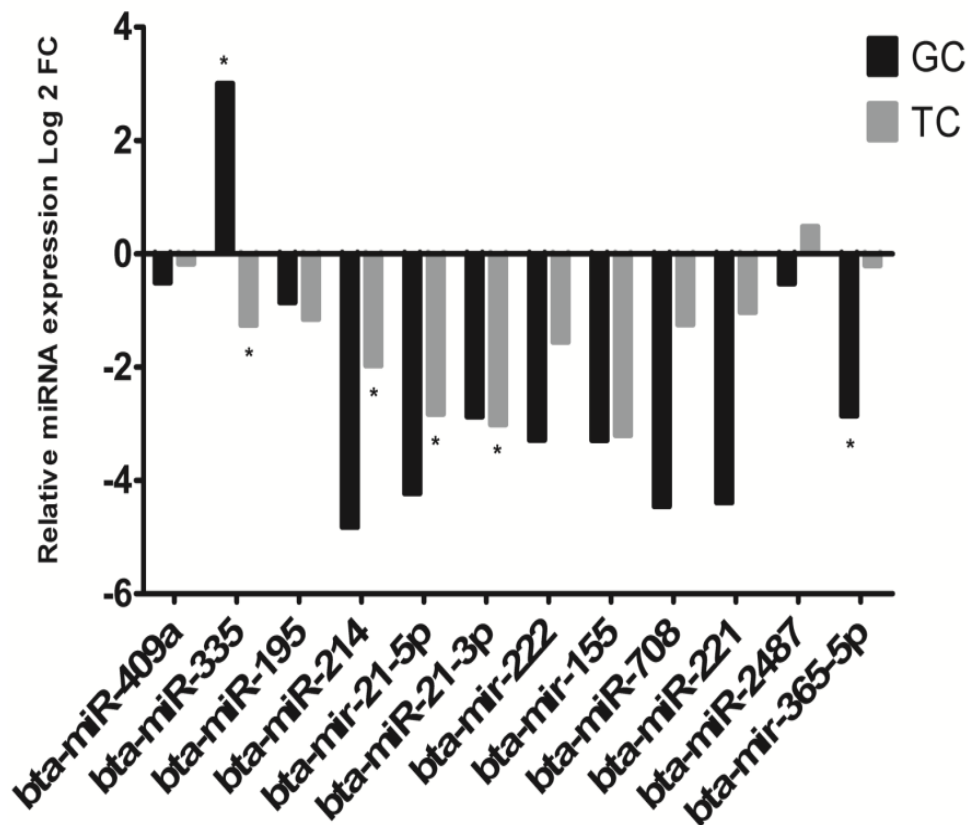
Figure 4.16: Expression pattern of miRNAS in granulosa and theca cells at day 7 of estrous (*P<0.05)

4.13 Expression of differentially expressed miRNAs in granulosa cells, theca cells and follicular fluid of dominant follicles between day 3 and day 7 of estrous cycle

The relative abundance of selected candidate miRNAs was determined in granulosa cells, theca cells and follicular fluid from DF at two different time points of estrous cycle. Results showed that nine selected miRNAs (bta-miR-155, bta-miR-221, bta-miR-222, bta-miR-21-3p, bta-miR-708, bta-miR-21-5p, bta-miR-365-5p and bta miR-214) were higher in granulosa cells, theca cells and follicular fluid of DF at day 3 of estrous cycle with the counter part of day 7 of estrous cycle (Figure 4.17). While, bta-miR-195 and bta miR-335 was enriched in theca cells and lower in granulosa cells and follicular fluid. Moreover, bta-miR-409a was lower in granulosa cells and theca cells and enriched in follicular fluid.
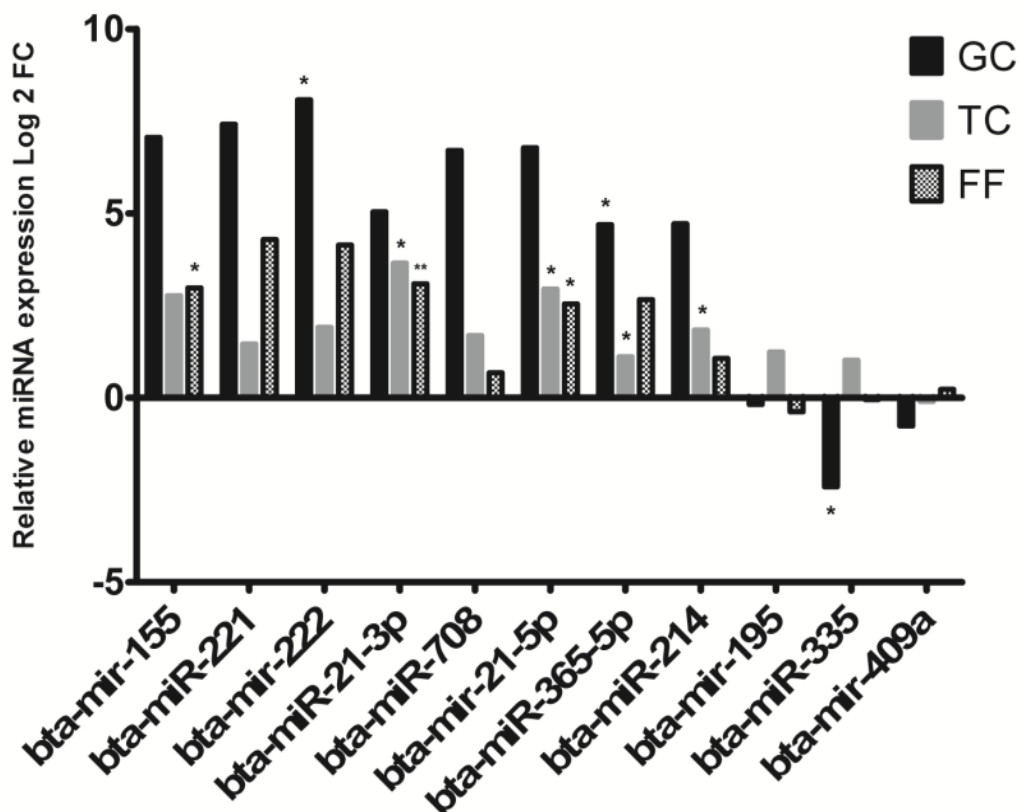
Figure 4.17: Expression pattern of selected miRNAS in different follicular cells of dominant follicles between day 3 and day 7 of estrous cycle (* P<0.05, ** p<0.02).

5 Discussion

During follicular development, miRNAs are obligatory to accomplish vital roles in the course of folliculogenesis. Follicles are the most commanding units of the ovary. Advancement of follicles within the ovary is a dynamic process which follows throughout the estrus cycle and comprises recruitment of follicles into the growing pool, physiological selection of an ovulatory follicle, and ovulation or regression. Though the ultimate fate for utmost of the follicles is atresia, which occurs at any stage but the dominant follicle survives and become the part of healthy pool of antral follicles, continually developing and eventually ovulation took place. These sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis, are reoccurring on a cyclical basis within the ovary and resulting in the development of a number of ovulatory follicles (Hunter et al. 2004). The capability of an ovulation of a selected dominant follicle from the cohort of same sized follicles is an acute transition in follicular development and the mechanisms underlying this are not well understood. Cattle deliver a very useful animal model for studies on follicular recruitment, selection, dominance, atresia and ovulation. Two or three consecutive waves of follicular development occur during the bovine estrous cycle, each generating a dominant follicle capable of ovulating if luteal regression occurs, whereas its sister subordinate follicles upland in growth and then degenerate (Fortune et al. 2001). The granulosa cells play a very important role during folliculogenesis. The expansion of oocyte and emerging into mature form is accompanied by the proliferation and differentiation of the surrounding granulosa cells during folliculogenesis (Eppig 1985). Therefore, it may be potential to envisage that the subordinate and dominant follicles have exclusive molecular signals that may alter contrarily the bidirectional crosstalk between the oocytes and the granulosa cells (Oktem and Urman 2010, Vanderhyden and Macdonald 1998).

Generating miRNA expression profiles in granulosa cells obtained from bovine subordinate and dominant follicles at defined developmental stages of estrous cycle using the Illumina deep sequencing approach is a prerequisite for a better understanding of their physiological involvement in relation to follicle recruitment, selection and maturation in the monovular bovine ovary and miRNA-mRNA interactions involved in follicular development. So, the major goal of this study was to understand the complete

miRNA transcriptome in the context of follicular development. The Illumina deep sequencing platform is effective for miRNA detection and is broadly used to produce small RNA profiles in numerous organisms. Deep sequencing of the small RNA fragment within cells produces a remarkably enormous quantity of data in the form of read counts, from which not only the expression levels of microRNAs are determined but the discovery of novel miRNAs also took place (Creighton et al. 2009). In the present study we investigated enrichment and degradation of miRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. For this, we have generated miRNA sequencing data from 12 granulosa samples derived from subordinate and dominant follicles both at day 3 and day 7 of the estrous cycle using high throughput sequencing technology. Meanwhile the sequencing data were generated from three biological replicates of each sample, we believe that the analysis has been robust and the findings could be repeatable. Furthermore, a definite miRNA was considered to be detected when it was expressed at least in two of the three biological replicates of each sample. Therefore, from our data, we detected numerous miRNAs in granulosa cells of subordinate and dominant follicles. Remarkably, 244 miRNAs were commonly detected in all samples irrespective of the follicle stage and days of the estrous cycle (Figure 4.2). For example, bta-miR-10b, bta-miR-26a, bta-miR-99b, bta-miR-27b, let-7 families (bta-let-7f, bta-let-7a-5p and bta-let-7i), bta-miR-92a, bta-miR-191, bta-miR-125a, bta-miR-143, bta-miR-30d and bta-miR-30a-5p were among the top 15 abundantly expressed miRNAs in both of the follicular groups and at each time point of the estrous cycle (Table 4.2). Certainly, the function of these miRNAs during folliculogenesis is still narrow, but the let-7 family members are thought to be involved in cell proliferation by targeting multiple genes (Johnson et al. 2007). Also, the let-7 miRNA family expressed abundantly in ovary and oocyte of bovine in several studies by cloning and deep sequencing techniques (Huang et al. 2011, Miles et al. 2012, Tesfaye et al. 2009, Tripurani et al. 2010), as well as in murine ovaries and testis (Reid et al. 2008). Similarly, miR-10b is stated to be involved in cell proliferation and growth by targeting the TGF-beta signaling pathway (Han et al. 2014). On the other hand bta-miR-143 is reported in the mouse ovary whose expression is insignificant in primordial follicles, but became readily detectable in the granulosa cells of primary, secondary and antral follicles. Thus by defining that the expression of bta-mir-143 is under the negative control of FSH which suggests that FSH regulates

folliculogenesis by a nowel pathway of miRNAs (Yao et al. 2009). Also, bta-miR-143 is highly expressed in the bovine testis and ovary and targets genes which participate in pathways associated with reproduction (Huang et al. 2011). Furthermore, miR-148a, miR-26a and miR-30d expression pattern has been documented in mouse and bovine gonads (Hossain et al. 2009, Mishima et al. 2008, Tripurani et al. 2010) . Moreover, miR-26a is stated to have anti-apoptotic effects in many cancers (Garzon et al. 2006, Lu et al. 2005, Saito and Jones 2006, Zhang et al. 2011). Similarly, mir-99b being a positive modulator of the TGF-β pathway regulates cell proliferation and also perform key roles in cancer development and progression, as well as in embryonic growth (Turcatel et al. 2012). The abundantly expressed miRNAs in SF and DF at different time points of estrous indicating their potential role in the follicular growth in the bovine and they may have important roles in female reproductive physiology. Therefore, the expression of these miRNAs in all cases could indicate their housekeeping cellular roles (Tripurani et al. 2010) during follicular recruitment, selection, dominance or follicular atresia.

5.1 At day 3 of the estrous cycle, the granulosa cells of subordinate follicle (SF) exhibited triggering of miRNAs equated to dominant follicles (DF)

Analyzing the spatiotemporal expression patterns of miRNAs would provide valuable information for their physiological functions (Wang et al. 2007). Differential expression of definite miRNAs at a certain stage and size in the somatic cells of bovine estrous cycle may deliver valued perception into their impending role in the development of a follicle for recruitment, selection, dominance, atresia and ovulation. Even though, 244 miRNAs were commonly detected in all sample groups, it does not necessary entail that they were likewise expressed. Therefore, to distinguish significant differences in the expression of miRNAs during the early luteal stage of the bovine estrous cycle, a differential miRNA expression analysis was directed between the granulosa cells of SF and DF at day 3 of the estrous cycle. On this day of the cycle, 14 out of 16 differentially expressed miRNAs were triggered in granulosa cells of SF compared to DF equivalents (Figure 4.3). Certainly, it is questionable to fully apprehend why the majority of the differentially expressed miRNAs were triggered in SF compared to DF. However, similar studies in gene expression at day 3.5 of estrous cycle showed that 11 of the 16

genes measured by qPCR were found to be activated in granulosa cells of SF compared to DF (Evans et al. 2004). In fact, the activation of miRNAs in granulosa cells of SF at day 3 of the estrous cycle could partly be linked to the repression of several genes associated with masking of the estrogen and progesterone production in granulosa cells. On this regard, (Evans and Fortune 1997) reported that at day 3 of the follicular wave, the granulosa cells of SF secreted lower estradiol levels and exhibited reduced level of FSH receptor expression compared to the granulosa cells of the DF. Thus, the reduction of estradiol and follicle stimulating hormone receptor (FSH) gene expression may also be associated with downregulation of several arrays of genes potentially targeted by activated miRNAs. With the connection of this, the current study specified a 2.3 to 16 fold change increase in the expression of 14 miRNAs in SF group. Among these, miR-449a and mir-449c were activated by 16 and 10.6 folds, respectively while miR-221 and miR-222 were increased by 3.5 and 6.5 folds, respectively. Similarly, the miR-21 family (bta-miR-21-3p and bta-miR-5p) are enriched in granulosa cells of subordinate follicle with 5 and 3.8-fold, respectively compared to the dominant follicle. Despite of these upregulated, two miRNAs (miR-183, miR-34c) were found to be downregulated. Although the role of these miRNAs in folliculogenesis needs to be expounded, the higher expression of miR-449a can be associated with growth arrests and apoptosis (Bou Kheir et al. 2011, Noonan et al. 2009, Noonan et al. 2010). Similarly, overexpression of miR-221/222 is suggested to affect the growth potential of cells by inducing a G1 to S shift in the cell cycle (Galardi et al. 2007). Moreover, an increased level of miR-222 could affect the cell motility by influencing the AKT signalling pathway (Wong et al. 2010) and its involvement in human endometrial cell differentiation has been described (Qian et al. 2009). On the other side, miR-183 which was down regulated in the SF group is believed to have an anti-apoptotic role and it can induce cell survival (Galardi et al. 2007, Zhang et al. 2010). This may propose while the triggering of miRNAs could be linked with cell arrest, its suppression might be related with cell proliferation and survival in SF groups. Gene ontology annotation and pathway analyses are capable to acquire an enhanced understanding from the cellular components, molecular functions and biological processes of target genes (Ji et al. 2012). The analysis of GO and KEGG, PANTHER and REACTOME analysis revealed that the theoretical target genes appear to be involved in a wide variety of biological processes. Ranging from genes encoding transcription factors involved in transcription,

regulation to genes involved in metabolism, genes regulating cell-cell adhesion, genes involved in programmed cell death, genes regulating biological adhesion and genes encoding cell fate commitment (Table 4.7). Apart from this, genes targeted by those miRNAs were found to be involved in main canonical pathways including Wnt signaling, TGF beta signaling, notch signaling, MAPK signaling, axon guidance, apoptosis, cadherin signaling and gap junction (Table 4.8). These results directed that these miRNAs might be involved in physiology of follicular development, and function in granulosa cell proliferation, apoptosis, and differentiation. Even though a bulky number of target gene candidates were predicted using bioinformatics tools, validation of the relationship between miRNAs and mRNA transcripts need further more biological experimental confirmations to exhibit downregulation of those pathways which are essential for follicular development in the granulosa cells of subordinate follicles.

5.2 The granulosa cells in subordinate follicle revealed a noticeable miRNA expression dysregulation at day 7 of the estrous cycle

Selection of a dominant follicle and productive hormonal influence of follicular wave growth is reliant on the understanding of the gonadotrophin provisions of independent wave follicles. The mechanism by which definite number of dominant follicles in mammals is designated from a morphologically alike cohort of follicles, while the remaining follicles of the cohort regress is not fully understood. SF and DF progress in the same endocrine environment, but it is probable that the selection process involves the differential expression of factors that mediate the actions of FSH and IGF-I (Ryan et al. 2007). After selection, the dominant follicle increased in diameter, higher follicular fluid concentrations of estradiol, insulin-like growth factor-binding protein (IGFBP) protease activity and free IGF, and lower IGFBP-4 concentrations (Fortune et al. 2004, Mihm et al. 2000). This phenomenon is synchronized by upregulation and downregulation of numerous arrays of genes which may have role in folliculogenesis. Thus observing into miRNAs associated with posttranscriptional regulation of genes in subordinate and dominant follicle during this period will improve the knowledge of molecular mechanism of folliculogenesis. In line to this, the current results discovered differences in the miRNA expression between the granulosa of SF and DF to be obvious

at day 7 than day 3 of the estrous cycle (Table 4.8, Figures 4.3, 4.12), intending that SF and DF could be molecularly distinct as the luteal phase of the estrous cycle progresses. This can be also explained by the fact that unlike to day 7, follicles may not reach the size of dominant at day 3 of the estrous cycle to exhibit remarkable difference between the two follicle sizes (subordinate and dominant) at that day of the estrous cycle. It is revealed that the greatest shifts in miRNA expression occur at dominance just after selection of dominant follicles. Interestingly, at day 7 of the estrous cycles, 108 differentially miRNAs are identified in granulosa cells of SF and DF follicles, out of which 51 miRNAs including bta-miR-2332, bta-miR-409a, bta-miR-2446, bta-miR-383, bta-miR-130a and bta-miR-148b were up regulated in the granulosa cells of SF while 57 miRNAs including bta-miR-184, bta-miR-365-5p, bta-miR-2487, bta-miR-143, bta-miR-185 and bta-miR-29 were found down regulated in the granulosa cells of subordinate follicles (Figure 4.4). Interestingly, at day 7 of the estrous cycles, 12 miRNA families were co-activated or co-repressed in SF compared to DF groups (Table 4.3). For example miR-199 family members namely, miR-199a-3p, miR-199a-5p, -miR-199b and miR-199c were repressed in granulosa cells of SF by a same regulation direction (FC=4-7.5) indicating that these miRNAs belonging to the same family could have a similar role during folliculogenesis. Among the miR-199 family members, higher expression of miR-199a-3p was found be associated with cell proliferation by imposing G1 cell cycle arrest (Fornari et al. 2010, Wang et al. 2012a). Similarly, miR-199a-5p along with miR-30d and miR-181a is believed to induce apoptosis by targeting the GRP78 gene (Su et al. 2013). On the other hand, increased expression of miR-199a significantly inhibited the ability of TGF-beta cell growth arrest and apoptosis in vitro (Zhang et al. 2012) suggesting the potential involvement of miR-199 family members in cellular development. Similar to miR-199 families, the miR-181 family members namely, miR-181a,-miR-181b, miR-181c, miR-181d were also downregulated in granulosa cells of SF. Previous study showed that miR-181a and miR-181b were found to be overexpressed in more aggressive breast cancers cells suggesting their anti-apoptotic role (Bisso et al. 2013) and miR-181a can influence cellular proliferation by targeting activin receptor IIA (Actr2a) (Zhang et al. 2013). In the current study, the bioinformatic analysis showed that Actr2a gene was found to be potentially targeted by all these miR-181 family members. Reduced level of miR-181a expression was found to result in reduced glucose deprivation induced apoptosis, mitochondrial dysfunction, and

loss of mitochondrial membrane potential in astrocyte cells (Ouyang et al. 2012) and implicated as a predictive biomarker for breast cancer metastasis and patient survival (Taylor et al. 2013). The other family member of miR-181, namely, miR-181b is also associated with cell proliferation and inhibited cell apoptosis in cervical cancer cells (Yang et al. 2014). Thus, alteration in the expression of miR-181 family may result in dysregulation of programmed cell death and cell proliferation in granulosa cells of the subordinate follicles. In a study it is reported that miR-195 was found to be repressed in colorectal cancer tissues compared to matched normal mucosa. This miR-195 is believed to target Bcl-2 and hence apply its pro-apoptotic function when physiologically regulated (Liu et al. 2010b). In addition, miR-202 was verified to be connected with reproductive hormone secretion (Bannister et al. 2011, Sirotkin et al. 2009). Similarly, a huge number of studies have shown that miR-143 (miR-143-3p) might be involved in mammalian reproductive activities (Hossain et al. 2009, Huang et al. 2011, Landgraf et al. 2007, Li et al. 2011, Mishima et al. 2008). A study reported that miR-21 was significantly up-regulated in murine granulosa cells prior and 4 h after the human chorionic gonadotropin (hCG)/LH surge and that it plays a role in stopping apoptosis in periovulatory granulosa cells as they passage to luteal cells. Further more, in-vivo experiments demonstrated that there was significant reduction in ovulation rate of miR21-LNA inhibitor treated ovaries compared to the controls. These results propose that miRNA 21 plays an important role in post transcriptionally regulating transcripts that are involved in preventing apoptosis in LH-induced terminally differentiating granulosa cells (Carletti et al. 2010, Fiedler et al. 2008). Other studies have revealed that miR-21 was repressed by estradiol in MCF7 cancer cells (Maillot et al. 2009, Wickramasinghe et al. 2009). In the present study, the miR-21 family (bta-miR-21-3p and bta-miR-21-5p) are enriched in granulosa cells of dominant follicle with 8 and 6.9-fold, respectively compared to the subordinate follicle, suggesting that miR-21 family plays an important role in development of folliculogenesis. Similarly in the current study, bta-miR-23a was regressed in the granulosa cells of SF with the counterpart DF. The bioinformatics analysis showed that pro-apoptotic gene casp-7 and antiapoptotic gene XIAP was found to be potentially targeted by bta-miR-23a. These findings suggest that mir-23a may play a role in the regulation of granulosa cell apoptosis. The mir-23a has been shown to play vital roles in numerous processes during normal and pathologic states and is firmly related to the cell cycle, proliferation, differentiation, apoptosis,

hematopoiesis, and cardiac hypertrophy (Chhabra et al. 2010, Huang et al. 2008). Studies have demonstrated that the mir-23a functions as a growth-promoting and antiapoptotic factor targeting the Smad pathway in hepatocellular carcinoma cells (Huang et al. 2008). On the other hand, a study proved that the mir-23a could induce caspase-dependent and caspase-independent apoptosis in human embryonic kidney cells (Chhabra et al. 2010). The pro-apoptotic and antiapoptotic nature of the mir-23a suggests that this may play diverse roles under different physiological and pathological conditions. It is of keen interest to recognize the function of mir-23a in granulosa cells and follicular atresia.

In order to further explore the function of the differentially expressed miRNAs at day 7 of the estrous cycle between granulosa cells of SF and DF, GO analysis and pathway annotation were used to evaluate their target gene pools. The gene ontology annotation and pathway analysis were performed to identify functional modules of the differentially expressed miRNAs at day 7 of the estrous cycle between granulosa cells of SF and DF. The results of GO term analysis from predicted miRNA target genes showed further significant biological processes classifications. In this study GO annotation enrichment results illustrates that these miRNAs are mostly related to the regulation of transcription, regulation of RNA metabolic process, regulation of transcription, DNA-dependent, regulation of cell proliferation, cell adhesion, posttranscriptional regulation of gene expression, regulation of apoptosis and apoptosis (Fig 4.9). The KEGG, PANTHER and REACTOME analysis showed that survival pathway (TGF-beta), significantly proliferative (ErbB, MAPK, Wnt, cell cycle) and apoptotic (apoptosis) signaling pathways, were utmost abundant among the significantly enriched ones (Table 4.11), which was in agreement with the GO analysis. Further investigation, based on the computational predictions revealed that cyclin D3 (CCND3) was defined as a potential target of bta-miR-13a, bta-miR-15b, bta-miR-195, bta-miR-497, bta-miR-592 and bta-miR-409a, which are downregulated in DF group, and that may induce cell cycle arrest in the granulosa cells of DF. This statement is in agreement with a study where its mentioned that CCND3 is expressed in nearly all proliferating cells and could promote the cell cycle (Lin et al. 2001). One of the study reported that miR-16 family could induce cell cycle arrest by targeting CCND3, CCNE1 and cyclin-dependent kinase (Cdk)-6 (Liu et al. 2008). The cell cycle is controlled by a family of

the CDKs and their stimulating partners (cyclins). The G1/S phase transition is controlled mainly by D-type cyclins (D1, D2 or D3) in complex with CDK4/CDK6 and E-type cyclins (E1 or E2) in complex with CDK2. These complexes collaborate in phosphorylating and avoiding Rb binding to E2F, thus triggering E2F-mediated transcription and driving cells from G1 into S phase (Grillo et al. 2006). CCND3 is communicated widely, signifying that it has numerous diverse cell functions. CCND3 had been specified to be involved in the advancement of cell growth by a number of research groups (Faast et al. 2004, Lin et al. 2001, Liu et al. 2008, Lu et al. 2011, Wang et al. 2011). Another study revealed that the downregulated miR-138 can regulate CCND3 and function as a tumor suppressor in hepatocellular carcinoma (HCC), thus by showing a useful therapeutic agent for miRNA-based HCC therapy (Wang et al. 2012b). On the basis of these current findings lead to postulate that these miRNAs are involved in the degenatration process of SF by targeting the CCND3 gene which is involved in cell cycle. Moreover, the functional identity recognized by diverse computational methods proposed that miRNAs may have regulatory effects on proliferation and apoptosis of granulosa cells by affecting the signaling pathways as mentioned above. Similarly, previous study on the gene expression analysis of small ($\leq$ 5 mm) and large (> 12 mm) follicles indicated altered expression of genes involving in TGF beta signaling, axonal guidance and protein trafficking (Hatzirodos et al. 2014b). Moreover, the gene expression pattern of granulosa cells from small healthy (3.1 $\pm$ 0.2 mm diameter) and atretic (4.2 $\pm$ 0.5 mm) bovine follicles also indicated the TGF-beta signaling and apoptosis pathways to be affected in atretic follicles (Hatzirodos et al. 2014a). Indeed, the role of TGF-beta in the bidirectional crosstalk between the granulosa cells and the oocyte has been reviewed (Knight and Glister 2003, Knight and Glister 2006). In addition to the common pathways enriched by genes targeted by miRNAs increased in SF or DF, there were also unique molecular pathways enriched by genes targeted by miRNAs only enriched in the granulosa cell of DF (Figure 4.10). For instance, the miRNA-gene interaction and pathway analysis showed that the target genes of by miR-142-5p, namely, ENAH, ROBO1, Rho GTPases (RHOA, RHOQ), NEO1 were found to be involved in axon guidance mediated by Slit/Robo. Among these, ROBO1 is one of the four Roundabout (Robo) which is believed to be interacting with the Slits (Huminiecki et al. 2002) and the Slit/Robo signaling can also be interacting with Rho GTPase activating proteins (Wong et al. 2001). The guidance

mediated by Slit/Robo is believed to involve in tissue growth, development, and remodeling (Dickinson and Duncan 2010). In addition, the Slit/Robo signaling induces cell adhesion, cell proliferation and survival (Dickinson and Duncan 2010). Given the gene expression and/or translation repression role of miRNAs, increased level of miR-142-5p in granulosa cells of DF may suggest downregulation of axon guidance by Slit/Robo pathway. Reducing the Slit/Robo activity may increase cell migration and reduce apoptosis activity (Dickinson et al. 2008). Indeed, the anti-apoptosis role of miR-142-5p has been also described by (Saito et al. 2012). This may suggest that miR-142-5p could be an important candidate for granulosa cell survival by targeting genes involving in guidance by Slit/Robo. On the other hand, the gene targeted by miRNAs enriched only in SF groups were found to be involved in O-glaycan biosynthesis, N-glycan biosynthesis, the metabolism pathways (D-glutamine and D-glutamate, vitamins and cofactors, phenyl alanine, biosynthesis of unsaturated fatty acids). The N-glycan generated by N acetylglucosaminyltransferase is encoded by mannosyl (alpha-1, 3) glycoprotein beta-1, 2-N acetylglucosaminyltransferase (Mgat1) (Shi et al. 2004). Our miRNA- target-gene analysis indicated 12 genes including Mgat1and Mgat2 to be the targets of miR-592 and miR-30e-5p, which were upregulated in granulosa cells of SF group. Previous studies, (Shi et al. 2004, Williams and Stanley 2009) have shown that targeted deletion of oocyte-specific Mgat1 gene to be associated with reduced cumulus cell number around the oocytes and reduction in ovulation rates. This may suggest aberrant expression of N-glycans could lead to abnormal folliculogenesis. In addition, lack of complex N- and O-glycans in oocytes may have a negative consequence on further embryonic development (Grasa et al. 2012). Therefore, increased level of miRNAs targeting genes involving in the N- and O-glycan biosynthesis in granulosa cells of SF groups may indicate a negative effect on follicle development in those follicular stages during the early luteal phase of the estrous cycle.

5.3 The temporal miRNA expression dynamics is bulging in granulosa cells of dominant follicle with the counterpart subordinate follicle at day 3 and day 7 of the estrous cycle

After identification of the differential expressed miRNAs between SF and DF, next attempt took place to recognize the temporal dynamic miRNA enrichment and degradation in granulosa cells within SF v*s* SF and DF vs DF at day 3 and day 7 of

estrous cycle. Results have displayed that the temporal miRNA expression profile changes between day 3 and day 7 of the estrous was appreciable in DF groups by which the expression profile of 131 miRNAs was found to be altered between day 3 and 7 of the estrous cycle. Conversely, in SF groups, only 5 miRNAs were differentially regulated between these two time points. This may conclude that follicles (subordinate and dominant) present during the early luteal phase act like nonovulatory follicles present during the follicular phase and become atretic. This idea of regression and follicular atresia could be associated to the influence of progesterone (Taylor and Rajamahendran 1991), and the decline in FSH commencement after day 2 of the estrous cycle which brings differential alterations in FSH dependent growth factors and hormones within the cohort of preselected follicles, which simultaneously inducing growth and boosting estradiol producing capacity of the DF and atresia of subordinate follicles (Mihm et al. 1997). The granulosa cells of DF displayed noticeable miRNA transcriptional activity variation as compared to SF in the current results (Figure 4.6). This may propose the presence of dynamic molecular shift in the follicles that could progress in size and to be entitled for dominance. The biological reason for this could be the element that bovine follicles are not adequate to attain the dominant stage at day 3 unlike to day 7 when the follicle can reach dominance stage even though they are not ovulated. Furthermore, as the estrous cycle progresses from day 3 to day 7, the follicles increase in size, the production of estradiol also elevates. The upsurge in estradiol concentrations in performance with inhibin are the main endocrine signals that suppress FSH concentrations from the anterior pituitary gland via negative feedback reducing FSH to basal concentrations (Ginther 2000, Ginther et al. 2000b, Sunderland et al. 1994). However, the increment of estradiol secretion usually does not occur more than 3 or 4 days and thus due to high levels of progesterone, the DF becomes atretic and estradiol production drops on day 6 of the estrous cycle (Mihm et al. 2002). Centered on this perception, the considerations were made to feature into the miRNAs whether their shift is associated with estradiol production. In fact, during folliculogenesis, steroidogenesis is one of crucial process that happened in the granulosa cells. For this, dehydroepiandrosterone (DHEA) is produced from pregnenolone, androstenedione from progesterone by cytochrome p450 enzyme called CYP17A1 occurs in theca cells. On the other hand, the conversion of androstenedione into estrone and conversion of testosterone into estradiol occurs in granulosa cells by aromatase gene (Craig et al.

2011). Agreeing to (Xu et al. 2011), aromatase gene (CYP19A1) is a validated target of miR-378. However, this miRNA was found to be increase in granulosa cells of DF at day 7 compared to day 3 of the estrous cycle suggesting a decrease in the level of aromatase genes expression. Similarly, the miRNAs which were found to be associated with inhibitory effect on estradiol production (Sirotkin et al., 2009) namely, miR-96b, miR-146, miR-28, miR-29a, miR-184, miR-32, miR-34a, miR-129, miR-132, miR-133a, and miR-150 were found to be increased at day 7 of the estrous cycle. Others, miR-24, miR-122, miR-145, miR-182, miR-143 and miR-150 which increased progesterone level in human granulosa cells after they were over expressed (Sirotkin et al. 2009) were found to be increased at day 7 of the estrous cycle. Other miRNAs which were dysregulated in DF between day 7 and day 3 of the estrous cycle namely miR-21, miR-132, miR-191 and miR-99b were also exhibited temporally expression alteration in mural granulosa cells collected from mice treated with eCG for 46 h followed by injection of hCG (Fiedler et al. 2008). This may suggesting some miRNAs whose expression was suppressed or enriched in granulosa cells of DF between day 3 and 7 of the estrous may involve in steroidogenesis or they may have some role in acquisition of follicular maturation. Recruitment and selection, the processes that give rise to the DF, and dominance, the physiological state of the mature DF, are important areas of basic research. Results of these basic studies on early luteal phase of follicular development need further validation to solve the problems in farm animal reproduction.

In conclusion, this thesis reported comprehensive understandings into the miRNA enrichment and degradation in the subordinate and dominant follicle during the luteal phase of the estrous cycle using high-throughput miRNA sequencing data generated from granulosa cells obtained from subordinate and dominant follicles at day 3 and 7 of the estrous cycle. In addition to novel miRNAs, the study identified large set of miRNAs which are found to be differentially expressed in granulosa cells of SF and DF and suggested to play a diverse role in various physiological processes associated with the two follicle types during the course of folliculogenesis. Further detail functional analysis of miRNAs is required to precisely determine the regulatory role of those noncoding small RNAs in bovine follicular development.

6 Summary

Follicular development is the outcome of multifarious hormonal and biochemical interactions that could be triggered or deactivated within the follicular environment in a spatiotemporal fashion. Mammalian ovaries consist of follicles as elementary functional units, and each follicle comprises of an oocyte enclosed by one or multiple layers of somatic granulosa cells and theca cells. The layer and number of granulosa cells may differ reliant on the size and phase of follicular development. It has been reported that, bidirectional crosstalk between the gonadal cell (oocyte) and the somatic cell type (granulosa and theca) affects the hormonal production and as well as the expression of genes associated with follicular development. The significance and involvement of miRNAs in the ovarian function, and follicular development has been described by several authors, but their existence, richness and sequential expression in the subordinate and dominant follicles during the bovine luteal phase of the estrous cycle needs to be revealed. In the recent years, next generation sequencing (NGS) technologies has enormously boosted the profiling of miRNA expression levels as well as identification of novel miRNA genes. So far, the involvement of miRNAs in specific ovarian somatic cell type was not investigated using high-throughput miRNA profile analysis. Therefore, this study was aimed to investigate the availability and abundance of miRNAs in bovine granulosa cells derived from subordinate and dominant follicles during early luteal phase of estrous cycle in the process of bovine follicular development. In addition, we predicted the potential targets of the differential expressed miRNAs in either of the cell type or at each time points and their potential involvement in biological pathways. In this study, 13 Simmental heifers were synchronized using optimized protocol and heifers were slaughterd at day 3 and day 7 of estrous cycle to collect ovarian samples.

At day 3 of the estrous cycle, follicles with follicular diameter of ≤ 6 mm (n = 43) were categorized as subordinate follicle (SF) while follicles with a diameter of 8-10 mm (n = 9) were considered as dominant follicle (DF). On the other hand, at day 7 of the estrous cycle, follicles with a diameter of ≤ 8 mm (n = 58) were considered as SF and those with 9-13 mm diameter (n = 3) were categorized as DF. The purity of granulosa cells was confirmed using cell-specific gene markers and showed that the contamination of

theca to granulosa cells was minimum. The subordinate and dominant follicles with the corresponding granulosa, theca, COCs and follicular fluid were then retrieved from the ovaries of each animal. The granulosa cells were subjected to miRNA-enriched total RNA isolation using the miRNeasy® mini kit (Qiagen GmbH, Hilden, Germany). The RNA integrity and quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies Inc, CA, USA) and Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc, DE, USA), respectively. Total RNAs from all groups were subjected to construction of tagged miRNA sequencing libraries (22 to 30 nt) according to Illumina Small RNA Sample Preparation protocol by GATC BioTech AG (Konstanz, Germany). The raw data from the deep sequencing was further analyzed and processed using miRDeep software package www.mdc-berlin.de/rajewsky/miRDeep. The miRNAs were considered to be differentially expressed when their fold change difference is ≥ 2, p-value ≤ 0.05 and false discovery rate is ≤ 0.1.

The detection analysis revealed that, at day 3 of the estrous cycle 291 and 318 miRNAs were detected in granulosa cells of SF and DF, respectively. Similar analysis showed that, at day 7, a total of 314 and 316 miRNAs were detected in granulosa cells derived from SF and DF, respectively. A total of 244 detected miRNAs were common to all follicle groups. Moreover, candidate miRNAs were validated for their expression in independent granulosa, theca, COCs and follicular fluid samples isolated from the two follicular catagories in both day 3 and day 7 of the estrous cycle.

We used state of the art NGS technology to explore the expression pattern of miRNAs in the granulosa cells of SF and DF at day 3 and day 7 of the estrous cycle. Accordingly, we found 16 miRNAs including bta-miR-449a, bta-miR-449c, bta-miR-212, bta-miR-21-3p, bta-miR-183 and bta-mir-34c were differentially expressed in granuolosa cell of subordinated follicle groups. Similarly, at day 7 of the estrous cycle, a total of 108 miRNAs including bta-mir-409a, bta-miR-2446, and bta-mir-383 were altered in their expression in granulosa cells of SF compared to DF. Interestingly, 9 miRNAs including bta-miR-21-3p, bta-miR-708, and bta-miR-335 were differentially expressed at both day 3 and 7 of the estrous cycle. Among these, bta-miR-335 showed similar expression pattern at day 3 and day 7 while other 8 miRNAs showed opposite pattern of expression. Expression analysis within the groups (day 3 SF vs day 7 SF and day 3 DF

vs day 7 DF) revealed that, 5 miRNAs including bta-miR-100 and bta-miR-155 to be differentially expressed at day 3 SF vs. day 7 SF group. On the other hand, a total of 131 miRNAs including bta-miR-409a, bta-miR-196a, bta-miR-505, bta-miR-16b, bta-miR-99a-5p, bta-miR-32, bta-miR-132, bta-miR-129 and bta-miR-346 to be differentially expressed at day 3 DF vs day 7 DF group.

To further understand physiological functions and biological processes of the differentially expressed miRNAs between granulosa cells of the subordinate and dominant follicles in the follicular development, *in silico* analysis of the candidate miRNAs was performed in order to identify the potential target genes. The gene ontological analysis indicated that the majority of the differentially expressed miRNAs in granulosa cells of subordinate and dominant follicles at day 3 and day 7 of the estrous cycle were found to be involved in regulation of programmed cell death and regulation of cell proliferation. Furthermore, the pathway analysis results at day 3 of estrous cycle showed that cadherin signaling, apoptosis, ErbB signaling, MAPK signaling, Ras, signaling by BMP, 5HT1 type receptor mediated signaling and adipocytokine signaling pathways were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of SF, whereas the focal adhesion, gap junction, histamine H1 receptor mediated signaling pathway and angiotensin II-stimulated signaling through G proteins and beta arrestin were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of DF. Interestingly, Wnt signaling pathway, TGF-beta signaling pathway, signaling by NGF, oxytocin receptor mediated signalin pathway, notch signaling pathway, axon guidance and adherens junction were enriched by genes targeted by miRNAs either enriched or repressed in granulosa cells of SF. Similar analysis for day 7 of estrous cycle showed that metabolic pathways (panthothenate and CoA biosynthesis, O-glycan and N-glycan biosynthesis, metabolism of vitamins and cofactors, D-glutamine and glutamate metabolism, biosynthesis of unsaturated fatty acids), Jak-STAT and cell cycle pathways were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of SF, whereas the Ras signaling pathway, lipids, lipoproteins, cysteine, methionine and carbohydrates metabolic pathways were enriched by genes potentially targeted by miRNAs enriched in granulosa cells of DF. Interestingly, axon guidance, Wnt signaling pathway, GnRH signaling pathway, MAPK-signaling, oocyte meiosis, TGF-beta signaling pathway and focal adhesion were

enriched by genes targeted by miRNAs either enriched or repressed in granulosa cells of SF. Although, miRNA expression profile shows very little similarity between groups, interestingly *in silico* predicted biological pathways were similar between groups. In addition to known miRNAs, novel miRNAs were identified from the sequenced data by using miRDeep2 novel miRNAs prediction tool. A total of 21 candidate novel miRNAs with base pair length of 17-22 nucleotides were identified and detected in granulosa cells of SF or DF at day 3 or day 7 of the estrous cycle.

In the present study, we provided detailed insights into the miRNA enrichment and degradation during the luteal phase of the estrous cycle using high-throughput miRNA sequencing data generated from granulosa cells obtained from subordinate and dominant follicles at day 3 and 7 of the estrous cycle. This study highlights the miRNA expression patterns of granulosa cells in subordinate and dominant follicles that could be allied with follicular recruitment, selection and dominance during the early luteal phase of the bovine estrous cycle. The functional identity by different bioinformatics methods suggested that the differentially expressed miRNAs at specific time points and groups may have regulatory effects on proliferation and apoptosis of granulosa cells by affecting the signaling patways. Together these observations exposed the importance of miRNAs in the regulation of follicular development during the bovine estrous cycle. However, further functional study based on this data is required to fully understand the role of each of the differentially expressed miRNAs during folliculogenesis.

7 Zusammenfassung

Die folikuläre Entwicklung ist das Ergebnis von vielfältigen hormonellen und biochemischen Interaktionen, die innerhalb der follikulären Umwelt in einer spatio-temporalen Form ausgelöst oder deaktiviert werden können. Die Eierstöcke von Säugetieren bestehen aus Follikel als elementare Funktionseinheiten und jeder Follikel besteht aus einer Eizelle die durch eine oder mehrere Schichten von somatischen Granulosa- und Thekazellen eingeschlossen wird. Die Schicht und Anzahl der Granulosazellen können abhängig von der Größe und der Phase der Follikelreifung sein. Es wurde berichtet, dass sich eine bidirektionale Kommunikation zwischen der Keimdrüsenzelle (Eizelle) und den somatischen Zelltyp (Granulosa-und Theka) auf die Hormonproduktion und somit auf die Expression von Genen, die mit der follikulären Entwicklung verbunden sind, auswirkt. Es wurde bereits von mehreren Autoren die Bedeutung und die Beteiligung von miRNAs an der Funktion des Eierstocks und der Follikelreifung beschrieben. Jedoch ihre Existenz, ihre Fülle Reichhaltigkeit und die sequentielle Expression in den untergeordneten und dominanten Follikel während der bovinen Lutealphase des Östruszyklus muss noch aufgeklärt werden. In den letzten Jahren haben die Next Generation Sequencing (NGS)-Technologien zu einem enormen Anstieg in der Erstellung von miRNA-Expressionsprofilen sowie zur Identifizierung neuer miRNA-Gene beigetragen. Bisher wurde die Beteiligung von miRNAs in bestimmten Eierstockzelltyp mittels Hochdurchsatz-miRNA-Profil-Analyse nicht untersucht. Daher war das Ziel dieser Studie die Untersuchung nach dem Vorkommen und der Fülle von miRNAs in bovinen Granulosazellen von untergeordneten und dominanten Follikel während der frühen Lutealphase des Östruszyklus beim Prozess der bovinen Follikelreifung. Weiterhin prognostizierten wir, die potenziellen Ziele der differenziell exprimierten miRNAs entweder in den Zelltypen oder an jedem Zeitpunkte und deren möglichen Beteiligung an biologischen Signalwegen. In dieser Studie wurden 13 Simmental Färsen nach optimiertem Protokoll synchronisiert und dann am Tag 3 und Tag 7 der Östruszyklus geschlachtet um Eierstockproben zu sammeln.

Am Tag 3 des Östruszyklus wurden Follikel mit einem follikulärem Durchmesser ≤ 6 mm (n = 43) als untergeordnete Follikel (SF) und Follikel mit einem Durchmesser von 8-10 mm (n = 9) als dominante Follikel (DF) eingeteilt. Auf der anderen Seite wurden

Follikel am Tag 7 des Zyklus mit einem Durchmesser von ≤ 8 mm (n = 58) als SF und solche mit einem Durchmesser von 9-13 mm (n = 3) als DF unterteilt. Die Reinheit der Granulosazellen wurde mit einem zellspezifischen Marker-Gen bestätigt und somit konnte gezeigt werden, dass Kontaminationen der Theka- und Granulosazellen minimal waren. Die untergeordneten und dominanten Follikel mit ihren entsprechenden Granulosa- und Thekazellen, dem COC und ihrer Follikelflüssigkeit wurden dann aus den Eierstöcken von jedem Tier isoliert. Von den Granulosazellen wurden miRNA-angereicherte Gesamt-RNA mittels des miRNeasy® Mini Kit (Qiagen GmbH, Hilden, Deutschland) isoliert. Die RNA-Integrität und Qualität wurde jeweils mit dem Agilent Bioanalyzer 2100 (Agilent Technologies Inc, CA, USA) und dem Nanodrop 8000 Spektralphotometer (Thermo Fisher Scientific Inc, DE, USA), bestimmt. Die Gesamt-RNA aller Proben wurde für die Konstruktion einer markierten miRNA-Sequenzierung Bibliotheken (22 bis 30 nt) eingesetzt. Dieses erfolgte nach dem Illumina Small RNA Sample Preparation Protokoll der Firma GATC BioTech AG (Konstanz, Deutschland). Die Rohdaten aus der Tiefensequenzierung (Deep Sequenzierung) wurde mit dem miRDeep Software-Paket www.mdc-berlin.de/rajewsky/miRDeep weiter analysiert und verarbeitet. Die miRNAs galt als differentiell exprimiert bei einer fold change ≥ 2, einem p-Wert ≤ 0,05 und bei einer false discovery rate ≤ 0,1.

Durch die Analyse konnten, für die Granolusazellen am Tag 3 des Östruszyklus 291 miRNAs in SF und 318 miRNAs in DF erfasst werden. Während für in Granulosazellen von Tag 7, insgesamt 314 miRNAs in SF und 316 in DF abgeleitet werden konnten. Insgesamt stimmten 244 miRNAs in alle Follikel-Gruppen überein. Zur Validierung wurde die Expression von Kandidaten miRNAs in unabhängigen Granulosa- und Thekazellen, COC und Follikelflüssigkeit von beiden follikulären Unterkategorien sowohl am Tag 3 als auch am Tag 7 des Zyklus untersucht.

Zur Untersuchung der Expressionsmuster von miRNAs in den Granulosazellen von SF und DF am Tag 3 und Tag 7 des Östruszyklus wurde die Technik NGS verwendet. Dementsprechend fanden wir 16 miRNAs einschließlich bta-miR-449a, bta-miR-449c, bta-miR-212, miR-bta-21-3p, bta-miR-183 und miR-bta-34c unterschiedlich exprimiert in Granuolosazelle von untergeordneten Follikeln. Am Tag 7 des Östruszyklus konnten insgesamt 108 miRNAs einschließlich bta-mir-409a, miR-bta-2446, und bta-mir-383

mit einer unterschiedlichen Expression in Granulosazellen von SF gegenüber DF beobachtet werden. Interessanterweise überschnitten sich neun miRNAs einschließlich bta-miR-21-3p, bta-miR-708 und miR-bta-335 sowohl bei Tag 3 und 7 des Östruszyklus. Davon zeigte bta-miR-335 ein ähnliches Expressionsmuster an Tag 3 und Tag 7, während die anderen acht miRNAs ein gegenläufiges Expressionsmuster aufwiesen. Expressionsanalyse innerhalb der Gruppen (Tag 3 SF vs SF Tag 7 und Tag 3 DF vs Tag 7 DF) ergab, dass fünf miRNAs einschließlich bta-miR-100 und miR-bta-155 unterschiedlich am Tag 3 SF vs Tag 7 SF exprimiert waren. Auf der anderen Seite, zeigten insgesamt 131 miRNAs einschließlich bta-miR-409a, bta-miR-196a, bta-miR-505, miR-bta-16b, bta-miR-99a-5p, bta-miR-32, bta -miR-132, miR-bta-129 und miR-bta-346 ein unterschiedliches Expressionsprofil am Tag 3 DF vs Tag 7 DF.

Um weitere physiologischen Funktionen und biologische Prozesse der unterschiedlich exprimierten miRNAs zwischen Granulosazellen der untergeordneten und der dominanten Follikel in der follikulären Entwicklung zu verstehen, wurde eine *in silico* Analyse von Kandidaten miRNAs durchgeführt, mit dem Ziel potentielle Ziel-Gene zu identifizieren. Die Analyse der Genontologie ergab, dass die Mehrheit der unterschiedlich exprimierten miRNAs in Granulosazellen von untergeordneten und dominanten Follikel die am Tag 3 und 7 des Östruszyklus ermittelt wurden, in der Regulierung des programmierten Zelltods und der Regulierung der Zellproliferation involviert sind. Zudem zeigten die Ergebnisse der Signalweg-Analyse, dass am Tag 3 des Östruszyklus die folgenden Signalwege: Cadherin, Apoptose, ErbB, MAPK, Ras, Signalisierung durch BMP, 5HT1 Typ rezeptorvermittelte Signalisierung und Adipocytokines; bei potentiellen miRNA Ziel-Genen in Granulosazellen der SF vorkamen. Wobei fokal Adhesion, Gab Junction, Histamin H1 rezeptorvermittelter Signalweg und Angiotensin II –stimulierter Signalweg durch G Proteine und beta Arrestin in potentiellen miRNA Ziel-Genen in Granulosazellen der DF ermittelt wurden. Interessanterweise traten folgende Signalwege: Wnt, TGF-beta, NGF, Oxytocin rezeptorvermittelter Signalweg, Notch, Axon Guidance und Adherens Junction bei miRNA Ziel-Genen die entweder in Granulosazellen der SF hoch oder runter reguliert waren auf. Ähnliche Analysen zeigten eine höherer aktivität von potentielle miRNA Ziel-Gene von hoch regulierten miRNA von Granulosazellen der SF an Tag 7 des Östruszyklus in folgende metabolische Signalwegen (Pantothenate und CoA

Biosynthese, O- Glykan und N- Glykan Biosynthese, Vitamin und Cofaktor Metabolismus, D-Glutamin und Glutamat Metabolismus, Biosynthese der ungesättigten Fettsäure), Jak-STAT und Zellzyklus Signalweg. Wohingegen die Signalwege Ras, Lipide, Lipoproteine, Cystein, Methionin Signalwege und der metabolischer Kohlenhydrat Signalweg durch potentielle miRNA Ziel-Genen in Granulosazellen von DF identifiziert wurden. Axon Guidance, Wnt Signalweg, GnRH Signalweg, MAPK, Meiose der Eizellen, TGF-beta Signalweg und focal Adhesion kamen interessanterweise in der Gruppe der miRNA Ziel-Gene die entweder in Granulosazellen von SF hoch oder runter reguliert waren, vor. Obwohl das Expressionsprofil der miRNAs gar keine Ähnlichkeit zwischen den beiden Gruppen zeigte, waren die *in silico* vorhergesagten biologischen Signalwege interessanterweise zwischen beiden Gruppen ähnlich. Zusätzlich zu den bekannten miRNAs wurden neue miRNAs mit Hilfe des neuen miRNA Prognosetool miRDeep2 identifiziert. Insgesamt wurden 21 neue Kandidaten-miRNAs mit einer Basenlänge von 17-22 Nukleotide in Granulosazellen der SF oder DF am Tag 3 oder am Tag 7 des Östruszyklus ermittelt.

In der vorliegenden Studie, geben wir einen detaillierten Einblick in die miRNA Anreicherung und ihren Abbau während der Lutealphase des Östruszyklus. High-throughput miRNA Sequenzierungs-Daten wurden aus Granulosazellen, die aus untergeordneten und dominanten Follikeln am Tag 3 und 7 des Östruszyklus gewonnen wurden, generiert. Diese Studie hebt miRNA Expressionsmuster von Granulosazellen aus untergeordneten und dominanten Follikeln hervor, die in Verbindung mit der follikulären Rekrutierung, Selektion und Dominanz während der frühen Lutealphase des bovinen Östruszyklus stehen. Die nach unterschiedlichen bioinformatischen Methoden ermittelte funktionelle aktivität zeigte, dass unterschiedlich exprimierte miRNAs zu bestimmten Zeitpunkten und Gruppen einen möglichen regulatorischen Effekt auf die Proliferation und Apoptose der Granulosazellen haben und dies durch die Beeinflussung von Signalwegen erzielen können. Zusammenfassend lässt sich sagen, dass die miRNAs in der Regulierung der follikulären Entwicklung während des bovinen Östruszyklus von Bedeutung sind. Dennoch sind weitere funktionelle Studien basierend auf diesen Daten notwendig, um vollständig die Rolle jeder unterschiedlich exprimierten miRNA während der Follikulogenese zu verstehen.

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9 Appendices

Appendix 1: List of differentially expressed miRNas between granulosa cells of
          subordinate and dominant follicles at day 7 of estrous cycle

miRNAs ID	FC	Pvalue	FDR
bta-miR-184	-23.5	1.67E-32	9.50E-30
bta-miR-365-5p	-19.2	0.00052605	0.003434445
bta-miR-2487	-18.3	0.013818341	0.059460738
bta-miR-2389	-18.1	0.022832071	0.08890562
bta-miR-483	-4.8	0.023635121	0.08890562
bta-miR-1249	-4.2	0.004415618	0.023009824
bta-miR-2411-5p	-4.0	0.011258	0.050350742
bta-miR-455-5p	-3.7	0.000212775	0.001469912
bta-miR-221	-3.7	3.23E-11	7.06E-10
bta-miR-708	-3.7	3.68E-13	1.49E-11
bta-miR-150	-3.6	0.002300316	0.012809605
bta-miR-34a	-3.6	4.04E-06	3.95E-05
bta-miR-346	-3.5	0.00165579	0.009796758
bta-miR-2285t	-3.5	0.008572809	0.039912751
bta-miR-142-5p	-3.3	0.003537107	0.018776418
bta-miR-155	-3.3	1.44E-11	3.55E-10
bta-miR-193b	-3.2	6.85E-12	1.77E-10
bta-miR-222	-3.2	5.02E-11	1.06E-09
bta-miR-455-3p	-3.1	7.52E-09	1.07E-07
bta-miR-23a	-3.1	1.23E-12	3.49E-11
bta-miR-21-3p	-3.0	4.68E-10	8.31E-09
bta-miR-21-5p	-2.8	1.26E-09	2.17E-08
bta-miR-214	-2.7	8.52E-11	1.73E-09
bta-miR-218	-2.6	0.02329968	0.08890562
bta-miR-149-5p	-2.6	6.98E-09	1.02E-07
bta-miR-199a-3p	-2.5	1.23E-17	7.12E-16
bta-miR-199c	-2.5	1.23E-17	7.12E-16

bta-miR-130b	-2.5	1.33E-10	2.52E-09
bta-miR-146b	-2.5	4.63E-06	4.41E-05
bta-miR-326	-2.5	0.008756066	0.040116175
bta-miR-27a-3p	-2.4	5.15E-13	1.75E-11
bta-miR-181a	-2.4	5.21E-13	1.75E-11
bta-miR-199b	-2.4	2.66E-07	3.08E-06
bta-miR-22-5p	-2.3	0.00071126	0.004488844
bta-miR-181b	-2.3	1.09E-12	3.25E-11
bta-miR-29b	-2.2	0.001886234	0.010822031
bta-miR-2425-5p	-2.1	0.007301522	0.035446706
bta-miR-199a-5p	-2.1	5.15E-09	8.01E-08
bta-miR-365-3p	-1.9	8.71E-06	7.61E-05
bta-miR-204	-1.9	6.05E-08	7.16E-07
bta-miR-24-3p	-1.9	2.44E-11	5.56E-10
bta-miR-146a	-1.9	0.000904744	0.005647193
bta-miR-2284aa	-1.8	0.007573989	0.035553933
bta-miR-3613	-1.8	0.008757757	0.040116175
bta-miR-181c	-1.8	8.56E-06	7.61E-05
bta-miR-28	-1.7	1.96E-05	0.000166227
bta-miR-451	-1.7	2.31E-05	0.000193158
bta-miR-23b-3p	-1.7	4.66E-06	4.41E-05
bta-miR-107	-1.5	0.00047124	0.003112378
bta-miR-181d	-1.5	0.019423357	0.078244443
bta-miR-486	-1.5	0.000100564	0.000771896
bta-miR-29a	-1.4	0.000147472	0.001073901
bta-miR-22-3p	-1.4	1.38E-06	1.43E-05
bta-miR-152	-1.4	0.001609023	0.009620264
bta-miR-874	-1.4	4.62E-05	0.000359681
bta-miR-185	-1.3	0.01497738	0.063963549
bta-miR-143	-1.3	0.000547562	0.003534266
bta-miR-2478	1.0	0.023055342	0.08890562
bta-miR-130a	1.0	0.000197757	0.001386741
bta-miR-148b	1.0	0.002868312	0.015665398

bta-miR-99b	1.1	0.002212934	0.012445016
bta-miR-16b	1.1	0.000158475	0.001125176
bta-miR-25	1.2	0.000138582	0.001035721
bta-miR-296	1.2	0.001738087	0.010177665
bta-miR-450b	1.2	0.006227416	0.030758019
bta-miR-2483-5p	1.2	0.021267183	0.084473845
bta-miR-99a-5p	1.3	1.18E-06	1.24E-05
bta-miR-128	1.3	4.27E-05	0.000341989
bta-miR-92a	1.4	6.24E-06	5.72E-05
bta-miR-423-3p	1.4	9.92E-07	1.06E-05
bta-miR-30c	1.4	4.28E-08	5.17E-07
bta-miR-769	1.4	0.000214793	0.001469912
bta-miR-15b	1.5	2.94E-05	0.000242189
bta-miR-6119-3p	1.5	0.026437804	0.0987939
bta-miR-1271	1.5	0.000260344	0.00176042
bta-miR-30e-5p	1.6	3.81E-09	6.35E-08
bta-miR-339a	1.6	1.24E-08	1.63E-07
bta-miR-339b	1.6	1.24E-08	1.63E-07
bta-miR-484	1.6	5.51E-06	5.13E-05
bta-miR-195	1.7	3.33E-06	3.38E-05
bta-miR-202	1.8	3.97E-06	3.95E-05
bta-miR-574	1.9	1.79E-08	2.31E-07
bta-miR-31	1.9	3.42E-07	3.89E-06
bta-miR-1296	1.9	0.000153302	0.00110222
bta-miR-592	2.0	0.009652084	0.043859069
bta-miR-497	2.0	6.54E-09	9.78E-08
bta-miR-505	2.0	0.000103734	0.000785609
bta-miR-2885	2.0	0.023405773	0.08890562
bta-miR-10b	2.1	5.15E-15	2.44E-13
bta-miR-744	2.1	3.50E-05	0.000284397
bta-miR-2285k	2.2	7.08E-07	7.85E-06
bta-miR-92b	2.2	4.55E-10	8.31E-09
bta-miR-2411-3p	2.4	0.005568101	0.027742821

bta-miR-1343-3p	2.8	8.83E-13	2.79E-11
bta-miR-2483-3p	3.0	0.003270191	0.017690178
bta-miR-452	3.3	0.000276098	0.001844984
bta-miR-3431	3.6	9.62E-11	1.88E-09
bta-miR-224	3.6	3.69E-14	1.61E-12
bta-miR-2344	3.6	0.001219755	0.00753066
bta-miR-190b	3.9	4.48E-05	0.000353812
bta-miR-6526	4.2	6.02E-18	6.84E-16
bta-miR-2284t-3p	4.4	0.001429003	0.008727672
bta-miR-335	5.5	5.64E-31	1.60E-28
bta-miR-2404	16.3	0.023504345	0.08890562
bta-miR-383	16.3	0.020668992	0.082675967
bta-miR-2446	16.4	0.013369487	0.057968464
bta-miR-409a	16.5	0.004178814	0.021977468
bta-miR-2332	17.9	0.000142205	0.001048992

Positive fold change means up-regulated in granulosa cells of subordinate follicles and negative fold change means down-regulated in granulosa cells of subordinate follicles compared to granulosa cells of dominant follicles at day 7 of estrous cycle (FC=Log$_2$ fold), P value and false discovery rate (FDR).

Appendix 2: List of differentially expressed miRNas between granulosa cells of dominant follicles at day 3 and day 7 of estrous cycle

miRNAs ID	FC	pvalue	FDR
bta-miR-409a	-18.8	0.00949551	0.03446748
bta-miR-2332	-18.6	0.00306952	0.01234431
bta-miR-196a	-18.4	0.01287771	0.04458695
bta-miR-6526	-5.0	4.66E-12	5.42E-11
bta-miR-2284t-3p	-4.9	3.02E-05	0.00017418
bta-miR-335	-4.2	2.26E-26	6.01E-25
bta-miR-3431	-3.7	3.78E-16	6.61E-15
bta-miR-592	-3.6	1.30E-10	1.37E-09
bta-miR-190b	-3.5	0.00030501	0.00155003
bta-miR-2346	-3.5	0.02084214	0.06734541
bta-miR-224	-3.5	4.94E-20	9.86E-19
bta-miR-2285k	-3.3	6.67E-17	1.28E-15
bta-miR-452	-3.3	5.86E-05	0.00033081
bta-miR-1343-3p	-3.2	1.24E-22	2.88E-21
bta-miR-1271	-3.0	1.25E-08	1.03E-07
bta-miR-2483-3p	-2.8	0.00188144	0.00821658
bta-miR-744	-2.5	9.51E-10	8.71E-09
bta-miR-92b	-2.5	1.08E-21	2.25E-20
bta-miR-202	-2.3	3.50E-09	3.16E-08
bta-miR-1296	-2.2	9.18E-07	6.58E-06
bta-miR-2285c	-2.2	0.0324179	0.09503858
bta-miR-497	-2.1	6.91E-15	1.04E-13
bta-miR-574	-2.0	2.07E-12	2.63E-11
bta-miR-505	-2.0	1.06E-05	6.87E-05
bta-miR-450b	-1.9	5.31E-09	4.49E-08
bta-miR-31	-1.7	2.98E-08	2.35E-07
bta-miR-15b	-1.7	4.48E-11	4.81E-10
bta-miR-99b	-1.7	2.32E-08	1.88E-07
bta-miR-195	-1.6	6.32E-07	4.65E-06

bta-miR-873	-1.6	0.00217441	0.00900367
bta-miR-2483-5p	-1.6	0.00058752	0.00280704
bta-miR-30e-5p	-1.6	3.43E-16	6.19E-15
bta-miR-148b	-1.5	8.41E-08	6.53E-07
bta-miR-769	-1.4	3.70E-09	3.28E-08
bta-miR-339b	-1.4	2.91E-10	2.86E-09
bta-miR-339a	-1.4	2.91E-10	2.86E-09
bta-miR-484	-1.3	3.87E-06	2.67E-05
bta-miR-374a	-1.3	7.46E-06	4.90E-05
bta-miR-194	-1.3	0.01693635	0.05635369
bta-miR-16b	-1.2	4.51E-09	3.94E-08
bta-miR-128	-1.2	1.32E-05	8.45E-05
bta-miR-10b	-1.2	7.06E-07	5.12E-06
bta-miR-1260b	-1.2	0.01945164	0.06321784
bta-miR-652	-1.1	1.48E-05	8.73E-05
bta-miR-191	-1.1	2.72E-10	2.82E-09
bta-miR-423-3p	-1.1	4.57E-07	3.40E-06
bta-miR-30c	-1.1	3.78E-10	3.52E-09
bta-miR-2478	-1.1	0.00113459	0.00515636
bta-miR-2285f	-1.1	0.00930551	0.03399855
bta-miR-99a-5p	-1.0	4.98E-09	4.28E-08
bta-miR-424-5p	-1.0	0.00504665	0.01919102
bta-miR-126-3p	1.0	0.0016148	0.00710766
bta-miR-185	1.2	0.01212062	0.04234643
bta-miR-874	1.2	6.42E-05	0.00035863
bta-miR-32	1.3	0.00274919	0.01113621
bta-miR-378	1.3	1.35E-05	8.54E-05
bta-miR-3432	1.3	0.00634198	0.02379308
bta-miR-2425-5p	1.4	0.02321531	0.07249919
bta-miR-345-3p	1.4	0.00574124	0.02168483
bta-miR-2419-5p	1.4	0.01114134	0.03941777
bta-miR-145	1.6	3.64E-10	3.45E-09
bta-miR-183	1.6	1.38E-05	8.54E-05

bta-miR-182	1.7	5.47E-06	3.68E-05
bta-miR-204	1.8	1.37E-07	1.05E-06
bta-miR-28	1.8	4.05E-14	5.80E-13
bta-miR-143	1.9	9.34E-07	6.61E-06
bta-miR-107	1.9	1.88E-05	0.00010925
bta-miR-22-5p	2.0	0.00422252	0.01627853
bta-miR-199b	2.2	2.52E-08	2.01E-07
bta-miR-23b-3p	2.2	1.02E-12	1.32E-11
bta-miR-29b	2.3	0.00317481	0.01258666
bta-miR-24-3p	2.4	4.58E-31	1.42E-29
bta-miR-24-3p	2.4	5.37E-31	1.58E-29
bta-miR-29a	2.4	4.10E-22	9.16E-21
bta-miR-34a	2.5	0.00058036	0.00279671
bta-miR-6524	2.5	0.02220839	0.06979809
bta-miR-181d	2.5	0.00013114	0.00068513
bta-miR-199a-5p	2.5	2.48E-15	3.86E-14
bta-miR-181c	2.6	3.52E-14	5.18E-13
bta-miR-132	2.7	4.34E-07	3.28E-06
bta-miR-365-3p	2.7	2.97E-10	2.86E-09
bta-miR-29c	2.7	0.01668064	0.05583519
bta-miR-760-3p	2.8	1.99E-06	1.39E-05
bta-miR-181b	2.9	4.29E-23	1.04E-21
bta-miR-455-3p	3.1	2.66E-11	2.92E-10
bta-miR-455-5p	3.2	0.00037138	0.00185361
bta-miR-146a	3.2	5.53E-09	4.62E-08
bta-miR-199a-3p	3.2	1.91E-49	9.68E-48
bta-miR-199c	3.2	1.42E-49	8.80E-48
bta-miR-149-5p	3.3	9.47E-14	1.32E-12
bta-miR-181a	3.3	2.33E-40	8.88E-39
bta-miR-27a-3p	3.3	1.50E-47	6.98E-46
bta-miR-6121-3p	3.4	0.01906656	0.06232871
bta-miR-130b	3.4	2.16E-26	6.01E-25
bta-miR-2284aa-1	3.4	1.43E-05	8.54E-05

bta-miR-2411-5p	3.5	0.01538145	0.05242823
bta-miR-223	3.5	0.00369432	0.01454312
bta-miR-96	3.6	0.00050302	0.00248839
bta-miR-338	3.6	0.02986021	0.09273253
bta-miR-330	3.6	0.03219727	0.09503858
bta-miR-214	3.8	7.48E-16	1.23E-14
bta-miR-133a	3.8	0.00129052	0.00577121
bta-miR-449a	4.0	0.00011613	0.00061241
bta-miR-449c	4.1	0.00092818	0.00425287
bta-miR-146b	4.1	2.73E-12	3.39E-11
bta-miR-483	4.2	0.00140628	0.00623899
bta-miR-23a	4.3	8.27E-25	2.10E-23
bta-miR-365-5p	4.5	0.00209543	0.00900367
bta-miR-2904	4.5	0.00085463	0.00394824
bta-miR-150	4.8	5.48E-05	0.00031252
bta-miR-142-5p	4.9	1.05E-11	1.18E-10
bta-miR-193b	5.0	1.05E-45	4.50E-44
bta-miR-326	5.5	1.44E-05	8.54E-05
bta-miR-21-5p	5.6	8.88E-82	4.96E-79
bta-miR-708	6.2	1.74E-58	2.43E-56
bta-miR-21-3p	6.6	2.68E-72	5.00E-70
bta-miR-222	6.9	9.81E-38	3.23E-36
bta-miR-221	7.0	3.54E-52	3.96E-50
bta-miR-155	7.2	4.74E-74	1.32E-71
bta-miR-184	9.0	3.78E-40	1.32E-38
bta-miR-363	16.3	0.03147695	0.09498658
bta-miR-29d	16.4	0.01292144	0.04458695
bta-miR-2389	16.5	0.01345579	0.04614592
bta-miR-142-3p	16.6	0.00260502	0.01062923
bta-miR-129-5p	16.6	0.00216743	0.00900367
bta-miR-129	16.6	0.00216743	0.00900367
bta-miR-449b	16.7	0.00077646	0.00367832
bta-miR-129-3p	17.8	0.00206603	0.00895281

bta-miR-1249	17.8	0.00015707	0.00081299
bta-miR-2285t	18.0	0.00023659	0.00121332
bta-miR-346	18.0	5.95E-06	3.96E-05

Positive fold change means up-regulated in granulosa cells of subordinate follicles and negative fold change means down-regulated in granulosa cells of subordinate follicles compared to granulosa cells of dominant follicles at day 7 of estrous cycle (FC=Log_2 fold), P value and false discovery rate (FDR).

Appendix 3: Top most enriched pathways of target genes for differentially expressed miRNAs between granulosa cells of subordinate and dominant follicles at day 7 of estrous cycle

miRNAs ID	Term	Genes
mir-214↓,mir-181b↓,mir-27a-3p↓,mir-346↓,mir-181a↓,mir-130a↑,mir-10b↑,mir-452↑,mir-204↓,mir-2332↑,mir-326↓,mir-148b,mir-146b↓,mir-155↓,mir-15b↑,mir-16b↑,mir-152↓,mir-222↓,mir-195↑,mir-1249↓,mir-497↑,mir-221↓,mir-2446↑,mir-1271↑,mir-505↑,mir-185↓	Wnt signaling pathway	FZD4, FZD7, WNT9A,WNT11,CSNK1G3,WNT16,FZD3, WNT1,WNT2B,WNT5A,WNT6, WNT10B,WNT7A,FZD10
mir-185↓,mir-181d↓,mir-1296↑, mir-24-3p↓,mir-21-5p↓,mir-204↓,mir-150↓,mir-128↑,mir-181b↓,mir-199b↓,mir-2404↑,mir-326↓,mir-193b↓,mir-199c↓,mir-15b↑,mir-16b↑,mir-1271↑,mir-142-5p↓,mir-181c↓,mir-195↑,mir-181a↓,mir-27a-3p↓,mir-452↑,mir-484↑, mir-365-3p↓,mir-1249↓,mir-2446↑	MAPK signaling pathway	MAPK14,MAP2K1,MAPK1,MAP3K8,MAP4K2, MAPKAPK3,MKNK1,MAPKAPK2, MAP3K3,MAP3K2,MAP2K7,MAPK13,MAPK8IP2, MAPK8IP3,MAPK8IP1,MAP3K11, MAP3K1,MAPK10,MAP2K4,MAP3K4,
mir-199a-5p↓,mir-218↓,mir-130a↑,mir-193b↓,mir-452↑, mir-202↑,mir-130b↓,mir-146a↓, mir-34a↓,mir-1296↑,mir-181b↓,	Axon guidance	EPHA7,SEMA6A,PAK6,EPHA4, FGFR1,NRAS, GNAI2, SEMA4F, EFNB1, MET, SEMA4C, NRP1,PAK7,RPS6KA5,NRP1,MET,ST8SIA4,

mir-148b↑,mir-497↑,mir-27a-3p↓,mir-142-5p↓,mir-181c↓,mir-218↓,mir-204↓,mir-181a↓,mir-152↓,mir-326↓,mir-484↑,mir-15b↑,mir-16b↑,mir-29b↓,mir-148b↑,mir-195↑,mir-339b↑,mir-152↓,mir-222↓,mir-214↓,mir-1271↑,mir-221↓,mir-29a↓,mir-1249↓,mir-199c↓,mir-185↓,mir-1249↓,mir-128↑,mir-31↑,mir-2446↑,mir-30c↑,mir-29a↓,mir-29b↓		SEMA6A,ROCK1, ROBO1,ROBO2, ARHGEF12 MAPK1,COL4A4, COL4A3, COL4A2, COL4A1, COL3A1, COL2A1, COL5A2, COL5A1, COL4A5, COL9A1, COL6A3, COL1A2, COL1A1, SPTAN1 COL4A4, COL4A3, COL4A2, COL4A1, COL3A1, COL2A1, COL5A2, COL5A1, COL4A5, COL9A1, COL6A3, COL1A2, COL1A1, SPTAN1
mir-199b↓,mir-1271↑,mir-92b↑, mir-148b↑,mir-107↓,mir-92a↑, mir-486↓,mir-2404↑,mir-152↓, mir-497↑,mir-150↓,mir-199c↓, mir-25↑,mir-484↑,mir-195↑, mir-185↓,mir-1249↓,mir-15b↑, mir-16b↑,mir-2446↑,mir-29b↓, mir-29a↓	Focal adhesion	PDGFRA, ITGA3, MAPK10, THBS1, FN1, ITGA6, ITGA5, ITGAV, COL1A2, PPP1R12A, RAP1B, COL11A1, PTEN, COL5A1,IGF1R, PDPK1, IGF1, ITGA4, PTEN,MAP2K1,MAP2K1,RAPGEF1, PIK3R1, AKT3, MYLK
mir-222↓,mir-221↓,mir-365-3p↓,mir-92a↑,mir-31↑,mir-21-3p↓,mir-148b↑,mir-218↓,mir-181b↓,mir-185↓,mir-181d↓,mir-181c↓,mir-214↓,mir-181a↓,mir-128↑,mir-195↑,mir-15b↑,mir-16b↑,mir-204↓,mir-497↑,mir-2446↑,mir-1271↑,mir-1249↓	Signalling by NGF	CDKN1B, RALA, FRS2, BCL2L11, PIK3R1, ITPR2, CDKN1B, PLCG1, ADAM17, RALA, BCL2L11, PIK3R1, ITPR2,SOS1, ADCY6, RIT1, RICTOR, FRS2, AKT3,ADCY3,PPP2R1B,MEF2A,RTN4, RICTOR, PRKCE, PRKCD, PIK3R1, ITPR2 IRS2,PRKAR2A,CALM1,MAPK

		1, PDPK1, PRDM4, ADORA2A, SQSTM1, PPP2CB, YWHAB, FOXO4, IKBKB, ITPR3, PTEN,CL2L11,PPP2R1A,MAP2K1, APH1A,SH3GL2,FOXO1, NFKB1, FOXO3
mir-155↓,mir-21-5p↓,mir-181a↓, mir-1249↓,mir-128↑,mir-130b↓, mir-15b↑,mir-16b↑,mir-181b↓, mir-3431↑,mir-199a-5p↓,mir-181c↓,mir-195↑,mir-346↓, mir-346↓,mir-214↓,mir-130a↑, mir-199c↓,mir-148b↑,mir-152↓, mir-2446↑	TGF-beta signaling pathway	SP1,GDF6,SMAD2,RPS6KB1,SMAD1,ACVR1C, SMAD7,BMPR2,SMURF2,ACVR1C,BMP8A, PPP2R1B,ACVR2A,INHBB,SMAD7, SMAD5, SMAD3, SMURF2, SMURF1, BMPR1A, BMP8A,TGIF1,ZEB2,BMPR1B, TGFBR1, CREBBP, BMPR2, RPS6KB1, DCN ACVR2A,INHBB,LTBP1, BMP7, BMP6
mir-15b↑,mir-16b↑,mir-2487↓, mir-497↑,mir-195↑,mir-128↑, mir-193b↓,mir-185↓,mir-27a-3p↓,mir-1271↑,mir-484↑, mir-181c↓,mir-2404↑,mir-181d↓,mir-181a↓,mir-181b↓, mir-1249↓,	GnRH signaling pathway	MAP3K4, MAP2K1, MAP3K3, MAP2K3, MAP2K4, PRKACA, ITPR1, CALM1,CDC42, PLD1,MAP3K4,MAP3K2,MAPK3,CDC42,EGFR,KRAS,CAMK2D, CALM3, GNRHR,SOS1, MAP2K4, HBEGF, PRKX, PRKCB,MAPK11, MMP14, MMP2, MAPK1, PRKCD, CALM1,ADCY1, PLD1

mir-339b↑,mir-199a-5p↓,mir-2404↑,mir-769↑,mir-30c↑, mir-128↑,mir-222↓,mir-181d↓, mir-181b↓,mir-181c↓,mir-181a↓,mir-221↓,mir-150↓, mir-1249↓,mir-2446↑	ErbB signaling pathway	PAK6, ERBB4, SOS1, ABL2,CDKN1B, KRAS, GSK3B, CBL, PIK3CD,MAP2K1, GRB2, MAP2K4, CRK, CAMK2A,CBLB, KRAS, EREG, PAK4, CAMK2D, PIK3R3, AKT3,PAK7, CDKN1B, ERBB4, PLCG1, CBL, MAPK10, PAK1, PIK3R1,PRKCA, EREG, CAMK2G, CBL, MAP2K4, ELK1, PAK1, AKT3, SHC4
mir-592↑,mir-326↓,mir-452↑, mir-193b↓,mir-181d↓,mir-181a↓,mir-15b↑,mir-16b↑, mir-2404↑,mir-181b↓,mir-195↑, mir-181c↓,mir-497↑,mir-185↓	Oocyte meiosis	MAPK1, RPS6KA3, ADCY1, ADCY2, RPS6KA1, IGF1, SMC1A, CAMK2A, PRKX, ITPR2,PRKACG, IGF1R, PPP2R5B, PPP3CB,RPS6KA3, ANAPC4, YWHAB, PPP2R5E, SKP1, CPEB1, YWHAE, PRKX YWHAZ, ADCY9, PPP2R5C, PRKACB, CALM1 CCNB1, MAPK1, RPS6KA3, ADCY1, YWHAG, MAP2K1, ADCY9, CAMK2G, CAMK2D, PPP3R1
mir-30c↑,mir-15b↑,mir-16b↑, mir-195↑,mir-146b↓,mir-185↓, mir-484↑,mir-1249↓	VEGF signaling pathway	KRAS, PLCG1,PLA2G12A,PIK3CD,NFAT5, PPP3CB, PPP3R1,PPP3CA,PIK3R2,CDC42, MAP2K1,VEGFA,PPP3CB,NFATC3,AKT3,PXN, PIK3R1, KDR,PRKCA,SPHK2,PIK3CD, MAPKAPK3,CHP2,PLA2G2F

mir-199c↓, mir-365-3p↓,mir-22-3p↓, mir-195↑,mir-143↓,mir-25↑, mir-2446↑,mir-218↓	Gap junction	TJP1, ADRB1, GNAI3, CSNK1D, MAP3K2, PLCB4, SOS1, ADCY6, PDGFRA, PRKG1, GRM5, NRAS, GNAI3, GUCY1B3, PRKCB PRKCA, ADCY3, ADRB1,ADCY3, GRM1, GRM5, MAPK1, ADRB1, KRAS, MAP3K2, SOS1, PDGFRA, GUCY1B3, EGF, HTR2A,GNAI2, ADCY9, GNAI1
mir-1271↑,mir-181c↓,mir-365-3p↓,mir-181b↓,mir-181b↓, mir-222↓,mir-221↓	Apoptosis	TRAF2,XIAP,DFFA,PPP3R1,ENDOD1,ATM, PRKAR2B,TNFRSF1A,TNFRSF10B,CASP9,RIPK1, PRKAR1A,CHP,MAP3K14,IKBKB,PIK3R1, CASP10
mir-181b↓,mir-744↑,mir-2446↑, mir-181d↓,mir-214↓,mir-592↑, mir-497↑,mir-30c↑,mir-365-3p↓, mir-346↓	Aldosterone-regulated sodium reabsorption	MAPK1,ATP1B1,IRS2,KRAS,ATP1B2, PIK3R3 IGF2,KCNJ1,IGF1,PDPK1,SGK1,MAPK3,ATP1B4, INSR, PIK3R1
mir-484↑	Cytokine-cytokine receptor interaction	CCL3, IL22RA1, PDGFA, CSF1, IL28RA, IL21R, CNTFR, CXCL12, ACVR1B, TNFRSF1A, TNFRSF1B, PLEKHO2, IL18R1, IL2RB, TGFBR1, FLT4, EDA2R, EDAR, IL6R, CD40, TNFSF9, CCL18, KDR, IFNAR1
mir-2446↑	Jak-STAT	GRB2, STAT5A, STAM2,

	signaling pathway	STAT5B, IL15, SPRY4, SPRY3, ZFP91, SPRY2, SPRY1, SOS1, IFNG, STAM, SPRED1, PIK3R1, IL6, IL2RA, IL7, CREBBP
mir-1296↑	Cell adhesion molecules (CAMs)	CLDN18, CD8A, CADM1, CDH1, CLDN11, PDCD1, SDC3, NRCAM, PVRL1, ICOS, PVRL2, CNTNAP2, CD6, HLA-DOA, HLA-DOB, ICAM1
mir-30c↑,mir-30e-5p↑	Cell Cycle, Mitotic	PPP2R1B, XPO1, E2F3, TAOK1, DBF4, CCNE2, RANBP2, CCNA1, PSMD7, STAG2, TFDP1
mir-1271↑	Angiotensin II-stimulated signaling through G proteins and beta-arrestin	PRKCA, MAP2K1, PRKCI, ADRBK2, GNG12, PRKCE, ITPR2, GRK6, RHOB, GRK4, PLCB2, GRK1, GNG7
mir-1249↓,mir-181c↓, mir-181a↓,mir-21-3p↓	Ras Pathway	HRAS, GRAP, GRB2, SRF, MAPK1, RPS6KA2, ETS1, GSK3A, JUN, MAPK14, MAPK3, MAP2K7, MAPK1, PAK7, RPS6KA3, PLD1, KRAS, MAP2K1, RPS6KA6, TIAM1, RPS6KA2, MAP3K1, MAP2K3
mir-218↓	Leukocyte transendothelial migration	RASSF5, ARHGAP5, VAV3, PLCG1, GNAI2, GNAI1, ACTN1, RAPGEF4, PXN, PIK3R1
mir-185↓	Integration of energy	PRKAR2A, ADCY2, GCK, CHKB, PPP2R5D, PRKAB2,

	metabolism	ADCY6, PRKAB1, GNG12, GNB3
mir-130b↓	Lysosome	CTSK, LIPA, GNPTAB, TPP1, AP1G1, PSAP, CLTC
mir-143↓	Metabolism of carbohydrates	GOT1, HK2, GYG2, PGK1, CALM1, PC

(↑) shows upregulation while (↓) indicate downregulation of differentially expressed miRNAs in the granulosa cells of SF compared to DF at day 7 of the estrous cycle, (P value < 0.05).

Appendix 4: The most enriched pathways of target genes for differentially expressed miRNAs between granulosa cells of dominant follicles at day 3 and day 7 of estrous cycle.

miRNAs,ID	Term	Genes
bta-miR-1271↓,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-185↑,bta-miR-326↑,bta-miR-133a↑,bta-miR-497↓,bta-miR-181a↑,bta-miR-181b↑,bta-miR-1249↑,bta-miR-221↑,bta-miR-330↑,bta-miR-155↑,bta-miR-214↑,bta-miR-222↑,bta-miR-183↑,bta-miR-2332↓,bta-miR-497↓,bta-miR-145↑, bta-miR-424-5p↓,bta-miR-204↑,bta-miR-182↑,bta-miR-96↑,bta-miR-129-5p↑,bta-miR-148b↓,bta-miR-27a-3p↑,bta-miR-449a↑,bta-miR-129↑,bta-miR-452↓,bta-miR-132↑,bta-miR-194↓,bta-miR-345-3p↑,bta-miR-2904↑,bta-miR-2285c↓,bta-miR-10b↓,bta-miR-346↑,bta-miR-146b↑	Wnt signaling pathway	WNT7B,WNT5A, WNT4,WNT1, WNT9A,WNT8B, WNT7A,WNT6, WNT5A,WNT4, WNT3A,WNT2B, WNT2,WNT16, WNT11,WNT10B, WNT1,WIF1
bta-miR-1271↓,bta-miR-1296↓,bta-miR-484↓,bta-miR-1249↑,bta-miR-96↑,bta-miR-181a↑,bta-miR-181c↑,bta-miR-182↑, bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-181b↑,bta-miR-330↑,bta-miR-424-5p↓,bta-miR-181d↑,bta-miR-27a-3p↑,bta-miR-199c↑,bta-miR-204↑,bta-miR-145↑,bta-miR-128↓,bta-miR-452↓,bta-miR-129↑,bta-miR-142-5p↑,bta-miR-326↑,bta-miR-338↑,bta-miR-185↑,bta-miR-150↑,bta-miR-21-5p↑,bta-miR-365-3p↑,bta-miR-24-	MAPK signaling pathway	MAP3K5,MAP2K7, FGF3,AKT3,AKT2 ,PRKCA,TAOK1, CACNG4,CACNG2, FLNB,CACNA1S, CACNA2D2,MAPK1, MAPK14,MAPK3, MAPK8IP3,MAP3K12

3p↑,bta-miR-193b↑,bta-miR-873↓,bta-miR-199b↑

bta-miR-1271↓,bta-miR-30c↓,bta-miR-1249↑,bta-miR-96↑,bta-miR-145↑,bta-miR-182↑,bta-miR-330↑,bta-miR-31↓,bta-miR-181a↑,bta-miR-181c↑,bta-miR-29b↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-181b↑,bta-miR-199c↑,bta-miR-29a↑,bta-miR-29b↑,bta-miR-29c↑,bta-miR-497↓,bta-miR-185↑,bta-miR-204↑,bta-miR-214↑,bta-miR-132↑,bta-miR-1296↓,bta-miR-484↓,bta-miR-130b↑,bta-miR-148b↓,bta-miR-326↑,bta-miR-34a↑,bta-miR-449a↑,bta-miR-129↑,bta-miR-142-5p↑,bta-miR-221↑,bta-miR-183↑,bta-miR-128↓,bta-miR-222↑,bta-miR-330↑,bta-miR-146a↑,bta-miR-199a-5p↑,bta-miR-27a-3p↑,bta-miR-96↑,bta-miR-338↑,bta-miR-148b↓,bta-miR-202↓,bta-miR-339b↓,bta-miR-193b↑,bta-miR-52↓,bta-miR-196a↓	Axon guidance	PAK7, GNAI3, GNAI2, SEMA6D, EFNB2, PPP3R1, SEMA3C, PAK1, EPHA4, EFNB3, SEMA4C, SEMA4B, NFATC4, COL4A4, COL4A3, COL4A2, COL4A1, COL3A1, COL2A1, COL5A2, COL5A1, COL4A5, COL9A1, COL6A3, COL1A2, COL1A1,
bta-miR-1271↓,bta-miR-1249↑,bta-miR-96↑,bta-miR-182↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-497↓,bta-miR-181a↑,bta-miR-181c↑,bta-miR-181b↑,bta-miR-181d↑,bta-miR-330↑,bta-miR-204↑,bta-miR-128↓,bta-miR-31↓,bta-miR-424-p↓,bta-miR-214↑,bta-miR-129↑,bta-miR-129-5p↑,bta-miR-185↑,bta-miR-32↑,bta-miR-148b↓,bta-miR-183↑,bta-miR-133a↑,bta-miR-142-3p↑,bta-miR-21-3p↑,bta-miR-	Signalling by NGF	SOS1, ADCY6, RIT1, RICTOR, FRS2, AKT3, MEF2C, MEF2A, ADCYAP1R1, CREB1, FOXO1, FOXO3, MAPK1, PDE1B, ADCY9, RALA, RAP1A, PIK3R1, CALM1, MAP2K5

345-3p↑,bta-miR-221↑,bta-miR-222↑,bta-miR-365-3p↑

bta-miR-484↓,bta-miR-29c↑,bta-miR-1271↓,bta-miR-29a↑,bta-miR-29b↑,bta-miR-1249↑,bta-miR-96↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-182↑,bta-miR-330↑,bta-miR-497↓,bta-miR-199c↑,bta-miR-185↑,bta-miR-107↑,bta-miR-148b↓,bta-miR-150↑,bta-miR-145↑,bta-miR-92b↓,bta-miR-363↑,bta-miR-196a↓,bta-miR-199b↑	Focal adhesion	HRAS, XIAP, GRB2, PXN, SRC, ITGB7, COMP, THBS1, PIK3R3, SHC2, RAPGEF1, AKT3, AKT2, COL4A4
bta-miR-484↓,bta-miR-1249↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-497↓,bta-miR-181a↑,bta-miR-181c↑,bta-miR-424-5p↓,bta-miR-181b↑,bta-miR-96↑,bta-miR-592↓,bta-miR-181d↑,bta-miR-182↑,bta-miR-330↑,bta-miR-214↑,bta-miR-128↓,bta-miR-150↑,bta-miR-199a-5p↑,bta-miR-193b↑,bta-miR-3431↓,bta-miR-769↓	Insulin signaling pathway	MAP2K1, GRB2, FLOT2, FOXO1, IRS1, KRAS, CRKL, PRKAR1A, MAPK9, SHC1, PRKACB, PIK3R1, SHC4
bta-miR-1249↑,bta-miR-182↑,bta-miR-96↑,bta-miR-330↑,bta-miR-145↑,bta-miR-181c↑,bta-miR-199c↑,bta-miR-145↑,bta-miR-132↑,bta-miR-148b↓,bta-miR-195↓,bta-miR-181a↑,bta-miR-181b↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-214↑,bta-miR-130b↑,bta-miR-199a-5p↑,bta-miR-128↓,bta-miR-155↑,bta-miR-21-5p↑,bta-miR-183↑,bta-miR-374a↓,bta-miR-346↑,bta-miR-345-3p↑,bta-miR-363↑,bta-miR-3431↓	TGF-beta signaling pathway	ACVR2A, MAPK1, ACVR2B, E2F5, RPS6KB1, DCN, SMAD7, MRAS, FOXK2, FOXO1, FOXO3, SMAD1, FOXO4, FOXN3

bta-miR-1271↓,bta-miR-484↓,bta-miR-1249↑,bta-miR-96↑,bta-miR-181a↑,bta-miR-181b↑,bta-miR-181c↑,bta-miR-182↑,bta-miR-181d↑,bta-miR-195↓,bta-miR-27a-3p↑,bta-miR-330↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-497↓,bta-miR-128↓,bta-miR-185↑,bta-miR-129↑,bta-miR-193b↑	GnRH signaling pathway	ADCY1, PLD1, MAP2K1, CAMK2G, MAP2K4, MMP14, MAPK1, KRAS, ADCY9, MAP3K3,
bta-miR-1296↓,bta-miR-1249↑,bta-miR-182↑,bta-miR-497↓,bta-miR-107↑,bta-miR-185↑,bta-miR-34a↑,bta-miR-449a↑,bta-miR-221↑,bta-miR-129↑,bta-miR-365-3p↑,bta-miR-223↑,bta-miR-10b↓	Angiogenesis	WNT5A, PTPRJ, ETS1, SOS1, FZD3, WNT5B, FGFR3, APC2, PDGFB, GRAP
bta-miR-484↓,bta-miR-1249↑,bta-miR-592↓,bta-miR-181a↑,bta-miR-30c↓,bta-miR-96↑,bta-miR-182↑,bta-miR-330↑,bta-miR-181d↑,bta-miR-204↑,bta-miR-129-5p↑,bta-miR-326↑,bta-miR-143↑,bta-miR-142-3p↑,bta-miR-196a↓	Calcium signaling pathway	ADCY1, PTGER3, SLC25A4, CAMK2G, PPP3R1, GRM5, ATP2B1, EDNRA
bta-miR-181c↑,bta-miR-330↑,bta-miR-497↓,bta-miR-181a↑,bta-miR-181b↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-181d↑,bta-miR-185↑,bta-miR-182↑,bta-miR-96↑,bta-miR-592↓,bta-miR-129↑,bta-miR-145↑,bta-miR-452↓,bta-miR-326↑,bta-miR-193b↑	Oocyte meiosis	ADCY1, MAP2K1, CAMK2G, PPP3R1, PPP1CB, PPP2R1A, MAP2K1, BTRC, CDC23, ITPR1
bta-miR-1249↑,bta-miR-195↓,bta-miR-27a-3p↑,bta-miR-96↑,bta-miR-29a↑,bta-miR-330↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-181a↑,bta-miR-181d↑,bta-miR-29b↑,bta-miR-129↑,bta-miR-129-5p↑,bta-miR-31↓,bta-miR-497↓,bta-miR-	Signaling by EGFR	MAPK1, HRAS, AP2A2, GRB2, MAPK3, CDC42, AP2A1, PAG1, SH3GL2, UBE2D3, SMAD7, BMPR2

221↑,bta-miR-145↑,bta-miR-133a↑,bta-miR-452↓,bta-miR-199a-5p↑,bta-miR-142-3p↑,bta-miR-374a↓

bta-miR-30c↓,bta-miR-30e-5p↓,bta-miR-23b-3p↑,bta-miR-424-5p↓,bta-miR-128↓,bta-miR-21-3p,bta-miR-224↓,bta-miR-194↓,bta-miR-146a↑,bta-miR-338↑,bta-miR-365-5p↑,	Ubiquitin mediated proteolysis	PRKCA, SPHK2, PIK3CD, MAPKAPK3, SOCS3, SOCS1,
bta-miR-484↓,bta-miR-1249↑,bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-30c↓,bta-miR-29c↑,bta-miR-330↑,bta-miR-185↑,bta-miR-146b↑,	VEGF signaling pathway	PRKCA, MAPK1, HRAS, FLT1, ETS1, CDC42, VEGFA,
bta-miR-1296↓,bta-miR-1249↑,bta-miR-96↑,bta-miR-128↓,bta-miR-183↑,bta-miR-769↓	Insulin/IGF pathway-protein kinase B signaling cascade	IGF1R, FOXQ1, FOXK2, FOXF2, FOXO1, IRS2, FOXJ2, FOXK1, PIK3C2B, RALGAPA2, FOXJ1
bta-miR-182↑,bta-miR-96↑,bta-miR-195↓,bta-miR-199c↑,bta-miR-143↑,bta-miR-365-3p↑,bta-miR-374a↓,	Gap junction	KRAS, PDGFRA, MAPK7, HTR2C,
bta-miR-15b↓,bta-miR-16b↓,bta-miR-195↓,bta-miR-199c↑,bta-miR-21-5p↑,bta-miR-181a↑,bta-miR-32↑,bta-miR-497↓,bta-miR-27a-3p↑,bta-miR-330↑,bta-miR-374a↓,	Signaling by BMP	SMAD9, SMAD5, SMAD4, SMAD1, ACVR2A, ACVR2B
bta-miR-27a-3p↑	Neuroactive ligand-receptor interaction	PTGER3, ADORA2B, PTGER4, CYSLTR2, GRIK3, GLRA2, GRIA3, GRIA4
bta-miR-1249↑,bta-miR-181a↑,bta-miR-181c↑,bta-miR-21-3p↑	Ras Pathway	HRAS, MAP2K7, AKT3, AKT2
bta-miR-181b↑	Apoptosis	MAP2K4, PRKCE,

	signaling pathway	PRKCD, CASP10, HSPA5, FAS, AKT3
bta-miR-96↑	Natural killer cell mediated cytotoxicity	MAP2K1, GRB2, PPP3R1, SH2D1A, KRAS, PLCG1, FYN
bta-miR-1249↑	Fc gamma R-mediated phagocytosis	PRKCA, MAPK1, GAB2, LIMK1, WASF2, MAPK3
bta-miR-133a↑, bta-miR-185↑	Integration of energy metabolism	DLST, STX1A, TPI1, PFKFB3, PPP2CA, PPP2R5D, ADCY6
bta-miR-185↑, bta-miR-146a↑	Tight junction	CDC42, TJP1, EPB41L1, ZAK
bta-miR-96↑, bta-miR-182↑	Fc gamma R-mediated phagocytosis	PLD1, CRKL, PLCG1, MAP2K1, WASF2, CFL1, RAC1, PAK1,
bta-miR-130b↑	Lysosome	CTSK, LIPA, GNPTAB, TPP1,
bta-miR-129-5p↑, bta-miR-149-5p↑, bta-miR-365-5p↑	Processing of Capped Intron-Containing Pre-mRNA	SFRS6, HNRNPA3, HNRNPK, SRRM1, SMC1A, HNRNPA1, PRPF4, SLBP
bta-miR-107↑, bta-miR-146a↑, bta-miR-142-3p↑	Fc gamma R-mediated phagocytosis	PTPRC, CRKL, VAV3, CFL1, RAF1, PRKCE, AKT3, PIK3R1,
bta-miR-183↑	Transcription	SSRP1, RNMT, POLR1C, POLR2D,
bta-miR-182↑	Axon guidance mediated by netrin	ABLIM1, PLCG1, RAC1, NTN4, NTNG1, UNC5D,
bta-miR-143↑	Metabolism of carbohydrates	GOT1, HK2, GYG2, PGK1, CALM1, PC
bta-miR-34a↑, bta-miR-449a↑	Notch	NOTCH2, NOTCH1,

	signaling pathway	DLL1, JAG1, NUMBL
bta-miR-183↑	Histamine H1 receptor mediated signaling pathway	PRKCA, PLCB4, GNB1, GNG4, GNG5
bta-miR-142-5p↑	Axon guidance mediated by Slit/Robo	ENAH, ROBO1, RHOA, RHOQ, NEO1
bta-miR-130b↑	Xanthine and guanine salvage pathway	GDA, HPRT1, PGM2L1
bta-miR-146b↑	Natural killer cell mediated cytotoxicity	KRAS, NFAT5, PPP3R2
bta-miR-130b↑	Metabolism of nitric oxide	WASL, CALM2, DNM2
bta-miR-24-3p↑	Pyridoxal phosphate salvage pathway	PDXK, PNPO
bta-miR-1296↓, bta-miR-202↓	Cell adhesion molecules (CAMs)	CLDN18, CD8A, CADM1, CDH1, CLDN11, PDCD1,
bta-miR-30c↓, bta-miR-30e-5p↓	Cell Cycle, Mitotic	PPP2R1B, XPO1, E2F3, TAOK1, DBF4,
bta-miR-1271↓	Angiotensin II-stimulated signaling through G proteins and	PRKCA, MAP2K1, PRKCI, ADRBK2, GNG12, PRKCE, ITPR2, GRK6, RHOB, GRK4, PLCB2, GRK1, GNG7

	beta-arrestin	
bta-miR-1296↓	Vitamin D metabolism and pathway	CYP27C1, CYP27B1, RARG, FDX1, CYP2D6, PML,
bta-miR-30e-5p↓, bta-miR-592↓	N-Glycan biosynthesis	MGAT2, TUSC3, MAN1A2, ALG10B,
bta-miR-202↓	5HT3 type receptor mediated signaling pathway	HTR3E, VAMP3, SLC18A1, SNAP23, KCNK3, HTR3D
bta-miR-30e-5p↓	O-Glycan biosynthesis	GALNT3, GALNT2, GALNT1, GALNT7, GALNT4
bta-miR-202↓	Phenylalanine metabolism	NAT6, MAOA, TAT, ALDH3B1

(↑) shows upregulation while (↓) indicate downregulation of differentially expressed miRNAs in the granulosa cells of DF at day 3 and day 7 of the estrous cycle, (P value < 0.05).

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