Institut für Tierwissenschaften, Abt. Tierzucht und Tierhaltung
der Rheinischen Friedrich – Wilhelms – Universität Bonn

Bovine microRNomics: Implications during oocyte maturation and pathophysiology of endometrium

I n a u g u r a l – D i s s e r t a t i o n

zur Erlangung des Grades

Doktor der Agrarwissenschaft

(Dr. agr.)

der

Hohen Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich – Wilhelms – Universität

zu Bonn

vorgelegt im März 2011

von

Dagnachew Worku Hailemariam

aus

West shoa, Äthiopien

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Tag der mündlichen Prüfung:	04 April, 2011

Dedicated to my parents

Bovines microRNomics: Bedeutung während der Eizellreifung und die Pathophysiologie des Endometriums

MikroRNAs (miRNAs), die bereits für die Regulierung von posttranskriptionalen Genen bekannt sind, konnte eine essentielle Rolle für die Entwicklung von Tieren und Krankheiten nachgewiesen werden. In dieser Studie wurden bovine miRNAs während der Eizellenreifung identifiziert und mittels verschiedener Methoden das Expressionsprofil untersucht. miRNAs, die eine neuartigen molekulare Signatur auf Grundlage einer subklinischen Endometritis zeigten wurden mit Hilfe eines integrativen Untersuchungsansatzes erforscht. Zuerst wurde die Identifikation und die Erstellung von Expressionsprofilen von miRNAs während der bovinen Eizellenreifung mittels des „miRCURY™ locked nucleic acids (LNA) array“ (Exiqon, Vedbeak, Denmark) durchgeführt. Dieser Microarray besteht aus 454 bekannten Sonden von Mensch, Maus und Ratten miRNAs. Die Ergebnisse zeigten 59 unterschiedlich exprimierte miRNAs, von denen 31 hauptsächlich in unreifen und 28 in reifen Eizellen überexprimiert wurden. Hierbei wurden mit verschiedenen Ansätzen 32 neue orthologe bovine miRNAs identifiziert. Des Weiteren wurden erste Versuche durchgeführt, um die spezifischen Funktionen von miR-99a und miR-100 innerhalb eines „in-vitro“ Kumuluszellen Modells zu untersuchen. Die Ergebnisse zeigten, dass miR-99a und miR-100 die Expression des bovinen tribbles homologue 2 Gens (TRB2) runter regulieren. Zum Anderen wurde mittels eines genomweitem RT² miRNA PCR Arrays, bestehend aus 354 gut beschriebenen humanen miRNA Primern, an uterinen Cytobrush-Proben von Kühen die entweder an subklinischer Endometritis erkrankt oder gesund waren, Expressionsanalysen durchgeführt. Das Ergebnis dieses Versuches zeigte abweichende Expressionen von 23 miRNAs in Geweben von Kühen mit subklinischer Endometritis im Vergleich zu den gesunden Tieren. Interessanterweise konvergieren die mittels der Igenuity Pathway Analyse(IPA), identifizierten Gennetzwerke, bekannten Pathways sowie biologische Funktionen mit den Signalwegen und zellulären Aktivitäten innerhalb des Endometriums während des Östruszyklus und der Trächtigkeit. Des Weiteren bestätigte der Luziferase Assay die vorangegangenen Informationen der bioinformatischen Auswertung und ermöglichte uns einen schlüssigen Zusammenhang zwischen der veränderten miRNA Expression und den Zielgenen abzuleiten. Zusammengefasst, zeigt die Identifikation sowie das dynamischen Expressionsmuster der bestimmten Klasse von miRNAs während der bovinen Eizellreifung ihren potentiellen Einfluss auf die frühe Embryonalentwicklung; wohingegen die unterschiedliche Expression der uterinen miRNAs bei Tieren mit subklinischer Endometritis möglicherweise die uterine Genregulation beeinträchtigt.

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Bovine microRNomics: Implications during oocyte maturation and pathophysiology of endometrium

MicroRNAs which are known for posttranscriptional gene regulation are evidenced for their essential role during animal development and disease. In this study, identification and expression profiling of bovine miRNAs during oocyte maturation are scrutinized using heterologous approach, while miRNA regulated novel molecular signature underlying bovine subclinical endometritis was dissected using an integrative approach. Primarily, identification and expression profiling of microRNAs during bovine oocyte maturation was investigated using miRCURY™ locked nucleic acids (LNA) array (Exiqon, Vedbaek, Denmark) microarray that consist of 454 capture probes for human, mouse and rat miRNAs. The result revealed differential expression of 59 miRNAs, of which 31 and 28 miRNAs were found to be preferentially expressed in immature and matured oocytes, respectively. Here, 32 new bovine orthologous miRNAs were identified using a heterologous approach. Furthermore, the preliminary attempt to dissect the specific function of miR-99a and miR-100 in invitro cumulus cell showed that both miRNAs down regulate bovine tribbles homologue 2 (TRB2). On the other hand, genome wide RT² miRNA PCR array consisting of 354 well characterized human miRNA primers was used to analyze miRNA expression in the uterine cytobrush samples taken from cows with subclinical endometritis and healthy. The result showed the aberrant expression of 23 miRNAs in cows with subclinical endometritis as compared to the healthy ones. Interestingly, the Ingenuity Pathway Analysis (IPA) for high ranking target genes of aberrantly expressed miRNAs identified gene networks, canonical pathways and biological functions that converged to array of signaling pathways and cellular activities inherent to the endometrium during estrous cycle and pregnancy. Furthermore, the luciferase assay data substantiated the primary information from bioinformatic prediction and enabled us to deduce a convincing link between the aberrantly expressed miRNAs and target genes. Taken together, identification and dynamic expression pattern of certain class of miRNAs during bovine oocyte maturation suggests their potential involvement in early embryo development; where as, aberrant expression of uterine miRNAs in animals with subclinical endometritis potentially interfere with the tight uterine gene regulation.

Table of contents Pages

Abstract		V
List of abbreviations		XI
List of tables		XIV
List of figures		XV
1	General introduction	1
1.1.	Bovine oocyte maturation	3
1.2.	Pathophysiology of the uterus in the context of bovine subclinical endometritis	5
2	Literature review	8
2.1.	Bovine folliculogenesis	8
2.1.1.	Oocyte somatic cell interaction during folliculogenesis	11
2.1.2.	Oocyte developmental competence	12
2.1.3.	Oocyte maturation	15
2.1.3.1.	Nuclear maturation	15
2.1.3.2.	Cytoplasmic maturation	16
2.1.4.	Transcription factors as mammalian oocyte gene expression regulators	18
2.1.4.1.	Oct-4: germ cell specific transcription factor	19
2.1.4.2.	FIGa: a transcription factor that regulates zona pellucida genes	19
2.1.4.3.	NoBox: oocyte-specific genes transcription regulator	20
2.1.4.4.	ALF: general transcription factor	20
2.2.	Discovery of microRNAs as posttranscriptional gene regulators	21
2.2.1.	MicroRNA biogenesis	22
2.2.2.	Principles of target recognition by miRNAs and mode of action	24
2.2.3.	MicroRNA involvement in early development	28
2.2.4.	Implication of miRNAs in mammalian fertility	30
2.2.5.	Role of miRNAs in immune system development	32

2.2.6.	MicroRNA and uterine pathophysiology	35
2.2.6.1.	Inflammation	36
2.2.6.2.	Cell growth, proliferation and apoptosis	37
2.2.6.3.	Angiogenesis	38
2.2.7.	Potential role of miRNAs in endometrial transcriptome dynamics and endometritis	39
3.	Part I: Identification and expression profiling of miRNAs during oocyte maturation	41
3.1.	Part I: Materials and methods	42
3.1.1.	Materials	42
3.1.1.1.	List of laboratory equipments used during the study	42
3.1.1.2.	List of chemicals, competent cells and kits	43
3.1.1.3.	Growth media and solutions	47
3.1.1.4.	List of soft wares and data bases used during the study	50
3.1.2.	Methods	51
3.1.2.1.	Heterologous approach	51
3.1.2.2.	Oocyte collection, in vitro maturation, sperm capacitation and IVF	51
3.1.2.3.	In vitro culture and embryo collection	52
3.1.2.4.	Brilliant cresyl blue (BCB) staining of COCs	52
3.1.2.5.	Total RNA isolation, miRNA amplification and invitro transcription	53
3.1.2.6.	miRNA labeling and hybridization	55
3.1.2.7.	MicroRNA array scanning and data analysis	56
3.1.2.8.	MicroRNA qRT-PCR for microarray validation	56
3.1.2.9.	Retrieving miRNA targets and chromosomal location	57
3.1.2.10.	Total RNA isolation and cDNA synthesis for analysis of target genes	57
3.1.2.11.	Real-time quantitative PCR for target genes	58
3.2.	Part I: Results	60
3.2.1.	MicroRNA detection during bovine oocyte maturation	60
3.2.2.	MicroRNA array validation	66
3.2.3.	MicroRNA expression profiling across pre-implantation embryo stages	67
3.2.4.	MicroRNA expression profiling in BCB+ and BCB- immature and matured COCs	68

3.2.5.	Predicted targets for some miRNAs	69
3.2.6.	Relative abundance of miRNAs and their target mRNAs	70
3.2.7.	miR-99a and miR-100 repress expression of bovine tribbles homologue 2 (TRB2)	73
3.2.7.1.	TRB2 is a predicted target for miR-99a and miR-100	73
3.2.7.2.	miR-99a and miR-100 have markedly higher expression in cumulus cells	74
3.2.7.3.	Inverse expression pattern of TRB2 with targeting microRNAs	75
3.2.7.4.	miR-99a and miR-100 directly target the 3'- UTR of TRB2	76
3.3.	Part I: Discussion	78
3.3.1.	Identification of new miRNAs using heterologous approach	78
3.3.2.	Expression analysis of miRNAs during oocyte maturation, fully grown and growing oocytes and pre-implantation embryos	78
3.3.3.	Potential role of miR-99a and miR-100 in bovine cumulus cells proliferation	82
4.	Part II: MicroRNA regulated noble molecular signature underlying bovine subclinical endometritis	84
4. 1.	Part II: Materials and methods	86
4.1.1.	Materials	86
4.1.1.1.	Laboratory equipments used during the study	86
4.1.1.2.	List of chemicals , competent cells and kits	87
4.1.1.3.	Media and solutions	91
4.1.1.4.	Data bases and soft wares used during the study	93
4.1.2.	Methods	94
4.1.2.1.	Endometrial cytobrush samples collection and cytology	94
4.1.2.2.	Total RNA isolation	95
4.1.2.3.	First strand cDNA synthesis and quantitative PCR (qPCR) assay	95
4.1.2.4.	Ingenuity path way analysis (IPA)	97
4.1.2.5.	Plasmid construction and transient transfection	98
4.1.2.6.	Quantification of selected miRNAs during estrous cycle	101
4.3.	Part II: Results	102
4.3.1.	Altered expression of microRNAs in cows with sub clinical endometritis	102

4.3.2.	Molecular networks and biological functions that are possibly affected by endometritis	104
4.3.3.	Canonical pathways	106
4.3.4.	Interaction between centre genes and targeting microRNA	107
4.3.5.	Validation of miRNA–center genes interactions using luciferase assay	109
4.3.6.	Expression profile for miRNAs during estrous cycle of healthy animals	113
4.4.	Part II: Discussion	115
4.4.1.	Endometrial miRNA detection	115
4.4.2.	Aberrant expression of microRNAs in the endometrium of cows with subclinical endometritis and implication of their genomic location	115
4.4.3.	Molecular networks and biological functions regulated by aberrantly expressed miRNAs	116
4.4.4.	Validation of some aberrantly expressed miRNAs and target genes	119
5.	Conclusion and future prospects	122
6.	Summary	123
7.	Zusammenfassung	125
8.	References	127

List of abbreviations

miRNA:	microRNA
rRNA:	Ribosomal RNA
A:	Adenine
ATP:	Adenosine triphosphate
ATPase:	Adenosine triphosphatase
BP:	Base pairs
BSA:	Bovine serum albumin
Bta :	Bos taurus
C:	Cytocine
cDNA:	Complementary DNA
CL:	Corpus luteum
ddH2O:	Distilled and deionized water
dNTP:	Deoxy ribonucleoside triphosphate
DMSO:	Dimethyl sulfoxide
DNA:	Deoxynucleic acid
DTT:	Dithiothreitol
E. coli:	*Esherichia coli*
A260:	UV light absorbance at 260 nm wave length
aRNA:	Amplified RNA
BCB+:	Brilliant cresyl blue positive oocytes
BCB-:	Brilliant cresyl blue negative oocytes
COC:	Cumulus oocyte complex
DEPC:	Diethylpyrocarbonate
ng:	Nanogram
n:	Number
EB:	Elution buffer
EDTA:	Ethylenediaminetetra acetic acid
G:	Guanine
GC:	Granulosa cell
GV:	Germinal vesicle
GVBD:	Germinal vesicle breakdown
hr:	Hour

IGF:	Insulin like growth factor
IPTG:	Isopropyl ß-D-thiogalactopyranoside
IV:	In vitro culture
IVF:	In vitro fertilization
IVM:	In vitro maturation
MII:	Metaphase II
µg:	Microgram
µl:	Micro litre
µM:	Micro molar
ml:	Millilitre
min:	Minute
mRNA:	Messenger RNA
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PE:	Protein extraction and lysis buffer
qRT-PCR:	quantitative reverse transcriptase-polymerase chain reaction
sec:	Second
RNA:	Ribonucleic acid
rpm:	Revolution per minute
rRNA:	Ribosomal RNA
TCM:	Tissue culture medium
TGFB:	Transforming growth factor beta
tRNA:	Transfer RNA
UV:	Ultra-violet light
v/v:	Volume per volume
w/v:	Weight per volume
X-gal:	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside
M7G:	7-methyl G-cap
RISC:	RNA induced silencing complex
Rpm:	Revolutions per minute
RT:	Reverse transcription
SNP:	Single nucleotide polymorphism
T:	Thymine
ZP:	Zona pellucida

LNA:	Locked nucleic acids
IPA:	Ingenuity path way analysis
RT-PCR:	Reverse transcriptase polymerase chain reaction
TRB2:	Tribbles homologue 2
LPS:	Lipopolysacharides
TLRs:	Toll-like receptors
MPF:	M-phase promoting factor
PI:	Prophase I
LH:	Luteinizing hormone
FSH:	Follicle stimulating hormone
OSFs:	Oocyte-secreted factors
MZT:	Maternal to zygotic transition
PMN:	Polymorphonuclear neutrophils
PGCs:	Primordial germ cells
TGFβ:	Transforming growth factor β
GDF-9:	Growth differentiation factor 9
BMP-15:	Bone morphogenetic Protein 15
cAMP:	Cyclic adenosine mono phosphate
ES:	Embryonic stem
UTR:	Untranslated region
DMEM:	Dulbecco's Modified Eagle's Medium
CPE:	Cytoplasmic polyadenylation element
PGCs:	Primordial germ cells
CPEB:	Cytoplasmic polyadenylation element binding protein
TBP:	TATA- binding protein
GAL:	GenePix® Array Lists
EC:	Endothelial cells

List of tables

Table 1: List of primers used for qRT-PCR quantification of target genes with corresponding sequences, product length and annealing temperatures 59

Table 2: The list of miRNAs highly abundant in immature oocytes compared to their matured counterparts with their corresponding chromosomal location and fold change 62

Table 3: The list of miRNAs highly abundant in matured oocytes compared to their immature counterparts with their corresponding chromosomal location and fold change 63

Table 4: MicroRNAs that are highly abundant in immature oocytes and not known so far in bovine but known orthologous microRNAs in either human, mouse or rat 64

Table 5: Bovine microRNAs (highly abundant in matured oocytes) which have no known targets in bovine but with known orthologous microRNAs at least in one of the three species (human, mouse and rat) 65

Table 6: List of primers with their sequences and annealing temperatures that were used for semi-quantitative PCR analysis of target genes. 98

Table 7: List of primers used to amplify binding sites of miRNAs in the 3' UTR of target genes 99

Table 8: Oligo nucleotide sequences with three nucleotide mismatches in the miRNA target site. The sense and anti sense nucleotide sequences were company synthesized and annealed to have a double strand DNA fragment and subsequently cloned into a pmirGLO vector to be used as a negative control. 100

Table 9: List of differentially regulated microRNAs in cows with subclinical endometritis as compared to the healthy counterparts 103

List of figures

Figure 2.1: The diagram summarizes the process of ovarian follicle development and growth, from oocyte nests present in the fetal ovary through follicle growth, ovulation and CL formation (adapted from Hernandez-Ochoa et al. 2009) 9

Figure 2.2: Diagrammatic representation of the canonical pathway of microRNA processing (Winter et al. 2009) 23

Figure 2.3: MicroRNA regulatory functions during human endometriosis lesion development. miRNA expression may play a role in these processes, regulating transcripts involved in hypoxia, inflammation, apoptosis, tissue repair, cellular proliferation, extracellular matrix remodeling and angiogenesis (adapted from Ohlsson Teague, 2009a) 36

Figure 2.4: The intronic miR-126 and its host gene, EGF-like-domain, multiple 7 (Egfl7), promote angiogenesis (adapted from Ohlsson Teague, 2009a) 38

Figure 3.5: Relative expression level of five microRNAs and 5S in non-amplified RNA from immature (IO) and matured (MO) (a) and amplified RNA from immature and matured oocytes analyzed using qRT-PCR (IO: immature oocytes, Mo: matured oocytes, Non-amp: non–amplified, Amp: amplified) 60

Figure 3.6: The distribution of species specific microRNA capture probes resulted in the detection of oocyte microRNAs which are not identified before in bovine 65

Figure 3.7: QRT-PCR quantification of eight miRNAs (miR-25, miR-125a, miR-130b, miR-208, miR-206, miR-127, miR-145 and miR-200c) in immature and matured oocytes (Figure 7a). The qRT-PCR product was loaded on 3% agarose gel with 50 bp molecular marker and confirmed the specificity of the product (Figure 7b) 66

Figure 3.8: The expression profile of miR-125a (a), miR-25 (b), miR-208 (c), miR-127 (d) and miR-145 (e) in bovine pre-implantation developmental stages analysed using qRT-PCR 67

Figure 3.9: The relative expression level of miR-125a, miR-127, miR-145 and miR-208 in BCB- and BCB+ immature (a) and matured (b) oocyte stages 68

Figure 3.10: The relative expression level of miR-125a, miR-127, miR-145 and miR-208 in cumulus cells derived from BCB- and BCB+ immature (a) and matured (b) oocytes (ICC: cumulus cells derived from immature COC, MCC: cumulus cells derived from matured COC) 69

Figure 3.11: The expression profile of six target genes in immature and matured oocytes as revealed by real time PCR. Among the six genes quantified, only SMARCC1 showed statistically significant (P≤0.05) expression level between immature and matured oocytes 71

Figure 3.12: The alignment of target genes and targeting microRNAs: MET 3´UTR and miR-130b (a), SNAPIN 3´UTR and miR-130b (b), IGF2BP3 3´UTR and miR-208 (c), SMARCC1 3´ UTR and miR-208 (d), ADAMTSL2 3´UTR and miR-125a (e), MAP3K11 3´UTR and miR-125a (f) 72

Figure 3.13: Bioinformatic prediction of miR-99a, miR-100, miR-487a and miR-350 binding sites on the 3'UTR of Bovine tribble homologue 2 (TRB2) 73

Figure 3.14: The expression profile of miR-99a, miR-100 and miR-487a in bovine oocytes and their companion cumulus cells at immature and matured stages. The analysis was conducted using qRT-PCR and the data was normalized with the mean value of three internal controls (U6, SNORD 44 and SNORD 48). IO = Immature oocyte, MO = Matured oocyte, ICC = Immature cumulus cells, MCC = Matured cumulus cells) 74

Figure 3.15: The mRNA expression profile of tribbles homologue 2 (TRB2) in bovine oocytes and their companion cumulus cells. The expression analysis was conducted using qRT-PCR (A) and semi-quantitative PCR (B). (IO = Immature oocyte, MO = Matured oocyte, ICC = Immature cumulus cells, MCC = matured cumulus cells) 75

Figure 3.16: Validation of miRNA-target interaction using luciferase reporter assay. The mean firefly / renilla ratio observed in four treatment groups (pmirGLO vector transfected, Construct vector transfected, Construct vector + mimic transfected and mismatch vector + mimic) showing a marked reduction in firefly/renilla ratio in miR-99a mimic co-transfected group (A). A pronounced firefly/renilla ratio was also observed in miR-100 mimic and construct vector co-transfected group (B). A modest reduction of luciferase activity was observed in miR-487a mimic and pmirGLO construct vector co-transfected group (C). Treatment groups with a star (*) indicate significant difference ($P < 0.05$) between them 77

Figure 4.17: Top three molecular networks enriched by the 680 genes that are predicted targets of the 23 aberrantly expressed miRNAs in subclinical endometritis as compared to healthy ones. A molecular network from the IPA analysis converged to NF-KB that controls gene expression (a), gene network that mediates cell death (b) and molecular network that dictates connective tissue development and function (c). In the graphical representation of a network, genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. Nodes are displayed using various shapes that represent the functional class of the gene product 105

Figure 4.18: A list of top biological functions in which the aberrantly expressed miRNA target genes are enriched 106

Figure 4.19: Top selected canonical pathways that were found to be most significant to the target gene list 107

Figure 4.20: The frequency and position of interaction between aberrantly expressed miRNAs and 3'UTR of target genes with central position in the top five gene networks 108

Figure 4.21: The semi-quantitative expression analysis of genes with central position in the top networks (ID3, INSR, NOS2, SP1, IKBKB, MAPK14 and JUN) and internal control (GAPDH) in endometrial samples at different time points of estrous 108

Figure 4.22: Luciferase activity assay for miR-503 - IKBKB 3' UTR and miR-503 – Jun 3' UTR interactions. The alignment of miR-503 with IKBKB and JUN (A & C) indicating the binding site that was cloned in the pmirGLO expression vector. Co-transfection of intact pmirGLO vector and construct cloned with IKBKB and JUN 3' UTR with miR-503 mimic in four treatment groups (B & D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them 110

Figure 4.23: The base complementarity between miR-27a-3p versus ID3 and SP1 where multiple miR-27a-3p binding sites on SP1 and ID3 were cloned into the pmirGLO expression vector (A & C). The mean firefly / renilla ratio observed in four treatment groups (pmirGLO vector transfected, construct vector transfected, construct vector + mimic transfected and mismatch vector + mimic) showing reduced firefly/renilla ratio in miR-27a-3p mimic co-transfected group (B & D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them 111

Figure 4.24: The response of luciferase reporter protein cloned with INSR 3' UTR following co transfection in cumulus cells in four treatment groups (A). The Watson and Crick complimentarity between INSR 3'UTR and miR-24-3p. All the three target sites were cloned in to pmirGLO expression vector (B). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them 112

Figure 4.25: Target validation for miR-196b – NOS2 and miR-423-3p – MAPK14 using luciferase reporter assay. The Watson and Crick complimentarity between miR-196b and NOS2 3'UTR (A) and miR-423-3p – MAPK14 (C). Transfection of pmirGLO vector, pmirGLO vector cloned with NOS2 3' UTR containing miR-196b target site, construct vector + miR-196b mimic in four treatment groups(B) similar transfection procedure was performed to validate the interaction between miR-423-3p and MAPK14 3' UTR (D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them 113

Figure 4.26: The mean ct values of internal controls across estrous cycle and pregnant group. The geometric mean of the internal controls (U6, SNORD44, and SNORD48) showed stable expression pattern across the groups that we compared and was used to normalize the qRT-PCR data for miRNAs 114

Figure 4.27: The expression pattern of six miRNAs during estrous cycle (day 0, day 3, day 7, day 14) and in pregnant cows.miR-223 and miR-24-3p showed more or less constantly lower expression during estrous cycle and a marked rise during pregnancy while the expression of miR-215 and miR-27a-3p showed a variable pattern which is lower at day 0 and day 7 . The expression of miR-619 linearly increased during estrous cycle (day3-day14) and a pronounced decrease during pregnancy. miR-423-3p showed more or less similar expression at different time points of estrous as well as pregnant animals 114

1. General introduction

The ability to improve reproductive efficiency in bovine is dependant up on a solid understanding of reproductive physiology. The capacity of a cow to conceive and maintain pregnancy mainly depends on the complex and coordinated actions of hypothalamic-pituitary-gonadal axis and its regulation of the ovarian, uterine and oviductal tissues (Carletti and Christenson 2009). The female reproductive tract responds to the cyclic changes in pituitary and ovarian hormones to provide an optimal environment for oocyte development, a suitable site for implantation and pregnancy. There is an ever increasing evidence that establishment of pregnancy and offspring health are strongly linked to oocyte developmental competence where follicular environments both prior to and during ovulation ultimately dictates the developmental competence of the pre-implantation embryo (Sirard et al. 1998, Sirard 2001, reviewed in Sirard et al. 2006). Once the blastocyst is in the uterus, successful embryo implantation is a marking event for the commencement of pregnancy establishment (Das 2009, Dey et al. 2004). This complex developmental process involves a reciprocal dialogue between the blastocyst and uterus (Wang and Dey 2006) which signals the necessity of both competent embryo and receptive uterine for successful pregnancy.

The developmental events during oocyte maturation and pre-implantation embryo development are synchronized with the proliferation and differentiation of specific uterine cell types, primarily under the direction of ovarian estrogen and progesterone that make the uterus conducive to accept the blastocyst for implantation (Dey et al. 2004, Paria et al. 2001, Red-Horse et al. 2004). The molecular mechanisms that operate behind developmentally competent oocyte/blastocyst and a receptive uterine is known to be complex and tightly controlled (Barnea 2001, Savaris et al. 2008, Sirard et al. 2006), where deviations are evidenced to contribute or solely cause poor quality oocyte /embryo and non-receptive uterus (Christiansen et al. 2006, Germeyer et al. 2010, Lee et al. 2007). The interplay between the ovary and uterus via estrogen and progesterone dictate the hierarchical instruction executed to effect highly regulated gene expression in an optimal time frame.

Gene expression regulation is an integral component of molecular program that drive mammalian development. A unique feature of preimplantation embryonic development

is the presence of maternally stored RNAs and proteins in mature, unfertilized eggs. As it is observed in mice, fertilization triggers the degradation of oocyte-stored transcripts, where 90% maternal transcript turnover is complete at the 2-cell stage (Tang et al. 2007). From this perspective, it appears compelling to hypothesize and test the dynamic expression of bovine microRNAs during oocyte maturation to meet the timely requirement of protein during pre-implantation embryo development and the necessity of maternal transcript clearance following embryonic genome activation.

Uterine diseases such as bovine subclinical endometritis perturb the hypothalamic and pituitary function thereby negatively affecting oocyte maturation and developmental competence, ovulation, pre-implantation embryo development and receptive uterine (Sheldon et al. 2006). Studies have shown that pathogen associated molecules disrupt endocrine function in the female reproductive tract of cattle (Herath et al. 2006, Herath et al. 2007, Herath et al. 2009a) in which the cumulative effect end up in compromised fertility. The molecular mechanism that leads to compromised fertility caused by bacterial infection during subclinical endometritis include direct interference of bacterial lipopolysacharides (LPS) with the hypothalamus pituitary secretion or induction of inflammatory response through Toll-like receptors (TLRs) that might cause premature luteusis (Herath et al. 2007, Herath et al. 2009a, Sheldon et al. 2009). This proposed model appears inconclusive as knowledge on the regulatory role of microRNAs in the intrinsic cellular processes of mammalian endometrium is increasingly building. Hence, we hypothesized the possibility of microRNA centered molecular mechanism governing the association of subclinical endometritis with reduced cattle fertility. In line with this, we propose microRNA regulated molecular roadmap that might alternatively lead to the disruption of the hypothalamus pituitary function and subsequently reduce cattle fertility.

Currently, enormous experimental evidences are building that unraveled the striking role of microRNAs in post-transcriptional gene regulation in the course of animal development and disease. Thus, with this work, identification and expression profiling of bovine miRNAs during oocyte maturation are scrutinized for the first time, while detection of aberrantly expressed microRNAs and associated pathways pioneered the move to unravel molecular mechanism underlying the pathophysiology of the uterus in the context of bovine subclinical endometritis from the miRNA perspective. Hence, we

apparently addressed constraints of bovine reproductive success with respect to post-transcriptional gene regulation by microRNAs from two complementary perspectives; oocyte maturation and pathophsylogy of the uterus.

1.1. Bovine oocyte maturation

The mammalian oocyte undertakes a highly complex journey during which it successively acquires a series of characteristics necessary for fertilization and the development of a healthy embryo (Sirard 2001, Sirard et al. 2006). Ovarian folliculogenesis begins during the pre-natal days with the breakdown of germ cell clusters and formation of primordial follicles which are the smallest ovarian follicle units continuously recruited to grow in to primary and more advanced ovarian follicles (Pepling and Spradling 2001). In bovine, these primordial follicles arrest at meiosis I of prophase (Sirard 2001) and the dogma is that the ovary is endowed with finite number of oocytes, resting in the primordial follicle.

As follicles grow and an antrum is formed, granulosa cells separate into two anatomically and functionally distinct sub-types: the cumulus granulosa cells (CC), those surrounding and in intimate metabolic contact with the oocyte; and the mural granulosa cells (MGC), the cells lining the follicle wall forming a stratified epithelium with the basal lamina. The cumulus – oocyte complex (COC) is then formed by highly specialized cumulus cells that have trans-zonal cytoplasmic processes which penetrate through the zona pellucida and abut the oocyte membrane (Albertini et al. 2001). Molecules that are necessary for oocyte growth pass from cumulus cells to the oocyte via gap junction where as oocyte secreted factors pass from the oocyte to the cumulus cells (Simon et al. 1997).

Sirard et al. (2006) precisely described different types of competence expressed by oocytes that include the ability to resume meiosis, to cleave upon fertilization, to develop into a blastocyst, to induce pregnancy and to generate healthy offspring. The follicular influence on oocyte competence could be dissected into two phases, namely, the pre-antral and growing phases (Sirard et al. 2006). Follicles from the preantral phase never complete meiosis as a result of incomplete meiotic, cytoplasmic and molecular maturation (Fair et al. 1995). However, a variable level of developmental competence

has been reported for the oocytes in the growing phase, where, limited (Blondin and Sirard 1995, Sirard 2001) and loss (Blondin and Sirard 1995) of competence were observed during the early growth phases and late atreitic phase respectively. The acquisition of developmental competence is the reflection of oocyte maturation which depends on the correct dynamics of chromosome separation, the redistribution of cytoplasmic organelles and the storage of mRNA, proteins and transcription factors (Albertini 1992, Hosoe and Shioya 1997, Rodriguez and Farin 2004, Stojkovic et al. 2001, Thibault et al. 1987). Transcription in cattle oocyte essentially ceases when the follicle reach 3 mm diameter (Fair et al. 1997). Short burst of transcription in the cumulus-oocyte-complex during the initial hours of maturation is required for spontaneous meiotic resumption, if the bovine oocyte is aspirated from an antral follicle with a diameter greater than 3 mm. Before chromatin condensation, some transcription can be detected in the germinal vesicle (GV) of the oocyte, but it is no longer detectable after GV breakdown (GVBD), whereas polyadenylation appears intense at least up to metaphase I (Tomek et al. 2002). Despite transcription is quiescent or less significant during the transition from GV stage to MII oocyte, translation of selected maternal transcripts is quite apparent which makes the role of microRNAs as post-transcription gene regulators inevitable and pressing. Fair et al. (2007) showed the differential expression of transcripts in invitro matured and immature bovine oocytes which underlines the tight temporal control of protein synthesis required for oocyte maturation and in preparation of subsequent fertilization and early embryo development. Except few documented evidences on the role oocyte microRNA in Zebra fish as means of maternal transcript turn over (Giraldez et al. 2006) and the necessity of maternal microRNA for mouse zygotic development (Tang et al. 2007), knowledge on bovine oocyte microRNAs remained open research area until this work pioneered the identification and expression profile of microRNAs during oocyte maturation. Therefore, this study was conducted with the following objectives: (i) to investigate the expression pattern of bovine miRNAs during oocyte maturation (ii) to identify new microRNAs which are not documented in bovine (iii) to trace some specific functions of selected microRNAs in invito cultured cumulus cells.

1.2. Pathophysiology of the uterus in the context of bovine subclinical endometritis

Reduced reproductive performance in dairy cattle is often caused by uterine disorders. Endometritis is one of the most commonly observed dairy cattle reproductive disorders that usually leads to reduced fertility (Knutti et al. 2000). Clinical endometritis is characterized by the presence of purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) uterine exudates in the vagina, 21 days or more post partum with out systemic signs of illness (LeBlanc et al. 2002) while, subclinical endometritis is described as inflammation of the uterus with out clinical signs but characterized by increased number of polymorphonuclear neutrophils (PMN) in the endometrium (Sheldon et al. 2006) with threshold level of 5 % (Gilbert et al. 2005). The incidence of clinical and subclinical endometritis in dairy cattle was reported to be 53 % at 40–60 days post partum, and often found to be associated with delayed conception and increased culling (Gilbert et al. 2005). The higher fertilization rate (90%) reported in bovine (Dalton et al. 2001, Pomar et al. 2005) is usually followed by magnificent proportion of embryo loss between day 8 and 17 of pregnancy (Humblot 2001, Thatcher et al. 2001) indicating implantation and maintenance of pregnancy are critical phases after fertilization.

Experimental evidences showed the negative impact of subclinical endometritis on dairy cattle fertility (Gilbert et al. 2005, LeBlanc et al. 2002). The inflammatory response to post partum bacterial infection during subclinical endometritis is associated with the expression of pro-inflammatory mediators (Gabler et al. 2009, Herath et al. 2009b) which affect embryonic development by acting either on the oocyte (Soto et al. 2003), developing embryo (Buford et al. 1996, Soto et al. 2003), endometrium (Davidson et al. 1995, Skarzynski et al. 2000) and/or hypothalamic-pitutary-gonadal axis (Alpizar and Spicer 1994, Petroff et al. 2001).

It is well known that successful embryo implantation is a gateway to pregnancy establishment and requires synchronized development of the embryo to the active stage of the blastocyst, differentiation of the uterus to the receptive state, and a ‘crosstalk’ between the blastocyst and uterine luminal epithelium (Dey et al. 2004). The attachment reaction coincides with the extensive proliferation and differentiation of uterine stromal cells into decidual cells (decidualization) at the site of implanting blastocyst. However,

luminal epithelial cells at the site of blastocyst apposition progressively undergo apoptosis with the succession of implantation. As the uterus is a dynamic physiological system in which cellular proliferation, differentiation, including the terminal differentiation and apoptosis occur in a temporal and cell-specific manner during pregnancy (Das 2009). Cell cycle is the most tightly regulated cellular phenomenon where normal operation involves complex interplay of cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors (Sherr and Roberts 1999). In vivo and in vitro animal model studies have shown the need for regulation of specific uterine genes both at transcriptional and translational levels (Achache and Revel 2006, Horcajadas et al. 2007). MicroRNAs emerged as potent regulators of gene expression in mammalian reproductive tract in normal as well as diseased condition. Aberrant miRNA expression is associated with a number of human diseases and fertility disorders of female reproductive tract. When functionally interpreting differential expression miRNAs in a certain diseased condition, it is important to recognize that up-regulation of a specific microRNA represses target mRNA translation, whereas down-regulation relieves the suppressive effect. The combined effect of multiple miRNAs will dictate a particular disease outcome.

Different animal models have shown the role of microRNAs in normal physiological processes of uterine (Chakrabarty et al. 2007, Hu et al. 2008). However, a number of experimental procedures using animal models have also identified numerous transcripts associated with mammalian uterine disease and theses transcripts encode for the proteins involved in broad signaling pathways. These pathways mediate inflammation, tissue remodeling, apoptosis, cellular proliferation, angiogenesis and wound healing (Arimoto et al. 2003, Hull et al. 2008, Matsuzaki et al. 2001). This implies that aberrant expression of miRNAs in the uterine likely result in the miss expression of target transcripts affecting the corresponding signaling pathways. With this line, studies elucidated the role of microRNAs in the development and progression of mammalian uterine diseases (Ohlsson Teague et al. 2009b, Pan and Chegini 2008).

Our knowledge regarding molecular mechanism underlying bovine subclinical endometritis was significantly limited and with this experiment, we intended to out line the underlying molecular mechanism potentially induced by subclinical endometritis and lead to reduced dairy cattle fertility. Thus, the present study tested the hypothesis

that aberrant expression of miRNAs and dys-regulation of their respective molecular networks and pathways could be triggered by subclinical endometritis. Hence, this study was conducted with the following major objectives: (i) to detect aberrantly expressed miRNAs in cows with subclinical endometritis (ii) to trace the target genes regulated by the aberrantly expressed microRNAs using bioinformatic prediction (iii) to figure out the molecular networks, biological functions and canonical pathways that these miRNAs potentially regulate (iv) to substantiate bioinformatically predicted microRNA and target genes interaction using luciferase reporter assay.

2. Literature review

2.1. Bovine folliculogenesis

Development of mammalian oocyte begins with the formation of primordial germ cells (PGCs) in the embryo and is followed by oogonial proliferation by mitosis, and initiation of meiosis I as primary oocytes (reviewed in Song and Wessel 2005). Gametes develop from PGCs that are established during early embryogenesis. In mammals, the PGCs appear to be induced de novo from other cells in early gasrulating embryo which have an extra gonadal origin and navigate through various tissues to reach the somatic gonad. The mechanisms of PGC migration are highly conserved in divergent animal classes and involve intrinsic and somatic cues, attraction and repulsion and amoeboid motility (Matova and Cooley 2001, McLaren 2003). Once in the gonad, the germ cells begin to actively divide mitotically and become either oogonia or spermatogonia. In most animal species, the oogonia divide to form cluster of interconnected cells. As the meiotic process is initiated, oogonia germ cells are referred as primary oocytes (Matova and Cooley 2001). Female germ cells begin entry into prophase I of meiosis and arrest in the diplotene stage of the first meiotic division until the resumption of meiotic division.

Female gametes are stored within the ovary in the form of primordial follicles, which are comprised of small non growing functionally immature oocytes surrounded by a single layer of flattened granulosa cells (Figure 2.1). In mammals, all the oocytes (eggs) that will be used over a female's lifetime are present in the ovary at birth in a finite pool (Hirshfield 1991). From the pool of primordial follicles, some are stimulated to grow and enter development into primary, secondary or antral follicles. Transition from primordial follicle to primary follicle is marked by an increase in oocyte diameter, companion granulosa cell proliferation and change of shape from flattened to cuboidal (Skinner 2005). Throughout the reproductive lifespan of most mammals, continuous trickles of primordial follicles are released from dormancy and enter the growing follicle pool. Once growth is initiated, the follicle embarks on a complex path of development during which the oocyte progresses through a series of highly co-ordinated phases of development that are necessary for the successful ovulation and fertilization. During postnatal life, ovarian follicles continue to grow, mature and either ovulate or

regress in a cyclic fashion. This process begins as soon as the pool of primordial follicles established in the ovary and continues until the pool is exhausted (Hutt and Albertini 2007).

It is now widely accepted that the oocyte plays a very active role in promoting follicle growth and directing granulosa cell differentiation (Gilchrist et al. 2004). The oocyte manipulates its own environment to ensure it is adequately supported throughout pre-antral, antral and pre-ovulatory development. The influence of the follicular environment on the oocytes acquisition of developmental competence is better understood when folliculogenesis is dissected into a number follicular phases that include; the preantral phase, the growing phase, the early atretic phase, the late atretic phase, the dominant phase, the plateau phase, the pre-ovulatory phase and the LH phase (Sirard et al. 2006).

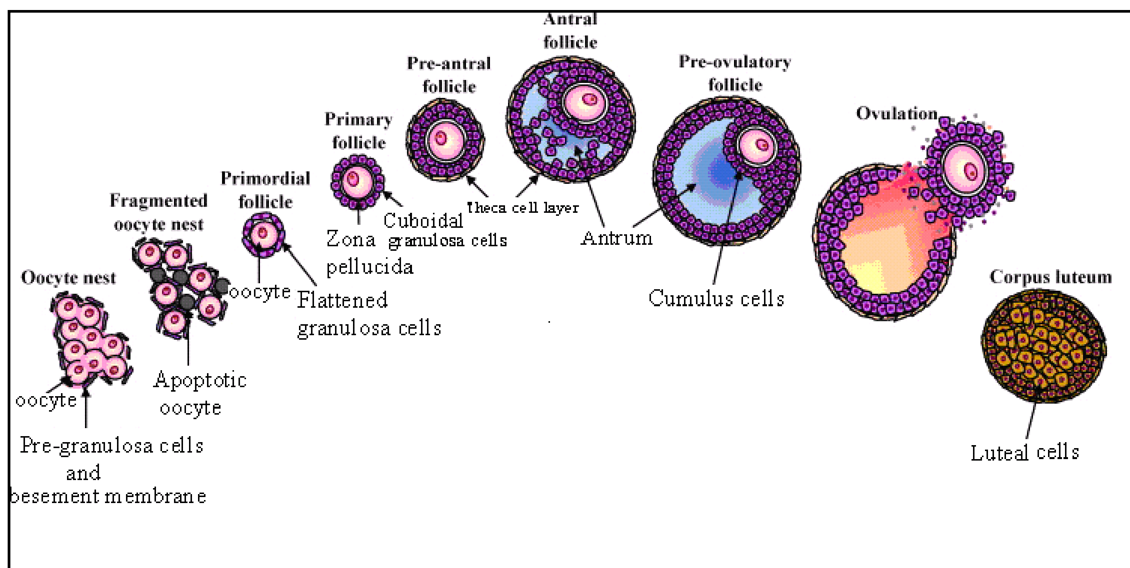

Figure 2.1: The diagram summarizes the process of ovarian follicle development and growth, from oocyte nests present in the fetal ovary through follicle growth, ovulation and CL formation (Hernandez-Ochoa et al. 2009).

The pre-antral phase of folliculogenesis is characterized by zona pellucida formation, granulosa cell proliferation, which is at first slow, the recruitment of theca cells to the follicular basal lamina and a dramatic increase in oocyte volume (Pedersen 1969). Pre-antral follicle growth occurs independently of extra-ovarian hormonal stimuli (Kumar et al. 1997) and its regulation predominantly involves direct interactions between

granulosa cells and oocytes and the local production of growth factors. In particular, two oocyte specific members of the transforming growth factor (TGF)β super family, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), have been shown to play important regulatory roles during pre-antral follicle development (Hanrahan et al. 2004).

The growing phase commences when the small antral follicles start to respond to FSH either in a normal estrous cycle leading to a single dominant follicle or in an ovarian stimulation scheme which leads to ovulation in a multi-dominant paradigm (Blondin and Sirard 1995). These follicles contain oocytes with 2-4 layers of cumulus cells and show very limited signs of atresia. The antral follicles that are under the influence of FSH, it is difficult to aspirtate from the living animals and when removed in the growing phase they display very limited competence (Blondin and Sirard 1995, Merton et al. 2003). However, once follicles reach a diameter of 8.5 mm in non-stimulated animals, they acquire LH receptor in the granulosa layers and become less dependant on the FSH support. In cows, only the follicle corresponding to the dominant follicle reaches this status during each follicular wave. It is possible to induce the production of several of these follicles with exogenous FSH support and, surprisingly, when oocytes are collected in the active growing phase, they also display low developmental competence even if their size often exceeds 7-9 mm (Ginther et al. 2000, Sirard 2001). In the early atretic phase, the subordinate follicles and the dominant follicle contain oocytes of relatively high developmental potential taking blastocyst rate as a measure. It is suggested that similarities between maturing dominant follicle and follicles in the early phase of atresia might send similar maturation promoting signal to the oocyte (Vassena et al. 2003).

The late atretic phase is characterized by the presence of oocytes with a disrupted cumulus layer which can be easily classified and result in a poor development rate to the blastocyst stage. The atretic follicles above 5 mm (large subordinates that are striving for dominance) often contain oocytes with partially expanded outer layers of cumulus (as if the oocyte was trying to mature) and consequently display the cumulus morphology change normally occurring after the LH surge (Blondin and Sirard 1995).

During the dominant phase, the dominant follicle shows a fast growth rate for few days and then reaches a slower growth rate correlated with higher estradiol out put which indicate further follicular differentiation (Ginther et al. 2000). Once these changes occur the developmental potential of the oocyte rapidly increases (Vassena et al. 2003). However, if there is a persistent corpus luteum that produces a high level of progesterone, the dominant follicle will not ovulate and the next follicular wave will soon start. Experimental evidence shows that prostaglandin injection can lead to ovulation of these follicles and subsequent pregnancy following insemination provides additional evidence that these follicles still contain good oocytes (Twagiramungu et al. 1995).

The plateau phase is a phase that occurs between the establishment of dominance and the resultant reduction of growth and the preovulatory period where progesterone concentration decreases dramatically with increasing LH pulsatility (Greve et al. 1983). This phase is followed by the preovulatory phase which is characterized by low progesterone concentrations and can be obtained with out stimulation on days 19-20 of the estrous cycle or with prostaglandin injection any time after wave emergence or with FSH stimulation and prostaglandin. The oocytes obtained from in these conditions have a competence level close to that of oocytes that mature in vivo (Humblot et al. 2005).

2.1.1. Oocyte somatic cell interaction during folliculogenesis

The mammalian oocyte is surrounded by layers of granulosa and theca cells, growth and development of the somatic and the oocyte occur in a highly coordinated and mutually interdependent manner (Gilchrist et al. 2004). During folliculogenesis, when antrum is formed, granulosa cells separate in to anatomically and functionally distinct sub-types: the cumulus granulosa cells (CC), those surrounding and in intimate metabolic contact with the oocyte; and the mural granulosa cells (MGC), the cells lining the follicle wall forming a stratified epithelium with the basal lamina. Cumulus – oocyte complex is formed as the highly specialized cumulus cells penetrate the zona pellucida with trans-zonal cytoplasmic processes and abut the oocyte membrane (Albertini et al. 2001). The oocyte achieves this by secreting soluble growth factors, oocyte-secreted factors (OSFs), which act on neighboring follicular cells to regulate a broad range of GC and CC functions (Buccione et al. 1990). Molecules that pass via gap junctions include

ions, metabolites, and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control oocyte development. This mode of communication in the ovary is essential for development and fertility, and is thought to play a key role in disseminating local and endocrine signals to the oocyte via the cumulus cells (Gilchrist et al. 2004).

Oocyte secreted factors regulate folliculogenesis by modulating a broad range of activities associated with the growth and differentiation of granulosa cells. Oocytes secrete potent mitogenic factors that promote mural granulosa and cumulus cell DNA synthesis and cell proliferation (Lanuza et al. 1998, Li et al. 2000). The oocyte mitogenes interact with key known granulosa cell regulators such as FSH, IGF-I and androgens augmenting their growth promoting activities (Hickey et al. 2004, Li et al. 2000). Oocytes potently regulate granulosa/cumulus cell differentiation. For instance, oocytes modulate FSH-induced progesterone and estradiol synthesis by mural and cumulus granulosa cells (Li et al. 2000) and suppress FSH-induced luteinizing hormone receptor (LHR) mRNA expression (Eppig et al. 2002). Oocytes also regulate the granulosa cell activin-follistatin-inhibin system (Lanuza et al. 1998). The implications of this communication loop are not yet fully clear, but it is likely to have local consequences for follicular growth/differentiation, and intriguingly, because inhibin acts primarily on the pituitary, the oocyte may indirectly regulate secretion of key endocrine hormones such as FSH. The potential broader implications for fertility are clearly profound.

2.1.2. Oocyte developmental competence

Studies have shown the influence of oocyte quality on the developmental potential of embryo in bovine. Sirard et al. (2006) described the levels of oocyte competence which characterize developmental potential of oocyte. This includes; ability to resume meiosis, ability to cleave following fertilization, ability to develop to the blastocyst stage, ability to induce pregnancy and bring it to term, ability to develop to term in good health.

When mammalian oocytes are removed from follicles, they spontaneously resume meiosis (Edwards 1965). Observation of first polar body extrusion marks meiotic

resumption. In bovine, the ability to form a metaphase plate is acquired by the oocyte before antrum formation when it reachs full size in the growing follicle (Sirard et al. 1998). Unlike in other species, the capacity to reach metaphase I and metaphase II seems to be acquired in bovine at the same time (Hampl and Eppig 1995, Sirard et al. 1998). In mouse, cAMP level within the oocyte has been shown to play a critical role in maintaining oocyte meiotic arrest. High levels of cAMP prevent oocyte spontaneous maturation in vitro, while a decrease in oocyte cAMP is associated with the resumption of meiosis (Schultz et al. 1983). It is observed that LH stimulation disrupts the interaction between oocyte and the follicle cells leading to a decrease in oocyte cAMP and subsequent re-entry into the cell cycle (Dekel 1988, Edry et al. 2006).

Fully grown mammalian oocyte has an intrinsic potential of the capacity to cleave following fertilization as simple activation stimulus such as electrical current and ethanol can trigger cleavage. Failure in cleavage doesn’t necessarily show the inability of the oocyte to undergo the first cell division as it may be also due to a dysfunctional sperm that failed to activate the oocyte (reviewed in Sirard et al. 2006). However, experimental evidences show that there is a significant difference in cleavage rate between fetal (36.7 %) and adult cow oocytes (49%) (Chohan and Hunter 2004) suggesting the possibility of maternal effect that could result in reduced cleavage rate.

Provided that a fertilized oocyte is maintained in the proper culture condition, it is supposed to reach the blastocyst stage with in 6-9 days to have a significant chance of inducing pregnancy and producing an offspring (reviewed in Sirard et al. 2006) . Large proportion of early embryos that do not reach the blastocyst stage are usually blocked at or close to the maternal to zygotic transition (MZT)-stage, which occurs at the eight-cell stage in bovine (Barnes and First 1991) suggesting that these incompetent oocytes fail to appropriately activate the embryonic genome. The early developmental program embedded in the oocyte through the accumulation of proteins and RNA is likely to be responsible for the proper execution of the embryonic genome activation. The use of transcription inhibitors such as a-amanitin during the first few days following fertilization results in normal cleavage until the four- to eight-cell stage. Thus, transcriptional activation of the new embryonic genome is required for development beyond MZT (Barnes and First 1991). Such activation may depend on the activation or translation of some maternal transcription factors already stored in competent oocytes

that make this activation possible (Vigneault et al. 2004). Blastocyst quality varies depending on different criteria such as number of cells, trophectoderm to inner - cell mass ratio, blastocoele expansion. The ability of the oocyte to survive cryopreservation and induce a pregnancy is also affected by their apparent morphology as well as by their origin (in vitro or in vivo). From this, one can predict that good blastocysts can result in pregnancy (reviewed in Sirard et al. 2006).

Peterson and Lee (2003) reported all blastocysts are not equal and do not always result in a pregnancy once transferred in suitable recipients, partly this failure is attributed to the recipient. Despite this fact, blastocysts that originate from oocytes matured in vitro result in lower rates of gestation compared to their in vivo counterparts (Peterson and Lee 2003). The ability to go to term is influenced by events occurring before the blastocyst stage and could be explained by either faulty culture conditions and/or by the incomplete oocyte programming before aspiration from its follicle.

Some studies indicate that induction of follicular differentiation by manipulation of the ovarian stimulation protocol, namely FSH starvation or coasting, can result in the recovery of germinal-vesicle stage (immature) oocytes where most are capable of developing to the blastocyst stage following completion of in vitro procedures. Moreover, the embryonic developmental rates obtained from this procedure equal or even surpass the blastocyst rates obtained with in vivo matured oocytes submitted to in vitro fertilization and culture although the two were not compared in the same experiment (Blondin et al. 2002). Overall, the comparison between the in vivo matured and the in vitro matured oocyte recovered at different times before the LH surge support a progressive influence of the follicular differentiation on oocyte competence.

There is now considerable evidence that production of bovine embryos in vitro can result in the large offspring syndrome (Young et al. 1998). The principal cause could be related to the oocyte maturation period, possibly due to an incomplete acquisition of developmental competence at onset of maturation as mentioned above, or as a consequence of sub optimal culture conditions. Therefore the follicular environment could have an influence not only on oocyte quality and female fertility but on the offspring's health as well. It is known that the uterine environment can affect fetal

development and impact the offspring's health but the ovarian influence is becoming an additional source of epigenetic influence that must be explored (Lucy 2003).

2.1.3. Oocyte maturation

Oogenesis is characterized by a unique process of cell division occurring only in gametes, called meiosis; whose goal is the production of haploid cells highly specialized for fertilization. In the majority of species, the oocyte arrests in different stages of meiotic division, in particular, the block occurring in the first meiotic prophase (PI) marks the state of immature oocyte characterized by a prominent nucleus called the germinal vesicle (GV), which contains de-condensed transcriptionally active chromatin (Voronina and Wessel 2003). Generally, meiotic resumption is manifested by germinal vesicle breakdown (GVBD) followed by progression by metaphase I (MI) or II (MII) where it undergoes a second arrest that is removed after successful completion (Sirard 2001).

Oocyte maturation is a long process during which oocytes acquire their intrinsic ability to support the subsequent stage of development in step wise manner, ultimately reaching activation of the embryonic genome (Ferreira et al. 2009). Eppig (1996) defined oocyte maturation as the period of progression from the first to the second meiotic arrest and involves coordinated nuclear and cytoplasmic maturation. Nuclear and cytoplasmic maturation are complex, distinct and appreciably interlinked that occur simultaneously at determined time, even though the molecular programming of the cytoplasm may have already started during the phase of oocyte growth (Ferreira et al. 2009).

2.1.3.1. Nuclear maturation

Nuclear maturation starts with the GVBD, ends at the meiosis exit, and is marked by the presence of the two polar bodies (reviewed in Sirard et al. 2006). In mammals, the luteinizing hormone surge initiates the transition from prophase I (PI) through MI to MII (Moor et al. 1981). In some species, including cattle, oocytes undergo maturation as soon as they are isolated from their follicles or the external milieu, suggesting that these elements contain substances preventing meiosis resumption of Prophase I arrested

oocytes (Tsafriri and Pomerantz 1986). Cytoplasmic maturation is a more obscure process and involves both morphological and molecular alterations (Tosti 2006). Another important factor responsible for meiosis resumption is the M-phase promoting factor (MPF). Nonetheless, most of the work on MPF has been carried out with frog and starfish oocytes and accumulated evidences demonstrate that this complex function exists in other animal models, such as mammals (Yamashita et al. 2000). Studies have provided evidence that meiosis arrest and resumption are modulated by numerous messengers. The involvement of cyclic nucleotides in the maintenance of meiotic arrest was shown where elevated levels of cyclic adenosine monophosphate and related substances such as GPR3, act by preventing spontaneous maturation and/or blocking GVBD in vitro reviewed in (Tosti 2006).

2.1.3.2. Cytoplasmic maturation

For deductive purpose, Ferreira et al. (2009) subdivided cytoplasmic maturation into three main events: redistribution of cytoplasmic organelles, dynamics of cytoskeletal filaments, and molecular maturation.

The redistribution of organelles such as mitochondria and the cortical granules occurs along with the progression of the oocyte to metaphase (Assey et al. 1994). The movement of mitochondria to areas of high energy consumption is crucial for the oocytes and the embryo blastomere during critical periods of the cell cycle (Hyttel et al. 1986). During oocyte maturation, mitochondria synthesize the ATP necessary for the synthesis of proteins where, the synthesized protein supports the completion of subsequent maturation processes and early embryo development (Stojkovic et al. 2001).
A study showed that mitochondria of invitro matured oocyte move from a more peripheral position to a more disperse distribution throughout the cytoplasm after 12-18 h of culture (Hyttel et al. 1986). During in vivo maturation, mitochondria movement also appear to have more peripheral distribution before the LH surge showing a clustered cortical formation in the final stages of nuclear maturation and a more dispersed distribution after the extrusion of the polar body (Hyttel et al. 1986). However, cortical granules show dispersed distribution in cluster through out the cytoplasm in GV stage oocyte (Hosoe and Shioya 1997) and when oocyte maturation advances to MII stage, the granules are distributed throughout the inner surface close to

the plasma membrane a pattern strategically arranged to await for spermatozoon entry and egg activation (Thibault et al. 1987)

The cytoskeletal filaments are dynamic and adaptable structures that can remain unchanged or undergo modifications according to the needs of the oocyte or the cell. This is a key process that is responsible for chromosome segregation during meiosis and mitosis and trafficking molecules and organelles inside somatic cells or oocytes. During the GV stage of oocyte growth, the spatial rearrangement of the organelles is related to the modified organization of the cytoskeleton that forms a network in which the organelles encased by a membrane move and occupy defined positions (Albertini 1992). When entering the M phase of the cell cycle (meiosis in the case of female gametes) microtubule asters appear close to the condensed chromatin in bovine oocytes after GVBD. Furthermore, during the transition from the GV stage to anaphase I, the microfilaments or actin filaments are distributed in the cortical area below the oolemma, without connecting to the microtubules (Li et al. 2005). In metaphase I (MI), the microtubules are nucleated by tubulin polymerization in the oocyte cytoplasm with the centrosome, forming the meiotic spindle and the metaphase plate in which the chromosomes are arranged in an equatorial manner (Albertini 1992). In MI, the metaphase plate is proportionally larger than that formed in MII and in this phase the actin filaments are abundantly distributed in the cortical region but are absent among the microtubules. The spindle is barrel-shaped and its poles are flattened (Li et al. 2005). Although the microfilaments are absent among the microtubules, there seems to be an interaction between these polymers since the polarized movement of the chromosomes also depends on processes mediated by actin filaments. When entering the M - phase of the cell cycle (meiosis in the case of female gametes) microtubule asters appear close to the condensed chromatin in bovine (Kim et al. 2000).

Molecular maturation corresponds to the phases of oocyte growth and maturation and it involves the transcription, storage and processing of the mRNAs expressed by the chromosomes, which will be further translated into proteins by the ribosomes. The proteins derived from these mRNAs are involved both in maturation and in subsequent cellular events such as fertilization, pronucleus formation and early embryogenesis. Thus these proteins are being stored until the appropriate time for their utilization. Gene expression ceases in oocyte after the resumption of meiosis and hence what was

produced during the growth phase will be metabolized at the appropriate time (Sirard 2001). The mRNA transcribed during the molecular maturation of the oocyte is accumulated in a stable and transiently inactive manner (Tomek et al. 2002). The biosynthetic machinery of the cytoplasm processes this mRNA into ribonucleoprotein particles where the mRNA will be protected from nucleolytic degradation and remains stored until the signals for translation are generated during maturation and early embryo development (Fulka et al. 1998).

2.1.4. Transcription factors as mammalian oocyte gene expression regulators

Development of the egg in mammals begins with the formation of primordial germ cells (PGCs) in the embryo and is followed by oogonial proliferation by mitosis and initiation of meiosis I as primary oocytes (reviewed in Song and Wessel 2005). In the course of development, oocyte must undergo several developmental transcriptions during which it acquires a specialized extracellular matrix and synthesizes a unique set of proteins in order to become a fertilizable egg. A hallmark of the oocyte across many species is its high level of transcription, reflecting the importance of maternal mRNAs and proteins that are crucial for supporting not only the growth of the oocyte but also the newly fertilized zygote (Wassarman and Kinloch 1992).

Transcriptional regulation of the oocyte was discovered following the investigations of structures with in the oocyte nucleus that led to our understanding of the transcriptional units (reviewed in Song and Wessel 2005). The observation of lamp brush chromosomes, readily observed in the growing oocytes of organisms that produce large eggs (reviewed in Song and Wessel 2005) highlighted the concept of gene expression for the first time. In many species maternal RNA has high complexity and sufficient to support all the needed protein biosynthesis required during early development. Until zygotic gene activation, the zygote is supported by maternal mRNAs transcribed and translated during oogenesis. The oocyte specific transcriptional mechanisms rely on differential regulation of shared or common factors that result in oocyte – specific activity (reviewed in Song and Wessel 2005).

2.1.4.1. Oct-4: germ cell specific transcription factor

Orthologs of Oct-4 in mice, bovine and human share highly conserved genomic organization and regulatory regions (Kurosaka et al. 2004) and during gonadal development, transcription of the germ cell specific transcription factor Oct-4 in mouse and human germ cells remain high until the onset of meiosis (Pesce and Scholer 2000). The transcription of Oct-4 in oogonia and spermatogonia is downregulated at specific stages of oogenesis and spermatogenesis (Kurosaka et al. 2004). In mouse oocytes, Oct-4 mRNA and protein are down-regulated when the oocyte enters prophase of the first meiotic division and are re-expressed near oocyte maturation (Pesce et al. 1998a). This suggests that Oct-4 may play a role in the growth or acquisition of meiotic competence of oocytes, and/or it may be involved in the transcriptional repression of oocyte-specific genes as Oct-4 is transcribed minimally during a time when the oocyte is undergoing an overall increase in transcriptional activity. Moreover, Oct-4 is transcribed abundantly at oocyte maturation when transcriptional activity in the oocyte decreases dramatically (Pesce et al. 1998b). Oct-4 mRNA is uniformly expressed through out the morula stage and becomes restricted to the inner cell mass of the blastocyst. However, after embryo implantation in mouse, its transcription is restricted to the epiblast and ES cells. The expression of this transcription factor is then progressively downregulated during gastrulation and eventually confined to the PGCs (Pesce and Scholer 2000). During mouse implantation, Oct-4 potentially up regulates Fibroblast Growth Factor- 4 (FGF-4) and Osteopontin (OPN) and in vivo models have shown that FGF-4 tends to stimulate ICM growth or maintenance (Wilder et al. 1997) and is involved in the establishment of the primitive endoderm and OPN is endodermal-specific, extracellular phosphoprotein that mediates adhesion by interacting with integrins (Guo et al. 2002).

2.1.4.2. FIGa: a transcription factor that regulates zona pellucida genes

Folliculogenesis specific basic helix-loop-helix (FIGa) is a germ cell specific transcription factor (in mice) that regulates the coordinated transcription of the three ZP glycoproteins, ZP1, ZP2 and ZP3 and may regulate additional pathways critical for ovarian development (Dean 2004). The ZP proteins form an extracellular matrix that surrounds the growing mammalian oocyte and are critical for sperm activation and for the block to polyspermy. Studies show that mouse ZP3 is composed of primary species

– specific sperm receptor of the oocyte, binding sperm via O-linked oligosaccharide and induce the sperm acrosome reaction. Mouse ZP2 acts as a secondary sperm receptor and plays a role in the prevention of polyspermy, while ZP1 cross-links the other two ZP proteins (Wassarman et al. 2004). FIGa is expressed in both the testis and ovary, but is expressed most abundantly in the ovary of mouse. Its expression is first detected in oocytes at E13.5 in mice and its transcript persists in oocytes into adulthood. Mice lacking FIGa are unable to express ZP genes or form primordial follicles, resulting in massive depletion of oocytes and sterility (Liang et al. 1997).

2.1.4.3. NoBox: oocyte-specific genes transcription regulator

NoBox is detected at the transcript level in maurine oocytes from primordial through antral follicles however this transcript is not observed in somatically derived granulosa cells, theca cells, and corpora lutea (Suzumori et al. 2002). NoBox protein localizes to the nuclei of germ cells and primordial follicles in the mouse ovary. Disruption of 90% of the NoBox coding region including the homeodomain in the mouse resulted in infertile female mice, while males were unaffected. Further examination indicated that in NoBox -/- ovaries, Oct-4, Rfpl4, Fgf8, Zar1, Dnmtlo, Gdf9, Bmp15, and H1oo transcripts were down – regulated (Rajkovic et al. 2004). As the transcription of NoBox precedes the transcription of the above mentioned genes, it is plausible that NoBox may directly or indirectly regulate these genes which are important for oocyte and early embryo development (Rajkovic et al. 2004).

2.1.4.4. ALF: general transcription factor

A set of general transcription factors are necessary for an accurate initiation of transcription. Alf (TFIIAT) is a counterpart of the large α/β subunit of the general transcription factor TFIIA and interacts with the small TFIIA subunit to form a hetrodimeric complex that stabilizes binding of TBP (TATA-binding protein) to core promoter DNA. In mice, ALF is expressed in ovary. In immature Xenopus oocytes, the maternal TFII α/β mRNAs are translationally repressed through a conserved 3' UTR, and ALF compensates for the maternal storage and inactivation of TFIIα/β mRNAs. When oocytes commit to meiosis, a transition from TFIIα/β to ALF occurs, and during

maturation, fertilization, and early embryogenesis, ALF is inactivated and replaced by somatic TFIIα/β (Han et al. 2003).

2.2. Discovery of microRNAs as posttranscriptional gene regulators

MicroRNAs (miRNAs) are small ~21-nucleotide-long noncoding RNAs that have emerged as key posttranscriptional regulators of gene expression in metazoan animals, plants, and protozoa. Lee et al. (1993) uncovered a novel form of gene regulation in C. elegans by pioneering the identification of lin-4 and its protein coding target lin-14. The lin-4 gene encodes a 21 nt small RNA that can partially base pair at multiple sites of lin-14 3'UTR causing diminished lin-14 protein level (Lee et al. 1993, Wightman et al. 1993). Since the lin-14 mRNA levels and polyribosome association appeared to be unaffected by lin-4 regulation, the model emerged that microRNAs inhibit protein expression at the translational level (Olsen and Ambros 1999). Let-7 was the second miRNA gene to be discovered and identified to regulate late larval development in C. elegans by inhibiting lin-41 expression (Reinhart et al. 2000, Slack et al. 2000). Based on the lin-4/lin-14 paradigm, it was predicted that let-7 recognition of let-7 complementary sites containing mRNAs would inhibit their translation (Slack et al. 2000). Shortly after the discovery of let-7 in worms, this gene was shown to be conserved broadly in many animal species (Pasquinelli et al. 2000). The term 'microRNA' was first coined in 2001 when tens of small RNAs with regulatory potential were discovered in C. elegans (Lau et al. 2001, Lee and Ambros 2001).

Forward genetics methods were instrumental in identifying the first miRNA genes, lin-4 and let -7. Analysis of a C. elegans mutant with a defective cell lineage indicated that a mutation in a small noncoding RNA, lin-4, was responsible for the phenotype (Lee et al. 1993). In contrast to lin-4, let-7 was found to be conserved in wide range of phylogentic taxa (Pasquinelli et al. 2000) indicating that miRNA- mediated gene regulation might be common biological phenomenon. In spite of lin-4 and let-7, forward genetics approaches yielded only four additional miRNAs; bantam, miR-14 and miR-278 in Drosophila melanogaster (Brennecke et al. 2003, Teleman et al. 2006, Xu et al. 2003) and lys-6 in C. elegans (Johnston and Hobert 2003). Only few miRNAs were identified using the forward genetics method and explanations include the small size of miRNAs and their potential tolerance to mutations that do not affect the seed sequence make

miRNA genes difficult – to – hit targets in mutagenesis. Besides, many miRNAs may not be recognized in a phenotype driven screen because of functional redundancy (Abbott et al. 2005). Hence, the preferred approach to de novo identification of miRNAs is to sequence size fractionated cDNA libraries.

2.2.1. MicroRNA biogenesis

Primary (Pri)-miRNA transcripts with stem-loop regions are usually transcribed by RNA polymerase II (Lee et al. 2004) and occasionally by RNA polymerase III (Borchert et al. 2006). The stem-loop precursor (pre)-miRNA is released following cleavage event catalyzed by the nuclear microprocessor complex that contains RNase III Drosha (Lee et al. 2003) and exported from the nucleus by exportin (Lund et al. 2004). The 60-90 nt miRNA precursors form the stem loop structure and ribonuclease class III enzyme Dicer excises miRNAs from the pri-miRNA in the cytoplasm and cleaves both strands to form a ~22 nt length double- stranded microRNA duplex (Hutvagner et al. 2001). The two strands become separated and one strand is incorporated in to the RNA Induced Silencing Complex (RISC). The core component of RISC is an Argonaute homologue (in mammals, this is called eIF2c; four eIF2c isoforms are expressed and all four are associated with RISC, though only eIF2c2 has “slicer” activity directed against dsRNAs). In turn, microRNA-loaded RISC binds to target mRNAs and regulates their translation by one of several different mechanisms (Pillai et al. 2007).

In contrast to the linear miRNA processing pathway that was initially thought to be universal for the biogenesis of all mature miRNAs (Figure 2.2) multiple discoveries led to the recognition of miRNA-specific differences that open a plethora of regulatory options to express and process individual miRNAs differentially. Drosha forms two different complexes a small microprocessor complex that contains only Drosha and DGCR8 and processes many pri-miRNAs, and a larger complex that contains RNA helicases, double-stranded RNA binding proteins, heterogeneous nuclear ribonucleoproteins and Ewing's sarcoma proteins (Gregory et al. 2004). The expression of several miRNAs were reduced in homozygous p68 -/- and p72 -/- (RNA helicases and part of large drosha complex) knockout mice, while, other miRNAs remained unaffected (Fukuda et al. 2007).

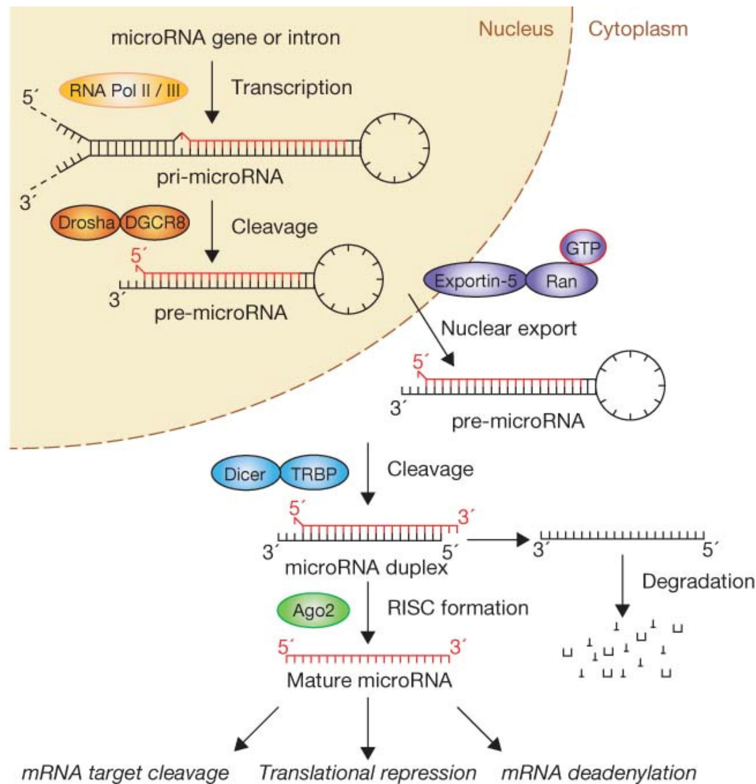

Figure 2.2: Diagrammatic representation of the canonical pathway of microRNA processing (Winter et al. 2009).

Individual miRNA can be also regulated by drosha mediated cleavage, for instance, the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) binds specifically to pri-miR-18a (Guil and Caceres 2007) and changes the hairpin conformation to create more favourable cleavage site for Drosha (Michlewski et al. 2008). As intron-derived miRNAs are released from their host transcripts after splicing, drosha-mediated processing of pri-miRNAs into pre-miRNAs is not always mandatory. If the intron resulting from the action of the splicing machinery and the lariat debranching enzyme has the appropriate size to form a hairpin resembling a pre-miRNA, it bypasses Drosha cleavage and is further processed in the cytoplasm by Dicer (Ruby et al. 2007). These miRNAs, called mirtrons, have been discovered in several species including mammals, D. melanogaster and C. elegans (Berezikov et al. 2007). The miRNA processing factors Drosha and DGCR8 are also regulated post-transcriptionally or post translationally. DGCR8 stabilizes Drosha through an interaction between its conserved carboxyl-terminal domains with the middle domain of Drosha. On the other hand, Drosha cleaves

two hairpin structures in the 5' untranslated region and the coding sequence of the Dgcr8 mRNA leading to DGCR8 degradation resulting in a negative feedback loop reducing Dgcr8 expression when sufficient microprocessor activity is available (Yeom et al. 2006). The discovery that Drosha can directly cleave hairpin structures in mRNAs also points to the possibility that the two Drosha complexes in the cell regulate mRNAs independently of miRNAs.

2.2.2. Principles of target recognition by miRNAs and mode of action

Animal miRNAs target transcripts most commonly through imperfect base pairing to the 3’ untranslated region (3’UTR). Base pairing between the 3’UTR of mRNA and the seed region of microRNA is critical for the mRNA and miRNA interaction. Moreover, 3’- end pairing is suggested to stabilize miRNA - mRNA hybrid formation, especially when there is weak Watson and Crick complementarity in the seed region (Brodersen and Voinnet 2009). Despite these facts, experiments with artificial sensor constructs in human cells showed that miRNA-guided repression is equally efficient when sites are located in the 5' or 3' UTRs of reporter transcripts, thereby demonstrating that no mechanistic requirement confines miRNA action to 3' UTRs (Lytle et al. 2007). Two studies in human cells have identified functional miRNA target sites in the ORFs of DNMT3B (Duursma et al. 2008) and CDKN2A (Lal et al. 2008). In microarray studies that involved miRNA transfection in human cells, transcripts with potential miRNA target sites in ORFs were noticed in repressed gene sets, although they were not pursued in depth because they were thought to be rare, weak or of uncertain importance in vivo (Lim et al. 2005).

Besides the complexity of the rule that govern miRNA- target mRNA interaction, studies have shown that a conserved seed ‘match’ consisting of bases 2-9 of the miRNA is a reliable predictor of interaction (Brennecke et al. 2005) while perfect base pair matching does not guarantee interaction between miRNA and target gene (Didiano and Hobert 2006) and wobble G:U base pairs are often tolerated in target sites (Vella et al. 2004). Furthermore, Hon and Zhang (2007) have shown that the number and arrangement of miRNA recognition sites can influence the degree and specificity of miRNA-mediated gene repression. Taking these principles into account, it has become a well established fact that an individual miRNA is able to control the expression of more

than one target mRNAs (Lewis et al. 2005, Lim et al. 2005) and that each mRNA may be regulated by multiple miRNAs (Brennecke et al. 2005, Enright et al. 2003).

Even if the requirements for microRNA-target gene interaction are fulfilled translation repression may not be possible unless the target site is accessible to the miRNAs. mRNA target site accessibility is influenced by the structure of RNA and its association with RNA binding proteins (RBPs) which consequently affect the extent of post transcriptional regulation by miRNAs (Brodersen and Voinnet 2009). The accessible mRNA target site can be regulated by the targeting miRNA loaded Argonaute (Ago) protein or regulation can be avoided if the target site participate in the secondary structure formation (Kertesz et al. 2007).

Eukaryotic cells contain two general and conserved pathways for the degradation of bulk mRNA, both of which require an initial removal of the 3′ poly (A) tail in a process referred to as deadenylation (Parker and Song 2004). In one case, deadenylation is followed by 3′- to-5′ exonucleolytic degradation by the exosome, a multimeric complex with 3′- to-5′ exonuclease activity. Alternatively, after deadenylation, mRNAs can be decapped by the Dcp1/Dcp2 decapping enzymes and degraded 5′- to-3′ by the abundant 5′- to-3′ exoribonuclease, Xrn1p (Valencia-Sanchez et al. 2006). Evidence suggests that following mRNA cleavage triggered by miRNAs, the 3′ fragment is degraded by major cellular 5′- to-3′ exonucleases. For instance, in Drosophila S2 cells in culture, Xrn1p is required for degradation of the 3′ cleavage product from RISC-mediated cleavage (Orban and Izaurralde 2005).

Another way that miRNAs silence mRNAs is by interfering with their translation. Silencing by a miRNA is observed with either no change in the mRNA level, or with a significantly smaller decrease in mRNA levels than is observed for protein (Cimmino et al. 2005). This was first suggested by the observation that the lin-4 miRNA reduced the amount of lin-14 protein, without reducing the amount of the lin-14 mRNA (Lee et al. 1993, Wightman et al. 1993). Never the less, another subsequent observation suggested that the lin-4 might also affect mRNA levels (Bagga et al. 2005). MicroRNAs also control post-transcriptional gene expression by directing endonuclease cleavage of the target mRNA. Such endonuclease cleavage activity was first demonstrated in cell cultures with exogenously provided dsRNAs (Hammond et al. 2000). It is observed that

some endogenous miRNAs in metazoans direct endolytic cleavage and it is generally favoured by perfect base-pairing between the miRNA and the mRNA, although some mismatches can be tolerated and still allow cleavage to occur (Yekta et al. 2004). However, studies in plants showed that extensive base-pairing between the miRNA and the mRNA is not always sufficient to induce cleavage, suggesting that there can be additional requirements for a RISC complex to catalyze endonucleolytic cleavage (Chen 2004).

The interaction between the miRNA and the target mRNA is complicated than it was thought. The observation in miR-122, a liver specific miRNA, showed that it binds to the 5'-non-coding region of the RNA HCV (hepatitis C virus) genome and induces the accumulation of viral RNAs and it seems that miR-122 can act on viral RNA replication rather than RNA translation or stability (Jopling et al. 2005). Another observation showed that miRNAs oscillate between repression and activation in the duration of a cell cycle, and they identified miR-369-3p in proliferating cells, which represses the translation of targets but switches to mediate activation process in cell-cycle arrest (G1/G0) (Vasudevan et al. 2007). miRNAs also appear to activate certain mRNA targets in stress conditions, such as hypoxia and nutrient deprivation (Leung and Sharp 2007). Whether these up-regulation phenomena happen in special conditions or in ubiquitous regulatory mechanisms remains to be further illustrated.

More than one-third of mRNAs in the mammalian genome are thought to be regulated by one or more miRNAs (Chaudhuri and Chatterjee 2007). Like the mRNAs, miRNAs themselves are co-ordinately modulated by different effectors. Experimental evidences demonstrated that miRNAs show developmental time and tissue specific regulation mechanisms although considerable amount of known miRNAs are expressed universally in various tissues and species (Biemar et al. 2005). It appears that precise regulation network exist to regulate the biogenesis and functions of miRNAs where the influence of cooperated multiple steps in miRNA biogenesis was reported (Kim 2005).

Single nucleotide polymorphisms (SNPs), created by changes in DNA sequences of miRNA-coding genes or in miRNA-binding site in mRNAs, are able to affect the biogenesis and function of miRNA. Many miRNA polymorphisms are shown to be associated with diseases, because a gain-of-function of an miRNA polymorphism may

recruit or enhance the combination of the miRNA to the targets, thereby strengthen the regulation effects, such as tumour suppressor genes; on the contrary, a loss-of-function may result in losing control of the mRNAs especially those oncogenes and drug targets (Mishra et al. 2008). A common G/C polymorphism within the pre-miR-146a sequence decreased the generation of pre- and mature miR-146a and led to less efficient inhibition of target genes involved in the Toll-like receptor and cytokine signaling pathway, which contribute to the genetic predisposition to papillary thyroid carcinoma (Jazdzewski et al. 2008). On the other hand, SNPs in the target sites of mRNAs may result in the escape of inhibition or degradation by an miRNA (Chin et al. 2008).

miRNA editing may represent a fine tuning process in miRNA biogenesis at different steps, resulting in variations of target mRNAs and providing another layer of regulatory controls within the complex network of RNA-mediated gene functions. RNA editing is a site-selective modification of RNA molecules at post-transcriptional level to yield a product differing from the DNA template. Approximately 16 % human pri-miRNAs are subject to A-to-I editing (Kawahara et al. 2008). A-to-I editing at specific positions of miR-142 and miR-151 blocks the Drosha/Dicer cleavage in the maturation of these miRNAs (Kawahara et al. 2007, Yang et al. 2006) indicating RNA editing displays a micro-regulatory role in controlling the miRNA processing machinery. In addition, edited miRNAs may silence a set of genes different from those targeted by the unedited miRNAs, extending the functional scope of miRNAs but increasing the complexity of analyses at the same time (Blow et al. 2006, Habig et al. 2007). Furthermore, RNA editing contributes to the diversity of miRNAs by generating different mature miRNAs from identical miRNA transcripts (Ohman 2007).

Some microRNA genes are affected by epigenetic inactivation due to aberrant hypermethylation, which is characterized as early and frequent events in cancer development (Ando et al. 2009). The expression of miRNA genes located near CpG island, tend to be affected readily by methylation (Lodygin et al. 2008). Besides, it is reported that DNA methylation can affect the expression of transcription factors so that it may control the miRNA expression in an indirect manner (Han et al. 2007).

More than, 45,000 miRNA target sites within human 3′ UTRs are conserved above background levels, and greater than 60% of human protein-coding genes have been

under selective pressure to maintain pairing to miRNAs (Friedman et al. 2009). It has been also known that considerable number of miRNAs is highly conserved among different organisms (Calin and Croce 2007) and experimental evidence supports the notion that miRNA is ancient regulatory mechanism evolved before the divergence of multicellular and unicellular organisms (Zhao et al. 2007). It is estimated that miRNAs constitute nearly 1% of all predicted genes in nematodes, flies and mammals (Lai et al. 2003, Lim et al. 2003a, Lim et al. 2003b). In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes (Friedman et al. 2009) and participate in the regulation of almost every cellular process investigated to date (Bushati and Cohen 2007, Ghildiyal and Zamore 2009) which include, developmental timing, cell differentiation, proliferation, apoptosis, tumorigenesis and host-pathogen interactions (Cho 2007, Liu 2008, Scaria et al. 2006).

2.2.3. MicroRNA involvement in early development

Since the discovery of lin-4 and let-7 in *C. elegans* there is increasing evidence that miRNAs are involved in mammalian development, including proliferation and differentiation of embryonic stem (ES) cells, lineage commitment during embryogenesis, maturation of multiple tissues. The first demonstration of the essentiality miRNA in early embryogenesis of mammals has been reported by Bernstein et al. (2003) where the disruption of the global miRNA biogenesis by ablation of Dicer caused embryo death before gastrulation. Later studies have also shown that ES cells isolated from dicer knock out mice have a slower proliferation rate and impaired differentiation, indicating the involvement of miRNAs in the self renewal and pluripotency of ES cells (Laurent et al. 2008). In undifferentiated ES cells, there is high expression of miR-290 and low expression of miR-21 was reported and proposed that miR-290 the protein that inhibit the expression of Oct 4 while miR-21 causes the inhibition of Oct-4 as a target (Sun et al. 2010). miR-290 cluster is highly expressed in ES cells and it is proposed that miR-290 suppresses the proteins that inhibit the expression of Oct 4 (Sinkkonen et al. 2008). miR-1 and miR-133 are expressed in skeletal muscle cells while miR-1 promotes skeletal muscle differentiation during myogenesis and miR-133 enhances the proliferation of myoblasts (Chen et al. 2006). The expression of miR-143 was increased in adiposites during adipogenesis and the inhibition of miR-143 effectively suppresses the differentiation process by a reduction

in triglyceride accumulation and a decreased expression of adiposite genes (Esau et al. 2004).

Oocytes lacking Dicer, a gene required for microRNA processing and essential for mouse development, lack maternal microRNAs and fail to pass the first cell division (Murchison et al. 2007, Tang et al. 2007). In mouse zygote, maternally inherited microRNAs are abundant with dynamic expression profile and, notably, some are downregulated by as much as 95% between the one-and two-cell stages. Zygotic transcription of microRNAs begin from the two-cell stages (Tang et al. 2007). miR-125a, an orthologue of C. elegans lin-4, is expressed in the two cell stage and its expression increases between the two cell and blastocyst stage. It is suggested that it may have a role in controlling early embryonic timing through the regulation of Ped gene (Byrne and Warner 2008). Furthermore, miR-93 is especially expressed in the trophectoderm and future primitive endoderm in a pattern complementary to Stat 3 gene which is important for early development (Foshay and Gallicano 2009, Takeda et al. 1997). During ES cell differentiation, miR-93 has been observed to interact with and down regulate Stat3 mRNA (Takeda et al. 1997). In recent study, it has been shown that depletion of miR-21 in cultured granulosa cells induced apoptosis. In vivo apoptosis increased in LNA-21-treated ovaries, and ovulation rate decreased in LNA-21-treated ovaries, compared with their contralateral controls (Carletti et al. 2010).

miRNAs, at least in *X. levis*, have a relevant role in Nodal signaling where miR-15 and miR-16 observed to inhibit Nodal signaling by reducing the expression of one of its receptors, Acvr2a. Over expression of miR-15 and miR-16 reduced Spemann's organizer and head structures where as blockage of these miRNAs increased Spemann's organizer and head structure. Moreover, miR-15 and miR-16 restored dorsal mesoderm induction in embryos in which Wnt/β-catenin signaling was suppressed (Martello et al. 2007). In zebra fish, miR-430, a highly abundant miRNA that is required for the clearance of maternal mRNAs, has been shown to directly decrease the expression of squint (sqt) a member of Nodal family (Choi et al. 2007). The temporal expression of miRNAs during embryonic development was determined by microarray analysis. Up to segmentation (12 hours post fertilization), most miRNAs could not be detected. Most miRNAs became visible 1 to 2 days after fertilization and showed strong expression when organogenesis is virtually completed (96 hpf). In adults, the majority of miRNAs

remained expressed. Further more, the expression of miRNAs in dissected organs of adult fish showed a high degree of tissue specificity at least for some miRNAs (Wienholds et al. 2005).

2.2.4. Implication of miRNAs in mammalian fertility

Impaired uterine receptivity is one of the major reasons for the failure of assisted reproduction techniques and defects in implantation and trophoblast invasion are presently considered the major challenges for the successful establishment of pregnancy (Edwards 1995, Herrler et al. 2003). The identification of microRNA expression in the uterus and the functional analysis of individual miRNAs, have shed light on to the physiological changes that occur in the uterus in response to steroids and pregnancy. Studies have shown that deletion of Dicer1 resulted in the reduction of the mouse uterus length and diameter by one-half as compared the wild type females. In addition, the Dicer1fl/fl; Amhr2Cre/+ uterus was one-third the weight of wild-type uteri. Histology indicated a decreased smooth muscle layer and decreased presence of uterine glands (Hong et al. 2008). In another study, Dicer inactivation in mullerian duct mesenchyme – derived tissues of the reproductive tract of the mouse resulted in female infertility but mutant males were normal and fertile. The dicer mutant female oviducts were less coiled forming cyst at the isthmus with unfertilized degenerated oocytes indicating defect in embryo transit. Furthermore, blastocysts transferred directly to the mutant uterus did not result in pregnancy (Gonzalez and Behringer 2009). However, the identification of endogenous small interfering RNA that require Dicer for their synthesis and a Dicer dependant miRNA biogenic pathway confounds the notion that deletion of Dicer is equivalent with deletion of miRNAs. Thus, deletion of individual miRNAs could give much more specific results.

Inline with this, miRNA microarray was performed on uteri from day 1 pregnant mice, during which the uterus is prereceptive to embryo implantation because estrogen is increased compared to uteri from day 4 pregnant mice, during which the uterus is receptive to embryo implantation because progesterone is increased. Two miRNA upregulated in the receptive stage, miR-101a and miR-199a*, are regulators of prostaglandin-endoperoxide synthase 2 (PTGS2). The expression of PTGS2 in the uterine luminal epithelium is necessary for embryo implantation. Thus, miR-101a and

miR-199a* may play a role in tightly regulating PTGS2 expression at the site of embryo implantation, thereby preventing unnecessary growth elsewhere in the uterus. Furthermore, PTGS2 expression is necessary for decidualization of the uterine stromal cells, and the expression profiles of miR-101a and PTGS2 are inversely correlated during decidualization. Therefore, miR-101a and miR-199a* are likely important regulators of PTGS2 during embryo implantation and the concurrent decidualization (Chakrabarty et al. 2007).

In mice, miRNA microarray was used to examine differential expression of miRNAs in the mouse uterus between implantation sites and inter-implantation sites. Eight microRNAs were upregulated at implantation sites as compared with inter implantation sites, specifically miR-21 was highly expressed in subluminal stromal cells at implantation sites on day 5 of pregnancy (Hu et al. 2008). Furthermore, the authors showed that REC is the target gene of miR-21 and suggested miR-21 may play a key role during embryo implantation (Hu et al. 2008).

Female mice with global miRNA deficiency as a consequence of germ line specific deletion of Dicer I are sterile from several causes including defects in oocyte function where oocytes from these mice are observed to have disorganized meiotic spindles and elevated abundance of maternal mRNA transcripts that are normally degraded during meiotic maturation. Many of these maternally derived oocyte transcripts are predicted targets of oocyte - specific miRNAs. These experiments show that DicerI controls oocyte and early zygotic development by enhancing miRNA repression of maternally-inherited cytoplasmic mRNAs. The sequential breakdown of maternally-derived cytoplasmic mRNAs in the oocyte appears to be vital to gamete production (Murchison et al. 2007, Tang et al. 2007). In human, morphologically similar blastocysts derived from patients with polycystic ovaries or male factor infertility exhibited a significant decrease in the expression of six microRNAs (has-let-7a, has-miR-19a, has-miR-19b, has-miR-24, has-miR-92, has-miR-93) in comparison with donor fertile control blastocysts. Hence, it appears that molecular signature of human embryo is not always reflective of morphology and this finding suggested that a possible role for embryonic microRNAs in the etiology of human infertility and potential subsequent implantation failure (McCallie et al. 2010).

It is reported that deletion of Dicer in mice has profound effects on uterine and oviductal development and function as well as ovarian function and oviductal defects are known to cause female infertility. Moreover, these mice exhibited reduced ovarian function as evidenced by decreased ovulation rate which was attributed to loss of miRNA with in the Amhr-2 expressing granulosa cells (Gonzalez and Behringer 2009, Nagaraja et al. 2008, Pastorelli et al. 2009). In another mouse model, female infertility was observed as a result of 80% reduction in Dicer expression. These female mice exhibited normal ovulation rates but the corpora lutea had reduced progesterone output and as a result the mice were unable to sustain pregnancies. These investigators went on to show that loss of Dicer decreased vascularisation of the corpora lutea and that exogenous administration of two miRNA (miR-17-5p and let-7b) could prevent this loss (Otsuka et al. 2008).

2.2.5. Role of miRNAs in immune system development

Mammals have evolved complex genetic programmes that simultaneously regulate the development and function of hematopoietic cells, resulting in the capacity to activate specific responses against invading foreign pathogens while maintaining self-tolerance. From recent studies, miRNAs are emerging as major players in the molecular circuitry that controls the development and differentiation of haematopoietic lineages (Cobb et al. 2006). Knocking-out Dicer activity in early B-cell progenitors determined a block at the pro-B cell stage during the differentiation process leading to mature activated B-cells. Gene-expression profiling revealed a miR-17-92 signature in the 3' UTRs of genes upregulated in Dicer-deficient pro-B cells; the proapoptotic molecule Bim, a top miR-17-92 target, was also highly upregulated. Surprisingly, B cell development was partially rescued by ablation of Bim or transgenic expression of the prosurvival protein Bcl-2 (Koralov et al. 2008). The pioneer knockout of miR-155 in mice (the first mouse knockout for a single miRNA) revealed an essential role in the acquired immunity for this miRNA. In fact, despite miR-155 null mice developed normally, immune system analysis revealed that miR-155 depletion led to pleiotropic defects in the function of B cells, T cells and dendritic cells. These mice were unable to gain acquired immunity in response to vaccination, demonstrating that miR-155 is indispensable for normal adaptive immune responses (Rodriguez et al. 2007).

Another functional example derives from the study of Ventura et al. (2008) who demonstrated that the miR-17-92 cluster is involved in controlling B-lymphocyte proliferation. Deletion of this miRNA cluster was lethal in mice resulting in lung hypoplasia, ventricular sept defects and impairment of the pro-B to pre-B transition. Absence of miR-17-92 led to increased levels of the pro-apoptotic protein Bim and inhibited B cell development at the pro-B to pre-B transition. Furthermore, while ablation of miR-106b-25 or miR-106a-363 (the two paralogous clusters) had no obvious phenotypic consequences, compound mutant embryos lacking both miR-106b-25 and miR-17-92 died at mid-gestation. On the contrary, over-expression of miR-17-92 cluster in mice led to lymphoproliferative and autoimmune diseases that were associated with self-reactive antibody production (Ventura et al. 2008).

MicroRNAs are implicated in a wide range of biological processes including development, differentiation, apoptosis and proliferation. miR-14 and batman were the first miRNAs to be associated with apoptotic function in Drosophila (Brennecke et al. 2003, Xu et al. 2003) and an increasing number of studies have clearly shown the link and role of miRNAs in apoptosis especially in cancer development. Dicer, an endonuclease, is required for miRNA maturation; loss or inactivation of Dicer leads to decreased levels of miRNAs such as let-7a, miR-16, and miR-21 that are implicated in apoptosis (Ghodgaonkar et al. 2009). Both pro-apoptotic and anti-apoptotic genes are potentially regulated by miRNAs. Since miRNAs can regulate hundreds of gene targets at a given time, a single miRNA can function both as pro-apoptotic and/or anti-apoptotic depending on the cellular context and the target gene. For instance, miR-21 predominantly functions as an anti-apoptotic miRNA and is upregulated in many cancer cell types and it also exerts its pro-apoptotic function when it targets tumour suppressor genes. Moreover, the level of miR-21 is inversely correlated with TPM1 and PTEN expression and apoptosis in tongue squamous cell carcinomas (Li et al. 2009). miR-15 and miR-16 regulate the anti-apoptotic factor BCL2 and are considered to be pro-apoptotic microRNAs. These miRNAs induce apoptosis and negatively regulate cell growth and cell cycle when exogenously expressed in tumour cell lines (Cimmino et al. 2005).

Even though there are cases where a particular miRNA acts as pro-apoptotic and anti-apoptotic, miR-17-92 were observed to target pro-apoptotic genes CDKN1A (p21) and

BIM in B-cell lymphoma (Inomata et al. 2009) and subsequently promote cell survival and proliferation. Up regulation of miR-106b-25 is detected in the majority of gastric cancer cases, where these miRNAs negatively regulate pro-apoptotic genes p21 and BCL2L11 protein expression in the TGFβ tumour suppressor pathway leading to tumour progression (Petrocca et al. 2008). miR-221 and miR-222 have been shown to regulate CDK1B (p27), a tumour suppressor gene that promotes apoptosis. These miRNAs behave as an anti apoptotic miRNAs by regulating CDK1B and their increase expression leads to cell proliferation and cell survival (Fornari et al. 2008).

Hull et al. (2008) hypothesized that TGFβ mediates a swith from an initially ischaemic and inflammatory environment that causes tissue damage and necrosis, to a healing milieu that promotes cellular proliferation and tissue remodeling during endometriotic lesion development. Experimental evidences have shown that TGFβ-1 and -2 mRNA translation is repressed by miR-21 and miR-141(Burk et al. 2008, Lu et al. 2009) and these miRNAs are downregulated in endometriosis (Pan et al. 2007). Dysregulation of miR-1, miR-21, miR-141 and miR-194 may synergetically enhance TGFβ signaling in endometriotic lesions by increasing TGFβ expression and restraining TGIF's. Besides, miR-1 and miR-194 mediated TGIF repression may have effects on retinoic acid signaling, promoting tissue remodeling, cell proliferation and invasiveness in endometriosis (Ohlsson Teague et al. 2009a).

Interleukin -1 beta and tumour necrosis factor alpha (TNF-α) in pro-inflammatory condition of the endometrium activate nuclear factor κB (NFκB) and HIFIα signaling leading to enhanced COX-2 transcription. Cox-2 is a rate limiting enzyme in the pro-inflammatory prostaglandin pathway that also demonstrates pro-angiogenic activity (Cao et al. 2005, Keenan et al. 1995, Wu et al. 2002). miR-199a* and miR-16 are known to repress translation of COX-2, moreover miR-199a* targets IκB kinase β (IKBKB) which is co-factor required for NFκB activation (Chakrabarty et al. 2007, Chen et al. 2008).

In study of various human cancers, tissue miRNA expression profiles identified both physiological and pathological conditions more accurately than mRNA abundance signatures (Lu et al. 2005). Potential miRNA biomarkers that display high sensitivity

and specificity have been identified for a variety of tumour types (Iorio et al. 2007). It is thus clear that cellular processes are regulated by miRNAs in a cell-lineage and disease dependant manner.

2.2.6. MicroRNA and uterine pathophysiology

Different animal models have shown the role of microRNAs in normal physiological processes of uterine (Chakrabarty et al. 2007). A number of experimental procedures using animal models have also identified numerous transcripts associated with mammalian uterine disease and these transcripts encode for the proteins involved in broad signaling pathways. These pathways mediate inflammation, tissue remodeling, apoptosis, cellular proliferation, and angiogenesis and wound healing (Arimoto et al. 2003, Hull et al. 2008, Matsuzaki et al. 2001). Many mRNA transcripts are differentially regulated in endometriotic lesions when compared with eutopic tissues (Arimoto et al. 2003, Hull et al. 2008), however, mismatch between the expression levels of transcriptomes and proteins associated with the endometriotic disease revealed the post-transcriptional regulatory role of miRNAs in endometriosis (Wren et al. 2007).

miRNA regulation at least partly share the post-transcriptional gene regulation, as it has been indicated by studies that identified differentially expressed miRNAs in endometriotic tissues (Ohlsson Teague et al. 2009b, Pan and Chegini 2008). Ohlsson Teague et al. (2009a) proposed a model for microRNA regulation in endometriotic lesion development utilizing miRNA microarray studies of eutopic and ectopic endometrium and further supplementing experimentally confirmed mRNA targets of endometriosis associated miRNAs in other diseases, including epithelial, fibroblast and myoblast cell lineages. However, these cells constitute endometriotic lesions and participate in pathological processes, such as cell proliferation, migration and angiogenesis that are common to human endometriosis, cancer and many other diseases (Figure 2.3).

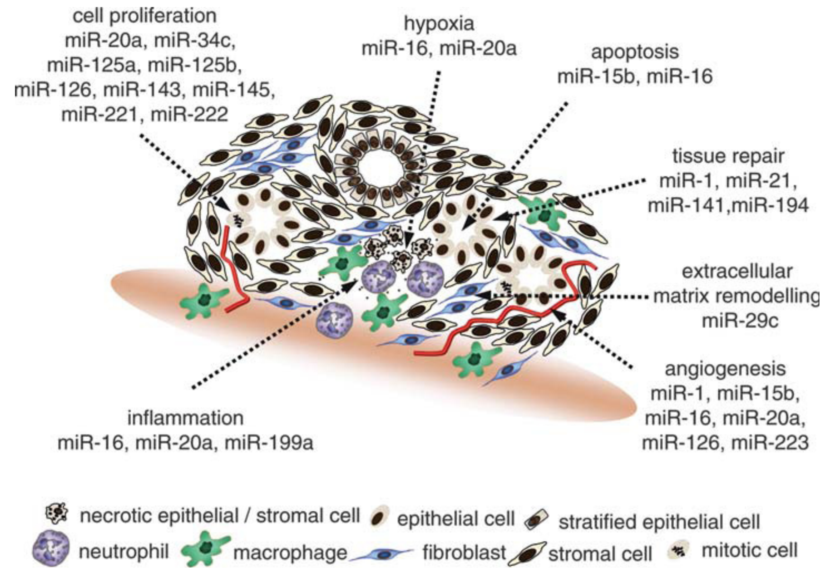

Figure 2.3: microRNA regulatory functions during human endometriosis lesion development. MicroRNA expression may play a role in these processes, regulating transcripts involved in hypoxia, inflammation, apoptosis, tissue repair, cellular proliferation, extracellular matrix remodeling and angiogenesis (Ohlsson Teague et al. 2009a).

2.2.6.1. Inflammation

Elevated levels of tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) elicit inflammatory response in the endometrium (Keenan et al. 1995, Mori et al. 1991). Endometrial synthesis of prostaglandins is under the control of several cytokines including TNF- α and IL-1β, which can increase $PGF_{2\alpha}$ secretion as it is observed cultured bovine endometrial stromal cells (Davidson et al. 1995, Skarzynski et al. 2000). In addition COX-2 demonstrates a pro-angiogenic activity (Smith and Dewitt 1996) and also participate in positive feed forward loop that enhances aromatase activity and local estradiol production there by promoting a proliferative local hormonal environment (Bulun et al. 2000). Cyclooxygenase-2 expression is posttranscriptionally regulated by mmu-miR-101a and mmu-miR-199a* and it is possible that whenever there is a situation that reduce the expression of these two microRNAs induce up-regulation of COX-2 protein level that may promote an inflammatory environment (Chakrabarty et al. 2007).

2.2.6.2. Cell growth, proliferation and apoptosis

Cellular growth is critically regulated at two particular transitions of cell cycle that is G1-S and G2-M. Mammalian cell proliferation is regulated by D-type cyclins (D1, D2 and D3) which are also known as G1 cyclines. The D-type cyclines accumulate during the G1 phase and their association with CDK4 or CDK6 is important to form holoenzymes that facilitate cell entry into S-phase. Retinoblastoma protein (RB) and its family members RBL1 and RBL2 are negative regulators of the D-cyclins. Inactivation of these regulators by phosphorylation, dependant on the cyclin/CDK complex activity, allows cell cycle progression through the G1 phase (Riley et al. 1994). By contrast, cyclins A and B are involved in progression from S to G2-M phase. A critical balance between the positive and negative cell cycle regulators is the key decision maker for cell division. Changes in the expression levels of D-type cyclins and CKls normally occur when quiescent cells are stimulated by mitogenic signals. MicroRNAs up-regulate cell cycle repressors that can lead to repression of cellular proliferation. The down regulation of microRNAs that target cell cycle repressors mRNA could lead to the accumulation of cell cycle inhibitor molecules, such as cyclin dependent kinase inhibitor 1A (CDKN1A/p21), CDKN1B and CDKN1C in endometrial cells (Hull et al. 2008, Inomata et al. 2009, Matsuzaki et al. 2001).

Moreover, cell proliferation is promoted by the cell cycle regulator insulin receptor substrate-1. The insulin receptor substrate-1 (IRS-1), a docking protein for both the type 1 insulin-like growth factor receptor (IGF-IR) and the insulin receptor, is known to send a mitogenic, anti-apoptotic, and anti-differentiation signal (White 1998). miR-126 and miR-145 target this mitogen protein and may inhibit endometrial cell proliferation (Shi et al. 2007). In addition, miR-143 may suppress cell proliferation by repressing mitogene-activated protein kinase 7 (MAPK7) (Esau et al. 2004). On the other hand, apoptotic resistance is mediated by intracellular proteins such as B-cell/lymphoma 2 (BCL2) that lead to enhanced survival of mammalian cells, and it has been observed that this gene promote survival of stressed endometrial cells in endometriosis (Meresman et al. 2000). As miR-15b/16 is confirmed to target BCL2 (Cimmino et al. 2005) upregulation of the miRNAs could induce apoptosis.

2.2.6.3. Angiogenesis

As a tissue that exhibit rapid cyclical growth throughout the reproductive life of a cow, the endometrium is subject to normal physiological angiogenesis as well as pathological situations such as wound healing and tumour growth. Angiogenesis is defined as the process whereby new blood vessels are created from the pre-existing vasculature. This process involves a number of steps which include activation of endothelial cells (EC) with in the existing vessel, breakdown of basement membrane, migration of the EC towards a stimulus, proliferation of the EC, fusion of two sprouts to form a continuous line of EC, tube formation and replacement of blood flow (Klagsbrun and D'Amore 1991). Variations on these steps have been also reported (Nagy et al. 1995, Vernon et al. 1995). Since several angiogenesis related transcripts are confirmed targets of microRNAs. For instance, miR-126 enhances VEGF and fibroblast growth factor (FGF) signaling via repression of inhibitors of these pathways, leading to neoangiogenesis and the development of mature vasculature (Wang et al. 2008). miR-126 is an overlapping transcript with EGF-like-domain, multiple 7 (EGFL7) gene and both transcripts were up-regulated in ectopic versus eutopic endometrium (Ohlsson Teague et al. 2009b) indicating that they are co-transcribed (Figure 2.4). The effect of miR-126 was enhanced by EGFL7 by inducing endothelial cell migration during neovascularisation (Schmidt et al. 2007).

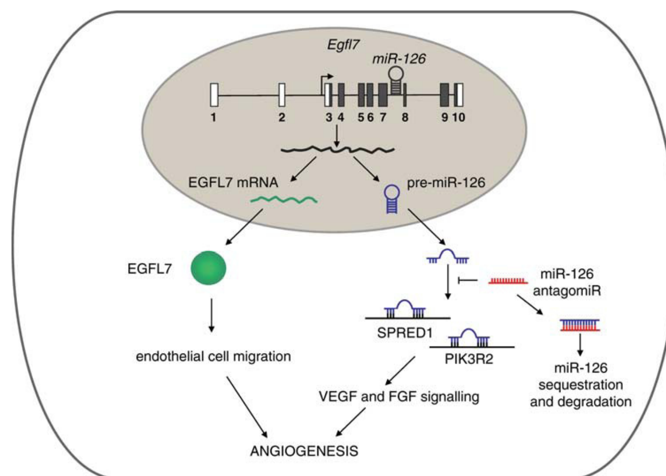

Figure 2.4: The intronic miR-126 and its host gene, EGF-like-domain, multiple 7(Egfl7), promote angiogenesis (Ohlsson Teague et al. 2009a).

2.2.7. Potential role of miRNAs in endometrial transcriptome dynamics and endometritis

Postpartum uterine health in dairy cows is compromised due to persistent bacterial infection causing uterine disease. Uterine infection often is associated with Arcanobacterium pyogenes, Escherichia coli, Fusobacterium necrophorum and Prevotella melaninogenicus (Bonnett et al. 1991, Sheldon et al. 2003). Endometritis is one of the most commonly observed dairy cattle reproductive disorders that usually lead to reduced fertility (Knutti et al. 2000). Clinical endometritis is characterized by the presence of purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) uterine exudates in the vagina, 21 days or more post partum with out systemic signs of illness (LeBlanc et al. 2002) while, subclinical endometritis is described as inflammation of the uterus with out clinical signs but characterized by increased number of polymorphonuclear neutrophils (PMN) in the endometrium (Sheldon et al. 2006) with threshold level of 5 % (Gilbert et al. 2005). The incidence of clinical and subclinical endometritis in dairy cattle was reported to be 53 % at 40–60 days post partum, and often found to be associated with delayed conception and increased culling (Gilbert et al. 2005). The higher fertilization rate (90%) reported in bovine (Dalton et al. 2001, Pomar et al. 2005) is usually followed by magnificent proportion of embryo loss between day 8 and 17 of pregnancy (Humblot 2001, Thatcher et al. 2001) indicating implantation and maintenance of pregnancy are critical phases after fertilization.

Uterine bacterial infections are important because they disrupt not only the function of the uterus, but also the ovary and the overarching higher control centers in the hypothalamus and pituitary. The innate immune system is principally responsible for combating bacterial contamination of the uterus by a range of anatomic, physiological, phagocytic and inflammatory defenses. Neutrophils are the earliest and most important phagocytic cell to be recruited from the peripheral circulation to the uterine lumen, killing internalized bacteria and contributing to the formation of pus when the phagocytes die. However, the functional capacity of neutrophils is reduced after parturition in many cattle (Zerbe et al. 2000); and this may predispose to the establishment of uterine disease. Later, macrophages are likely to be important in the uterine immune response. Immune cells detect bacterial components such as endotoxin and peptidoglycan via toll-like receptors, which activate down-stream signaling to

stimulate the release of cytokines including tumour necrosis factor-alpha (TNFα), and interleukins (IL-1, IL-6, IL-8) (Beutler et al. 2003).

Heat-killed E. coli or LPS provokes an inflammatory response by the endometrial cells, characterized by the increased expression of transcripts for tumour necrosis factor, nitric oxide synthase, and prostaglandin-endoperoxide synthase 2 (PTGS2, formerly COX-2) and the secretion of prostaglandins $F_{2\alpha}$ (PGF) and PGE (Herath et al. 2006). Heat-killed E. coli, LPS, A. pyogenes pyolysin, BoHV-4, bacterial DNA, and lipids also influence endometrial cell prostaglandin secretion, particularly stimulating the secretion of PGE rather than PGF in cattle (Herath et al. 2009a). LPS-induced PGE secretion by endometrial cells is important for fertility because prostaglandins have multiple roles in endometrial function, and luteolysis is initiated by PGF from oxytocin-stimulated epithelial cells (Poyser 1995). In addition, PGE has an important role in the mammalian immune response, acting through prostaglandin E receptors 2 and 4 (PTGER2 and PTGER4) to control inflammation (Sugimoto and Narumiya 2007).

Taken together, studies in mouse and invitro human uterine cell culture models have shown the implication of miRNAs in healthy as well as diseased condition of endometrium. Despite the suggestion of possible role of some transcripts in the development of subclinical endometritis in bovine, no attempt has been conducted to understand the involvement of miRNAs in bovine pre-implantation embryo development and uterine disorders.

3. Part I: Identification and expression profiling of miRNAs during oocyte maturation

Bovine oocyte maturation involves the resumption and completion of the first meiotic division from germinal vesicle (GV) stage to metaphase II (MII) with corresponding cytoplasmic maturation. Optimal storage and timely availability of mRNA during oocyte maturation and early embryo development are essential for oocyte quality and developmental competence. Fair et al. (2007) showed the differential expression of transcripts in invitro matured and immature bovine oocytes which underlines the tight temporal control of protein synthesis required for oocyte maturation and in preparation of subsequent fertilization and early embryo development. Like the mRNA, microRNA expression in mouse shows a dynamic change during oogenesis and large proportion of maternal genes are directly or indirectly under the control of miRNAs (Tang et al. 2007) and the turn over of substantial subset of maternal transcripts is induced by the binding of proteins and microRNAs to the 3' untranslated region (UTR) of target mRNAs.

Despite the fact that knowledge on the role of microRNAs in development in different species is increasingly building; discovery, expression profiling and specific functions of microRNAs during bovine early development remained to be little explored. Given the emerging roles of microRNAs in development, identifying the differentially expressed microRNAs during in vitro oocyte maturation is an important first step to investigate the function of miRNAs in the course of early bovine development. In this study, differentially expressed microRNAs during in vitro bovine oocyte maturation were generated with their matured and immature oocyte stage preference using miRNA microarray technology and quantitative RT-PCR. Moreover, new bovine miRNAs were identified using heterologous approach. Besides, as the function of a specific microRNA can be thought of as a product of the genes that it regulates; we retrieved the potential targets for some micro-RNAs and quantified using qRT-PCR.

3.1. Part I: Materials and methods

3.1.1. Materials

During the laboratory analysis, a diverse list of laboratory experiments, chemicals, growth media and solutions were used. Besides, during the entire study period a broad list of data bases and soft wares were used.

3.1.1.1. List of laboratory equipments used during the study

Equipments	Manufacturer
Laminar flow chamber	Heraeus, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
ABI PRISM® 7000 SDS	Applied Bio systems
Millipore apparatus	Millipore Corporation, USA
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Inverted fluorescence microscope DM IRB	Leica, Germany
Stereomicroscope SMZ 645	Nikon, Japan
Centrifuge	Hermel, Wehing
Electrophoresis chamber	BoRad, Munich
My Cycler Thermal cycler	Bio-RadLaboratories, CA, USA
SHKE6000-8CE refrigerated Stackable Shaker	Thermoscinentific, IWA, USA
CEQ^{TM} 8000 Genetic Analysis	BeckmanCoulter,Krefeld, Germany
Ultra low freezer (-80 ° C)	Labotect GmbH, Gottingen, Germany
Centrifuges (small, medium, large)	Heraeus, Hanau, Germany
MAXQ6000 shacking incubator	Thermo scientific, Germany
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Hybridization chamber	GFL, Dülmen, Germany
Hybridization cassette	(TeleChem International, Inc., Sunnyvale, CA).
Nanodrop 8000 Spectrophotometer	Thermo Fisher Scientific, DE, USA

Axon GenePix 4000B scanner	Axon Instruments, Foster City, CA
Four well dishes	Thermo Fisher Scientific, Nunc, Roskilde, Denmark
Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR	STARLAB GmbH (Ahrensburg)

3.1.1.2. List of chemicals, competent cells and kits

Chemicals and competent cells	Manufacturer/Supplier
Fetal Bovine serum (FBS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
10x PCR buffer	Promega, WI, USA
2x rapid ligation buffer	Promega, WI, USA
Fetal Calf serum (FCS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
E. coli competent cells	Stratagene, Amsterdam, The Netherlands
5x First-Stand buffer	Invitrogen Life Technologies, Karlsruhe
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich
Ligase cocktail	System Bioscience (SBI), Mountain View, CA
Control RNA	System Bioscience (SBI), Mountain View, CA
RNase-free Water	System Bioscience (SBI), Mountain View, CA
3’ adaptor	System Bioscience (SBI), Mountain View, CA
3’adaptor primer	System Bioscience (SBI), Mountain View, CA
5’ adaptor	System Bioscience (SBI), Mountain View, CA
5’ adaptor primer	System Bioscience (SBI), Mountain View, CA
Chloroform	Roth , Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Roth , Karlsruhe, Germany
dNTPs	Roth , Karlsruhe, Germany

DTT	Invitrogen Life Technologies, Karlsruhe, Germany
Dye terminator cycle sequencing s(DTCS)	Beckman Coulter, Krefeld, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Hy5™ fluorescent label	Exiqon, Vedbaek, Denmark
Hy3™ fluorescent label	Exiqon, Vedbaek, Denmark
Labeling enzyme	Exiqon, Vedbaek, Denmark
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
Reverse transcriptase	System Bioscience (SBI), Mountain View, CA
5x Reverse transcriptase buffer	System Bioscience (SBI), Mountain View, CA
QIAzol lysis reagent	Qiagen, Hilden, Germany
Buffer RWT	Qiagen, Hilden, Germany
Buffer RPE	Qiagen, Hilden, Germany
Hybridization buffer	Exiqon, Vedbaek, Denmark
20x Salt buffer	Exiqon, Vedbaek, Denmark
10 % Detergent solution	Exiqon, Vedbaek, Denmark
Isopropyl -D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
Igepal	Roth, Karlsruhe, Germany
Isopropyl β-D-thiogalactoside (IPTG)	Roth, Karlsruhe, Germany
L-Glutamine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
ROX	Bio-Rad, Hercules, CA
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany

BCB	Sigma-Alderich, Taufenkirchen, Germany
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
Pepton	Roth , Karlsruhe, Germany
Penicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen
pGEM®-T vector	Promega, WI, USA
Phenol red solution (5% in D-PBS)	Sigma-Aldrich Chemie GmbH , Munich
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
DNA size ladder	Bio-Rad laboratories, Munich, Germany
Random primer	Promega, WI, USA
Ribo-nuclease inhibitor (RNasin)	Promega, WI, USA
FSH	Sigma-Aldrich Chemie GmbH, Munich, Germany
RQ1 RNase-free DNase	Promega, WI, USA
RNasin	Promega, WI, USA
Sample loading solution (SLS)	Beckman Coulter, Krefeld, Germany
Sequagel XR Sequencing Gel	Beckman Coulter, Krefeld, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Inc, MO, USA
Sodium hydrogen sulphate	Sigma-Aldrich Inc, MO, USA
Sodium lactate solution (60%)	Sigma-Aldrich Inc, MO, USA
Sodium pyruvate	Sigma-Aldrich Inc, MO, USA
Streptomycin sulphate	Sigma-Aldrich Inc, MO, USA
Superscript II reverse transcriptase	Invitrogen, CA, USA

iTaq SYBR Green Supermix with ROX	Bio-Rad laboratories, Munich, Germany
T4 DNA ligase	Promega, WI, USA
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
Tris	Roth ,Karlsruhe, Germany
Triton X-100	Roche Diagnostics GmbH, Mannheim, Germany
Resuspension solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
Lysis solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
Neutralization solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
Column preparation solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
Optional wash solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
Wash solution concentrate	Sigma –Aldrich Chemie GmbH, Munich, Germany
Elution solution	Sigma –Aldrich Chemie GmbH, Munich, Germany
X-Gal (5-bromo-4-chloro-3-indolylbeta-D-galactopyranoside)	Roth, Karlsruhe, Germany
miRCURY™ LNA Array microarray kit	Exiqon, Vedbaek, Denmark
miRCURY™ LNA Array labeling kit	Exiqon, Vedbaek, Denmark
miRNeasy Mini kit	Qiagen, Hilden, Germany
Global microRNA amplification kit	System Bioscience, Mountain View, CA
A T7 based in vitro transcription kit	Epicentre Technologies, Oldendorf, Germany
mirVana qRT-PCR kit	Ambion, Austin, TX
QIAquick PCR Purification	Quigen, Hiden, Germany
GenElute™ Plasmid Miniprep Kit	Sigma–Aldrich Chemie GmbH, Munich, Germany

3.1.1.3. Growth media and solutions

CR1-aa culture medium (50 ml)	Hemi-calcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl
	Sodium hydrogen carbonate	0.080 g
Modified parker medium	HEPES	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Gentamicin	500 µl
	Medium 199	99 ml
	Hemi calcium lactate	0.06 g
	added to water	110 ml
Capacitation medium (50 ml)	Sodium chloride	0.2900 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogene phosphate	0.0017 g
	Hepes	0.1190 g
	Magnesium chloride dihydrate	0.0115
	Calcium chloride dihydrate	0.0145 g
	Sodium lactate solution (60 %)	184 µl
	Phenol red solution (5 % in D-PBS)	100.0 µl
	Water up to	50.0 ml
Fertilization medium	Sodium chloride	0.3300 g
	Potasium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin	0.0032 g

	Magnesium chloride hexahydrate	0.0050 g
	Calcium chloride dihydrate	0.0150 g
	Sodium lactate solution (60%)	93.0 µl
	Phenol red solution (5 % in D-PBS)	100.0 µl
	Water up to	50.0 ml
LB-agar	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0µl
	ddH_2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
BSA (3%)	Bovine serum albumin	0.15 g
	added to PBS+PVA	5 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml
Lysis buffer (100 µl)	Igepal (0.8%)	0.8 µl
	RNasin	5 µl
	DTT	5 µl
	added to water	100 µl
TAE (50x) buffer, pH 8.0	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH_2O added to	1000.0 ml
TE (1x) buffer	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
X-gal	ddH_2O added to	1000.0 ml
	X-gal	50.0 mg
(16%) Para formaldehyde (10 ml)	N, N'-dimethylformamide	1.0 ml

	Para formaldehyde	1.6 g
	added to water	10 ml
PBS + PVA (50 ml)	Polyvinyl alcohol (PVA)	300 mg
	PBS added to	50 ml
Permeabilizing solution (10 ml)	Triton X-100	5 µl
	Glycine + PBS added	10 ml
Physiological saline solution	Sodium chloride	9 g
	added to water	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
dNTP solution	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH_2O added to	400.0 µl
Wash buffer A	20x Salt buffer	20 ml
	10 % Detergent solution	4 ml
	Nuclease free water	176 ml
Wash buffer B	20x Salt buffer	10 ml
	Nuclease free water	190 ml
Wash buffer C	20x Salt buffer	2 ml
	Nuclease free water	198 ml
IPTG solution	IPTG	1.2 g
	ddH_2O added to	10.0 µl
3M Sodium Acetate, pH 5.2	Sodium Acetate	123.1 g
	ddH_2O added to	500 ml
1M EDTA, pH 8.0	EDTA	37.3 g
	ddH_2O added to	1000 ml
Phenol Chloroform	Phenol : Chloroform	1 : 1 (v/v)
0.2% Triton-X100:	Triton	2 ml
	10x PBS : added to	1,000.0ml

0.3% BSA in PBS	BSA	3 g
	10x PBS : added to	1,000.0 ml
3% BSA in PBS	BSA	30g
	10x PBS : added to	1,000.0 ml

3.1.1.4. List of soft wares and data bases used during the study

Soft wares and data bases	Source of soft wares and data bases
miRBase v.10	http//microrna.sanger.ac.uk/
Significance Analysis for Microarray (SAM)	(http://www-stat.stanford.edu/;tibs/SAM/)
GPRocessor 2.0a software	(http://bioinformatics.med.yale.edu/softwarelist)
GenePix Pro (ver. 4.0)	Axon Instruments, Foster City, CA
Entrez Gene	http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
EndNote X1	Thomoson
Primer Express ® software	Applied Biosystems, Foster city, CA, USA
BLAST program	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Prism for windows (ver.5.0)	Graph Pad software, Inc.
NCBI	http://www.ncbi.nlm.nih.gov
Weight to molar quantity converter	http://www.molbiol.ru/eng/scripts/01_07.html
miTarget	http://cbit.snu.ac.kr/~miTarget/
Manupulate and display DNA sequence	http://www.vivo.colostate.edu/molkit/manip/
Alibee - multiple alignment	http://www.genebee.msu.su/services/malign_reduced.html
ENSEMBL	http://www.ensembl.org).
Patrocles database	www.patrocles.org

3.1.2. Methods

3.1.2.1. Heterologous approach

As most microRNAs are evolutionarily conserved across related species and the bovine microRNAs discovered and registered in the miRBase release 8.0 at The Wellcome Trust Sanger Institute are too small, we used microarray slides spotted with miRNA probes from human, mouse and rat. For this, miRCURY ™ array (Exiqon, Vedbaek, Denmark) that consists of control probes, mismatch probes and 454 capture probes was used to hybridize total RNA containing microRNA isolated from immature and invitro matured oocytes. After sequentially washing and scanning, we used species-specific GenePix® Array Lists (GAL) files of human, mouse and rat alternatively to figure out the differentially expressed microRNAs. As we alternatively analysed the array data using the three gal files, we detected the same number of miRNAs. The number of known miRNAs varies with species and hence a specific probe could belong to a known miRNA at least in one or all of the three gal files. We systematically assessed each differentially expressed miRNA in each data set (human, mouse and rat) and observed that a given capture probe was designed to serve for all the three species specific gal files by exploiting the inter species sequence conservation feature of miRNAs. Therefore, we deduced those miRNAs that are differentially expressed and known at least in one of the three species gal files and not included in the miRBase v.10 under bovine microRNAs as bovine miRNA not known before.

3.1.2.2. Oocyte collection, in vitro maturation, sperm capacitation and IVF

Bovine ovaries were obtained from a local slaughter house and transported to the laboratory in a thermoflask containing 0.9% saline solution. The cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8mm in diameter) with an 18-gauge needle and COCs with multiple layers of cumulus cells were selected for this experiment. Part of these oocytes was used as immature oocytes, while the others allowed to mature. To get matured oocytes, selected oocytes were washed in maturation medium (MPM, modified parker medium) (MPM) supplemented with 15% estrus cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/ml gentamycin sulfate, and 10 µl/ml FSH (Folltropin, Vetrepharm, Canada) before set into culture. The COCs

were cultured in groups of 50 in 400 ml of maturation medium under mineral oil in four-well dishes (Nunc, Roskilde, Denmark). Maturation was performed for 24 hr at 39°C under a humidified atmosphere of 5% CO_2 in air. Both immature and matured oocytes were snap frozen after removal of the surrounding cumulus cells.

Two sperm straws were thawed at a temperature of 39 °C for 8 seconds in a water bath, and separated by means of the ´swim up´ technique (Parrish et al. 1988). The matured oocytes in groups of 50 were transferred to fertilization medium and inseminated with spermatozoa at a concentration of $1x10^6$ cells/ml. Fertilization was initiated during coincubation of spermatozoa and matured oocytes for 20 hrs under the same condition as used for maturation (Parrish et al. 1988).

3.1.2.3. In vitro culture and embryo collection

Cumulus free zygotes were washed and transferred to CR1aa culture media (Rosenkrans and First 1994) supplemented with essential and non essential amino acids. Further culture in culture medium was performed in the same incubator and under the same conditions as used for maturation and fertilization. Cattle oocytes and embryos were collected from the culture at specific time points after maturation or fertilization. Matured oocytes were collected at 24 hr after maturation, while 2-cell, 4-cell, 8-cell, morula, and blastocyst stage embryos were collected from the culture at 28, 48, 70, 120 and 168 hr of post-insemination (hpi) respectively. The oocytes and embryos collected were washed once with PBS and snap-frozen in cryotubes containing lysis buffer 0.8% Igepal (Sigma, Taufkirchen, Germany), 40 U RNasin (Promega, Mannheim, Germany), and 5 mM dithiothreitol (DTT) (Promega). Until RNA isolation, all frozen oocytes and embryos were stored at -80 ^{o}C.

3.1.2.4. Brilliant cresyl blue (BCB) staining of COCs

The procedure of BCB staining was done as described in previous studies (Alm et al. 2005, Bhojwani et al. 2007). Briefly, immature and matured oocyte complexes (COC) were subjected to 26 µM BCB (B-5388, Sigma-Alderich, Taufenkirchen, Germany) diluted in mDPBS for 90 min at 38.5 °C in humidified air atmosphere. After washing, the stained COCs were examined under stereomicroscope and categorized into two

groups according to their cytoplasm coloration: oocytes with any degree of blue coloration in the cytoplasm (BCB+) (n = 300) and oocytes without visual blue coloration (BCB-) (n = 300). From each group the denuded oocytes and corresponding cumulus cells were snap frozen and stored in -80°C.

3.1.2.5. Total RNA isolation, miRNA amplification and invitrotranscription

Total RNA containing microRNAs was isolated from three independent pools of immature and in vitro matured oocytes (each containing 100 oocytes) using miRNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol. RNA isolation was performed at three time points in whole experiment. First, RNA isolation was performed from three independent pools of immature and invitro matured oocytes each containing 100 oocytes to be used for miRNA array hybridization. Secondly, three independent pools of oocytes (IO and MO) and cumulus cells from BCB+ and BCB- groups were used for RNA isolation to test some of the candidates' expression in fully grown (BCB+) and growing (BCB-) oocytes. At last RNA isolation was also performed from three independent pools of pre-implantation stage embryos that includes matured oocytes (n = 400), 2-cell (n = 100), 4-cell (n = 80), 8-cell (40), morula (20) and blastocysts (10) to profile the expression of some candidate miRNAs in early developmental stages.

The need of microgram amount of total RNA for microRNA microarray analysis led us to amplify low yield of RNA (nanogram range) obtained from both immature and matured oocytes. Total microRNA was then linearly amplified using global microRNA amplification kit (System Bioscience, CA, USA) and amplification was performed according to manufacturer's procedure. Briefly, prior to adaptor ligation, 100 ng of total RNA from three pools of immature and matured oocytes was incubated at 65°C for 5 min. 3' adaptor ligation was performed in a 10 µl reaction with 2 µl RNase free water, 5 µl ligase buffer (warmed to 37°C before use), 0.5 µl 3' adaptor, 2µl total RNA and 0.5 µl ligase cocktail added together. The reaction was incubated at 37 °C for 1 hour and then kept on ice until the RT-step. For the first strand cDNA synthesis, primarily, 4.0 µl RNase free water, 0.5 µl 3' adaptor primer and 1 µl ligation were added together and incubated at 65°C for 1 min and at 42°C for 5 min. After incubation the reactions were kept on ice. Then after, a master mix was prepared by adding 2.0 µl of 5X reverse

transcriptase buffer, 1 µl dNTP mix, 0.5 µl 5' adaptors, 0.5 µl Dithiothreitol (DTT) and 0.5 µl reverse transcriptase. From this mix, 4.5 µl was added to the previous one and incubated for 5 min at 42 °C and for 10 min at 95 °C. Second strand cDNA synthesis and amplification was done by adding 67 µl RNase free water, 10 µl 10X PCR buffer, 2 µl dNTP mix, 4 µl 3' adaptor primer, 4 µl 5'adaptor primer and 3 µl PCR polymerase. The cycling conditions were, initial denaturation at 95 °C for 2 min , 38 cycles of denaturing at 95°C for 20 seconds, annealing at 55°C for 15 sec and extension 72 °C for 15 sec followed by final extension of 72°C for 30 sec and hold at 15°C. The amplified cDNA was purified using QIAquick nucleotide removal kit (Qiagen, Hilden, Germany) as suggested by the manufacturer and after subsequent washing the cDNA was eluted with 30 µl elution buffer.

A T7 based invitro transcription kit (Epicentre technologies, Oldendorf, Germany) was used according to manufacturer's instruction to invitro transcribe the amplified cDNA in to RNA. Briefly, 2 µl of 10X reaction buffer, 6 µl dNTP (100 mM each of ATP, CTP, GTP and UTP), 2 µl of DTT and 2 µl of T7 RNA polymerase were added to 8 µl of purified cDNA, mixed well and incubated at 37 ˚C for 2 hrs. At the end of incubation, 1 µl of DNase I (Qiagen, Hilden, Germany) has been added and incubated at 37 ˚C for 15 minutes to degenerate the DNA. Then the amplified RNA (aRNA) was purified using miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Finally, the aRNA was eluted with 30 µl RNase free water from which 8 µl was taken to estimate the yield and purity of aRNA by UV absorbance reading at A260/280 using Ultrospec™ 2100 pro UV/Visible Spectrophotometer (Amersham Bioscience, Freiburg, Germany).

In order to validate if the amplified pool of microRNA was representative of the initial population, we took the starting total RNA for amplification from the same sample of non-amplified total RNA and quantified five microRNAs (miR-127, mir-145, miR-206, mir-130b, miR-208) and 5S using qRT-PCR.

3.1.2.6. miRNA labeling and hybridization

Three independent pools amplified miRNAs from both immature and matured oocytes each with 8 µg amount were used for labelling and subsequent hybridization. MicroRNA labeling was done using miRCURY™ locked nucleic acids (LNA) array (Exiqon, Vedbaek, Denmark) labeling kit where miRNA from immature oocytes were labeled with Hy5™ fluorescent label and miRNA from matured oocytes with Hy3™ fluorescent label. Labeling was performed in a two step protocol, where by, a fluorescent label was attached enzymatically to the 3'- end of the miRNAs. Then, this was followed by an enzyme inactivation step after which the sample was ready for hybridization. Synthetic small RNA oligonucleotides designed to hybridize to miRCURY™ array labeling control capture probes were spiked into a miRNA sample to serve as a positive labeling control.

The labeled RNAs from immature and matured oocytes were hybridized in three independent slides with capture probes using miRCURY™ locked nucleic acids (LNA) array (Exiqon, Vedbaek, Denmark) that consist of 454 capture probes for human, mouse and rat miRNAs as registered and annotated in the miRBase release 8.0 at The Wellcome Trust Sanger Institute. Briefly, 10 µl of completely dissolved 2x hybridization buffer was added to the labeled samples. After mixing by vortexing and brief spinning, the samples were incubated at 95°C for 3-5 min. Then, the labelled targets were centrifuged at maximum speed for two min. The slides were placed in the slide chamber and target samples were added over the spotted area. The arrays were covered with glass cover slips and fixed well in the hybridization cassette (TeleChem International, Inc, CA, USA). Hybridization was done by incubating the casset in a hybridization chamber (GFL, Dülmen, Germany) at 60 °C for 16 hours.

The microarray slides were washed with wash buffer A (20ml of 20X salt buffer, 4ml of 10% detergent solution and 176 ml nuclease free water) for 1 min and briefly immersed in wash buffer B (10ml of 20X salt buffer and 190 ml nuclease free water) to avoid transfer of detergent to the next wash step. After subsequently washing the slides with wash buffer B in new coupling jar (at room temperature), washing with buffer C (2ml 20X salt buffer and 198 ml of nuclease free water) was performed by plunging gently

for two min at room temperature. Finally, slides were dried by centrifugation for 2 min at 2000 rpm.

3.1.2.7. MicroRNA array scanning and data analysis

The miRNA array slides were scanned using Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA). The GenePix® Pro 4.0 software (Axon Instruments, CA, USA) was used to process the images, find spots, integrate the alternative GAL files and finally to create reports of spot intensity data. The LOWESS normalization of microarray data was performed using GPRocessor 2.0a software (http://bioinformatics.med.yale.edu/softwarelist). The normalized data was then calculated for the replicates to obtain one value per clone. For microarray data analysis the Hy5/Hy3 ratios were log2 transformed and submitted to SAM (Significance Analysis for Microarray), a free software developed at Stanford University (http://www-stat.stanford.edu/;tibs/SAM/). We finally took a threshold level of ≥ 1.5 fold changes as a cut off value and significant level of $p \leq 0.05$ to screen differentially expressed miRNAs.

3.1.2.8. MicroRNA qRT-PCR for microarray validation

To validate the results of the microarray, individual miRNAs were quantified in three independent miRNA samples of immature and matured oocytes using mirVana qRT-PCR kit (Ambion, Austin, USA), which is designed to detect mature miRNA sequences in a two-step process according to manufacturer's protocol. In the first, RT-primer was used to reverse transcribe and in the second the RT-product was used as template for quantification using miRNA specific PCR primers. Each qRT-PCR primer set includes a primer for reverse transcription and a PCR primer pair optimized for sensitive amplification of specific miRNAs which preferentially detects mature miRNA. Primer set that amplifies 5S rRNA was used as internal control to normalize miRNA content among different experimental samples. Three biological replicates were used for the qRT-PCR to quantify the expression level of the miRNAs in question.

The relative abundance of miRNAs was calculated using a comparative threshold cycle (ct) method. Where, Relative abundance $=2^{-\Delta\Delta ct}$

Δct = average ct target miRNA – average ct of normalize∗

$\Delta\Delta ct = \Delta ct$ target miRNA - Δct of the calibrator∗∗

Expression level (fold change) = $2^{-\Delta\Delta ct}$

∗ 5S was used as internal control to normalize each target microRNA expression

∗∗ the one with the highest Δct value among the groups was used as a calibrator

3.1.2.9. Retrieving miRNA targets and chromosomal location

The potential targets for all microRNAs discovered and annotated so far are predicted and found in the miRBase. We selected three microRNAs that showed consistent pattern of expression in microarray and qRT-PCR platform and retrieved their potential targets. With the aim of generating supplemental evidence for the predicted microRNA targets, we quantified the expression of some target genes (IGF2BP3, SMARCC1, MAP3K11, ADAMTSL2, MET and SNAPIN) and compared their expression with the targeting miRNAs.

We also traced the chromosomal location of microRNAs that were found to be differentially regulated during bovine oocyte maturation from ENSEMBL (http://www.ensembl.org). As limited number of bovine microRNAs annotated in the miRBase, we used chromosomal location of human, mouse and rat to get an insight about bovine miRNA genomic location.

3.1.2.10. Total RNA isolation and cDNA synthesis for analysis of target genes

A total of three pools each containing 100 oocytes both in immature and matured oocytes were used for total RNA isolation using miRNeasy mini kit (Qiagen, Hilden, Germany) as described before. The RNA was then reverse transcribed to cDNA using superscript II reverse transcriptase (Invitrogen). Briefly, 10 µl of total RNA from each sample, 1 µl of random primer and 1µl of oligo (dT) 23 were mixed and incubated at 70 °C for 3 min and then the samples were kept on ice for 2 min. The RNA samples were reverse transcribed in a 20µl reaction volume containing 4 µl 5x first strand buffer, 2µl

DTT, 1µl dNTP, 0.3µl RNasin and 0.7 µl of RT enzyme. The cDNA was then used for the quantitative real time PCR to quantify the target genes (IGF2BP3, SMARCC1, MAP3K11, ADAMTSL2, MET and SNAPIN) using sequence specific primers.

3.1.2.11. Real-time quantitative PCR for target genes

Quantification of IGF2BP3, SMARCCA1, SNAPIN, MAP3K11, ADAMTSL2, MET and GAPDH (endogenous control) mRNAs in each pool of cDNA samples from immature and matured oocytes were assessed by quantitative PCR. The ABI Prism 7000 apparatus (Applied Biosystems, Foster City, USA) was used to perform the quantitative analysis using iTaq™ SYBR® Green Supermix with ROX (Bio-Rad, CA, USA). Quantification of all transcripts was done in comparison with GAPDH as an endogenous control and was run in separate wells. The primer sequences (Table 1) were designed for PCR amplification according to the bovine cDNA sequences using Primer Express software v 2.0 (Applied Biosystems, Foster City, USA). Standard curves were generated for all target genes and the endogenous control using serial dilution of plasmid DNA (10^1-10^9 molecules). All PCRs were performed in a 20 µl reaction volume containing 10µl iTaq SYBR® Green; optimal levels of forward and reverse primers and 2µl of oocyte cDNA samples. During each PCR reaction, samples from the same cDNA source were run in duplicate to control the reproducibility of the results. A universal thermal cycling parameter with initial denaturation step at 95°C for 3 min, 40 cycles of PCR cycling at 95°C for 15 sec and 60°C for 45 sec was used to run the PCR. Finally, quantitative analysis was done using the relative standard curve method in which the ratio of the quantity of target genes to that of the endogenous control (GAPDH) with in the same sample was calculated and expressed as relative levels of mRNA expression.

Table 1: List of primers used for qRT-PCR quantification of target genes with corresponding sequences, product length and annealing temperatures.

S.N.	Primer sequences	Product length	Annealing temperature
1	ADMTSL2 F:CAGATCGTGGAGCGGAAGA ADMTSL2 R:TTGCCATTTTGATTCCACACC	232	56 °C
2	IGF2BP3 F:TGAGGACCAGGCAACTTTCG IGF2BP3 R:AAGCCAATTTCTGGCATCATG	225	56°C
3	MET F:TGGATTCCCACCCTGTGTCT MET R:GAGCATTCCTCCAATCGCAC	213	56°C
4	MAP3K11F:CAAAGGCAGCGATGTCTGG MAP3K11R:TGAAGGATGGAGGCGAAGTC	221	56°C
5	SMARCC1F:GGCCAAGCTAGTCTTTATGGGAA SMARCC1R:CGCCTGTCGTGACTGTTTCTT	219	54°C
6	SNAPIN F:CCACCGAACTGTGCCGTAT SNAPIN R:TCTCCTTCGGGCTGTTTCC	178	55°C
7	GAPDH F:AATGAAAGGGCCATCACCATC GAPDH R:GTGGTTCACGCCCATCACA	240	57°C

3.2. Part I: Results

To ascertain if the amplified pool of microRNA was representative of the initial population, we conducted qRT-PCR analysis of five microRNAs (miR-127, mir-145, miR-206, mir-130b, miR-208) and 5S in amplified and non-amplified total RNA containing microRNAs in both immature and matured oocytes. Here, we found out that all the microRNAs investigated and the 5S were detected and maintained similar trend of expression in immature and matured oocytes when compared between non-amplified and amplified samples. This could subsequently testify the representativeness of the amplified microRNA species in immature and matured oocytes after amplification (Figure 3.5).

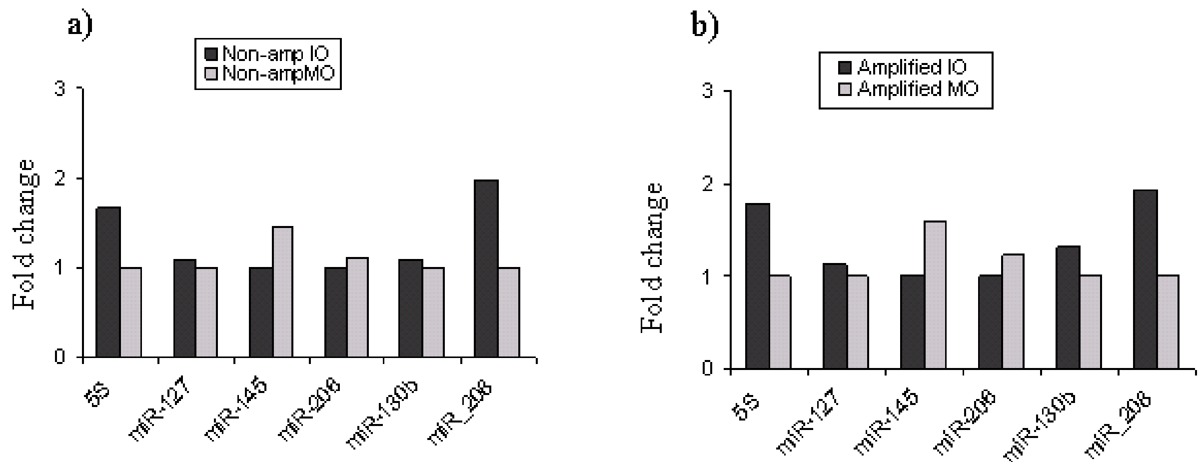

Figure 3.5: Relative expression level of five microRNAs and 5S in non-amplified RNA from immature (IO) and matured (MO) (a) and amplified RNA from immature and matured oocytes analysed using qRT-PCR (IO: immature oocytes, Mo: matured oocytes, Non-amp: non – amplified, Amp: amplified).

3.2.1. MicroRNA detection during bovine oocyte maturation

Here we used a heterologous array with microRNA capture probes that are perfectly matched for all human, mouse and rat miRNAs as registered and annotated in the miRBase release 8.0 at The Wellcome Trust Sanger Institute to analyse conserved miRNAs in bovine oocytes. Analysis of the data using each of the three species specific gal files showed the differential expression (≥1.5 fold changes) of 59 micoRNAs out of

the total of 454 multi-species capture probes. Among the microRNAs that showed altered expression during oocyte maturation, 31 microRNAs showed relative preferential expression in immature oocytes (Table 2) where as the remaining 28 were more abundant in matured ones (Table 3). The expression of seven microRNAs (miR-496, miR-297, miR-292-3P, miR-99a, miR-410, miR-145 and miR-515-5p) in matured and two microRNAs (miR-512-5p and miR-214) in immature showed ≥ 2 fold higher abundance in relative terms. miR-496 (5.2 fold change) and miR-512-5p (2.3 fold change) revealed the maximum fold change up regulation in matured and immature oocytes respectively.

Expression profiling of bovine microRNAs using human, mouse and rat probes during oocyte maturation led us to identify 32 microRNAs which are not so far identified and annotated in bovine. As most microRNAs show high degree of cross-species sequence conservation, miRCURY^{TM} LNA slides were spotted with multi-species miRNA capture probes (human, mouse and rat) in such a way that alternative application of the respective gal files help to investigate the expression of all target miRNAs for the existing probes. For instance, probe ID 11020 stands for hsa-miR-22, mmu-miR-22 and rno-miR-22 as the nucleotide sequence for this microRNA remain the same in all the three species and hence analysis of microarray image data with GenePix® 6.0 species-specific array list results in the detection of inherently similar number of microRNAs.

After the analysis of the hybridized image data, we systematically compared the three (human, mouse and rat) differentially expressed miRNA data sets to screen those microRNAs that are not identified in bovine so far but correspond to a known miRNA in any of the three species having the same probe ID. This comparative approach enabled us to identify and deduce orthologous microRNAs that are not so far identified in bovine but identified and annotated in either human, mouse or rat. By then, only 117 microRNAs have been identified and annotated for bovine in the miRBase v.10. Our result revealed the presence and expression of 32 bovine microRNAs not identified before. Among these, 18 were abundant at higher level in immature oocytes (Table 4) and the remaining 14 were more enriched in matured oocytes (Table 5).

Table 2: The list of miRNAs highly abundant in immature oocytes compared to their matured counterparts with their corresponding chromosomal location and fold change.

microRNA	Chromosomal location	Fold change
miR-512-5p	19q13.42 (H)	2.28
miR-214	Chr.16 (B)	2.02
miR-200c	Chr.5 (B)	1.90
miR-298	2H3 (M)	1.87
miR-208	14q11.2 (H)	1.87
miR-469	5F (M)	1.86
miR-375	2q35(H)	1.84
miR-541	12 F2 (M)	1.79
miR-127	Chr.21 (B)	1.78
miR-381	14q32.31 (M)	1.78
miR-25	Chr.25 (B)	1.77
miR-372	19q13.42(H)	1.76
miR-207	4A5 (M)	1.70
miR-124a	Chr.8 (B)	1.69
miR-545	Chr.X (B)	1.67
miR-433-5p	12F2 (M)	1.67
miR-468	6 D1 (M)	1.63
miR-29b	Chr.16 (B)	1.63
miR-382	14q32.31(H)	1.62
miR-130b	22q11.21(H)	1.61
miR-378	5q32 (H)	1.61
miR-125a	Chr.18 (B)	1.60
miR-192	Chr.29 (B)	1.58
miR-138	Chr. 18(B)	1.58
miR-519e*	19q13.42 (H)	1.56
miR-470	XA6 (M)	1.55
miR-98	Chr.X (B)	1.55
miR-542-5p	X q26.3 (H)	1.53
miR-339	7p22.3 (H)	1.53
miR-547	X A6 (M)	1.52
miR-10a	Chr. 19 (B)	1.51

Table 3: The list of miRNAs highly abundant in matured oocytes compared to their immature counterparts with their corresponding chromosomal location and fold change.

microRNA	Chromosomal location	Fold change
miR-496	14q32.31(H)	5.19
miR-297	10 q 32.1(R)	3.35
miR-292-3p	7A1(M)	3.16
miR-99a	Chr.1 (B)	2.37
miR-145	Chr.7 (B)	2.16
miR-515-5p	19 q13.42(H)	2.05
miR-410	14q32.31(H)	2.03
miR-206	6p12.2 (H)	1.91
miR-423	Chr.19 (B)	1.85
miR-188	X p11.22 (H)	1.82
miR-22	17p13.3 (H)	1.75
miR-34c	Chr.15 (B)	1.75
miR-181c	Chr.7 (B)	1.73
miR-467a	2 A1(M)	1.72
miR-190	15q22.2 (H)	1.71
miR-153	12F2(M)	1.70
let 7d	9q22.32 (H)	1.68
miR-122a	18q21.31(H)	1.65
miR-182*	7q32.2 (H)	1.62
miR-140*	8D2 (M)	1.60
miR-365	Chr.25 (B)	1.60
miR-128a	2q21.3(H)	1.56
miR-380-5p	Chr.21 (B)	1.54
miR-518b	19q13.42 (H)	1.53
miR-519e	19q13.42 (H)	1.53
miR-484	Chr.25 (B)	1.52
miR-128b	3p22.3 (H)	1.50
miR-223	Xq12 (H)	1.50

The letter in parenthesis for the chromosomal location indicate the species for which the map was done (H= human, M=mouse, R=rat, B= Bovine).

Table 4: MicroRNAs that are highly abundant in immature oocytes and not known so far in bovine but known orthologous microRNAs in either human, mouse or rat.

Probe ID	Human	Mouse	Rat
11145	hsa-miR-512-5p	No-known-mmu-target	No-known-rno-target
11220	No-known-hsa-target	mmu-miR-298	rno-miR-298
11255	No-known-hsa-target	mmu-miR-469	No-known-rno-target
14306	hsa-miR-381	mmu-miR-381	rno-miR-381
11208	No-known-hsa-target	mmu-miR-207	rno-miR-207
5730	hsa-miR-208	mmu-miR-208	rno-miR-208
11088	hsa-miR-375	mmu-miR-375	No-known-rno-target
11084	hsa-miR-372	No-known-mmu-target	No-known-rno-target
14290	No-known-hsa-target	mmu-miR-541	rno-miR-541
10936	hsa-miR-130b	mmu-miR-130b	rno-miR-130b
11092	hsa-miR-378	mmu-miR-378	rno-mir-378
11097	hsa-miR-382	mmu-miR-382	rno-miR-382
11256	No-known-hsa-target	mmu-miR-470	No-known-rno-target
11245	No-known-hsa-target	mmu-miR-433-5p	No-known-rno-target
11068	hsa-miR-339	mmu-miR-339	rno-miR-339
11254	No-known-hsa-target	mmu-miR-468	No-known-rno-target
14291	No-known-hsa-target	mmu-miR-542-5p	rno-miR-542-5p
13784	No-known-hsa-target	mmu-miR-547	No-known-rno-target

Among the bovine microRNAs identified as a result of heterologous approach (not identified in bovine before), six microRNAs (miR-512-5p, miR-372, miR-515-5p, miR-182*, miR-518b, miR-519e) are only known in human and another six (miR-469, miR-470, miR433-5p, miR-468, miR-547 and miR-467a) are also known only in mouse. However, miR-297 was found to be differentially expressed in bovine oocytes and its identification and assessment of expression in bovine was possible as result of rat miRNA capture probe. The remaining ten microRNAs were identified in all the three species, four microRNAs in human and mouse and five microRNAs in mouse and rat (Figure 3.6).

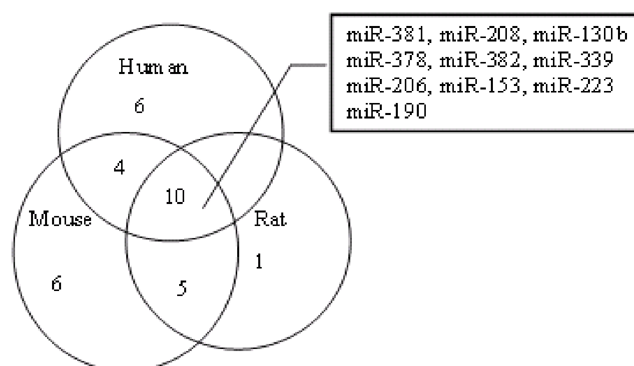

Figure 3.6: The distribution of species specific microRNA capture probes resulted in the detection of oocyte microRNAs which are not identified before in bovine.

Moreover, we assessed these newly detected bovine microRNAs for single nucleotide polymorphism (SNP) from Patrocles database (www.patrocles.org). Out of the 32 miRNAs, only four miRNAs were observed to have single nucleotide polymorphism in human (miR-339 with G/A and miR-223 with -/C) and mouse (miR-206 with A/G and miR-468 with T/C).

Table 5: Bovine microRNAs (highly abundant in matured oocytes) which have no known targets in bovine but with known orthologous microRNAs at least in one of the three species (human, mouse and rat)

Probe ID	Human	Mouse	Rat
11128	hsa-miR-496	mmu-miR-496	No-known-rno-target
11262	No-known-hsa-target	No-known-mmu-target	rno-mir-297
11215	No-known-hsa-target	mmu-miR-292-3p	rno-mir-292-3p
11102	hsa-miR-410	mmu-miR-410	No-known-rno-target
11149	hsa-miR-515-5p	No-known-mmu-target	No-known-rno-target
11007	hsa-miR-206	mmu-miR-206	rno-miR-206
10961	hsa-miR-153	mmu-miR-153	rno-miR-153
10981	hsa-miR-188	mmu-miR-188	No-known-rno-target
10976	hsa-miR-182*	No-known-mmu-target	No-known-rno-target
11156	hsa-miR-518b	No-known-mmu-target	No-known-rno-target
11164	hsa-miR-519e	No-known-mmu-target	No-known-rno-target
11024	hsa-miR-223	mmu-miR-223	rno-miR-223
11253	No-known-hsa-target	mmu-miR-467a	No-known-rno-target
10984	hsa-miR-190	mmu-miR-190	rno-miR-190

3.2.2. MicroRNA array validation

The result from the qRT-PCR analysis revealed that miR-25, miR-125a, miR-130b, miR-208 miR-127 and 200c were more abundant in immature as compared to their matured counterparts. On the other hand, real time PCR analysis has validated the expression of miR-206 and miR-145 to be higher in matured as compared to the immature ones. Interestingly, the magnitude of expression for five microRNAs (miR-25, miR-125a, miR-130b, miR-145 and miR-200c) in both microarray and qRT-PCR platforms was found to be comparable. However, miR-208 revealed a much higher abundance in immature relative to the matured ones (4.72 fold change) in qRT-PCR as compared to 1.9 fold change in microarray. Despite the detection of expression in both microarray and qRT-PCR platforms, we observed a discordant expression pattern for miR-122a, miR-382 and miR-375. Comparison of the magnitude of increased expression in immature oocytes (among the microRNAs profiled using qRT-PCR and confirmed the array result) showed that miR-208 was the most abundant, while miR-125a was the least. We found out that miR-206 and miR-145 (among the miRNAs validated the array result) were more abundant in matured oocytes relative to the immature ones (Figure 3.7a). Furthermore, the specificity of the product was reaffirmed by loading the qRT-PCR product on agarose gel (Figure 3.7b).

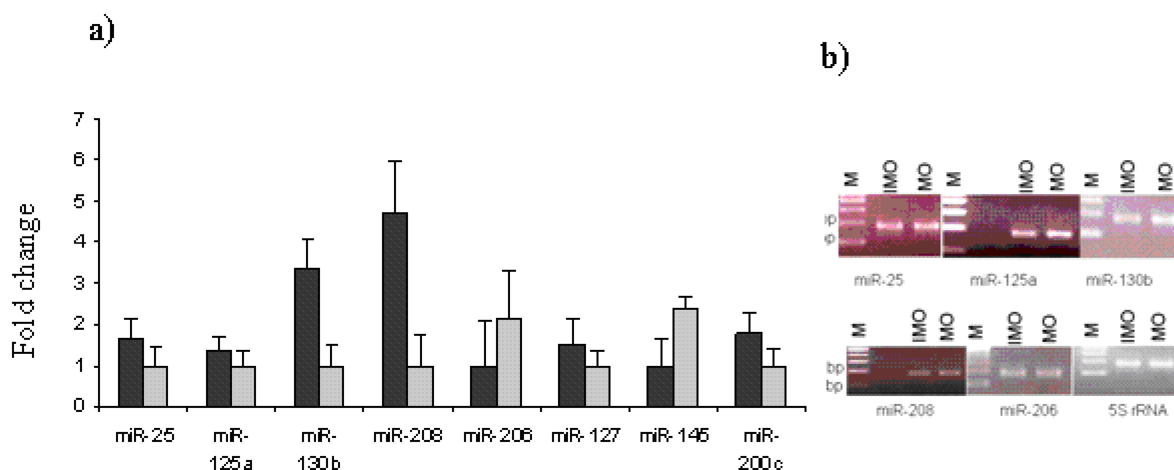

Figure 3.7: qRT-PCR quantification of eight miRNAs (miR-25, miR-125a, miR-130b, miR-208, miR-206, miR-127, miR-145 and miR-200c) in immature and matured oocytes (a).The qRT-PCR product was loaded on 3 % agarose gel with 50 bp molecular marker and confirmed the specificity of the product (b).

3.2.3. MicroRNA expression profiling across pre-implantation embryo stages

With the aim of searching for more comprehensive and wider dimension of information on the expression profile of some microRNAs that validated the array result, we investigated the expression pattern of five microRNAs (miR-125a, miR-25, miR-127, miR-208 and miR-145) in bovine pre-implantation developmental stages (oocyte, 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocyst). The result revealed that all the five microRNAs have a variable trend of expression from oocyte to blastocyst stage (Figure 3.8a-f). miR-125a has the lowest expression level in oocyte and the maximum at 4-cell stage with a slight decline until blastocyst. miR-25, however, revealed opposite trend of expression as that of miR-125a , which showed the lowest expression level at 8-cell stage with decreasing trend from oocyte to 8-cell and increasing trend from 8-cell to blastocyst. Moreover, miR-127 and miR-145 have the maximum expression level at 8-cell stage with relatively lower expression level in pre-and post 8-cell stages and miR-208 revealed an increasing trend of expression through pre-implantation developmental stages with relatively decreased level at blastocyst.

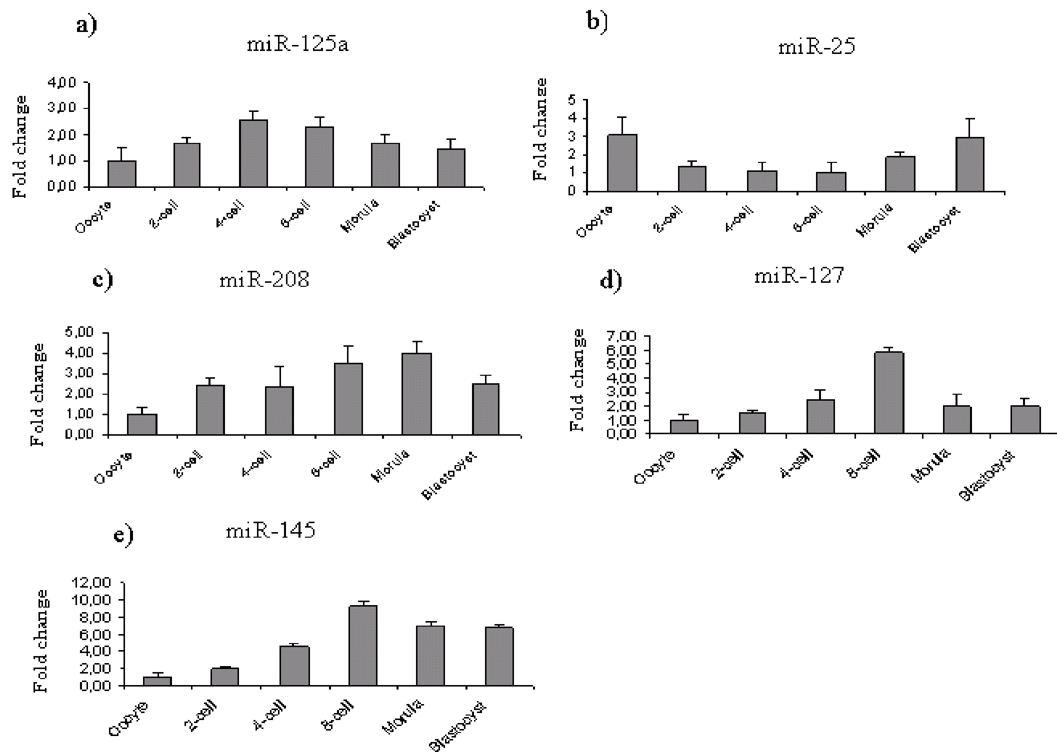

Figure 3.8: The expression profile of miR-125a (a), miR-25 (b), miR-208 (c), miR-127 (d) and miR-145 (e) in bovine pre-implantation developmental stages analysed using qRT-PCR.

3.2.4. MicroRNA expression profiling in BCB+ and BCB- immature and matured COCs

As BCB staining enables us to differentiate between fully grown and growing oocytes, here we aimed to investigate the expression of some microRNAs during oocyte growth (Figure 3.9a & b). For this, we conducted qRT-PCR analysis for four microRNAs (miR-125a, miR-127, miR-145 and miR-208) in BCB+ (fully grown oocytes with low G6PDH activity) and BCB-(growing oocytes with high G6PDH activity) oocytes. Our result in BCB+ and BCB- immature oocytes seem to indicate that the expression level of all microRNAs investigated was higher in BCB- immature oocyte as compared to their BCB+ counterparts. Similarly, we investigated the expression level of the same microRNAs in BCB+ and BCB- matured oocytes. Our result showed that the expression of miR-125a and miR-127 was higher in BCB- while the expression of miR-145 and miR-208 was higher in BCB+ matured oocytes.

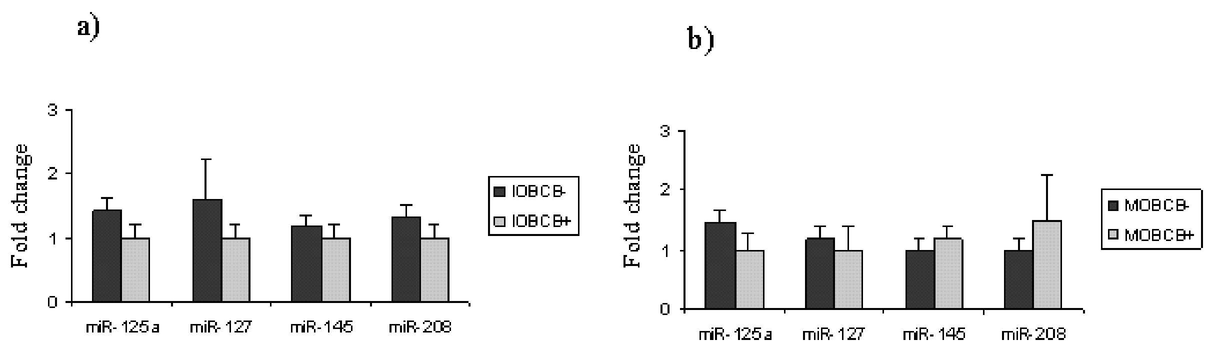

Figure 3.9: The relative expression level of miR-125a, miR-127, miR-145 and miR-208 in BCB- and BCB+ immature (a) and matured (b) oocyte stages.

As bovine oocyte maturation is strongly influenced by cumulus cells expansion and subsequent communication between the cumulus and the oocyte, we investigated the relative abundance of miR-125a, miR-127, miR-145 and miR-208 in the cumulus cells denuded from BCB+ and BCB- immature and matured COCs (Figure 10a & b). Interestingly, the result in BCB stained immature cumulus cells revealed that the expression levels of miR-125a, miR-127, miR-145 and miR-208 were higher in BCB- immature cumulus cells as compared to their BCB+ counterparts. However, in BCB stained matured cumulus cells only miR-127 and miR-145 showed higher relative expression level in BCB- matured cumulus cells as compared to the BCB+, while, a

similar expression level in both BCB- and BCB+ matured cumulus cells was observed for miR-125a and miR-208.

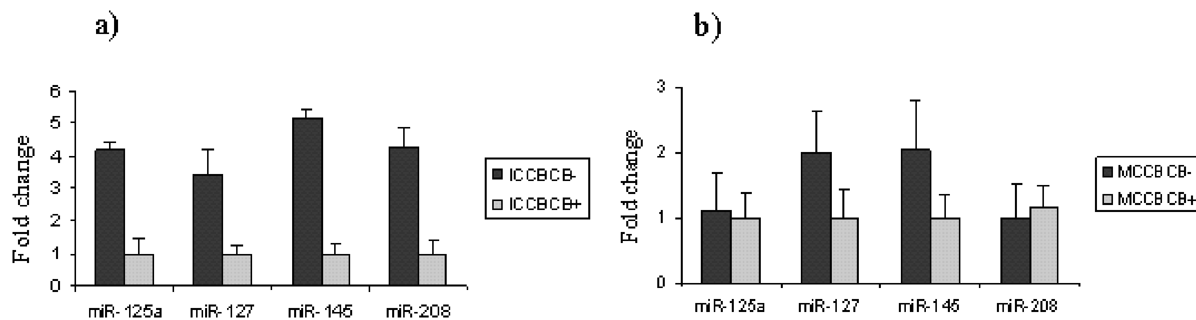

Figure 3.10: The relative expression level of miR-125a, miR-127, miR-145 and miR-208 in cumulus cells derived from BCB-and BCB+ immature (a) and matured (b) oocytes (ICC: cumulus cells derived from immature COC, MCC: cumulus cells derived from matured COC).

3.2.5. Predicted targets for some miRNAs

With the aim of functional characterization, we retrieved for the mRNA targets of five selected miRNAs which are predicted using MIRANDA algorithm and made available at http//microrna.sanger.ac.uk/. Even though, there are too many potential target genes predicted for a particular microRNA, we selected some targets to present in this study based on the score and their relevance to the biological process. Given miR-25, miR-125a, miR-130b, miR-208 and miR-206 showed consistent differential expression pattern between microarray and qRT-PCR, we filtered the corresponding target hits on the basis of their score, free energy and evolutionary conservation in the MIRANDA prediction as well as their potential relevance during mammalian oocyte maturation. Among the microRNAs that we selected to trace their targets, miR-25 and miR-125a were primarily identified in bovine (Coutinho et al. 2007) and target bovine mRNA search was possible, however, miR-130b, miR-208 and miR-206 were not previously identified in bovine and target bovine mRNA search using MIRANDA from the miRBase was not possible. Hence, their targets were retrieved from their human homolog.

Some miR-125a target genes like Tripartite motif–containing protein 71 (Lin-41 homolog) (TRIM71), Argonaute 2 (EIF2C2), Aminopeptidase (LOC407138), 5-hydroxy tryptamine 6 receptor (HTR6), Potassium voltage gated channel subfamily H

member 6 (KCNH6), Mitotic spindle assembly check point protein MAD1 (MAD1L1), ADAMTS like 2 (ADAMTSL2) are known to be expressed in the process of mammalian oogenesis.

Some of the targets for miR-130b includes hepatocyte growth factor receptor precursor (MET), low-density lipoprotein receptor-related protein 2 precursor (LRP2), potassium voltage-gated channel subfamily A member 1 (KCNA1), trinucleotide repeat-containing gene 6A protein (TNRC6A), proactivator polypeptide precursor (PSAP), relaxin receptor 2 (RXFP2), condensin complex subunit 1 (NCAPD2), growth arrest and DNA-damage-inducible protein GADD45 alpha (GADD45A).

The chromosomal locations for differentially expressed microRNAs during bovine oocyte maturation were presented in table 1 and 2. The search for chromosomal locations of these microRNAs in human mouse and rat showed the distribution miRNAs in most of the chromosomes.

3.2.6. Relative abundance of miRNAs and their target mRNAs

Despite, nearly all microRNA targets were predicted by bioinformatics analysis, only a hand full of microRNA targets are experimentally validated. Moreover, it appears more important and complete to study microRNAs with their targets so as to investigate their subsequent role in diverse cellular processes. We then quantified six genes (targets for some selected microRNAs) with the intention to compare the expression pattern of the microRNA and the corresponding targets in immature and matured oocytes (Figure 3.11).

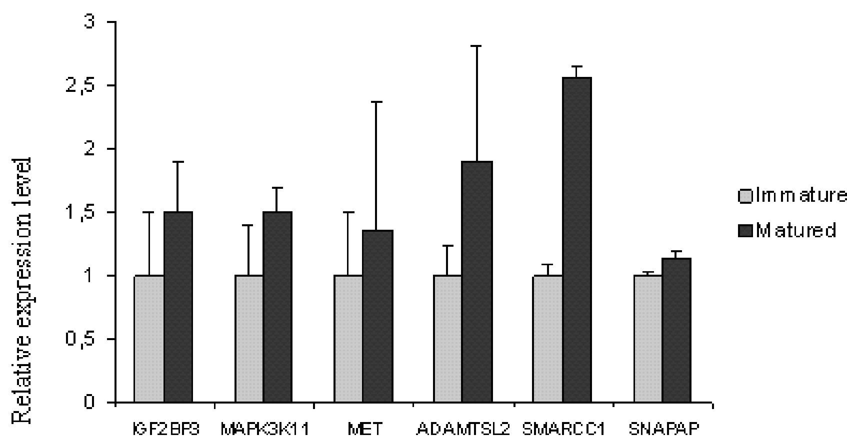

Figure 3.11: The expression profile of six target genes in immature and matured oocytes as revealed by real time PCR. Among the six genes quantified, only SMARCC1 showed statistically significant (P< 0.05) expression level between immature and matured oocytes.

We selected three microRNAs and six target genes (two targets for each microRNA) namely; insulin like growth factor 2 mRNA binding protein 3 (IGF2BP3) and SWI/SNF-related matrix – associated actin dependent regulator of chromatin subfamily C member 1(SMARCC1) as potential targets for miR-208, hepatocyte growth factor receptor precursor (MET) and SNARE-associated protein snapin (SNAPIN) as targets for miR-130b, mitogen activated protein kinase kinase kinase 11 (MAP3K11) and ADAMTS-like 2 (ADAMTSL2) as targets for miR-125a. As we utilized the data base of mRNA target prediction for human, alignment of the microRNAs and corresponding targets of bovine mRNA (3' UTR region) confirmed that these genes harbor complementary sequences with their targeting miRNAs as it was observed in human (Figure 3.12). The result revealed that the expression pattern of the three microRNAs in both immature and matured oocytes is in a reciprocal manner with their corresponding targets. The microRNAs showed higher expression in immature as compared to matured where as the targets showed higher expression in matured as compared to the immature.

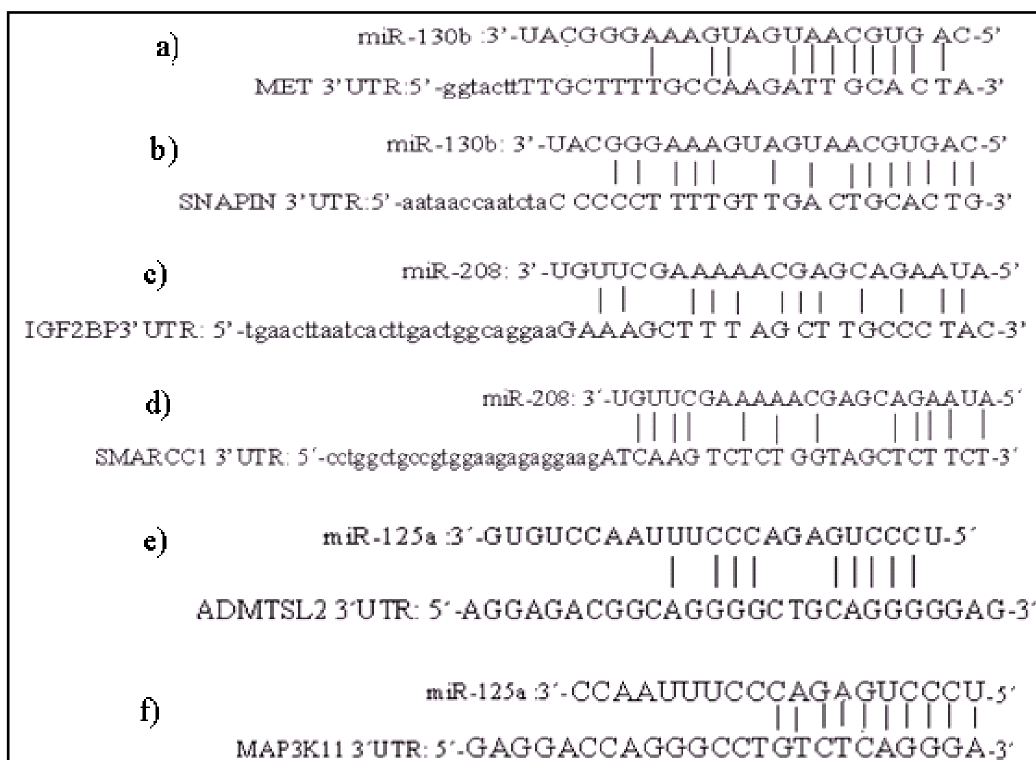

Figure 3.12: The alignment of target genes and targeting microRNAs: MET 3´ UTR and miR-130b (a), SNAPIN 3´ UTR and miR-130b (b), IGF2BP3 3´UTR and miR-208 (c), SMARCC1 3´ UTR and miR-208 (d), ADAMTSL2 3´ UTR and miR-125a (e), MAP3K11 3´ UTR and miR-125a (f).

3.2.7. miR-99a and miR-100 repress expression of bovine tribbles homologue 2 (TRB2)

3.2.7.1. TRB2 is a predicted target for miR-99a and miR-100

miR-99a and miR-100 are family of miRNAs that differ in a single nucleotide and are predicted to target bovine tribbles homologue 2 (TRB2). miR-99a is observed to be differentially regulated at GV stage oocyte as compared to MII stage oocyte while miR-100 is differentially expressed in companion cumulus cells at both immature and matured stages. Exceptionally, TRB2 is predicted to be targeted by only four microRNAs (miR-99a, miR-100, miR-487a and miR-350) (Figure 3.13). Among these four miRNAs miR-99a, miR-100 and miR-487a are identified in bovine, where as, miR-350 is discovered only in *Mus musculus*. Experimental evidences showed that Bovine tribbles homologue 2 is a widely conserved gene evidenced to regulate cell cycle negatively. From this preliminary information, we hypothesised that miR-99a and miR-100 might involve in the promotion of cell cycle by repressing TRB2 protein which interact and negatively regulate CDC25C, a phosphatase protein that dephosphorylate maturation promoting factor and regulate G2/M transition.

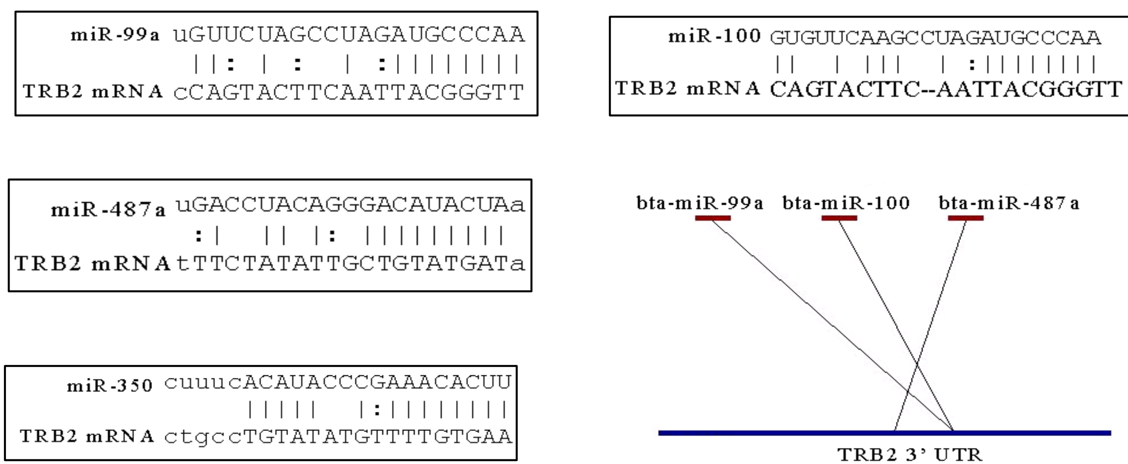

Figure 3.13: Bioinformatic prediction of miR-99a, miR-100, miR-487a and miR-350 binding sites on the 3'UTR of Bovine tribbles homologue 2 (TRB2).

3.2.7.2. miR-99a and miR-100 have markedly higher expression in cumulus cells

The expression profile of miR-99a, miR-100, miR-387a and miR-350 was conducted in immature and matured oocytes and their corresponding cumulus cells using qRT- PCR. Quantification of miR-99a, miR-100 and miR-387a was performed using primers designed for the quantification of their human orthologues. However, quantification of miR-350 in bovine cumulus cells and oocyte samples using the human orthologue primer showed unspecific amplification and removed from the analysis.

Interestingly, miR-99a and miR-100 which are family of microRNAs showed a markedly higher expression in cumulus cells as compared to oocytes at both immature and matured stages. However, miR-487a revealed a higher expression at GV stage oocyte as compared to the matured one (Figure 3.14).

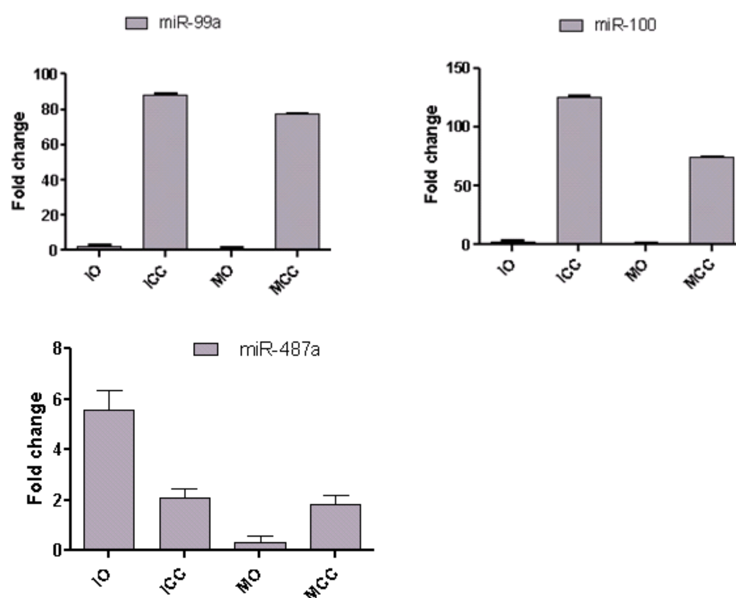

Figure 3.14: The expression profile of miR-99a, miR-100 and miR-487a in bovine oocytes and their companion cumulus cells at immature and matured stages. The analysis was conducted using qRT-PCR and the data was normalized with the mean value of three internal controls (U6, SNORD 44 and SNORD 48). (IO = Immature oocyte, MO = Matured oocyte, ICC = Immature cumulus cells, MCC = matured cumulus cells).

3.2.7.3. Inverse expression pattern of TRB2 with targeting microRNAs

We analysed the expression pattern of tribbles homologue 2 (TRB2) mRNA in oocytes and corresponding cumulus cells using qRT-PCR and semi-quantitative PCR. GAPDH was used as an internal control. We observed a striking inverse expression pattern between TRB2 mRNA expression and the expression of miR-99a and miR-100. We quantified the expression level of both the targeting miRNA and target genes from the same sample. The expression analysis of TRB2 using qRT-PCR revealed markedly higher expression level in oocytes as compared to their companion cumulus cells both at GV and MII stages and this is the exact opposite pattern with the targeting miRNAs (miR-99a and miR-100) (Figure 3.15a). The semi-quantitative PCR analysis has confirmed the fact that TRB2 mRNA is expressed in much higher level in oocytes as compared to the cumulus cells (Figure 3.15b). TRB2 showed markedly lower band intensity in cumulus cells as compared to the oocytes. Here, we are confident that the expression data quality is not compromised by comparing oocytes and somatic cells as the internal control (GAPDH) showed a stable expression with markedly variable expression of TRB2. Besides, the mRNA of TRB2 showed a comparable expression level in immature and matured oocyte where as a much higher expression in immature cumulus cells as compared to the matured one.

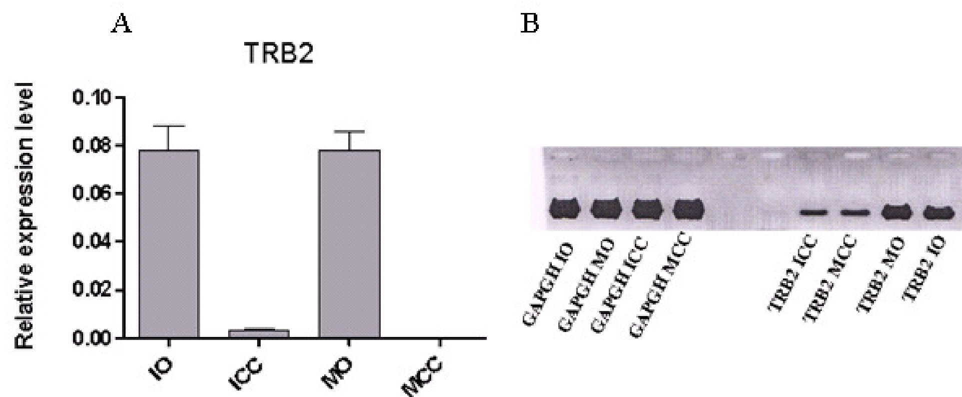

Figure 3.15: The mRNA expression profile of Tribbles homologue 2 (TRB2) in bovine oocytes and their companion cumulus cells. The expression analysis was conducted using qRT-PCR (A) and semi-quantitative PCR(B). (IO = Immature oocyte, MO = Matured oocyte, ICC = Immature cumulus cells, MCC = matured cumulus cells)

3.2.7.4. miR-99a and miR-100 directly target the 3'- UTR of TRB2

To confirm that miR-99a and miR-100 directly bind and repress the expression of TRB2 transcript, we used a luciferase reporter assay system. We cloned the binding site of miR-99a and 100 down stream of the luciferase reporter gene into the pmirGLO vector. After we checked the cloning of the right sequence in the correct orientation by sequencing, we conducted transfection into cumulus cells in four treatment groups namely, cells transfected with pmirGLO vector, cells transfected with pmiRGLO vector construct containing miR-99a and 100 target site, cells co-transfected with construct vector and mimic, cells co-transfected with mismatch construct vector and mimic. Exogenous miR- 99a resulted in 48 % luciferase activity reduction as compared to those cells transfected with construct vector. Overexpression of miR-99a in the negative control (three nucleotide mismatches in the seed region) showed a slight reduction of luciferase activity but not statistically different from the positive control group (Figure 3.16a).

The direct interaction between miR-100 and TRB2 3' UTR and subsequent effect of translation repression was assessed using same procedure and treatment groups as it was used for miR-99a (Figure 16b). Co-transfection of miR-100 mimic and pmirGLO vector cloned with miR-100 target site into cumulus cells revealed a pronounced luciferase activity reduction (73%). The precision and conclusiveness of the result has been supported by slight reduction of the miR-100 mimic in the negative control group. Moreover, miR-487a is one of the three bovine miRNAs that target bovine tribbles homologue 2 (TRB2) and target validation using luciferase reporter assay. The result from the luciferase assay experiment showed that miR-487a showed a modest luciferase activity reduction (29%) indicating minimal role of this miRNA in the regulation of the target TRB2 gene (Figure 3.16c). Hence, these data show that miR-99a and miR-100 directly target the 3' UTR of TRB2 mRNA.

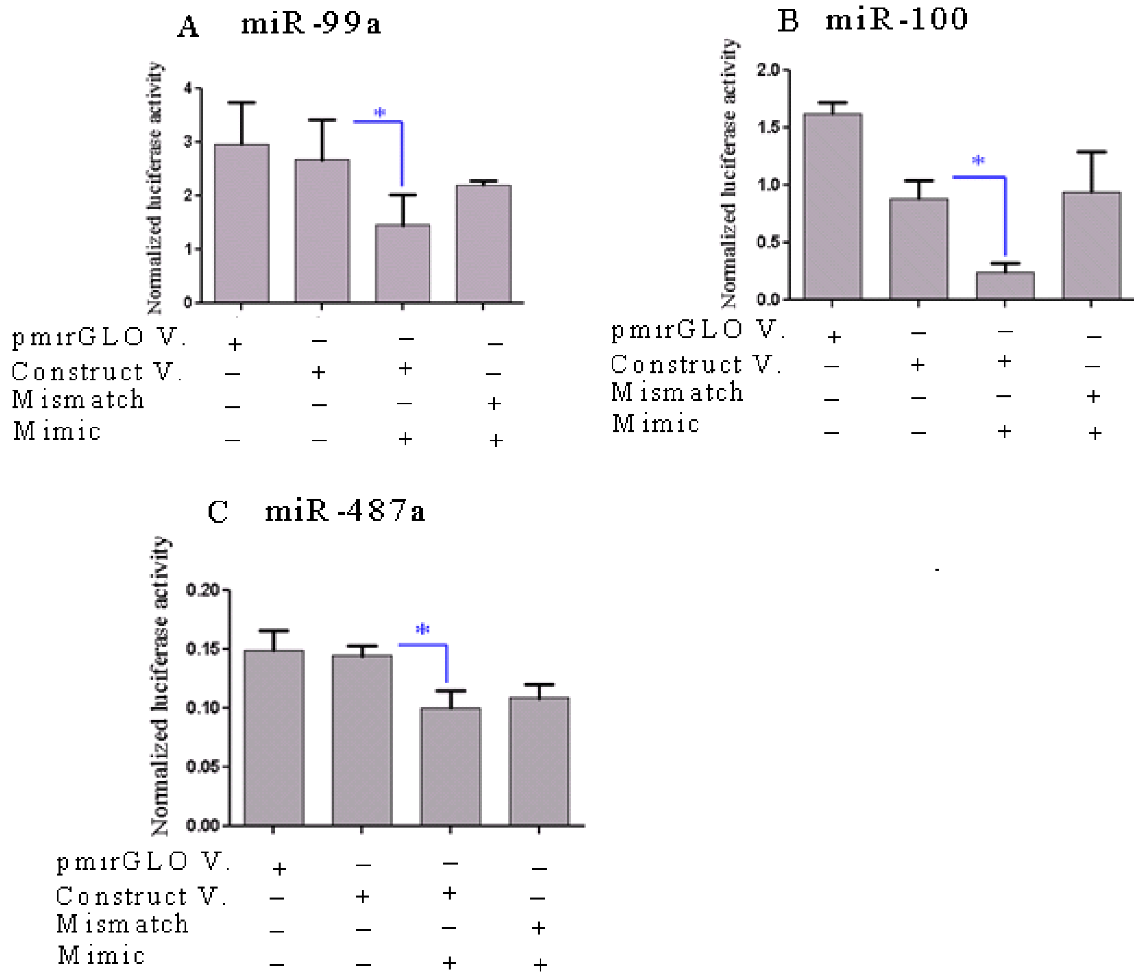

Figure 3.16: Validation of miRNA-target interaction using luciferase reporter assay. The mean firefly / renilla ratio observed in four treatment groups (pmirGLO vector transfected, construct vector transfected, construct vector + mimic transfected and mismatch vector + mimic) showing a marked reduction in firefly/renilla ratio in miR-99a mimic co-transfected group (A). A pronounced firefly/renilla ratio was also observed in miR-100 and construct vector co-transfected group (B). A modest reduction of luciferase activity was observed in miR-487a mimic and pmirGLO construct vector co-transfected group (C). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them.

3.3. Part I: Discussion

3.3.1. Identification of new miRNAs using heterologous approach

The use of a heterologous microRNA microarray platform (human, mouse and rat) to analyse the expression profile of bovine microRNAs during invitro oocyte maturation enabled us to detect 400 miRNAs out of 454 probes. This reaffirms the fact that most microRNA sequences are conserved across related animal species (Griffiths-Jones et al. 2006, Niwa and Slack 2007) and assures the validity of the approach we used for miRNA identification and expression analysis. Some miRNAs differ from each other by only a single nucleotide which imposes ambiguity on the accurate detection and expression profiling. To circumvent this problem we used locked nucleic acid (LNA)-modified capture probes designed in such a way that it could produce uniform, high-affinity hybridizations, yielding highly accurate signals able to discriminate between single nucleotide differences and, hence, between closely related miRNA family members (Castoldi et al. 2006). We, therefore, exploited the strong mismatch discriminatory feature of the LNA capture probes on one side (Castoldi et al. 2006) and the cross species conservation of miRNAs (Bentwich et al. 2005) on the other to asses the expression profile as well as identify those miRNAs differentially expressed during bovine oocyte maturation. The nomenclature of miRNAs also takes the cross species conservation features into account and orthologous miRNAs only differ in the first three letters indicating the same microRNA sequence but found in different species (Griffiths-Jones et al. 2006). Hence, it appears reasonable to deduce those miRNAs differentially expressed in the course of oocyte maturation and not known before in bovine are essentially new miRNAs.

3.3.2. Expression analysis of miRNAs during oocyte maturation, fully grown and growing oocytes and pre-implantation embryos

The results of this study showed a differential miRNA expression pattern during bovine oocyte maturation which is in agreement with the report by Tang et al. (2007) where they showed a dynamic change of miRNA during mouse oogenesis. With this regard, our data indicated that bovine oocyte maturation involves a wave of expression of some classes of miRNAs suggesting their possible role during oocyte maturation. The qRT-

PCR for miR-200c, miR-145, miR-127, miR-25, miR-125a, miR-130b, miR-208 and miR-206 revealed a similar expression pattern with the microarray result for the same microRNAs but a bit different magnitude of expression which can be explained by differential sensitivity of the two techniques.

The findings of the qRT-PCR analysis for five microRNAs (miR-125a, miR-25, miR-127, miR-208 and miR-145) across pre-implantation embryo developmental stages (oocyte, 2-cell, 4-cell, 8-cell, 16-cell, morula and blastocyst) suggests the possible role of microRNAs in bovine pre-implantation embryo development. The expression profile of miR-125a showed an increase in 4-cell and 8-cell followed by a decline during morula and blastocyst. This tends to be different from the findings observed in mice (Byrne and Warner 2008) where, miR-125a expression increases during pre-implantation embryo development. The progressive decrease in the expression of miR-25 from oocyte to 8-cell and subsequent increase from 8-cell to blastocyst, that we observed, is in agreement with the findings of Tang et al. (2007) in which they showed the down regulation of total amount of microRNAs by 60% between the one cell and two cell stages of mouse embryonic development. The increased expression level of miR-127 and miR-145 at 8-cell stage with increasing pre-8-cell stages and decreasing post 8-cell stages during pre-implantation embryo development might indicate their maternal origin and their potential involvement in maternal transcript turn over and maternal-to-zygotic transition as it was observed for miR-430 in zebra fish (Giraldez et al. 2006).

The differential expression of miRNAs between immature and matured oocytes may indicate their natural turnover or their involvement in the process of oocyte maturation. In order to investigate the expression of some candidate miRNAs during oocyte growth, we used independent model involving the use of brilliant cresyl blue (BCB). Previous findings demonstrated that brilliant cresyl blue (BCB) can be used for the selection of competent oocytes of cattle (Alm et al. 2005, Bhojwani et al. 2007, Pujol et al. 2004). Our data on the expression profile of four microRNAs (miR-125a, miR-127, miR-145 and miR-208) in growing (BCB-) and fully grown (BCB+) immature oocytes and corresponding cumulus cells showed increased expression of all microRNAs in BCB- as compared to BCB+ ones. This is in accordance with their expression pattern between immature and matured oocytes except for miR-145. This suggests that the increased

expression of these microRNAs might correlate with decreased rate of mRNA and protein accumulation as microRNAs could degrade their target mRNA or inhibit translation.

Stable over expression of miR-200c in A549 cells leads to reduced expression of transcription factor 8 and increased expression of E-cadhrin (Hurteau et al. 2007) indicating transcription factor 8 to be a target for miR-200c. Our result revealed the preferential expression of miR-200c in immature oocyte as compared to matured ones which is in agreement with the notion that during mouse preimplantation development, the components of the E-cadherin-catenin complex are derived from both maternal and zygotic gene activity and increasingly accumulated in a non-functional form ready to be used for compaction and the formation of trophectoderm cell layer (Ohsugi et al. 1996). Hence, increased expression of miR-200c in immature oocytes in this study may have led to decreased expression of transcription factor 8 and subsequent accumulation of E-cadherin at germinal vesicle stage in non-functional form.

We also observed that miR-145 showed one of the most altered expressions during oocyte maturation with matured stage preferential abundance. It was confirmed that miR-145 targets the insulin receptor substrate-1 (Shi et al. 2007) known to send unambiguous mitogenic, anti-apoptotic and anti-differentiation signal when activated by insulin like growth factor receptor (White 1998). The elevated expression level of miR-145 in immature oocytes in our study might be to regulate the expression of insulin receptor substrate-1 during oocyte maturation.

Recent reports show that microRNAs may be associated with fragile sites and cancer associated genomic regions. Huppi et al. (2008) reported the identification of miRNAs in genomically unstable region of human chromosome 8q24, while, Sevignani et al. (2007) showed significant association between the chromosomal location of microRNAs and those of mouse cancer susceptibility loci that influence the development of solid tumors.

The role of miRNAs as posttranscriptional regulators is well documented (Ambros 2004, Bagga et al. 2005, Bartel 2004). Many studies suggested that posttranscriptional down regulation of mRNAs by partially complementary miRNAs was entirely

attributed to decreased translation (Lee et al. 1993, Moss et al. 1997) but other studies have shown these miRNAs reduce cellular concentration of their mRNA targets (Bagga et al. 2005, Wu et al. 2006). This shows that microRNA expression and their corresponding targets abundance could be an inversely related cellular phenomenon and measuring the expression profile of the microRNA and the predicted target in the same sample could give an additional evidence for the miRNA target relation. The differential expression of miRNAs in immature or matured stage may fine tune their corresponding target genes in which their expression need to be maintained at a certain threshold level.

The quantitative RT-PCR results showed a reciprocal expression pattern between the miRNA and the targets in bovine oocytes which is in agreement with (Tian et al. 2008) that showed reciprocal expression of a microRNA and a predicted target within a physiological context and suggested the presence and relevance of a microRNA–target pair. Moreover, alignment of the 3´ UTR of the target gene and the targeting microRNA revealed base complementarity especially between the miRNA seed region and the target mRNA (Figure 3.12) which may suggest the regulatory mechanism of the miRNA on the relative abundance of the target mRNA. Schulman et al. (2005) also reported reciprocal expression of lin-41 and the microRNAs let-7 and miR-125 during mouse embryogenesis. Despite the fact that the reciprocal expression of miRNAs and predicted targets imply the microRNA-target pair, we couldn’t rule out the involvement of other miRNAs in expression regulation of the target as multiple miRNAs could have binding sites on single target (Kuzin et al. 2007).

Su et al. (2007) showed the selective destruction of transcripts during the maturation of mouse oocyte (GV to MII transition) in which transcripts involved in processes that are associated with meiotic arrest at the GV stage and the progression of oocyte maturation such as oxidative phosphorylation, energy production and protein synthesis and metabolism were dramatically degraded while transcripts involved in protein kinase pathway were the most prominent among the stable transcripts. miR-430 accelerates the clearance of several hundred target messenger RNAs suggesting this miRNA facilitates the deadenylation and clearance of maternal mRNAs during early zebra fish embryogenesis (Giraldez et al. 2006). Hence, it is plausible to suggest the up regulation of some miRNAs in matured oocytes might correlate with the maternal transcript turnover to effect the maternal-zygotic transition as it was observed in other species.

3.3.3. Potential role of miR-99a and miR-100 in bovine cumulus cells proliferation

With the intention of shift from expression profiling to investigation of specific microRNA functions in cumulus oocyte complex, we selected miR-99a and miR-100 that belong to miR-99 family. The bioinformatic prediction of target genes for these microRNAs revealed that a range of genes with extremely diverse role could be affected by miR-99 family. Of special interest, bta-miR-99a and bta-miR-100 target tribbles homolog 2 (TRB2) and only bta-miR-487a was also shown to co-target TRB2. Hence, our data on these three miRNAs (bta-miR-99a, bta-miR-100 and bta-miR-487a) and TRB2 3' UTR interaction is more informative since the confounding effect as a result of too many microRNA targeting a single transcript is avoided.

An obvious hurdle to the understanding of microRNA function is identifying the target genes they regulate. Experiments indicate that animal miRNAs generally display only limited pairing to their targets and seven contiguous base pairs between the target 3' UTR and miRNA 'seed' region is supposed to be suffice for regulation (Brennecke et al. 2005, Doench and Sharp 2004, Lai et al. 2005). The contiguous base pairs between the TRB2 UTR and the 5' end of miR-99a and miR-100, substantial base pairing at the 3' end and conservation across multiple species (Lewis et al. 2005, Xie et al. 2005) suggest the regulatory importance of these microRNAs.

TRB2 and targeting microRNAs (bta-miR-99a and bta-miR-100) showed a striking spatial shift in expression in germ cell (oocyte) and companion cumulus cells in exact reciprocal manner. This consolidates the rationale that animal microRNA could reduce the expression of their target mRNA when they appear in the same cells or tissue (Lim et al. 2005). It seems also apparent that the higher expression of miR-99a and miR-100 in oocyte tend to keep lower expression of TRB2 and this might help the oocyte to trigger the G2/M transition and subsequently mature. On the contrary, markedly higher expression of miR-99a and miR-100 and the lower expression of TRB2 in cumulus cells support the idea that cumulus cell proliferation is promoted (Mata et al. 2000, Seher and Leptin 2000) during oocyte maturation. This might be attained by deregulating the cell cycle negative regulator TRB2 protein through miR-99a and miR-100 mediated posttranscriptional regulatory process.

Next, we wanted to confirm if bovine tribbles homologue 2 (TRB2) is a physiological target for miR-99a, miR-100 and miR-487a and subsequently used the luciferase assay technique. As miRNAs constitute a prominent class of regulatory genes in animals, posttranscriptional regulatory mechanisms that are mediated by these small RNAs are ubiquitous (Lagos-Quintana et al. 2001, Lewis et al. 2005). For this purpose, we co-transfected invitro cultured cumulus cells with target site cloned pmiRGLO vector and microRNA mimic. microRNA mimic is small, chemically modified double-stranded RNA molecule that mimic endogenous mature miRNA molecule which is incorporated into RNA-induced silencing complex up on transfection into the cells. The introduction of miR-99a and miR-100 into cumulus cells resulted in 48% and 73% luciferase reporter protein reduction, respectively, suggesting that these miRNAs are potent regulators of TRB2. The mild repressive ability observed for miR-487a confirms the fact that miRNA vary in the extent of their target genes regulation which is attributed to the variation of the molecular architecher in the 3' UTR (Brennecke et al. 2005).

Taken together, this data suggests that miR-99a and miR-100 may promote bovine cumulus cells proliferation by down regulating the negative regulator of cell cycle, bovine tribbles homologue 2 (TRB2).

4. Part II: MicroRNA regulated noble molecular signature underlying bovine subclinical endometritis

In vivo and in vitro animal model studies have shown the need for regulation of specific uterine genes both at transcriptional and translational levels (Achache and Revel 2006, Horcajadas et al. 2007, Makker and Singh 2006). MicroRNAs (~ 22nt length) have emerged as key regulators of posttranscriptional gene expression by interacting with the 3' UTR of target mRNAs and subsequently influencing the translation or stability of transcripts. Animal miRNAs are known to regulate diverse biological functions and evidenced to have central roles in the development of some diseases and biological disorders where their aberrant expression has been associated with establishment and progression of various disorders in mammalian uterine (Pan and Chegini 2008).

In cattle, a significantly higher expression of L-PGDS, IL-1α and IL-1-RN has been reported in the endometrium of cows with subclinical or clinical endometritis compared with healthy. Besides, a two fold lower cPGES mRNA expression was observed in subclinical endometritis as compared to the healthy ones (Gabler et al. 2009). Differential expression of 48 miRNAs in the human endometrium has been reported suggesting the importance of endometrial microenvironment on the regulation of microRNAs (Pan et al. 2007). Furthermore, miR-21 was confirmed to target Reck gene suggesting its key role during embryo implantation (Hu et al. 2008). Qian et al. (2009) showed the involvement of hsa-miR-222 in differentiation of endometrial stromal cells in to decidual cells invitro. Abnormalities of decidualization in human result in many pregnancy disorders such as recurrent miscarriages and infertility (Gellersen and Brosens 2003).

Despite the fact that experiments evidenced the negative impact of subclinical endometritis on dairy cattle fertility (LeBlanc et al. 2002; Gilbert et al. 2005), our knowledge regarding molecular mechanism underlying bovine subclinical endometritis was significantly limited. Therefore in this experiment, we aimed to investigate the underlying molecular mechanism potentially affected by subclinical endometritis from microRNA perspectives. Thus, we hypothesized that aberrant expression of miRNAs and dysregulation of their respective molecular networks and pathways could be triggered by subclinical endometritis. Hence, we identified aberrantly expressed

miRNAs in uterine cytobrush samples taken from cows with subclinical endometritis as compared to the healthy ones. Then after, we screened high ranking target genes for the 23 miRNAs from miRBase and used Ingenuity Pathway Analysis (IPA) to figure out the molecular networks, biological functions and canonical pathways that these miRNAs potentially regulate. Further more, to substantiate the miRNA target gene interaction beyond bioinformatic prediction, we validated some miRNAs and key target genes that control top networks using luciferase assay technique.

4. 1. Part II: Materials and methods

4.1.1. Materials

4.1.1.1. Laboratory equipments used during the study

Equipment	Manufacturer
Laminar flow chamber	Heraeus, Geramny
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
ABI PRISM® 7000 SDS	Applied Bio systems
Millipore apparatus	Millipore Corporation, USA
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Inverted fluorescence microscope DM IRB	Leica, Germany
Stereomicroscope SMZ 645	Nikon, Japan
Centrifuge	Hermel, Wehing
Electrophoresis chamber	BoRad, Munich
My Cycler Thermal cycler	Bio-RadLaboratories, CA, USA
SHKE6000-8CE refrigerated Stackable Shaker	Thermoscinentific, IWA, USA
CEQ™ 8000 Genetic Analysis	BeckmanCoulter,Krefeld, Germany
Ultra low freezer (-80 °C)	Labotect GmbH, Göttingen, Germany
Centrifuges (small, medium, large)	Heraeus, Hanau, Germany
MAXQ6000 shacking incubator	Thermo scientific, Germany
Carbon dioxide incubator (MCO-17AI)	Sanyo, Japan
Nanodrop 8000 Spectrophotometer	Thermo Fisher Scientific, DE, USA
Axon GenePix 4000B scanner	Axon Instruments, Foster City, CA
Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR	STARLAB GmbH (Ahrensburg)

4.1.1.2. List of chemicals, competent cells and kits

Chemicals and competent cells	Manufacturer/Supplier
pmirGLO Dual-Luciferase miRNA target expression vector	Promega, Madison, Wisconsin, USA
Attractene transfection reagent	QIAGEN Gmbh, Hilden, Germany
Restriction endonuclease	New England Biolabs, Inc., U.S.A
Hemacolor staining set	Merck, Darmstadt, Germany
DNase I	Qiagen, Hilden, Germany
Cytobrush	Medscand Inc., Malmö, Sweden
10x PCR buffer	Promega, WI, USA
2x rapid ligation buffer	Promega, WI, USA
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
E. coli competent cells	Stratagene, Amsterdam, The Netherlands
5x First-Stand buffer	Invitrogen Life Technologies, Karlsruhe
Acetic acid	Roth, Karlsruhe, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich
Ampicillin	Roth , Karlsruhe
BME (essential amino acids)	Gibco BRL, life technologies, Karlsruhe
Boric acid	Roth , Karlsruhe, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, Germany
Calcium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany

Chloroform	Roth , Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Roth , Karlsruhe, Germany
dNTPs	Roth , Karlsruhe, Germany
DTT	Invitrogen Life Technologies, Karlsruhe, Germany
Dye terminator cycle sequencing (DTCS)	Beckman Coulter, Krefeld, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Ethylenediaminetetra acetic acid	Roth , Karlsruhe, Germany
Formaldehyde	Sigma-Aldrich Chemie GmbH, Munich, Germany
QuantiTect SYBR Green PCR Buffer	QIAGEN, Austin, USA
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
Hemi-calcium lactate	Promega, WI, USA
Hydrochloric acid	Roth, Karlsruhe
Hepes	Sigma-Aldrich Chemie GmbH, Munich
Hydroxylamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
dNTP mix, including dUTP	QIAGEN, Austin, USA
SYBR Green I	QIAGEN, Austin, USA
Igepal	Roth, Karlsruhe, Germany
ROX	QIAGEN, Austin, USA
L-Glutamine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
MEM (non essential amino acids)	Gico BRL, life technologies, Karlsruhe

Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
Oligonucleotide primers	MWG Biotech, Eberberg, Germany
Pepton	Roth , Karlsruhe, Germany
Penicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen
5x miRNA RT Buffer	SABiosciences, Frederick, MD, USA
Phenol red solution (5% in D-PBS)	Sigma-Aldrich Chemie GmbH , Munich
Potassium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany
Random primer	Promega, WI, USA
2x RT^2 SYBR Green PCR master mix	SABiosciences, Frederick, MD, USA
RNA later	Sigma-Aldrich, MI, USA
RQ1 RNase-free DNase	Promega, WI, USA
RNasin	Promega, WI, USA
Sample loading solution (SLS)	Beckman Coulter, Krefeld, Germany
Sequagel XR Sequencing Gel	Beckman Coulter, Krefeld, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium chloride	Roth , Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Inc, MO, USA
Sodium hydrogen sulphate	Sigma-Aldrich Inc, MO, USA
Sodium lactate solution (60%)	Sigma-Aldrich Inc, MO, USA
Sodium pyruvate	Sigma-Aldrich Inc, MO, USA
Streptomycin sulphate	Sigma-Aldrich Inc, MO, USA
Superscript II reverse transcriptase	Invitrogen, CA, USA
miRNA RT Primer & ERC Mix	SABiosciences, Frederick, MD, USA

T4 DNA ligase	Promega, WI, USA
Taq DNA polymerase	Sigma-Aldrich Inc, MO, USA
Tris	Roth ,Karlsruhe, Germany
Triton X-100	Roche Diagnostics GmbH, Mannheim, Germany
Yeast extract	Roth, Karlsruhe, Germany
QIAzol lysis reagent	Qiagen, Hilden, Germany
Buffer RWT	Qiagen, Hilden, Germany
Buffer RPE	Qiagen, Hilden, Germany
miRNeasy mini kit	Qiagen, Hiden, Germany
RT^2 miRNA first Strand kit	SABiosciences, Frederick, MD, USA
Genome wide RT^2 miRNA PCR array kit	SABiosciences, Frederick, MD, USA
miScript SYBR Green PCR Kit	QIAGEN, Austin, USA
miScript Reverse Transcription Kit	QIAGEN, Austin, USA
GenElute™ Plasmid Miniprep Kit	Sigma–Aldrich Chemie GmbH, Munich, Germany
Dual – Luciferase Reporter Assay kit	Promega, WI, USA

4.1.1.3. Media and solutions

LB-agar	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
Dulbecco's Modified Eagle Medium (D-MEM)	DMEM 1x	425 ml
	Sodium pyruvate	5 ml
	MEM	5 ml
	L-glutamine	5 ml
	Penicillin- Streptomycin	5 ml
	Amphotericin	5 ml
	Merkaptoethanol	5 µl
BSA (3%)	Bovine serum albumin	0.15 g
	added to PBS+PVA	5 ml
DEPC-treated water (1000 ml)	DEPC	1 ml
	added to water	1000 ml
Lysis buffer (100 µl)	Igepal (0.8%)	0.8 µl
	RNasin	5 µl
	DTT	5 µl
	added to water	100 µl
TAE (50x) buffer, pH 8.0	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml
	ddH_2O added to	1000.0 ml
TE (1x) buffer	Tris (1 M)	10.0 ml

	EDTA (0.5 M)	2.0 ml
X-gal	ddH_2O added to	1000.0 ml
	X-gal	50.0 mg
(16%) Para formaldehyde (10 ml)	N, N'-dimethylformamide	1.0 ml
	Para formaldehyde	1.6 g
	added to water	10 ml
PBS + PVA (50 ml)	Polyvinyl alcohol (PVA)	300 mg
	PBS added to	50 ml
Permeabilizing solution (10 ml)	Triton X-100	5 µl
	Glycine + PBS added	10 ml
Physiological saline solution	Sodium chloride	9 g
	added to water	1000 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
dNTP solution	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH_2O added to	400.0 µl
IPTG solution	IPTG	1.2 g
	ddH_2O added to	10.0 µl
3M Sodium Acetate, pH 5.2	Sodium Acetate	123.1 g
	ddH_2O added to	500 ml
1M EDTA, pH 8.0	EDTA	37.3 g
	ddH_2O added to	1000 ml
Phenol Chloroform	Phenol : Chloroform	1 : 1 (v/v)
0.2% Triton-X100:	Triton	2 ml
	10x PBS : added to	1,000.0ml
0.3% BSA in PBS	BSA	3 g
	10x PBS : added to	1,000.0 ml
3% BSA in PBS	BSA	30g

10x PBS : added to 1,000.0 ml

4.1.1.4. Data bases and softwares used during the study

Soft wares and data bases	Source of soft wares and data bases
miRBase v.14	http//microrna.sanger.ac.uk/
Entrez Gene	http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
EndNote X1	Thomoson
Primer Express ® software	Applied Biosystems, Foster city, CA, USA
BLAST program	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Prism for windows (ver.5.0)	Graph Pad software, Inc.
NCBI	http://www.ncbi.nlm.nih.gov
Weight to molar quantity converter	http://www.molbiol.ru/eng/scripts/01_07.html
miTarget	http://cbit.snu.ac.kr/~miTarget/
Manupulate and display DNA sequence	http://www.vivo.colostate.edu/molkit/manip/
Alibee - multiple alignment	http://www.genebee.msu.su/services/malign_reduced.html
ENSEMBL	http://www.ensembl.org).

4.1.2. Methods

4.1.2.1. Endometrial cytobrush samples collection and cytology

For the investigation of the miRNA expression in the endometrium of cows with subclinical endometritis and healthy, endometrial cytobrush samples were collected from lactating cows using the cytobrush technique at day 7 of estrous cycle. Cytobrush samples were collected from cows at the second parity and 60-80 days postpartum. Briefly, a brush (Gynobrush, Heinz Herenz, Hamburg, and Germany) with 20 mm length and 6 mm diameter, screwed on a 70-cm long rod, protected by a metallic catheter was inserted via the cervix into the uterine body. Cells were collected by rotating the cytobrush in a clockwise direction while in contact with the uterine wall. Here, two cytobrush samples were collected from each animal: for cytological analysis and miRNA expression study. Cytological analysis was done by rolling the cell-containing cytobrush on a clean glass microscope slide. The slides were immediately fixed on farm and stained (LT-SYS®, Labor und Technik, Berlin, Germany) in the laboratory. A total of 300 cells were counted under a microscope (× 400 magnifications) to determine the proportion of PMN. On the basis of PMN proportion in the endometrial samples, cows were categorized in to two groups. Healthy group included 0 % PMN where as cows with subclinical endometritis included ≥ 5% PMN count as described by Gilbert et al.(2005). The other cytobrush sample for each cow was immediately transferred into reaction tubes filled with 500 µl RNA later (Sigma, Deisenhofen, Germany) and transported to the laboratory. Finally, samples were stored at -20 °C until miRNA extraction.

4.1.2.2. Total RNA isolation

Total RNA containing microRNAs was isolated from uterine cytobrush samples from healthy (n = 6) and cows with sub-clinical (n = 6) using miRNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol. Briefly, cytobrush samples were thawed at room temperature and the RNA later was removed from the cells. Then after, cells were lysed by adding 700 µl QIAzol lysis reagent. After adding 140 µl chloroform samples were thoroughly mixed and centrifuged for 15 min at 12000xg at 4°C. The upper aqueous phase was collected to a new collection tube and 525 µl ethanol was added. Total RNA containing microRNA was bound on column by centrifuging at ≥ 8000xg for 15 sec. at room temperature. On-column DNA digestion was subsequently done using DNAse I (Qiagen, Hilden, Germany) to remove any DNA contamination. After subsequent washing with washing buffers, total RNA was eluted with 30 µl elution buffer.

4.1.2.3. First strand cDNA synthesis and quantitative PCR (qPCR) assay

From the total RNA, fractionation of small RNAs and large RNA was done using RT^2 qPCR-Grade miRNA isolation kit (SABiosciences, Frederick, MD). In all RNA samples, the fractionated small miRNAs and large RNAs were checked for their concentration and integrity (based on 260/280 nm measurement) using Nanodrop 8000 instrument (peqLab Biotechnologies GmbH, Erlangen, Germany). Equal amount of RNAs from both healthy (PMN = 0) and cows with subclinical endometritis (PMN ≥ 5) groups were used for first strand cDNA synthesis using RT^2 miRNA first Strand kit (SABiosciences, Frederick, MD) following manufacturer's protocol. Briefly, 50 ng of small RNA was incubated with a mix containing 2 µl of 5x miRNA RT buffer, 1 µl miRNA RT primer, 1 µl DTT (100 mM) and miRNA enzyme mix in a 10 µl reaction volume. Reaction incubation was done at 37 °C for 2 hrs followed by heating at 95 °C for 5 min to inactivate the reverse transcriptase. The resulting cDNA samples were diluted before use as template for miRNA qPCR assay.

Expression profiling of miRNAs in endometrial cytobrush samples from healthy and subclinical dairy cows was conducted using 96-well Genome wide RT^2 miRNA PCR array (SABioscience, Frederic, MD) following manufacturers protocol. The 96-well

whole Genome RT^2 miRNA qPCR array is designed to quantify a total of 352 well characterized human microRNAs in four different sets of 96-well plates each containing 88 miRNAs, four housekeeping small RNAs (SNORD44, SNORD47, SNORD48 and U6) and RT negative controls. Prior to real time PCR profiling, 90 µl DNase/RNase-free water was added to each of the 10 µl first strand cDNA product from each biological replicates of endometrial cytobrush samples from healthy and sub-clinical dairy cows. A PCR master mix was prepared using the 100 µl diluted cDNA template, 1275 µl 2x RT^2 SYBR Green PCR master mix and 1175 µl DNase/RNase-free water. Twenty four micro liter of this mix was distributed to each well of the 96-well plate containing sequence specific miRNA primer sets and the respective controls. Following brief centrifugation, the plate was loaded onto ABI prism 7000 real time PCR apparatus and run with a thermal program of initial heating at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The specificity of amplification was controlled using a melting curve generated at the end of the PCR protocol.

The expression of microRNAs in clinical and healthy cytobrush samples was then analysed using ΔΔCT method from the PCR array data analysis web portal (http//www.sabiosciences.com/pcrarraydataanalysis.php). A 96-well template was used for each four set separately and the final result has been combined.

Prior to miRNA and their corresponding target interaction validation, we ascertained the expression of those target genes (MAPK14, NOS2, JUN, IKBKB, INSR, SP1, ID3) in bovine endometrium samples collected across estrous cycle using semi quantitative RT-PCR. Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) was quantified from the same sample and used as a reference gene (Table 6).

4.1.2.4. Ingenuity path way analysis (IPA)

With the aim of tracing the molecular networks and biological functions that the aberrantly expressed microRNAs involve, we filtered the miRBase microcosm predicted target genes according to their score, free energy and evolutionary conservation. The high ranking target genes with known gene identifiers (Known symbols) were uploaded into the web-based pathways analysis tool IPA (Ingenuity Systems, www. Ingenuity.com) to identify molecular networks and biological functions underlying bovine endometritis as described previously (Li and Capuco 2008). This web-based entry tools allows for the mapping of gene expression data into relevant pathways based on their functional annotation and known molecular interactions. The knowledge coming from published, peer reviewed scientific publications is stored in the Ingenuity Pathway Knowledge Base (IPKB), and is continuously updated. A molecular network of direct or indirect physical, transcriptional and enzymatic interactions between mammalian orthologs was computed from the knowledge base. High ranking target genes for the differentially regulated microRNAs were imported and the system compares the list of genes with IPKB and produce relevant networks, biological functions and canonical pathways as out put.

Each gene identifier was mapped to its corresponding gene object in the Ingenuity Path Way Knowledge Base. Networks of focus genes were then algorithmically generated based on their connectivity and assign a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input data set and it takes into account the number of focus genes in the network and the size of the network to approximate the relevance of the network to the original list of focus genes.

A functional analysis of a network then identified the biological functions and/or diseases that were most significant to the genes in the network. Canonical pathways were identified from the IPA library of canonical pathways based on two parameters: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map the canonical path way and (2) a P value calculated using Fisher's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

Table 6: List of primers with their sequences and annealing temperature that were used for semi-quantitative PCR analysis of target genes.

Gene name	Sequence	Annealing temperature (°C)
MAPK14	F-GTGGTACAGGGCTCCTGAGATC	TD: 60-56
	R- TGGCTTGGCATCCTGTTAATG	
NOS2	F-CAACAACGGCAACATCAGGTC	TD: 58-56
	R- CTAGAGGCAACACGTCGAAGC	
JUN	F-AGAGGAAGCGCATGAGGAAC	63
	R-CTGCGTTAGCATGAGTTGGC	
IKBKB	F- AGTGCGAGTGATTTATACGCAGC	57
	R-GTTCATACTGTCCGGGCTTCC	
INSR	F-GCCTGCGTCACTTTACTGGC	TD: 57-55
	R-CAATCAGACCGTTGGGTTCC	
SP1	F-ACACCTACGGGCACACTTGC	TD: 57-55
	R-TTATTCTGGTGGGTCTTGATAATGC	
ID3	F-TGACTTCCCCAAACCCCTG	TD: 56-54
	R- ACCCCGTACACCTCCACACA	

TD = touchdown PCR

4.1.2.5. Plasmid construction and transient transfection

After identifying the aberrantly expressed miRNAs, we used their respective bioinformatically predicted high ranking targets for the IPA analysis. Bioinformatic prediction of target mRNA and miRNA interactions is not by itself a solid evidence unless confirmed by further laboratory experiments. As central genes in the network (from the IPA analysis) are key genes that control the entire network and subsequent biological functions, we decided to experimentally verify whether the central genes in the top networks (IKBKB, JUN, ID3, Sp1, INSR, NOS2 and MAPK14) are in vivo targets of miRNAs (miR-423-3p, miR-196b, miR-24-3p, miR-27a-3p, miR-503). To attain this, we amplified miRNA binding site(s) from the 3'UTR of the central genes from genomic DNA by PCR using sequence specific primers indicated in Table 7. The amplified PCR products were then cloned into pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, Wisconsin, USA) digested by NheI and

SalI restriction enzymes. Cloning of the right sequence was confirmed by sequencing from the construct plasmid vector. As negative controls, we used synthesized oligos that harbor the miRNA target recognition site with three nucleotide mismatches at the seed region (Table 8). Furthermore, the negative controls were designed in such a way that when annealed and ligated into the pmirGLO vector result in the miRNA target region in the correct 5´ to 3´ orientation. The overhangs created at the ends of the annealed oligonucleotides were made complementary to those generated by restriction enzyme digestion of the pmirGLO Vector.

Table 7: List of primers used to amplify binding sites of miRNAs in the 3' UTR of target genes.

Gene name	Primer sequence	Annealing temperature (°C)
MAPK14	F -CTAGTGTGGGAGGGTAAAACATGATG	60
	R- TCGACGTCTACTCCGGATTCACACG	
NOS2	F - CTAGGGAACTTAATGATGGCACCCA	TD: 62-59
	R - TCGAGAGTGGCGTGACAGCGC	
JUN	F- CTAGCAAGTTGCGAGAGGGAAACAG	TD: 61-57
	R -TCGAATCCTTTCTGGAATTTTCAGAAACA	
IKBKB	F- CTAGACCCCTCCTGCTCTCCAAAG	62
	R- TCGACCGTGCTGTCCTTCTGTTACAC	
INSR	F - CTAGGTGGCCTTTTGCTTGGTCTTC	61
	R - TCGACCTTAGCGGTTTCTCCTCTGG	
SP1	F - CTAGGCTGGGAGGAGGAAGGAGAC	TD: 64-60
	R- TCGATGGAGATGTTCAAAAGAGGAAGTG	
ID3	F - CTAGCCTCCAGAACGCAGGTGC	61
	R - TCGACATCGCATGGTTACAGAAAGTCAC	

TD = touchdown PCR

Bovine cumulus cells were co-transfected with miRNA target sites cloned pmirGLO vector and miRNA mimic using attractene transfection reagent (QIAGEN Gmbh, Hilden, Germany) in a 24 well plate in four treatment groups (pmirGLO vector, pmirGLO – 3'UTR reporter vector, pmirGLO – 3'UTR reporter vector + mimic, pmirGLO – mismatch reporter vector) according to manufacturer's protocol. Briefly,

0.4 µg of pmirGLO vector and mimic was diluted in a medium with out serum and proteins, to a total volume of 60 µl. After adding 1.5 µl of attractane transfection reagent and short centrifugation, the solution was kept at room temperature for 10-15 minutes to allow transfection complex formation. In the mean time, cells from the monolayer were harvested by trysinization and suspended in culture medium containing serum and antibiotics. Then after, cells were seeded in a 24-well plate at the density of 0.4 - 1.6 x105 in 500 µl medium. Next, transfection complexes in the respective treatment groups were added to each well and the cells with transfection complexes were incubated at 37 °C with 5 % CO_2 for 48 hours. Cell extracts were prepared 48 hours after transfection and the luciferase activity of firefly and Renilla was measured with the Dual-Luciferase Reporter Assay System (Promega).

Table 8: Oligo nucleotide sequences with three nucleotide mismatches in the miRNA target site. The sense and anti sense nucleotide sequences were company synthesized and annealed to have a double strand DNA fragment and subsequently cloned into a pmirGLO vector to be used as a negative control.

miRNA target mismatch	Nucleotide sequence
miR-423-3p	Sense 5'-CTAGCTAGCGGCCGC TAGTACTGAGGGGCCTCAGAGGCGCTG-3'
	Antisense 5'-TCGACAGCGCCTCTGAGGCCCCTCAGTACTAGCGGCCGCTAG-3'
miR-196b	Sense 5´-CTAGCTAGCGGCCGCTAGTCCCAACAACAGGAAACTTGGAG-3'
	Antisense 5'- TCGACTCCAAGTTTCCTGTTGTTGGGACTAGCGGCCGCTAG-3'
miR-24	Sense 5' – CTAGCTAGCGGCCGCTAGTCTGTTCCTGCTGAACTGCGGAG-3'
	Antisense 5' – TCGACTCCGCAGTTCAGCAGGAACAGACTAGCGGCCGCTAG-3'
miR-503	Sense 5'- CTAGCTAGCGGCCGCTAGTCTGCAGAACTGTTCCCGGTCGAG-3'
	Antisense 5'- TCGACTCGACCGGGAACAGTTCTGCAGACTAGCGGCCGCTAG-3'
miR-27a	Sense 5'- CTAGCTAGCGGCCGCTAGTGCGGAACTTAGCCAGTCTCAG-3'
	Antisense 5'- TCGACTGAGACTGGCTAAGTTCCGCACTAGCGGCCGCTAG-3'

4.1.2.6. Quantification of selected miRNAs during estrous cycle

Total RNA containing small RNAs was purified from endometrial epithelium samples collected at different time points of estrus cycle (day 0, day 3, day 7, day 14) and three pregnant cows. Samples were collected after slaughtering the cows and the three pregnant cows were at day 50 pregnancy. cDNA synthesis from equal amount (690 ng) of total RNA containing small RNAs was performed using miScript Reverse Transcription Kit (QIAGEN, Austin, USA) according to manufacturer's protocol. Following cDNA synthesis, quantitative real time PCR analysis in three biological replicates for six selected miRNAs was conducted using miScript SYBR Green PCR Kit (QIAGEN, Austin, USA) following manufacturer's procedure.

The relative abundance of miRNAs at different time points of estrous cycle and pregnancy group was calculated using a comparative threshold cycle (ct) method. The data was normalized by geometric mean of multiple endogenous controls.

Where, Relative abundance $=2^{-\Delta\Delta ct}$

Δct = average ct target miRNA – average ct of endogenous controls*

$\Delta\Delta ct$ = Δct target miRNA - Δct of the calibrator**

Expression level (fold change) = $2^{-\Delta\Delta ct}$

* geometric mean of endogenous controls (U6, SNORD44 and SNORD 48) was used to normalize each target microRNA expression at different time points of estrous.

** the one with the highest Δct value among the groups was used as a calibrator

4.3. Part II: Results

4.3.1. Altered expression of microRNAs in cows with subclinical endometritis

Out of a total of 352 microRNAs in which their expression was assessed, we found 23 microRNAs to be differentially expressed in cytobrush samples taken from cows with subclinical endometritis as compared to their healthy counterparts. Among the differentially expressed microRNAs 15 were upregulated and 8 were downregulated in cows with subclinical endometrititis. miR-423-3p exceptionally showed higher (1341 fold change) expression level where as, miR-215 showed a higher fold change down regulation in cows with subclinical endometritis. Among the differentially expressed miRNAs, 12 were found to be intergenic where as five miRNAs (miR-25, miR-423-3p, miR-98, miR-339-5p and miR-215) were intronic and the other five (miR-511, miR-643, miR-619, miR-503 and miR-210) were not yet identified in bovine. However, miR-194 was observed to have both intergenic and intronic genomic location. We detected two miRNA clusters; miR-27a and miR-24 clusters on bovine chromosome 7 and were highly expressed in cows with subclinical endometritis where as miR-362-3p and miR-502-5p were miRNA clusters on bovine X chromosome and were found to be downregulated in cows with subclinical endometritis (Table 9).

Table 9: List of differentially regulated microRNAs in cows with subclinical endometritis as compared to the healthy counterparts.

microRNA	P-value	Fold Change	Genomic location
miR-16	0.0488	4	Intergenic (Chr.12)
miR-21	0.0186	2	Intergenic (Chr.19)
miR-126	0.0599	5	Intergenic (Chr.11)
miR-27a	0.0265	2	Intergenic (Chr.7)
miR-24	0.0370	2	Intergenic (Chr.7)
miR-424	0.0135	4	Intergenic (Unknown)
miR-25	0.0251	2	ENSBTAT00000003728 (Chr.25)
miR-223	0.0314	16	Intergenic (Chr. X)
miR-196b	0.0435	6	Intergenic (Chr.4)
miR-194	0.0184	2	Intergenic (Chr. 29) ENSBTAT00000061457 (Un.)
miR-125a-5p	0.0209	2	Intergenic (Chr. 18)
miR-423-3p	0.0677	1341	ENSBTAT00000025760 (Chr. 19)
miR-210	0.0012	-3	Not known
miR-196a	0.0311	3	Intergenic (Chr.5 & 19)
miR-503	0.0679	5	Not known
miR-98	0.0046	3	ENSBTAT00000008060 (Chr. X)
miR-339-5p	0.0714	-3	ENSBTAT00000043431 (Chr.25)
miR-362-3p	0.0445	-2	Intergenic (Chr. X)
miR-215	0.0591	-47	ENSBTAT00000061457 (Unknown)
miR-502-5p	0.0420	-5	Intergenic (Chr. X)
miR-619	0.0189	-9	Not known
miR-643	0.0183	-9	Not known
miR-511	0.0324	-6	Not known

4.3.2. Molecular networks and biological functions that are possibly affected by endometritis

The identification of aberrantly expressed miRNAs in cows with subclinical endometritis led us to hypothesize that tracing target genes for the miss expressed miRNAs and subsequently identifying pathways and biological functions that these target genes are enriched with, will help us to understand the underlying molecular mechanisms potentially induced and/or affected by subclinical endometritis. To test this hypothesis, we filtered the high ranking predicted target genes (680) for the aberrantly expressed miRNAs (23) and uploaded into the Ingenuity Pathway Analysis (IPA). The result indicated molecular networks, biological functions and the molecular pathways that these target genes potentially constitute in the context of subclinical endometritis in bovine. The IPA identified 28 significant molecular networks with a score greater than 10 (10^{-10} chance that the gene in the network associated solely due to random events) underlying the biological functions which we exemplified top five networks and corresponding miRNAs that target genes with central position in the network. The first molecular network (score = 42) converged on nuclear factor kappa B (NF-kB) a key transcription factor that mediates gene expression and represent gene expression, haematological system development and function, cellular development. The second molecular network (score = 33) constituted by the 680 target genes has B cell lymphoma 2 (Bcl2), mitogen-activated protein kinase 14 (MAPK14), nitric oxide synthase 2 (NOS2) and interleukin 12 complex (IL12) at the central positions mediating cell death. The third network (score = 32) represents connective tissue development and function, nervous system development and function, skeletal and muscular system development and function with Jun as a focus gene. Cell signaling, amino acid metabolism, post translational modification (score = 31) and cell cycle, connective tissue development and function, cancer (score = 31) represent the fourth and the fifth networks respectively (Figure 4.17).

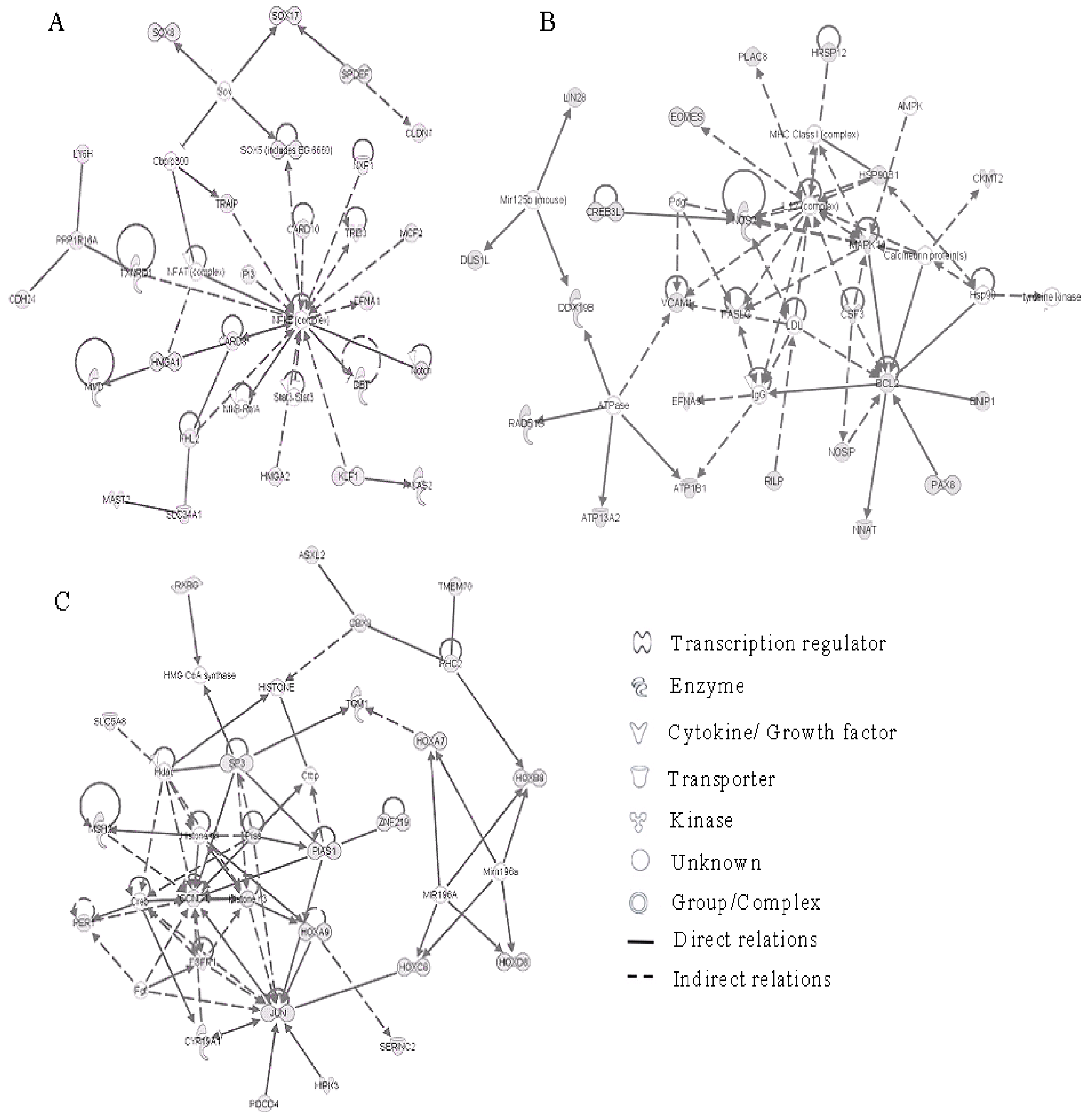

Figure 4.17: Top three molecular networks enriched by the 680 genes that are predicted targets of the 23 aberrantly expressed miRNAs in subclinical endometritis as compared to healthy ones. A molecular network from the IPA analysis converged to NF-KB that controls gene expression (A), gene network that mediates cell death (B) and molecular network that dictates connective tissue development and function (C). In the graphical representation of a network, genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. Nodes are displayed using various shapes that represent the functional class of the gene product.

The target genes were significantly enriched for diverse biological functions known to be fundamental for uterine dynamism and receptivity, organ development and reproductive organ diseases. This includes: cellular growth and proliferation, cell cycle, gene expression, cellular movement, cellular development, organ morphology, cell death, organismal development, haematopoiesis, cell morphology, cellular function and maintenance, cell mediated immune response, embryonic development, reproductive system development and function, connective tissue development and function and reproductive system disease (Figure 4. 18).

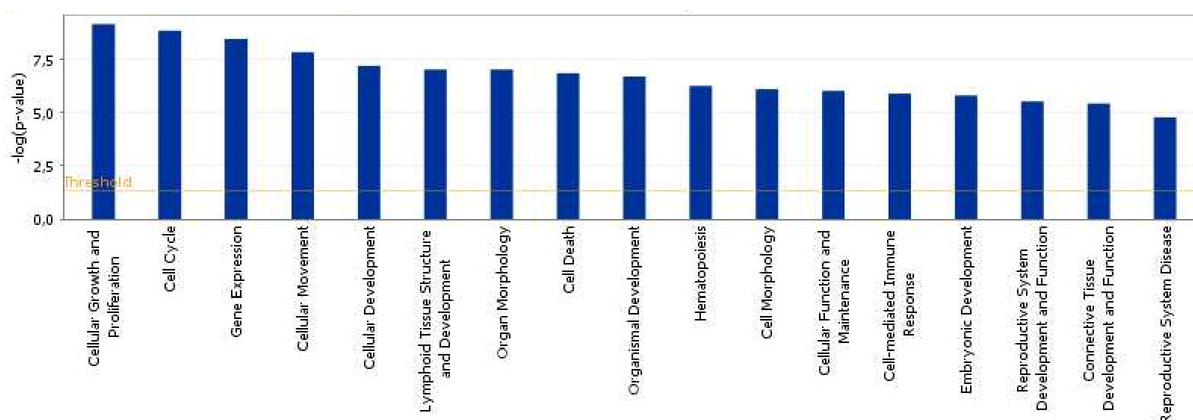

Figure 4.18: A list of top biological functions in which the aberrantly expressed miRNA target genes are enriched.

4.3.3. Canonical pathways

The canonical pathway analysis was generated using the IPA to understand whether the 23 dysregulated miRNAs in subclinical endometritis and their corresponding target genes are significantly correlated to previously defined signaling or metabolic pathways. We identified pathways in which the 680 target genes for the 23 aberrantly expressed miRNAs are significantly correlated to canonical pathways library in the IPA as compared to randomly selected genes from the reference gene list (IKB). The analysis generated pathways known to induce female reproductive abnormalities, cellular proliferation and immune response that includes; aryl hydrocarbon receptor signaling, cell cycle : G1/S check point regulation, PTEN signaling, P53 signaling, Myc mediated apoptosis signaling, RhoA signaling, cyanoamino acid metabolism, cell cycle regulation by BTG family proteins, regulation of actin-based mobility by Rho, CCR5 signaling in macrophages, cytotoxic T-lymphocyte mediated apoptosis of target cells,

melanoma signaling, TGF-β signaling , ILK signaling, JAK/Stat signaling, Wnt/β catenin signaling and IL-8 signaling (Figure 4.19).

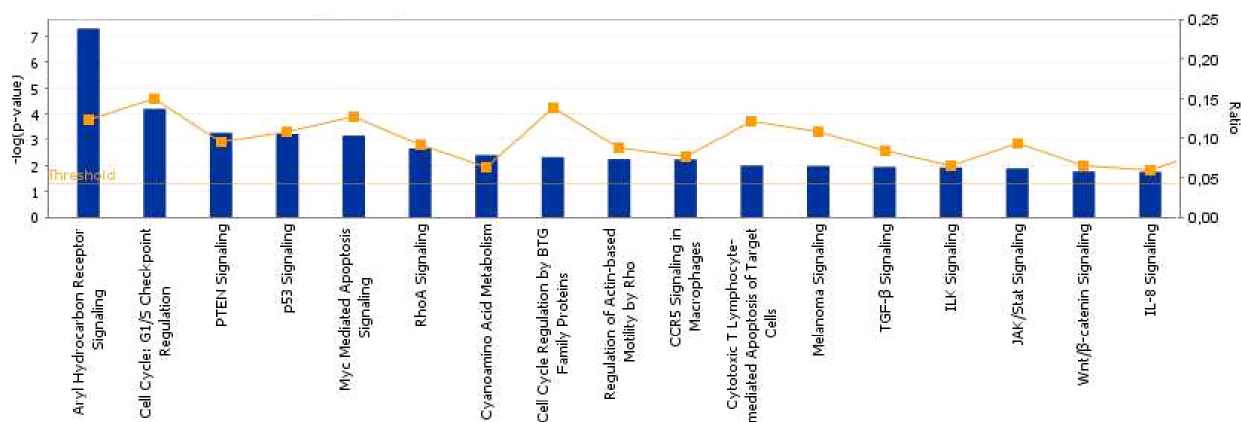

Figure 4.19: Top selected canonical pathways that were found to be most significant to the target gene list.

4.3.4. Interaction between center genes and targeting microRNA

The IPA identified biological functions and /or diseases that were most significant to the genes in the network. With this line, the genes with central position in the network have critical role in dictating the underlying biological functions. Hence, scrutinizing the interaction of the aberrantly expressed miRNAs and the central genes of the network enabled us to exemplify and elucidate the magnitude of regulation exerted by the aberrantly expressed miRNAs. The bioinformatic analysis of some of the aberrantly expressed miRNAs and 3' UTR of bovine mRNA (centre genes for the top networks) showed stringent and repetitive miRNA recognition sites (Figure 4.20). miR-423-3p has recognition sites in IKBKB, JUN, INSR, MAPK14 and ID3 3'UTR of the respective mRNA. miR-196b appears to regulate four central genes (IKBKB, INSR, MAPK14 and NOS2) where as miR-24-3p seems to exert a stronger posttranscriptional regulation on INSR as it harbors three recognition sites. Further more, miR-24-3p seems to extend its regulatory effect on IKBKB, SP1, MAPK14 and NOS2. miR-27a-3p has recognition sites on SP1, MAPK14 and ID3 while MAPK14 and JUN posses double recognition sites for miR-503.

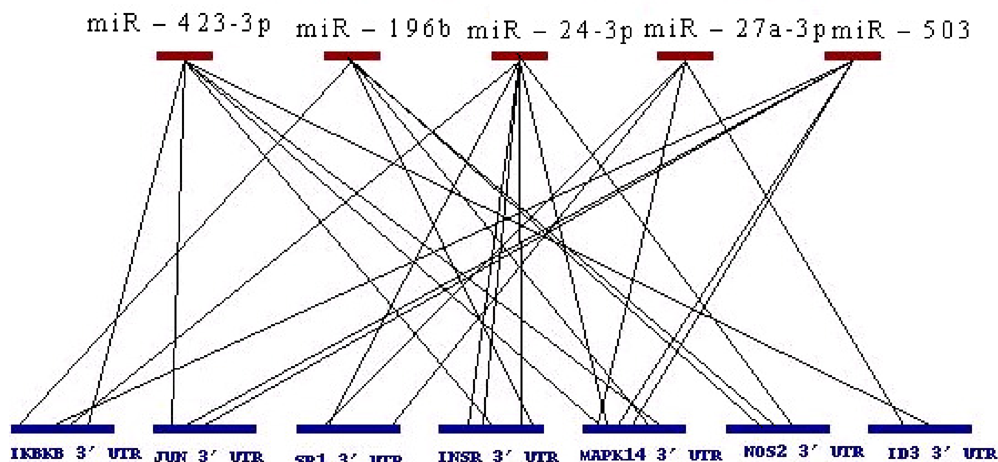

Figure 4.20: The frequency and position of interaction between aberrantly expressed miRNAs and 3'UTR of target genes with central position in the top five gene networks.

Moreover, the expression analysis of genes with central position of the top five gene networks (ID3, INSR, NOS2, SP1, IKBKB, MAPK14 and JUN), in which their interaction with their respective targeting miRNAs was validated using luciferase reporter assay, was conducted in endometrial cytobrush samples (taken at different time points of estrous cycle) using semi quantitative polymerase chain reaction method. The result revealed that all the seven genes are expressed during estrous cycle (Figure 4.21).

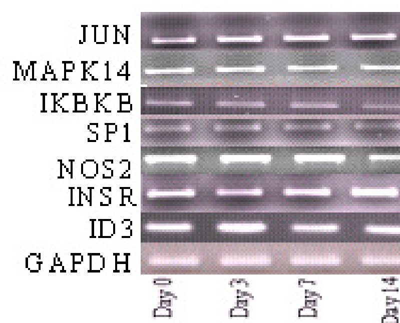

Figure 4.21: The semi-quantitative expression analysis of genes with central position in the top networks (ID3, INSR, NOS2, SP1, IKBKB, MAPK14 and JUN) and internal control (GAPDH) in endometrial samples at different time points of estrous.

4.3.5. Validation of miRNA–center genes interactions using luciferase assay

Next, to verify if bioinformatically predicted miRNA target sites are real physiological targets, we cloned 3' UTR containing target sites at the down stream of the firefly luciferase gene (luc2) in the pmirGLO dual-luciferase miRNA target expression vector and quantitatively evaluated the reduction in firefly luciferase expression that indicates the binding and translation repression of endogenous or the introduced miRNAs to the cloned miRNA target sequence. The bioinformatic analysis indicated that a particular miRNA could target multiple key genes of the networks and a single miRNA could have a repetitive target sites on a particular 3' UTR (Figure 4.20). As validation for all target sites is too much to handle, we selected seven representative target sites for five miRNAs. Prior to luciferase activity assay, we ascertained the expression of the target genes in bovine endometrial epithelium samples using semi quantitative PCR. The interaction of the miRNAs and the 3'UTR revealed by bioinformatic analysis usually lacks the expression pattern of both miRNA and target gene in time and space. Thus, it seems quit apparent to unravel the expression of the miRNA and predicted target genes in the endometrial epithelium as in most cases miRNAs repress the expression of their target genes.

To directly asses the impact of miR-503 on IKBKB and JUN, we cloned 599 and 655 bp of the 3' UTR into firefly luciferase (pmirGLO vector) respectively. IKBKB 3' UTR was found to harbor miR-503 target site at 483 position while JUN 3' UTR has recognition site for this miRNA at 406 position after the stop codon. We performed transfection in four treatment groups (pmirGLO vector, pmirGLO – IKBKB 3'UTR reporter vector, pmirGLO – IKBKB 3'UTR reporter vector + mimic, pmirGLO – mismatch reporter vector + mimic). Co-transfection of the construct vector (pmirGLO - IKBKB 3'UTR reporter vector) and miR-503 mimic in cumulus cells reduced luciferase activity by 46% as compared to those groups transfected with only construct vector. Here, the level of luciferase reduction is solely attributed to the exogenously introduced miR-503 mimic as the endogenous effect is ruled out by systematic inclusion of transfection only construct vector in the treatment groups (Figure 4.22A & B). On the other hand, exogenous miR-503 introduction resulted in 33% luciferase activity reduction in cumulus cells transfected with pmirGLO vector with JUN 3'UTR and miR-

503 mimic as compared to control miR-negative (pmirGLO-JUN 3’ UTR vector) transfected cells (Figure 4.22C & D).

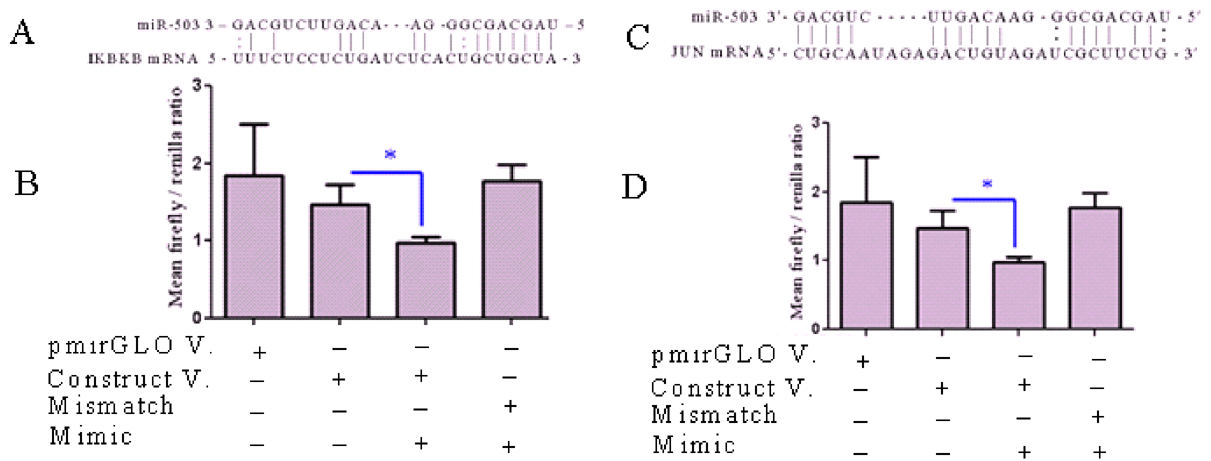

Figure 4.22: Luciferase activity assay for miR-503 - IKBKB 3’ UTR and miR-503 – Jun 3’ UTR interactions. The alignment of miR-503 with IKBKB and JUN (A & C) indicating the binding site that was cloned in the pmirGLO expression vector. Transfection of intact pmirGLO vector, construct cloned with IKBKB and JUN 3’ UTR with miR-503 mimic in four treatment groups (B & D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them.

To confirm that miR-27a-3p can bind and cause translational repression to the 3’ UTR of Inhibitor of DNA binding 3, dominant negative helix- loop-helix protein (ID3) and SP1 transcription factor (SP1), we cloned 449 and 779 base pairs of 3’ UTR, respectively into pmirGLO dual-luciferase miRNA target expression vector. Besides, miR-27a-3p was observed to have two binding sites in SP1 3’ UTR (position 1052 and 1679) and one binding site in ID3 3’ UTR at position 177 from the stop codon. Transfection was performed in four treatment groups as described previously. As shown in figure 4.23, the intensity of luciferase fluorescence was markedly reduced (63%) in the group (pmirGLO-SP1 3’ UTR reporter vector + miR-27a-3p mimic) as compared to the control group (pmirGLO-SP1 3’ UTR reporter vector). miR-27a -3p mimic has shown no effect on the luciferase activity in the miR-27a-3p mismatch cloned vector (pmirGLO-SP1 3’UTR mismatch + miR-27a-3p mimic). Similarly, we conducted transfection experiment for ID3 and observed a pronounced luciferase activity reduction (52%) in the group (pmirGLO - ID3 3’ UTR reporter vector + miR-27a-3p mimic)

compared to the control (pmirGLO-ID3 3'UTR reporter vector) with no miR-27a-3p effect on the luciferase activity in the negative control group (pmirGLO-ID3 3'UTR mismatch + miR-27a-3p mimic). These facts indicate that miR-27a-3p could bind to the 3'UTR of both SP1 and ID3 mRNA and mediate posttranscriptional gene expression repression. Furthermore, the data substantiates the bioinformatic prediction that SP1 and ID3 are direct targets of miR-27a-3p.

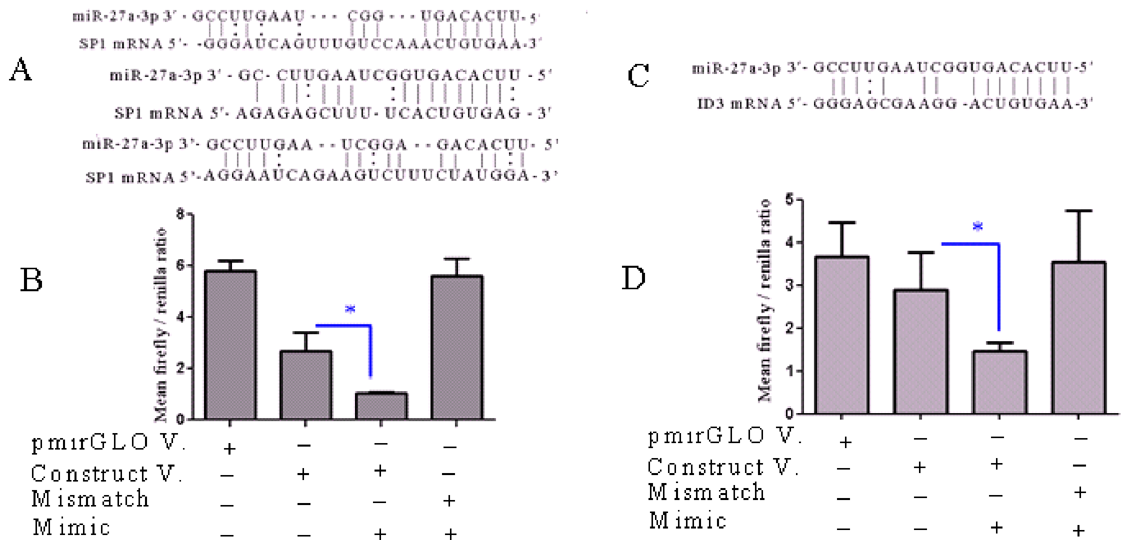

Figure 4.23: The base complementarity between miR-27a-3p versus ID3 and SP1 where multiple miR-27a-3p binding sites on SP1 and single binding site on ID3 were cloned into the pmirGLO expression vector (A & C). The mean firefly / renilla ratio observed in four treatment groups (pmirGLO vector transfected, Construct vector transfected, Construct vector + mimic transfected and mismatch vector + mimic) showing reduced firefly/renilla ratio in construct vector and miR-27a-3p mimic co-transfected group (B & D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them.

We next examined whether the multiple miR-24a-3p binding sites with in the 3' UTR of Insulin receptor (INSR) form miR-24a-3p - INSR 3'UTR duplex and mediate translational repression. Here, we cloned 500 base pairs of INSR 3' UTR that harbor three binding sites of miR-24-3p into pmirGLO dual-luciferase miRNA target expression vector and transfected into cumulus cells in four treatment groups as described previously. Interestingly, co-transfection of miR-24-3p mimic and pmirGLO - INSR 3' UTR reporter vector into cumulus cells markedly reduced the Luciferase/

Renilla ratio by 55% when compared to pmirGLO - INSR 3’ UTR reporter vector only cloned group. Mismatches in the miR-24-3p binding sites considerably avoided translational repression by miR-24-3p mimic (Figure 4.24). Taken together these findings show that the multiple miR-24-3p binding sites on INSR 3’UTR could cause a pronounced protein expression reduction possibly by allowing miR-24-3p - INSR 3’UTR duplex formation. Thus, INSR appears to be a plausible physiological target of miR-24-3p.

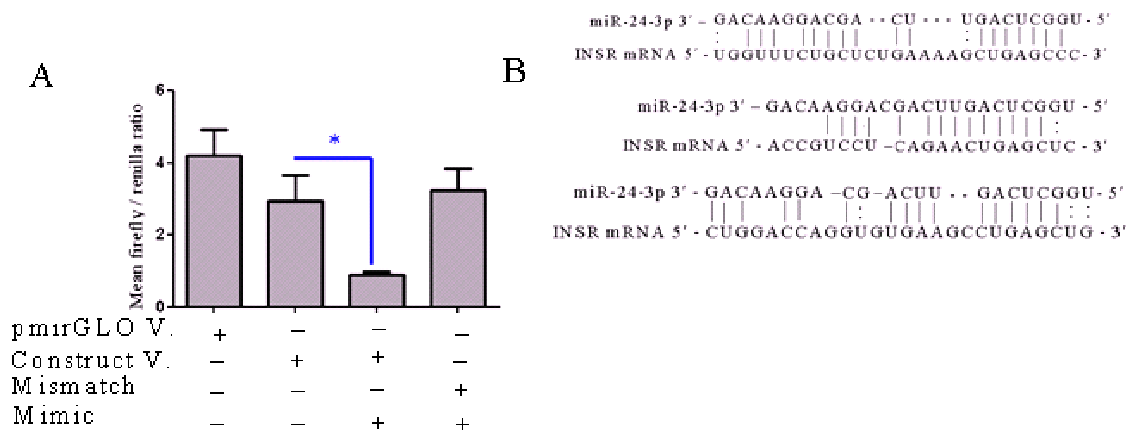

Figure 4.24: The response of luciferase reporter protein cloned with INSR 3’ UTR following co-transfection in cumulus cells in four treatment groups (A). The Watson and Crick complimentarity between INSR 3’UTR and miR-24-3p. All the three target sites were cloned in to pmirGLO expression vector (B). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them.

We cloned 325 and 764 base pair fragments of NOS2 and MAPK14 3’UTRs into pmirGLO expression vector, downstream of the luciferase gene. Upon co-transfection of miR-196b and miR-423-3p mimics and corresponding constructs (pmirGLO vector cloned with the target sites) into cumulus cells, we observed repressed luciferase protein expression compared to the control (wt target site cloned construct in the absence of miRNA mimic) in both experiments (Figure 4.25). Unlike the other miRNAs analyzed, we observed a mild protein repression effect for both miR-196b (25%) and miR-423-3p (37%) on NOS2 and MAPK14 3’ UTRs respectively. Co-transfection of mismatch target site cloned vector with corresponding mimic didn’t significantly reduce luciferase activity showing specificity of the protein repression effect to the introduced miRNA mimics.

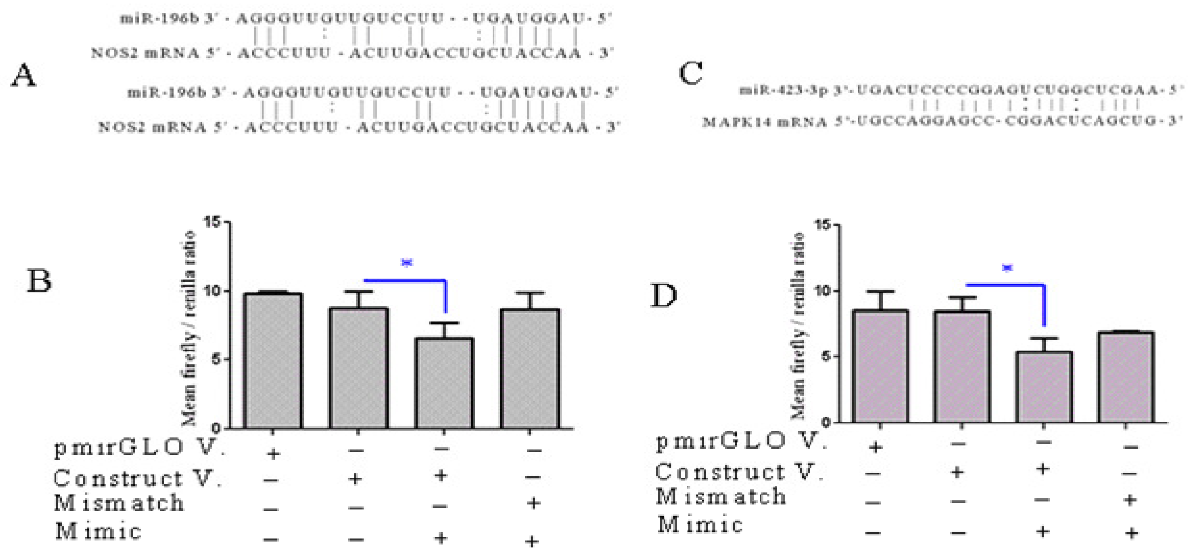

Figure 4.25: Target validation for miR-196b–NOS2 and miR-423-3p–MAPK14 using luciferase reporter assay. The Watson and Crick complimentarity between miR-196b and NOS2 3'UTR (A) and miR-423-3p–MAPK14 (C). Transfection of pmirGLO vector, pmirGLO vector cloned with NOS2 3' UTR containing miR-196b target site, construct vector + miR-196b mimic in four treatment groups (B) similar transfection procedure was performed to validate the interaction between miR-423-3p and MAPK14 3' UTR (D). Treatment groups with a star (*) indicate significant difference (P< 0.05) between them.

4.3.6. Expression profile for miRNAs during estrous cycle of healthy animals

With the intension of investigating the temporal expression pattern of the differentially regulated miRNAs, we quantified the expression of six miRNAs (miR-24, miR-215, miR-27a, miR-223, miR-619, miR-423) in endometrial epithelium samples using qRT-PCR at four time points of estrous cycle (day 0, day 3, day 7, day 14) including pregnant animals. U6, SNORD44 & SNORD48 were quantified during estrous cycle and pregnant animals. The geometric mean ct value of the three internal controls was observed to be stable across estrous cycle and used to normalize the miRNA real time data (Figure 4.26). miR-24 and miR-223 showed a similar expression pattern where we observed a magnificent increase during pregnancy with lower and constant expression pattern during estrous cycle. miR-619 and miR-423-3p also showed a similar expression pattern with a progressive increase from the beginning to the end of estrous cycle

followed by decline during pregnancy. miR-619 exceptionally showed a pronounced decrease in expression level in the pregnant animal endometrial epithelium. The last group of miRNAs (miR-215 and miR-27a) revealed more or less similar pattern of expression with a slight increase at day3 of estrous cycle and subsequent decline at day 7 and day 14 and finally followed by a marked increase at pregnancy (Figure 4.27).

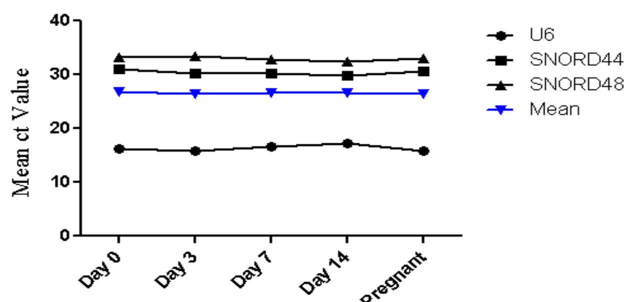

Figure 4.26: The mean ct values of internal controls across estrous cycle and pregnant group. The geometric mean of the internal controls (U6, SNORD44 and SNORD48) showed stable expression pattern across the groups that are compared and was used to normalize the qRT-PCR data for miRNAs.

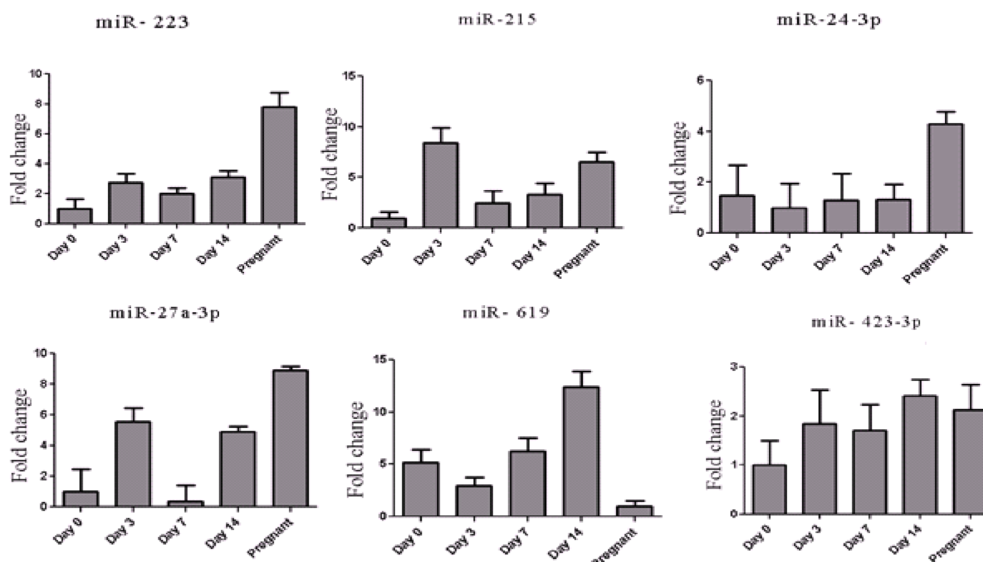

Figure 4.27: The expression pattern of six miRNAs during estrous cycle (day 0, day 3, day 7, day 14) and in pregnant cows.miR-223 and miR-24-3p showed more or less constantly lower expression during estrous cycle and a marked rise during pregnancy while the expression of miR-215 and miR-27a-3p showed a variable pattern which is lower at day 0 and day 7. The expression of miR-619 linearly increased during estrous cycle (day3-day14) and a pronounced decrease during pregnancy. miR-423-3p showed more or less similar expression at different time points of estrous cycle as well as pregnant animals.

4.4. Part II: Discussion

4.4.1. Endometrial miRNA detection

We used whole genome human microRNA PCR array platform which represent 352 microRNAs in a four set of 96- Well plate to assess the miRNA abundance in endometrium of cows with subclinical endometritis and healthy ones. As there is no ready to use bovine array, we used human RT^2 PCR array and interestingly resulted in a strong signal detection with minimal negative calls. This is mainly attributed to the cross species conservation feature of miRNAs between human and bovine as it has been observed in wide range of species (Bentwich et al. 2005). To complement this evidence, we aligned the human miRNAs from the array used with the currently known bovine counterparts and we observed 100 % conservation in most cases. The real time based RT^2 PCR array combines the principles of qRT-PCR and microarray which enabled us asses the expression of large number of bovine miRNAs at a time with out compromising accuracy as we have tried to use appropriate data analysis and normalization procedures.

4.4.2. Aberrant expression of microRNAs in the endometrium of cows with subclinical endometritis and implication of their genomic location

Although so far there is no solid evidence linking aberrant miRNA expression to sub-clinical endometritis, microRNAs are implicated in a number of diseases in human. Studies have shown altered expression of mRNA transcripts in endometriotic lesions when compared with eutopic tissues (Ohlsson Teague et al. 2009a) while, 22 miRNAs were found to be differentially expressed in seven paired ectopic versus eutopic peritoneal endometrial tissues from woman with rAFS stage II-IV endometriosis (Ohlsson Teague et al. 2009b). In our study we identified 23 differentially expressed miRNAs in cows with subclinical endometritis as compared to the healthy ones suggesting the aberrantly expressed miRNAs might have a role in the patho - physiology of subclinical endometritis.

The genomic location of six differentially expressed miRNAs (miR-25, miR-194, miR-423-3p, miR-98, miR-339-5p and miR-215) were observed to be intronic (with in the

introns of protein coding genes) and several studies showed that intronic miRNAs are co-expressed with host genes (Baskerville and Bartel 2005, Lagos-Quintana et al. 2001, Wang and Lee 2009) implying that both derived from a common transcript (Rodriguez et al. 2004) or the intronic miRNAs and their host genes may share common regulatory elements, such as common promoters (Kim and Nam 2006). miR-126, upregulated in cows with subclinical endometritis, in human it is located with in the intron 7 of EGFL7 which is highly expressed in endothelial cells and vascularised tissues was found to control an important step in vasculogenesis (Fitch et al. 2004, Parker et al. 2004). On the other hand, miR-196 is genomically located in the intergenic regions in the Hox clusters of vertebrates and known to direct the cleavage of HoxB8 mRNA in mouse embryos and also regulates the expression of HoxC8, HoxD8, and HoxA7 (Yekta et al. 2004). The intronic location of the aberrantly expressed miRNAs in cows with subclinical endometritis indicates the possible miss expression of host genes co-expressed with this class of miRNAs.

Similar expression pattern was observed for miRNA clusters where miR-27a and miR-24 showed high expression, where as miR-362-3p and miR-502-5p showed a lower expression in cows with subclinical endometritis as compared to healthy counterparts. This is consistent with the fact that proximally paired miRNA genes located up to 50 kb apart are frequently co-expressed (Baskerville and Bartel 2005).

4.4.3. Molecular networks and biological functions potentially regulated by aberrantly expressed miRNAs

Altered expression of miRNAs and improper repression of their targets can have diverse effects as these genes may involve in different molecular networks and biological functions. Assignment of the target genes to different functional groups depending on the signaling pathways that they have been mapped to, gives us important insight to the uterine functions that are potentially affected by the incidence of subclinical endometritis. This highlights a clue for the association of incidence of endometritis with reduced conception rate from miRNA perspective. Based on our data, the most significant biological functions generated from the IPA such as cellular growth and proliferation, cell cycle, gene expression, cellular movement and cell death strikingly overlap with array of cellular activities that are intrinsic to the uterus during estrous

cycle and pregnancy (Bigsby and Cunha 1986, Kurita et al. 1998). It is well established fact that successful pregnancy out come is affected by the quality of the embryo as well as endometrial function (Christiansen et al. 2006, Rai and Regan 2006). Several studies attempted to examine the effect of embryo and endometrium on implantation at cellular and molecular levels (Barnea 2001, Savaris et al. 2008, Strowitzki et al. 2006). The mammalian uterus is a dynamic physiological system in which cellular proliferation, differentiation, apoptosis occur in a temporal and cell-specific manner during pregnancy (Das 2009). Recently, Germeyer et al. (2010) found out that inadequate endometrial growth marked by reduced cell proliferation in endometrium could contribute to reproductive failure. Moreover, defective cell growth as a result of aberrantly expressed genes controlling cell cycle is likely to contribute to implantation failure (Lee et al. 2007). In another study, a positive association between pregnancy rate and endometrial volume and /or thickness has been shown (Raga et al. 1999) which is likely dependent on the rate of cell proliferation at specific reproductive stages. In spite of the identification of miRNA-regulated biological functions intrinsic to cellular activities in the uterine, the canonical pathway analysis from the IPA showed pathways which are essential for successful pregnancy in mammals. Aryl hydrocarbon signaling pathway, the most significant pathway generated from the IPA, is evidenced to involve in the normal function of the endometrium, possibly by modulating cellular proliferation in response to hormones, by regulating the expression of upstream genes involved in the function of the endometrium, and/or by controlling uterine secretary function (reviewed in Hernandez-Ochoa et al. 2009).

Besides, some of the key genes that have been shown to have central positions in the top five networks are implicated in bovine endometritis. Li et al. (2005) reported higher expression of NOS2 mRNA in endometrial biopsies from cows with subclinical endometritis, while Kim et al. (2005) demonstrated the interaction of IKBKB with NOS2 in endothelial cells that activation of IKBKB plays a critical and novel role to mediate the deleterious effect of high glucose on the insulin mediated activation of eNOS. Zhao et al. (2007a) showed the expression of INSR in the endometrial carcinoma cells and incubation with insulin stimulated a dose and time dependent proliferation and the apoptosis rate of carcinoma cells was decreased gradually with increasing concentration of insulin. Moreover, Takano et al. (2007) showed INSR as one of the most upregulated FOXO1 dependent transcriptional target and decidua–specific

transcriptome in human endometrium. Sp1 is one of the transcription factors that have been shown to be regulated by progesterone receptor in human endometrial adenocarcenoma cells (Gao et al. 2001). Cheng et al. (2006) revealed the expression of HSD17B2 in endometrial epithelial cells where estrogen inactivation is regulated by SP1 and SP3 which are down stream target of progesterone-dependent paracrine signals originating from endometrial stromal cells.

NF-kB plays a crucial role in immune and inflammatory responses through the regulation of genes encoding inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes such as cyclo-oxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (Baldwin 2001, Barnes and Karin 1997, Chen et al. 1999). Pro-inflammatory mediators (TNF-α, IL-1β, IL-6 and NOS2) affect embryonic development by acting either on the oocyte, developing embryo or endometrium. Bovine oocyte in vitro matured with TNF-α showed reduced blastocyst rate, while addition of TNF-α increased the proportion of blastomeres that were apoptotic (Soto et al. 2003). IL-6 blocks follicle stimulating hormone-induced estradiol secretion from bovine granulosa cells (Alpizar and Spicer 1994) and TNF - α is cytotoxic to bovine luteal cells (Petroff et al. 2001). Studies have shown that endometrial expression of prostaglandin F2α is under the control of several cytokines including TNF - α that increase PGF2α from cultured bovine stromal endometrial cells (Skarzynski et al. 2000) and IL-1β that induce PGF2α from endometrial stromal and epithelial cells (Davidson et al. 1995). Prostaglandin F2α has been reported to have a negative effect on embryo development in cattle where removal of the corpus luteum reduced the embryotoxic effects of PGF2α suggesting that PGF2α induces secretion of the embryotoxic molecule of luteal origin (Buford et al. 1996). Furthermore, pro-inflammatory cytokines like IL-1β exert effects on endometrial tissue that interfere with endometrial receptivity and subsequent embryo development by reducing proliferation of endometrial stromal cells (Davidson et al. 1995). Elevated expression of NOS2 mRNA was observed in infertile cattle as a result of persistent endometritis (Herath et al. 2009b) where as IL-1α and IL-RN were highly expressed in subclinical endometritis (Gabler et al. 2009). Thus, it is more likely that dysregulation of uterine miRNAs during subclinical endometritis result in the up regulation and translocation of NF-kB leading to expression of cytokines that could induce endometrial PGF2α release and subsequent pre-mature corpus luteum

regression. The ultimate negative effect of pro-inflammatory mediators on endometrial receptivity, oocyte maturation and blastocyst development could cause non-receptive endometrium, developmentally incompetent oocyte and/or blastocyst which is associated with frequent embryo loss and extended calving interval. Hence, at least partly, this explains the molecular mechanism that operates behind the association of subclinical endometritis with reduced reproductive performance in bovine.

4.4.4. Validation of some aberrantly expressed miRNAs and target genes

Luciferase assay method have been widely used (Ben-Ami et al. 2009, Le et al. 2009, Silber et al. 2008, Yao et al. 2010) and it is becoming the standard and preferred means for the experimental validation of gene regulation by miRNAs in mammalian cells. In our study, we used pmirGLO expression vector which is designed to quantitatively evaluate miRNA activity by the insertion of miRNA target sites downstream of the firefly luciferase gene. To make the assay method as efficient as possible and increase experimental confidence, we incorporated control samples into the experimental design where we designed our experiment in four treatment groups (pmirGLO vector, pmirGLO – 3'UTR reporter vector, pmirGLO – 3'UTR reporter vector + mimic, pmirGLO – mismatch reporter vector). The basis of this experiment lies on the over expression (exogenous introduction of miRNA mimic) of the miRNA in question despite the possible expression of same miRNAs in the cells. However, comparison of firefly/renilla ratio in the construct group with no insert reveals the effect of endogenous miRNA while comparison of the construct group with construct + mimic could tell the exogenous miRNA effect systematically avoiding the confounding effect of endogenous miRNA. In the negative control group, introduction of three mismatches in the seed region and co-transfection with target miRNA showed no significant reduction in luciferase activity highlighting the specificity of luciferase reduction to the miRNA analysed and abrogating the possibility of global luciferase reduction effect from miRNA mimic introduction.

The magnitude of luciferase assay reduction and the possible regulation of target genes by targeting miRNAs are observed to be variable for different miRNAs and target genes. We observed the maximum luciferase activity reduction (63%) for miR-27a-3p-Sp1 3'UTR interaction followed by miR-27a-3p – ID3 mRNA interaction (52%) while

the minimum was for miR-196b-NOS2 interaction (25%). The differential repressive power of the miRNAs considered in this study might be attributed to the variation in the molecular architecture of target genes' 3' UTR which subsequently affected the miRNA - mRNA interaction. Besides the complexity of the rule that govern miRNA- target mRNA interaction, studies have shown that a conserved seed 'match' consisting of bases 2-9 of the miRNA is a reliable predictor of interaction (Brennecke et al. 2005) while perfect base pair matching does not guarantee interaction between miRNA and target gene (Didiano and Hobert 2006) and wobble G:U base pairs are often tolerated in target sites (Vella et al. 2004). Furthermore, Hon and Zhang (2007) have shown that the number and arrangement of miRNA recognition sites can influence the degree and specificity of miRNA-mediated gene repression. In agreement with this, Sp1 3'UTR harbors three miR-27a-target sites with comfortable seed region and 3' end complimentarily, that may caused the pronounced repression of reporter gene protein following transfection by miR-27a mimic. Moreover, ID3 has the shortest 3'UTR (481 nt) when compared with the genes (mRNA) considered in this study and this may be the reason for the boost in repression as it is confirmed that shorter size 3'UTR induce higher repression as compared to the larger ones (Hon and Zhang 2007). Interestingly, NOS2 and MAPK14 showed less stringent complementarity with the seed region of miR-196b and miR-423-3p, respectively and this in agreement with the fundamental principles of miRNA- target gene interaction (Brennecke et al. 2005). Taken together, the data substantiates the primary information from bioinformatic prediction that the miRNAs we have studied mediate reporter gene protein repression in variable magnitude suggesting the genes are physiological targets for the miRNAs considered. Thus, the data enabled us to deduce a convincing link between the aberrantly expressed miRNAs and target genes which keep our attempt to trace subclinical endometritis induced miRNA miss expression and potential dysregulation of molecular pathways and functions straight and reasonable.

Collectively, this data suggests that incidence of subclinical endometritis induces altered expression of uterine miRNAs which at least partly regulate genes that involve in uterine cell proliferation, cell cycle, differentiation, apoptosis and gene expression. Extensive studies showed the need for tight regulation of cellular processes in the mammalian uterine in which deviations could compromise fertility. To articulate the link, miss expression of miRNAs could cause dysregulation of the fundamental cellular

processes of the uterus that negatively affect uterine receptivity and result in reduced fertility. It is more likely that these abnormal molecular mechanisms are informative of the mechanism that functions behind the correlation of subclinical endometritis with reduced conception rate.

5. Conclusion and future prospects

In conclusion, our data showed the temporal expression of a certain class of microRNAs in the course of bovine oocyte maturation with a possible role during early embryonic development. The heterologous approach also enabled us to detect and profile microRNAs which are not so far identified in bovine. Moreover, miR-99a and miR-100 which are expressed in a reciprocal manner with their predicted target, bovine homologue tribbles 2 (TRB2), in bovine oocytes and companion cumulus cells could bind the 3' UTR of TRB2 mRNA and mediate posttranscriptional gene expression repression. This suggests that miR-99a and miR-100 promote cumulus cell proliferation by repressing the TRB2 protein which is a negative regulator of cell cycle.

MicroRNAs potentially constitute a central role in the molecular mechanism underlying the development and progression of subclinical endometritis. This unravels a new play ground for scientists in the quest to develop miRNA-based therapy on the hypothesis that the use of miRNA inhibitors and mimics could help to rescue dysregulated molecular pathways and biological functions triggered by the incidence of subclinical endometritis.

The primary attempt to detect and elucidate miRNA expression during oocyte maturation produced a remarkable list of new bovine microRNAs and set of miRNAs with dynamic expression pattern during oocyte maturation. Hence, it appears important to dissect the specific functions of individual miRNAs with a motive to use this information in the breeding purpose so that to tackle the ever declining dairy cattle fertility. The investigation on the involvement of uterine miRNAs in the development of subclinical endometritis revealed microRNAs as the core component of molecular mechanism that explain the association of the incidence of subclinical endometritis with reduced dairy cattle fertility. As the approach used was basically integrative, there is a need to sharpen down the investigation to the most important molecular pathways and biological functions from the perspective of the aberrantly expressed miRNAs. This could be attained partly by using invitro endometrial cell culture model.

6. Summary

The developmental process in mammals begins during oogenesis and oocyte maturation is a decisive process during which oocytes acquire their intrinsic ability to support the subsequent stages of development in a stepwise manner. This process involves complex events of nuclear and cytoplasmic maturation which generally involves storage of mRNAs, proteins, transcription factors and chromosomal segregation. As there is selective transcription and translation of transcripts in the course of oocyte maturation, the role microRNAs as posttranscriptional gene expression regulators seems quite apparent. MicroRNA identification and expression analysis are the first and key steps in the long journey to understand the role of these posttranscriptional regulators in bovine pre-implantation embryo development. In line with this, identification and expression profiling of microRNAs during invitro oocyte maturation was performed using a heterologous microRNA microarray platform (human, mouse and rat). As miRNAs are posttranscriptional gene regulators, identification and expression profiling constitute core elements of miRNA studies but it appears less informative of their specific role in diverse biological processes. With this notion, the bioinformatically predicted target genes for selected miRNAs were systematically filtered from on line data bases and investigation of the spatio - temporal expression pattern of the miRNAs and the predicted targets in the same sample was conducted and gave a clue for the miRNA-mRNA interaction. Then after, the attempt to dissect the specific functions of miR-99a and miR-100 commenced with the validation of their interaction with Bovine homologue tribbles 2 (TRB2) using luciferase reporter assay. The result indicated that miR-99a and miR-100 potentially down regulate TRB2 gene which is the negative regulator of the cell cycle, and by so doing these miRNAs may promote cumulus cell proliferation.

In parallel and complementary to the above experiments, the potential role of microRNAs in the pathophysiology of the uterus in the context of bovine subclinical endometritis was investigated as there is precisely orchestrated interplay between the ovary and the uterus through the gonadotropin hormones. Subclinical endometritis is one of the most prevalent uterine diseases which is associated with reduced fertility in cattle. It is a well established fact that the inflammatory response to post partum bacterial infection during subclinical endometritis is associated with the expression of

pro-inflammatory mediators which affect embryonic development by acting either on the oocyte, developing embryo, endometrium and/or hypothalamic-pitutary-gonadal axis. The already suggested model for the development and progression of subclinical endometritis appears inconclusive with the emerging role of miRNAs as potent regulators of uterine gene expression in human and mouse models. Then, hypotheses was set and tested using genome wide RT² miRNA PCR array consisting of 354 well characterized human miRNA primers and uterine cytobrush samples taken from cows with subclinical endometritis and healthy. The approach that was used to unravel the molecular mechanism that lead incidence of subclinical endometritis to loss of fertility, from the miRNA perspective was entirely integrative. Specific class of miRNAs were observed to be aberrantly expressed in endometrium of cows with subclinical endometritis. These aberrantly expressed miRNAs were evidenced to target set of genes that constitute pathways and biological functions which are inherent to the endometrium during estrous cycle and pregnancy. This has an interesting implication in that up or down regulation of miRNAs during subclinical endometritis could result in dysregulation of target genes that may trigger malfunctioning of the pathways and biological functions that these genes constitute. Once the tight uterine gene regulation is in peril, imbalance in hypothalamic-pitutary-gonadal axis could occur and negatively affect folliculogenesis, oocyte maturation, embryo implantation and development and uterine receptivity in which the combined or individual effect describe loss of fertility in cattle.

7. Zusammenfassung

Der Entwicklungsprozess von Säugetieren beginnt bereits während der Oogenese. Die Eizellen Maturation ist dabei ein maßgebender Prozess, bei dem die Eizelle ihre wesentlichen Fähigkeiten zur schrittweisen Unterstützung der nachfolgenden Entwicklungsstufen erlangt. Dieser Prozess ist an den komplexen Ereignissen der nuklearen und cytoplasmatischen Maturation beteiligt, die sowohl an der Speicherung von mRNAs, Proteinen und Transkriptionsfaktoren einschließt als auch die chromosomale Segregation. Während der Eizellen Maturation tritt eine selektive Transkription und Translation von Transkripten auf, wobei die Rolle der miRNAs als posttranskriptionelle Genexpression Regulatoren ziemlich offensichtlich zu sein scheint. Dabei sind die ersten und wichtigsten Schritte die Identifikation sowie die Expressionsanalysen der miRNAs, um die Rolle dieser posttranskriptionellen Regulatoren während der präimplantären Embryonalentwicklung beim Rind besser zu verstehen. Deshalb wurde die Identifikation und das Expressionsprofiling der miRNAs während der invitro Maturation der Eizelle mit Hilfe von heterologen miRNA Microarrayplattformen (Mensch, Maus und Ratte) durchgeführt. Da die miRNAs posttranskriptionelle Genregulatoren sind, stellen sowohl die Identifikation als auch das Expressionsprofiling die Kernelemente der miRNA Studien dar. Allerdings scheinen diese weniger informativ für ihre spezifische Rolle, während der unterschiedlichen biologischen Prozesse, zu sein. Daher wurden die bioinformativ vorhergesagten Targetgene für die ausgewählten miRNAs systematisch aus online Datenbanken gefiltert. Zudem wurde eine Untersuchung des räumlich- zeitlichen Expressionsmodells der miRNAs und ihren vorhergesagten Targets in denselben Proben durchgeführt, dies soll Rückschlüsse auf die miRNA- mRNA Interaktion geben. Ein weiteres Experiment wurde durchgeführt, um die spezifischen Funktionen von miR-99a und miR-100 zu analysieren, dabei wurde die Interaktion mit dem Bovinen Homologen Tribbles 2 Gen (TRB2) mittels eines Luciferease Reporterassays überprüft. Die Ergebnisse zeigen, dass miR-99a und miR-100 möglicherweise TRB2, einen negativen Regulator des Zellzyklus, herunter regulieren. Somit könnten die miRNAs die Kumuluszellproliferation anregen.

Parallel und ergänzend zu dem vorherigen Experiment, wurde die mögliche Rolle der miRNAs bei der Pathophysiologie des Uterus im Zusammenhang mit der subklinischen

Endometritis des Rindes untersucht. Da ein präzise instrumentalisiertes Wechselspiel zwischen den Ovarien und dem Uterus durch das Gonadotropine Hormon bestehen könnte. Die subklinische Endometritis ist eine der am häufigsten verbreiteten uterinen Krankheit, die in Verbindung mit einer reduzierten Fruchtbarkeit beim Rind steht. Ein bekannte Tatsache ist, dass die Entzündungsreaktion auf bakterielle Infektionen post partum während einer subklinischen Endometritis mit einer Expression von pro-entzündlichen Mediatoren einhergeht, die die embryonale Entwicklung beeinflusst, da sie sowohl auf die Eizelle, die Entwicklung des Embryos, das Endometrium als auch die hypothalamische- hypophysäre- gonadäre Achse einwirken. Das bereits existierende Modell zur Entwicklung und Verlauf der subklinischen Endometritis scheint mit dem Auftreten der miRNAs als mögliche Regulatoren der uterinen Genexpression im menschlichen sowie Mausmodell nicht mehr ausreichend zu sein. Daher wurde eine neue Hypothese aufgestellt und diese mittels eines RT^2 miRNA PCR Arrays, das aus 354 Vetiefungen besteht, die menschlichen miRNA Primer enthalten, an uterinen Cytobrush Proben, die sowohl von gesunden Kühen als auch Kühen mit subklinischer Endometritis stammen, überprüft. Der Ansatz, der hier genutzt wurde, um die molekularen Mechanismen zu entschlüsseln, die zu einer subklinischen Endometritis und somit zu einem Verlust der Fruchtbarkeit führen, war völlig integrativ vom Gesichtspunkt der miRNA aus. Spezifische Klassen von miRNAs zeigten eine abweichende Expression im Endometrium von Kühen mit subklinischer Endometritis. Mit Hilfe dieser abweichenden Expression der miRNAs konnte gezeigt werden, dass diese eine Reihe von Genen steuern, die die Pathways und biologische Funktionen unterstützen, welche zum Endometrium während dem Östruszyklus und der Schwangerschaft gehören. Eine interessante Schlussfolgerung lässt sich daher aufstellen. Die hoch oder herunter Regulierung der miRNAs während der subklinischen Endometritis könnte zu einer Dysregulierung der Targetgene führen, weshalb die Pathways und biologischen Funktionen, für die diese Gene verantwortlich sind, versagen könnten. Wenn die enge uterine Genregulation in Gefahr geraten, könnte ein Ungleichgewicht in der hypothalamischen- hypophysären- gonadären Achse auftreten und die Entwicklung der Follikel, die Eizellen Maturation, die Embryo Implantation, dessen Entwicklung sowie die uterine Aufnahmefähigkeit negativ beeinflussen, so dass diese einzeln oder kombiniert zu einem Verlust der Fruchtbarkeit beim Rind führen könnten.

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Acknowledgement

I am sincerely grateful to my advisor Prof. Dr. Karl Schellander for his intellectual and material support to make this work possible. His remarkable vision for scientific future and tremendous passion for work makes him my great mentor and advisor. Besides, his morally sound guidance and caring behavior that are optimally combined with delicacy for science and dedication for work, I believe, helped me to transform my self. I have also enjoyed his genuine advice, critics, inspiration and help which often go beyond academic mentoring. I truly cherish every moment I had with Prof. Dr. Karl Schellander.

I would like to thank Prof. Dr. Brigitte Petersen for her willingness to co-supervise this study.

I like to take this opportunity to express many thanks to PD. Dr. Dawit Tesfaye for every comment and support during my study. It is also my pleasure to acknowledge the discussions we made and the ideas he raised constitute basic elements for successful accomplishment of this study. Moreover, I appreciate his kind cooperation and technical guidance during the course of my research.

A special note of appreciation is extended to Dr. Ernst Tholen for his valuable comments to help me follow appropriate statistical procedure and draw a valid inference which constitute the core of every scientifically sound experiment. I have also enjoyed his open minded and fun loving personality which usually stimulates a healthy working environment.

I am deeply indebted to Prof. Dr. Christian Looft for his persistent motivation, advice and constructive scientific comments to refine my work to the level now it is. Moreover, I am very much thankful for every academic and non-academic help I often get. I also appreciate his friendly and motivating approach to help me attain better in my level.

I am sincerely grateful to Dr. Michael Hölker and Ms. Franca Rings for providing valuable experimental samples. Many thanks go to Ms. Helga Brodeßer for her sincere and dedicated help in the cell culture lab. Especial thanks goes to all technical

assistants: Ms. Nadine Leyer, Ms. Katrin Bauch, Ms. Steffi Heußner, Ms. Jessica Gonyer, Ms. Birgit Koch-Fabritius for providing me laboratory assistance and creating good working environment.

I would like to extend many thanks to Ms. Christine Große-Brinkhause and Ms. Hanna Heidt not only for translating the abstract and summary of my dissertation from English to German but also for wellcoming me with a spirit of coopertation for every help I required.

As molecular biology is always a team work , I would like to thank all my colleagues: Dr. Nasir Ghanen, Dr. Parinya Wilaiphan, Dr. Abdullah Mohammadi-Sangchesmeh, Dr. Saowaluck Yammuen-art, Dr.Alemu Regassa, Ms. Anke Brigs, Mr. Heiko Buschbell, Dr. Dessie Salilew, Dr. Munir Hossein, Dr. Ulas Mehmer Cinar, Ms. Simret Betsha, Ms. Christiane Neohoff, Ms. Maren Julia Pröll, Ms. Walaa Abd-El-Neby, Mr. Ahmed Yehia Gad, Mr. Luc Frieden, Ms. Eva Held, Mr. Huitao Fan, Mr. Ijas Ahmed, Mr. Muhammad Jasim Uddin, Ms. Pritam Bala Sinha, Ms. Autchara Kayan, Mr. Watchara Laenoi, Ms. Kanokwan Kaewmala, Mr. Ariful Islam, Mr. Md. Mahmodul Hasan Sohel and Sina Seifi for sharing experiences, challenges and also for having good times together.

My thanks go to Mr. Peter Müller who always provided me solution for every problem I had with my computer and network during my study. I extend my sincere thanks to Ms. Bianca Peters and Ms. Ulrike Schröter for every administrative support they kindly offered me.

I am deeply indebted to my parents for their unlimited moral and material support to keep me in the right academic truck. My mother deserves my heart felt appreciation for trying to install in my childhood mind a roadmap of achievable long journey to be ‘*academician’*. My especial appreciation also goes to my father for his persistent attempt to pass on to me his own life philosophy such as commitment to goals, respect for others and ‘never give up’. I also extend my appreciation to my sisters and brothers for their moral and material support.

I owe my great thanks to my wife, Ms. Abebayehu Workie, who make marvellous contribution to my scientific career and her constant support makes what happens now. I extend many thanks to my lovely daughter, Fenet Dagnachew, for the priceless love and happiness she used to bring to me along with this work.

Moreover, I want to thank Dr. Tiegist Dejene and Mr. Tsegaw Seyoum for sharing ideas, views and also having good time together.

I would like also to extend many thanks to Ambo University, the university where I have been working in Ethiopia, for every support I was offered to finalize my study. My last but most important thanks go to German Academic Exchange Service (DAAD) for the financial support which covered all living expenses for me and my family. I would like to extend special thanks to Ms. Jasmin Ramershoven (DAAD staff) for her unlimited support to relocate my family (my daughter and wife) from Ethiopia to Germany.

Above all, I thank the almighty GOD.

4. Publications

Tesfaye D, **Worku D,** Rings F, Phatsara C, Tholen E, Schellander K, Hoelker M. (2009) Identification and expression profiling of microRNAs during bovine oocyte maturation using heterologous approach. Mol. Reprod. Dev. 76:665-77

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5. Trainings

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