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der Rheinischen Friedrich – Wilhelms – Universität Bonn

MicroRNAs in the bovine ovary and placentas derived from in vivo, in vitro and nuclear transfer pregnancies

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 am 23. September 2010

von

Md. Munir Hossain

aus

Barisal, Bangladesh

Zugl.: ITW; Bonn, Univ., Diss., 2010

D 98

Referent	: Prof. Dr. Karl Schellander
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Tag der mündlichen Prüfung	: 3rd of December 2010

Dedicated to my family

MicroRNAs in the bovine ovary and placentas derived from in vivo, in vitro and nuclear transfer pregnancies

MicroRNAs are the major class of gene regulating molecules playing pivotal roles at post-transcriptional level. Identification and expression profiling are the initial steps to understand their regulation of biological processes. Despite increasing efforts in miRNAs characterization in different species, little is known in the bovine reproductive tissues especially in ovary and placenta. Two subsequent studies were carried out to the expression of miRNAs in bovine ovary and Day-50 placenta derived from different sources of pregnancy. The first study aims to identify and characterize miRNAs in bovine ovary through cloning, expression analysis and target prediction. The constructed miRNA library revealed cloning of 50 known and 24 novel miRNAs. Among these, 38 were new miRNAs which were derived from 43 distinct loci with characteristic secondary structure. Most of the miRNAs were cloned multiple times and thereby reflecting their expression level and potential role in the ovary. Analysis of identified miRNAs in different intra-ovarian structures and other tissues reveals their stage and tissue specific expression patterns. Furthermore, in silico target prediction and Gene Ontology analysis of the targets genes identified several biological processes and pathways underlying the ovarian function. Results of this study suggest the presence of miRNAs in the bovine ovary; thereby elucidate their potential role in regulating diverse mechanisms underlying the ovarian functionality.

The second study aimed to elucidate the difference in expression profile of miRNAs in the placenta at day 50 derived from Somatic cell nuclear transfer (SCNT), in vitro production (IVP) and artificial insemination (AI) pregnancies by quantifying 377 miRNAs. The study reveals a massive deregulation of miRNAs which were poorly reprogrammed and affected as large chromosomal cluster as well as miRNA families in the NT and IVP placenta compared to that of AI. Furthermore, cell specific localization miRNAs in the expanded blastocysts and expression profiling in different developmental stages of embryos and placenta identified that the major deregulation of miRNAs arises at day 50 of NT and IVP pregnancies. This deregulation were found to be less dependent on global DNA methylation, rather aberrant miRNA processing molecules were evidenced. Among them, observed down regulation of AGO2 could be a reason for global down regulation of miRNAs in the NT or IVP placenta. Identified deregulation of miRNAs might associate to the abnormal placentogenesis in NT or IVP pregnancies, which are the results of aberrant genetic and epigenetic modification. Result of this study will help to move one step closer towards improving the efficiency of nuclear transfer pregnancy.

Altogether, the present study has discovered miRNAs in the bovine ovary and elucidated the pattern of expression of miRNAs along with their regulatory mechanism in the placenta derived from pregnancies of various origins.

Untersuchung von MicroRNAs in bovinen Ovarien und Plazenten aus geklonten, in vivo und in vitro erzeugten Trächtigkeiten

MicroRNAs gehören zur großen Klasse der genregulierenden Moleküle und spielen eine bedeutende Rolle auf der posttranskriptionellen Ebene. Die Identifikation und die Erstellung von Expressionsprofilen sind die ersten Schritte für ein besseres Verständnis der miRNA und ihrer Beteiligung an der Regulierung von biologischen Prozessen. Trotz der zunehmenden Charakterisierung der miRNA in verschiedenen Spezies, sind kaum Untersuchungen in bovinen Ovarien und Plazenten bekannt. In zwei Studien wurde die miRNA Expression in bovinen Ovarien und in Plazenten am Tag 50 der Trächtigkeit, in unterschiedlich erzeugten Schwangerschaften untersucht. Das Ziel der ersten Studie war die Identifizierung und Charakterisierung von miRNAs in klonierten bovinen Ovarien, Expressionsanalysen sowie Target-Vorhersagen. Die konstruierte miRNA-Bibliothek ergab 50 bekannte und 24 neue miRNAs. Unter diesen waren 38 neue bovine miRNAs die von 43 eindeutigen Loci abgeleitet werden konnten, die eine charakteristische sekundäre Struktur zeigten. Durch die mehrfache Klonierung der meisten miRNAs, konnte ihr Expressionsniveau in den Ovarien erfasst werden. Analysen von miRNAs in unterschiedlichen intra-ovariellen Strukturen sowie anderen Geweben zeigten ihr phasen- und gewebsspezifisches Expressionsmuster. Des Weiteren konnte durch bioinformatische Auswertungen und Gen Ontology Analysen der Target Gene verschiedene biologische Prozesse und Pathways der Ovarienfunktion identifiziert werden. Die Ergebnisse dieser Studie deuten auf das Vorhandensein von miRNAs in bovinen Ovarien hin und verdeutlichen die potenzielle Bedeutung in der Regulierung diverser Mechanismen der Ovarienfunktion. Die zweite Studie sollte die Unterschiede von Expressionsprofilen von miRNAs in Plazenten am Tag 50 der Trächtigkeit von SCNT, IVP und AI Schwangerschaften durch Quantifizierung von 377 miRNAs aufklären. Die Studie zeigte eine starke reduzierte Expression und eine geringe Reprogrammierung der miRNAs in NT und IVP im Vergleich zu AI. Diese miRNAs gehören vermutlich zu einer miRNA Familie in einem chromosomalen Cluster. Des Weiteren zeigten zellspezifische Lokalisationen von miRNAs in der expandierenden Blastozyste und Expressionsprofile von unterschiedlichen Entwicklungsstadien im Embryo und in der Plazenta, dass die wichtigsten Deregulierungen von miRNAs am Tag 50 der Trächtigkeit in NT und IVP entstehen. Diese Deregulierung erwies sich als weniger abhängig von einer globalen DNA-Methylierung, vielmehr wurde eine Abweichung in den miRNA-Prozessgenen belegt. Von diesen könnte die reduzierte Expression von AGO2 die Ursache für die globale Deregulierung der miRNAs in NT oder IVP Plazenten sein. Die identifizierten Deregulationen der miRNAs könnten im Zusammenhang mit abnormaler Plazentogenese in NT oder IVP Trächtigkeiten stehen. Die Ursachen für Abnormalitäten in der Plazentogenese liegen in genetischen und epigenetischen Modifikationen.

Zusammenfassend konnte durch diese Studien miRNAs in bovinen Ovarien identifiziert werden und Expressionsmustern der miRNAs sowie ihre regulierenden Mechanismen in der Plazenta von unterschiedlichen Trächtigkeiten beschrieben werden.

List of abbreviations

AGO	: Argonaute
AI	: Artificial insemination
ATP	: Adenosine triphosphate
ATPase	: Adenosine triphosphatase
BNC	: Binucleate cell
BSA	: Bovine serum albumin
Bta or btau	: Bos taurus
cDNA	: Complimentary DNA
Ch	: Chromosome
CL	: Corpus luteum
CLSM	: Confocal laser scanning microscope
CSH	: Chorionic somato mommotropin hormone
ddH_2O	: Distilled & deionized water
ddNTP	: Dideoxyribonucleoside triphosphate
dH_2O	: Deionized or distilled water
DIG	: Digoxigenin
DMAP	: Dimethyl amino pyridine
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: deoxyribonucleoside triphosphate (usually one of dATP, dTTP, dCTP and dGTP)
dPAGE	: Denaturing Polyacrylamide gel electrophoresis
dsRBD	: Double stranded RNA binding domain
DTT	: Dithiothreitol
E.coli	: *Escherichia coli*
ECM	: Endothelial extra cellular matrix
EDTA	: Ethylenediaminetetraacetic acid (powder is a disodium salt)
eNOS	: Endothelial nitric oxide synthetase
EST	: Expressed sequence tags
ET	: Embryo transfer
EtBr	: Ethidium bromide
EtOH	: Ethanol

GCC : Germ cell cluster
GO : Gene ontology
G1 : Gap 1
hCG : Human chorionic gonadotropin
ICM : Inner cell mass
IGF2 : Insulin-like growth factor
INFt : Interferon tau
IPTG : Isopropylthio-ß-D-galactoside
ISGs : Interferon tau stimulated genes
ISH : In situ hybridization
IVC : In vitro culture
IVF : In vitro fertilization
IVP : In vitro production
kb : Kilobases
KIT : c-kit proto-oncogene
KO : Knock out
LH : Lutinizing hormone
LOS : Large offspring syndrome
M : Molar
m7G : 7-methyl G-cap
mA : Milliamperes
mg : Milligrams
$MgCl_2$: Magnesium chloride
mII : Metaphase II
miRNA : MicroRNA
miRNP : Micro-ribonucleoprotein
mM : Milimolar
mmole : Milimole
MMPs : Matrix metalloproteinases
mRNA : Messenger RNA
MRP : Maternal recognition of pregnancy
MW : Molecular weight
MZT : Maternal zygotic transition
ncRNA : Non-coding RNA

NT : Nuclear transfer
PCR : Polymerase chain reaction
PE : Pre-aclampsia
PGF2α : Prostaglandin F2 alfa
pmol : Picomolar
rapiRNA : Repeat associated piwi interacting RNA
rasiRNA : Repeat associated small interfering RNA
RISC : RNA induced silencing complexes
RNA : Ribonucleic acid
RNP : Ribonucleoprotein
rpm : Revolutions per minute
rRNA : Ribosomal RNA
RT : Reverse transcription
santRNA : Small antisense RNA
SCNT : Somatic cell nuclear transfer
SDS : Sodium dodecyl sulfate
siRNA : Small interfering RNA
SNP : Single nucleotide polymorphism
TAE : Tris-acetate buffer
TBE : Tris- borate buffer
TE : Trophectoderm
TEB : Tris- EDTA buffer
Thr : Threonine
tncRNA : Tiny non-coding RNA
tRNA : Transfer RNA
UTR : Untranslated region
UV : Ultra-violet light
v/v : Volume per volume
W : Watts
w/v : Weight per volume
X-gal : 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside
ZP : Zona pellucida
5mC : 5-methyl cytosine

List of tables

List of figures

1 General introduction

1.1 miRNAs in the bovine ovary

Folliculogenesis is the result of series of complex and coordinated processes, which include morphological and functional changes in different types of follicular cells and their interactions. Sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis are dynamically regulated events that occur on a cyclical basis within the ovary. These processes are under control of closely coordinated endocrine and paracrine factors to develop a number of ovulatory follicles that are species and breed dependent (Hunter et al. 2004). All those events entail substantial changes and balance between many processes such as the cell cycle, cellular growth, proliferation, differentiation, angiogenesis, steroidogenesis and atresia to determine the ultimate fate of follicles. All of these steady state cyclic changes are controlled by tightly regulated expression and interaction of a multitude of genes in different compartments of the ovary (oocyte, cumulus granulosa, mural granulosa cells and theca cells) to facilitate oocyte development (Bonnet et al. 2008).

In oogenesis and embryo development, there are different mechanisms regulating gene expression at the post-transcriptional level. These include events of mRNA adenylation and deadenylation, the CAP structure at the 5' end of the mRNA and the effective action of mRNA binding factors (Eichenlaub-Ritter and Peschke 2002, Piccioni et al. 2005). Recently, a new post-transcriptional gene regulation is opened up after promising discovery of hundreds of miRNAs in different mammalian species. Diverse expression pattern of miRNAs and high number of their potential target mRNAs suggests their involvement in the regulation of various developmentally related genes at post-transcriptional level (Alvarez-Garcia and Miska 2005, Ambros 2004, Bartel 2004, Chen and Rajewsky 2007, Lai 2003, Lau et al. 2001, Plasterk 2006). The tiny (18-24 nt in length) and single-stranded miRNAs are derived from primary transcripts termed as "pri-miRNAs", which have an RNA hairpin structure of 60-120 nt with a mature miRNA in one of the two strands. This hairpin in turn is cleaved from the pri-miRNA in the nucleus by the double-strand-specific ribonuclease, Drosha (Lee et al. 2002). The resulting precursor miRNA (or pre-miRNA) is transported to the cytoplasm via a process that involves Exportin-5 (Yi et al. 2003) and subsequently cleaved by Dicer

(Lee et al. 2003) to generate a short, double-stranded (ds) RNA duplex. One of the strands of the miRNA duplex is incorporated into a protein complex termed RNA induced silencing complex (RISC). RISC is guided by the incorporated miRNA strand to mRNAs containing complementary sequences in 3´ untranslated region, which primarily results in inhibition of mRNA translation (Pillai et al. 2005). Those mRNAs which are repressed by miRNAs are further stored in the cytoplasmic foci called P-bodies (Liu et al. 2005a, Liu et al. 2005b, Rehwinkel et al. 2005).

Several studies have shown the involvement of miRNAs in animal development. Inhibition of miRNA biogenesis has resulted in developmental arrest in mouse and fish (Bernstein et al. 2003, Giraldez et al. 2005, Wienholds et al. 2003). Similarly, loss of important miRNA processing machinery, Dicer1 resulted in female infertility in mouse (Otsuka et al. 2007, Otsuka et al. 2008). Targeted knockdown of mir-17-5p and let-7p in wild type mice revealed impaired corpus luteum (CL) angiogenesis and decreased serum progesterone levels. In the same study, injection of these miRNAs revealed the restoration of vasculature within the CL and increased progesterone levels (Otsuka et al. 2008). In addition to loss-of-function approach, efforts have been done to identity miRNAs by cloning. For example, small RNA-cDNA libraries from the ovaries of 2-wk-old and adult mice have generated a number of miRNAs with potential role in ovarian function (Ro et al. 2007b). Subsequent study on ovarian miRNAs in mouse showed the post-transcriptional regulation of CtBP1 gene by miR-132 and miR-212 in cultured granulosa cells (Fiedler et al. 2008). In addition to miRNA, several other non-coding small RNAs including rapiRNAs, napiRNAs, rasiRNAs and tncRNAs are identified and reported in different species (Ambros et al. 2003b, Aravin et al. 2003, Reinhart et al. 2002).

Bio-informatic approaches and construction of small RNA-cDNA libraries from bovine adipose tissue, mammary gland, embryo, thymus, small intestine, mesenteric lymph node and abomasum lymph node have identified most of presently annotated bovine miRNAs (Coutinho et al. 2007, Gu et al. 2007). The number of bovine miRNAs (117) in comparison to Human (695), Chicken (475), Mouse (488), Chimpanzee (595), Rhesus Monkey (463) in miRBase 12.0 are inadequate to disclose global miRNAs regulation of gene expression for various biological functions and disease conditions. Recently, the dynamics of miRNAs expression during bovine oocyte maturation in vitro

have been studied using heterologous approach (Tesfaye et al. 2009). This together with previous report in mouse supports the possible role of miRNAs during follicular development and oocyte growth. Identifying entire set as well as ovary-specific miRNAs may lead to understanding miRNA-guided gene regulation in the ovary. So, the present study has been conducted with the objective to get insight into the miRNA population present in bovine ovary by investigating their characteristics, expression pattern and features of their target genes.

1.2 miRNAs in the placentas derived from in vivo, in vitro and somatic cell nuclear transfer pregnancies

Animal Cloning is a break-through technology with emerging potential applications in agricultural and biomedical research, but the technology is hindered by very low rates of live birth due to high incidence of placental abnormalities leading to embryonic losses (De Sousa et al. 2001, Hill et al. 2000, Humpherys et al. 2002, Ogura et al. 2002, Ono et al. 2001, Stice et al. 1996, Yang et al. 2007). The major source of these abnormalities is thought to be due to genetic and epigenetic modifications arise from improper reprogramming of the donor cell after nuclear transfer, but the effect last long throughout the embryonic development (Jouneau and Renard 2003, Latham 2004, Piedrahita et al. 2004, Santos and Dean 2004, Smith and Murphy 2004, Tamada and Kikyo 2004).

Incomplete epigenetic reprogramming of the donor cell nucleus by the cytoplasm of the oocyte during early development leads to postimplantation lethality with ultimate placental defects (Lambertini et al. 2008, Niemann et al. 2008, Wagschal and Feil 2006, Yang et al. 2007). Key mechanisms underlying this epigenetic reprogramming are DNA methylation, histone remodeling and telomere maintenance, which are involved in the control of gene expression, X chromosome inactivation and genomic imprinting (Holmes and Soloway 2006, Li et al. 1993, Perecin et al. 2009), but all mechanisms are not contributing equally to the embryonic and extra-embryonic lineage (McGrath and Solter 1984, Oudejans et al. 1997, Surani et al. 1984, Wagschal and Feil 2006). Specially, genomic imprinting has been shown to be less or not dependent on DNA methylation in the placenta for its somatic maintenance than in the embryo. Rather, placenta-specific imprinting involves repressive histone modifications and non-coding

RNAs (Brockdorff 2002, Higashimoto et al. 2002, Lewis et al. 2004, Li et al. 1993, Sado et al. 2000, Sado and Ferguson-Smith 2005, Yatsuki et al. 2004).

When considering non-coding RNAs, miRNAs a recently discovered class of small RNAs appeared as first and foremost epigenomic tool or modifier that regulate gene expression epigenetically at the post-transcriptional or transcriptional level and were found to play important roles including but not restricted to cell proliferation, apoptosis, diseases and differentiation during mammalian development (Ambros 2004, Bartel 2004, Kloosterman and Plasterk 2006). They were found to be targeted by epigenetic modification and eventually controlling epigenetics and some imprinted miRNAs found to undergo subsequent epigenetic reprogramming in mouse embryos (Cui et al. 2009, Kircher et al. 2008, Williams et al. 2007). Many of the miRNAs have been predicted and found to have pivotal roles in controlling DNA methylation, regulating chromatin structure and controlling telomere recombination (Benetti et al. 2008a, Benetti et al. 2008b, Fabbri et al. 2007, Guil and Esteller 2009, Lewis et al. 2005, Lujambio and Esteller 2007, Ting et al. 2008, Valeri et al. 2009). Interestingly, they could be imprinted like genes and many of X-linked microRNAs escape meiotic sex chromosome inactivation (Kanellopoulou et al. 2009, Song et al. 2009).

Taking all promising information together, this experiment has been conducted to answer even more deep question by quantifying 377 miRNAs in the placenta derived from artificial insemination (AI), in vitro produced (IVP), nuclear transfer (NT) pregnancy at day 50 and in the donor fibroblast cells along with their detailed characterization. Present study hypothesized that aberrant expression of transcriptional regulatory miRNA molecules during redifferentiation for placentogenesis leading to abnormal genetic and epigenetic modification in the placenta of cloned conceptuses are likely caused by re-programming errors after cloning and in vitro culture. The objectives of the study were to identify the expression pattern of miRNAs in the placentas derived from in vitro and nuclear transfer pregnancies compared to the placenta from the in vivo or artificial insemination pregnancies.

2 Literature review

This chapter addresses the fundamental aspects and current knowledge on miRNAs mediated regulation of bovine ovarian development, folliculogenesis, ovarian steroidogenesis, fertilization, early embryonic development, implantation and placental functions in addition to the overview of miRNAs biosynthesis and their mechanisms of regulation of gene expression. Sound ovarian physiology and folliculogenesis is leading to ovulation of competent oocyte, which is directly related to successful fertilization, embryogenesis and subsequently pregnancy outcome. So, beside the major investigations on miRNAs which are carried out in the ovary and placenta derived from artificial insemination, in vitro production and nuclear transfer pregnancy for this dissertation, miRNAs in other associated sequential events are also presented to maintain logical flow and their inter-relationship. However, a large portion of this review is focused on basics of ovary and placenta in relation to miRNAs. The review concludes in the end of respective sections with a problem statement and the rationale for conducting the research contained within this dissertation.

2.1 Brief overview of miRNAs

Genomic research over the past few years has exposed a striking result in the discovery of noncoding RNAs (ncRNAs) as a representation of a substantial component of all metazoan genomes. Among the noncoding RNAs, microRNAs (miRNAs), so called because of their tiny size (~18-24 nucleotides long) currently represent a new class of small RNA molecules, those are receiving the highest interest as they inhibit translation and/or induce degradation of protein-coding mRNAs that contain complementary sequences to miRNAs (Gu et al. 2007, Ying and Lin 2006). miRNAs are first identified simply as a part of 'junk' RNA, in 1993 by Victor Ambros and his colleagues Rosalind Lee and Rhonda Feinbaum at Harvard University, while studying developmental mutants of the nematode *Caenorhabditis elegans*. During analyzing a putative 'protein', lin-4, a sequence regulating heterochronic temporal control of development, the research team has identified that lin-4 has no protein, but actually a short hairpin RNA. Since the discovery of the founding members of the microRNA family, lin-4 and let-7 (Lee et al. 1993, Reinhart et al. 2000, Wightman et al. 1993), hundreds of miRNAs have been identified in insects, plants, animals and viruses by small RNA cDNA library

construction, advance sequencing techniques and bioinformatic analysis (Berezikov et al. 2006, Lau et al. 2001, Lee et al. 2001, Stark et al. 2007). miRNAs were found to down regulate gene expression by base pairing with the 3′ untranslated regions (3′ UTRs) of target messenger RNAs (mRNAs) (Lee et al. 1993, Reinhart et al. 2000, Wightman et al. 1993). Advances in the analysis of this widespread class of miRNA molecules after initial discovery, has opened up a new layer of gene regulation in many organisms. Mechanism for the generation of miRNAs in the cells, their mode of regulation as well as the available methodological approaches to study such miRNAs mediated post transcriptional gene regulation are presented bellow.

2.1.1 Biogenesis of miRNAs and their regulation of gene expression

Following sections highlight the synthesis of miRNAs, from primary transcription up to formation of mature functional state through a group of processing factors and miRNAs mediated genetic or epigenetic regulation of post transcriptional gene expression.

2.1.1.1 Biogenesis of miRNAs

Most miRNA genes are transcribed by RNA polymerase II (Pol II) to generate a primary transcript called ‘primary miRNA’ (pri-miRNA), which can range in size from several hundred nucleotides (nt) to tens of kilobases (kb) (Cai et al. 2004, Lee et al. 2004) (Figure2.1). Like mRNAs, Pol II transcribed pri-miRNAs contain 5’ cap structures, are polyadenylated and may be spliced (Bracht et al. 2004, Cai et al. 2004). The tiny (18-24 nt in length) and single-stranded miRNA molecule, derived from this pri-miRNAs, having a RNA hairpin structure of 60-120 nt with a mature miRNA in one of the either two strands (Figure 2.1). The pri-miRNA is processed within the nucleus by a multi-core protein complex called the Microprocessor, which is composed of the RNAse III enzyme Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8/Pasha (Denli et al. 2004, Gregory et al. 2004, Han et al. 2004, Landthaler et al. 2004, Lee et al. 2003). This microprocessor cleaves the hairpin from the pri-miRNA in the nucleus (Lee et al. 2002). The resulting precursor miRNA (pre-miRNA) is transported to the cytoplasm via a process that involves binding of Exportin-5 to pre-miRNA through recognizing the 2-nt 3’overhang produced by RNAse III mediated cleavage (Yi et al. 2003). This pre-miRNA undergoes a cleavage mediated by

another RNAse III enzyme, Dicer, which interacts with the dsRBD proteins TRBP to generate the mature ~22nt miRNA:miRNA* duplex (Chendrimada et al. 2005, Forstemann et al. 2005, Hutvagner et al. 2001, Jiang et al. 2005a, Ketting et al. 2001, Lee et al. 2003, Lee et al. 2006, Saito et al. 2005). Subsequently, TRBP/Loquacious recruits the Argonaute protein and together with Dicer they form a trimeric complex that initiates the assembly of the RNA-induced silencing complex (RISC), a ribonucleoprotein (RNP) complex (Gregory et al. 2005, Maniataki and Mourelatos 2005). Based on the relative stability of the two ends of the duplex, the miRNA strand, with relatively unstable base pairs at the 5' end, remains incorporated into the RISC, whereas the passenger strand, or miRNA* strand, is degraded (Leuschner and Martinez 2007, Matranga et al. 2005, Schwarz et al. 2003).

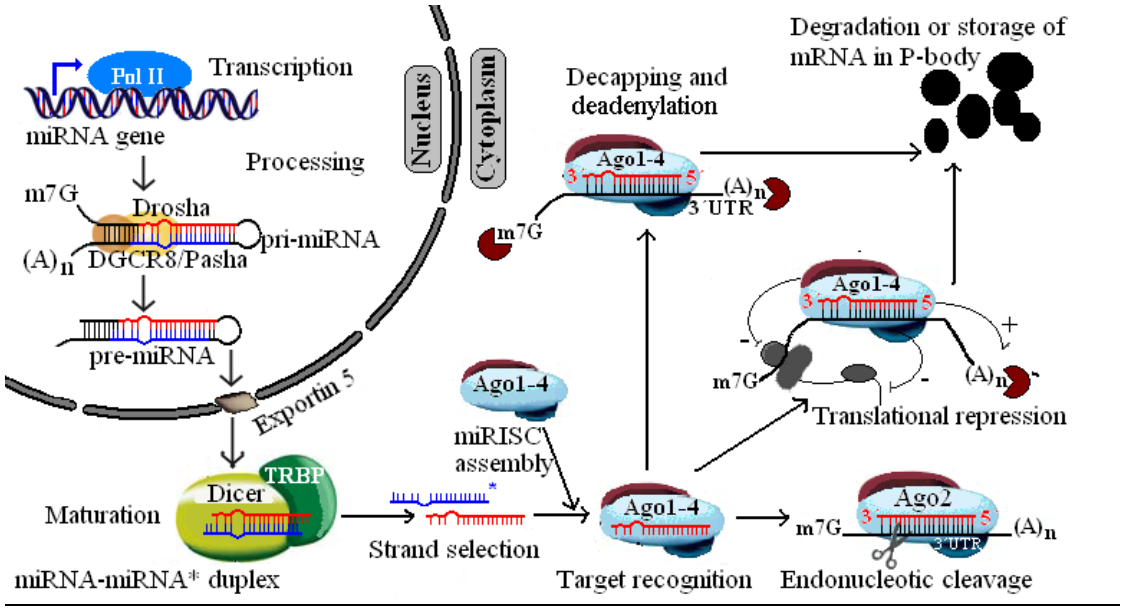


Figure 2.1: Biogenesis of miRNAs and their mechanism of gene regulation.
Argonaute proteins 1-4 (Ago1-4), inhibition of initiation of translation (-), Promoting deadenylation (+), 7-methyl-G cap (m^7G)

Recently, it is proposed that an alternative pathway also involved in microRNA biogenesis from the pre-miRNA hairpin to the mature functional miRNA (Diederichs and Haber 2007)(Figure 2.2) where, Ago2 has been found as a highly specialized member of the Argonaute family with an essential nonredundant Slicer-independent function within the mammalian miRNA pathway (O'Carroll et al. 2007). Ago2-mediated pre-miRNA cleavage has been observed in the processing of miRNAs derived from the 5′-arm of the pre-miRNA hairpin having no mismatches at the immediate

cleavage site (Diederichs and Haber 2007, Han et al. 2006). The product of Ago2-mediated pre-miRNA cleavage (ac-pre-miRNA), was found to be an intrinsic on-pathway intermediate during miRNA biogenesis and hence a substrate to Dicer, or it could be a byproduct which cannot be further processed toward the mature miRNA. These data identify the ac-pre-miRNA as a pathway intermediate in miRNA biogenesis that is generated from the pre-miRNA by Ago2 and serves as a substrate for Dicer to mature into the active miRNA. Although some microRNAs are processed by Drosha (also known as Rnasen), its maturation does not require Dicer. Instead, the pre-miRNA becomes loaded into Ago and is cleaved by the Ago catalytic centre to generate an intermediate 3′ end, which is then further trimmed and processed into mature miRNA (Cheloufi et al. 2010). So, Ago2-mediated cleavage of pre-miRNAs, followed by uridylation and trimming, generates functional miRNAs independently of Dicer (Cifuentes et al. 2010).

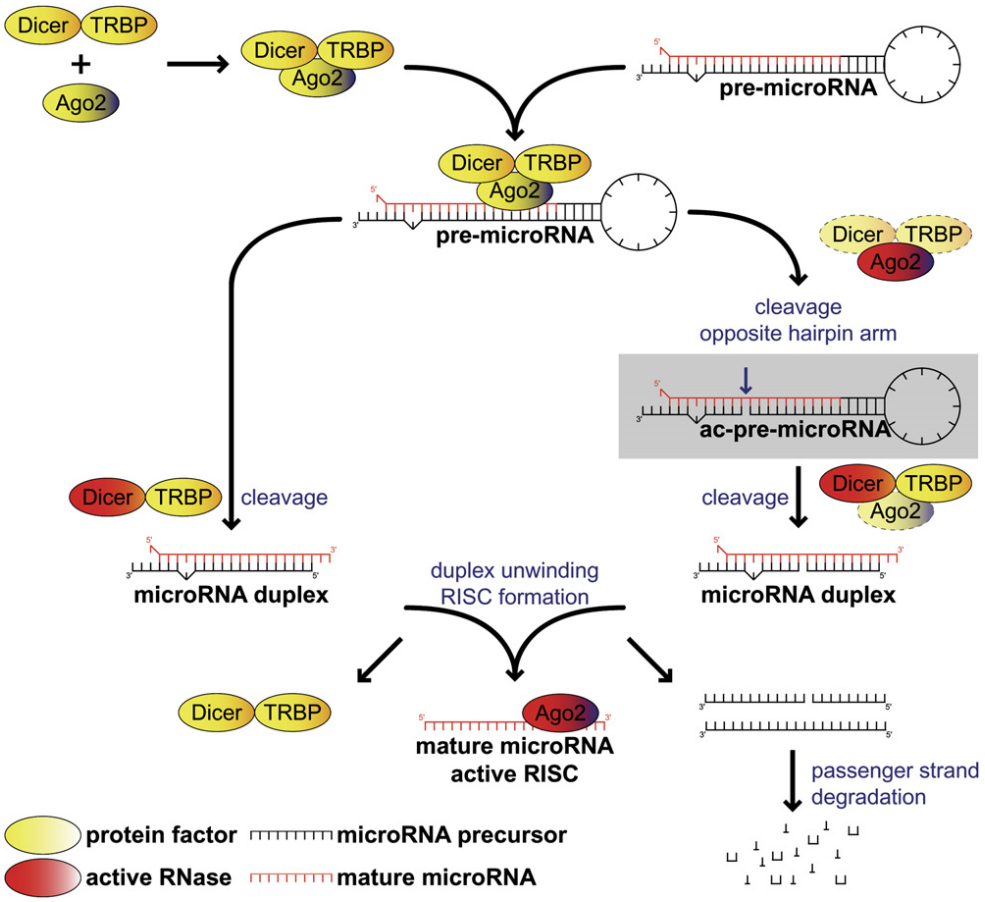


Figure 2.2: Alternative model of miRNA processing including the ac-pre-miRNA. After nuclear export, the pre-miRNA binds to a preformed complex of Dicer, TRBP and Ago2 to build the RISC-Loading Complex (RLC). This model of miRNA processing includes an additional endonuclease step in

which Ago2 cleaves the pre-miRNA within the RLC generating the nicked ac-pre-miRNA hairpin (shown in gray box). The impact of Dicer and TRBP on the Ago2 cleavage step, as well as the influence of Ago2 to Dicer cleavage, have not been determined, these proteins are depicted with a dashed outline. Since miRNA maturation is diminished but not completely abrogated in Ago2-KO MEF cells, a less efficient salvage pathway is likely to exist that either omits the ac-pre-miRNA (left arm of the pathway) or generates it by using a different, unknown RNase. The left arm of the pathway is also likely used for miRNAs derived from the 3´ arm of the pre-miRNA and miRNAs with mismatches at the cleavage site. After cleavage by Dicer, the resulting miRNA duplex is unwound, Dicer and TRBP dissociate, the passenger strand of the miRNA duplex is degraded and the mature miRNA forms the RISC together with Ago2. The stabilization of mature miRNAs by Ago proteins is independent of this Ago2-specific processing step (modified from Diederichs and Haber 2007).

2.1.1.2 miRNA mediated regulation of gene expression

miRNAs incorporated within the RISC complex, direct RISC to downregulate expression of target mRNAs containing complementary sequences in 3´ untranslated region to 7- to 8-nt region of 5´ end of miRNA called seed sequence (Pillai et al. 2005) (Figure 2.1). Depending on the degree of complementarity between the miRNA and the target sequence, mRNAs are either cleaved or degraded (perfect or near perfect complementarity) or their translation is repressed (imprecise complementarity) (Hutvagner and Zamore 2002, Martinez and Tuschl 2004). The miRNP complex which is loaded onto the target mRNA exhibits direct or indirect effect in translational repression. Direct effects occur either through inhibition of initiation of translation through binding of Ago2 to m^7G (7-methyl-G cap) results in prevention of ribosome association with the target mRNA, or through inhibition of translation post-initiation, which includes premature ribosome drop off, slowed or stalled elongation and co-translational protein (Figure 2.1).

In addition to direct effects on translation (or protein accumulation), miRNPs can have other effects on targeted mRNAs, including promoting deadenylation, which might

result in degradation (increased turnover) (Nilsen 2007). Recent reports have also indicated that miRNA with or without perfect sequence complementarity, can cause an increase in mRNA degradation by endonucleolytic cleavage or deadenylation, respectively (Jackson and Standart 2007) or changes in proteins associated with RISC can cause a shift from translational inhibition to translational enhancement (Orom et al. 2008, Vasudevan et al. 2007). Translational repression and/or deadenylation occurs followed by decapping and exonuclease mediated degradation if base-pairing is partially complementary or in the case of perfect complementarity and provided the miRNP contains specifically Ago2, may result in endonucleolytic cleavage of the mRNA at the site where the miRNA is annealed (Standart and Jackson 2007). Those mRNAs which are repressed by miRNAs are further stored in the cytoplasmic foci called P-bodies (Liu et al. 2005a, Liu et al. 2005b, Rehwinkel et al. 2005). miRNAs have found to play an integral part of animal gene regulatory networks as one of the most abundant classes of gene regulators. Roughly 30% of all animal genes are predicted to be targeted by miRNAs. An algorithm which attempts to identify miRNA target sites without relying on cross-species conservation or miRNA sequences (Miranda et al. 2006) predicts even higher numbers of miRNA regulated genes.

2.1.1.3 Regulation of genes by miRNA mediated epigenetics

The term epigenetics refers to all heritable changes in gene expression that are not associated with concomitant alterations in the DNA sequence. Reversible DNA methylation and histone modifications are known to have profound effects on controlling gene expression. Correct DNA methylation patterns are paramount for the generation of functional gametes with pluripotency states, embryo development, placental function and the maintenance of genome architecture and expression in somatic cells. Aberrancies in both the epigenetic and in the miRNA regulation of genes have been documented to be important in diseases and early development. However, little is known about the miRNAs mediated epigenetic processes or epigenetic control of miRNAs expression, which could be potentially involved in regulating reproduction and early development. The potential role of Dicer has been postulated in heterochromatin formation (Fukagawa et al. 2004). In addition, Dicer-deficient mutants are shown to reduce epigenetic silencing of expression from centromeric repeat sequences as a result of alterations in DNA methylation and histone modifications

(Kanellopoulou et al. 2005). As contradictory to this, no apparent changes were observed in the centromeric heterochromatin later on (Murchison et al. 2005). However, result in the recent study has shown the Dicer deficient stem cells to have reduced levels of both de novo DNA methylation and DNA methyltransferases (Dnmts) (Benetti et al. 2008b, Sinkkonen et al. 2008) as well as increased telomere recombination and elongation (Benetti et al. 2008b). This result supports a model in which the miR-290 cluster maintains ES cells by controlling de novo DNA methylation via Rbl2 and indirectly telomere homeostasis and by repressing the self-renewal program through modulating the epigenetic status of pluripotent genes upon differentiation [reviewed in (Wang et al. 2009)].

Epigenetic regulation by the miRNAs has opened up a new dimension of mode of regulation from translational suppression and classical RNAi degradation. In addition to regulation of gene expression at the posttranscriptional level in the cytoplasm, recent findings suggest additional roles for miRNAs in the nucleus. miRNAs which are encoded within the promoter region of genes could be involved in silencing such genes at transcriptional level epigenetically. Such cis-regulatory roles of miRNAs have been observed in transcriptional silencing of POLR3D expression and endothelial nitric oxide synthase (eNOS) promoter activity (Kim et al. 2008, Zhang et al. 2005). Moreover, miR-122 has been shown to facilate replication of hepatitis C viral RNAs without affecting mRNAs translation or RNA stability (Jopling et al. 2005).

Recently, aberrant epigenetic reprogramming of imprinted miR-127 in cloned murine embryos has been reported in relation to the aberrant epigenetic reprogramming of the mouse retrotransposon-like gene Rtl1 (Cui et al. 2009). miRNA mediated switching of chromatin remodeling complexes in neural development by repression of BAF53a has been observed in mouse (Yoo et al. 2009). This repression is accomplished through the 3′ UTR of BAF53a and mediated by the simultaneous activities of miR-9* and miR-124. Repressor-element-1-silencing transcription factor participates in this switch by repressing miR-9* and miR-124, thereby permitting BAF53a expression in neural progenitors. Interestingly, the aberrant DNA methylation and histone modifications were found to simultaneously induce silencing of miRNAs in colorectal cancer (Bandres et al. 2009). The relation of miRNA and epigenetics is presently being elucidated. So, much less is known about the specific miRNA and their targets to

regulate epigenetic machinery or epigenetic regulation of specific miRNAs that are required for normal physiological condition or for any phenotypic effects, but this area of research is rapidly moving forward.

2.1.2 Methodological approaches to discover, expression profiling and functional study of miRNAs

Substantial attempts have been made to recognize new miRNAs like molecules since their early discoveries. The approaches, which were effectively used to identify new miRNA sequences, can be classified in to four major groups. Those are - computational approach, through library construction, heterologous approach and finally by advanced nucleotide sequencing methods. The least proficient advance is the identification of miRNAs through computational approach or genetic screening. In addition to lin-4 and let-7 miRNAs, founder member of miRNAs family, hundreds of miRNAs in different species has been identified by forward genetic screening. But now-a-days the most efficient way of miRNA discovery is through construction of cDNA libraries of endogenous small RNAs (Ambros and Lee 2004, Berezikov et al. 2006). Different approaches to construct small RNAs cDNA library are presented in the figure 2.3. Briefly, size-fractioned RNA from diverse sources is ligated to 5' and 3' adapter molecules, which are then, reverse transcripted and subsequently amplified by PCR in order to construct a cDNA library. The PCR products are concatemerized by DNA ligation and cloned into the sequencing vectors for identification (Berezikov et al. 2006). Small RNA is discovered by cDNA cloning, is categorised as a miRNA when it meets up certain criteria. First, the small RNA sequence should be at the arm of a 60-80 nucleotide hairpin RNA structure.

Second, if the small RNA is not cloned repeatedly, the expression of the small RNA should be established experimentally, classically by Northern blotting or by other defined methods. The sequence in most cases should also be phylogenetically conserved between related species (Ambros et al. 2003a). Third, the heterologous approach refers detection of known miRNAs from known species in a new species followed by detailed characterizations. This approach is carried out either by real time qRT-PCR or through microarray based technologies or by the both methods. However, the approach is limited to only conserve miRNAs families and requires further validation either by

sequencing or by northern blotting. So, one of the limitations of heterologous approaches is the requirement of prior sequence information, to be used for probe design. Until recently, this method has been limited mostly to that found in public databases (i.e. miRBase), having been gathered mainly through a combination of bioinformatics and extensive cloning experiments. Forth, instead of individual clone sequencing, deep sequencing of small RNA libraries is an effective approach to uncover rare and lineage- and/or species-specific miRNAs in any organism by utilizing massively parallel sequencing, generating millions of small RNA sequence reads from a given sample. Deep sequencing technology is becoming more available to researchers studying microRNAs and the analysis of profiling data by deep sequencing may be carried out using both publicly available and custom-made software. This approach is not dependent on any prior sequence information instead, providing information about all RNA species in a given sample and allowing for discovery of novel miRNAs or other types of small RNAs. Thus providing an excellent tool for those studying species where limited sequence information is currently available. Additionally, new sequence information provided by deep sequencing can be used to design microarray probe content for future large scale expression studies.

Diverse approaches have been followed to profile miRNA expression, such as Northern blotting with radiolabeled probes (Sempere et al. 2004, Valoczi et al. 2004), oligonucleotide macroarrays (Krichevsky et al. 2003), quantitative PCR-based amplification of precursor or mature miRNAs (Jiang et al. 2005b, Schmittgen et al. 2004, Shi and Chiang 2005), bead-based profiling methods (Barad et al. 2004, Lu et al. 2005) and DNA microarrays spotted onto glass surfaces (Babak et al. 2004, Baskerville and Bartel 2005, Liu et al. 2004, Miska et al. 2004). The most extensively used technique to profile the expression of hundreds of miRNAs are miRNA microarrays (Babak et al. 2004, Barad et al. 2004, Baskerville and Bartel 2005, Krichevsky et al. 2003, Liu et al. 2004, Miska et al. 2004, Sun et al. 2004, Wienholds et al. 2005).

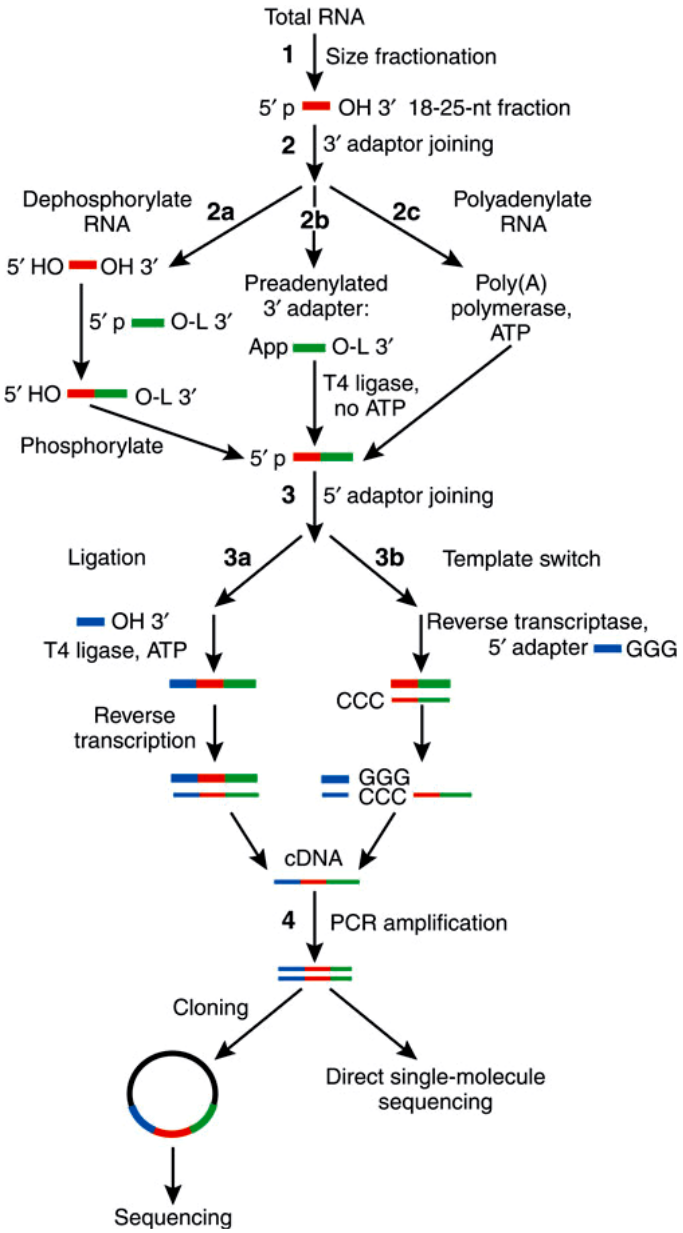


Figure 2.3: Approaches to discover miRNA by small RNAs-cDNA library construction. In 1, total RNA is separated on a polyacrylamide gel and the fraction corresponding to RNAs of 18–25 nt is recovered. In 2, a 3′ adapter can be introduced in different ways: the adapter can be ligated to a dephosphorylated RNA, which is then phosphorylated (2a); a preadenylated adapter can be ligated to RNA without free ATP in the reaction (2b); or the RNA can be polyadenylated by poly(A) polymerase (2c). In 3, a 5′ adapter is introduced either by ligation (3a), or by template switching during reverse transcription (3b). In 4, cDNA is amplified by PCR and cloned into a vector to create a library. Alternatively, PCR products can be sequenced directly by single-molecule sequencing methods (massive parallel sequencing) (adapted from Berezikov et al. 2006).

The usual microarray systems are not favourable to detect miRNAs as short targets. Consequently most of the miRNA microarray systems targeted pre-miRNA expression instead of mature miRNAs (Barad et al. 2004). Nevertheless, miRNA processing can be regulated and pre-miRNA expression may not always correlate to the mature miRNAs (Obernosterer et al. 2006). Recent microarray studies for detecting mature miRNAs by specific probes (like LNA modified probs), though closely sequence-related miRNAs might also be cross-hybridized. This constitutes an efficient methodology using locked nucleic acid (LNA)-modified oligonucleotides to screen in a parallel fashion for the expression of a large number of miRNAs through extensive sample collections. Where, there is no need for RNA size fractionation and/or amplification and that can discriminate among closely related miRNA family members (Castoldi et al. 2006). For sensitive miRNA array analysis, to compare the miRNA expression profiles of tumor and normal adjacent tissues from lung, colon, breast, bladder, pancreatic, prostate or thymus cancer microarray platform has already been used. For absolute quantification of miRNA expression, Northern blot assay is preferred for some cases over microarray. Several miRNAs has already profiled in different samples related to diseases, development and differentiation. Studies on miRNAs expression profiles in cancer samples have identified a handful of miRNAs that are differentially regulated in tumors, suggesting a possible link between miRNAs and oncogenesis (Calin et al. 2002).

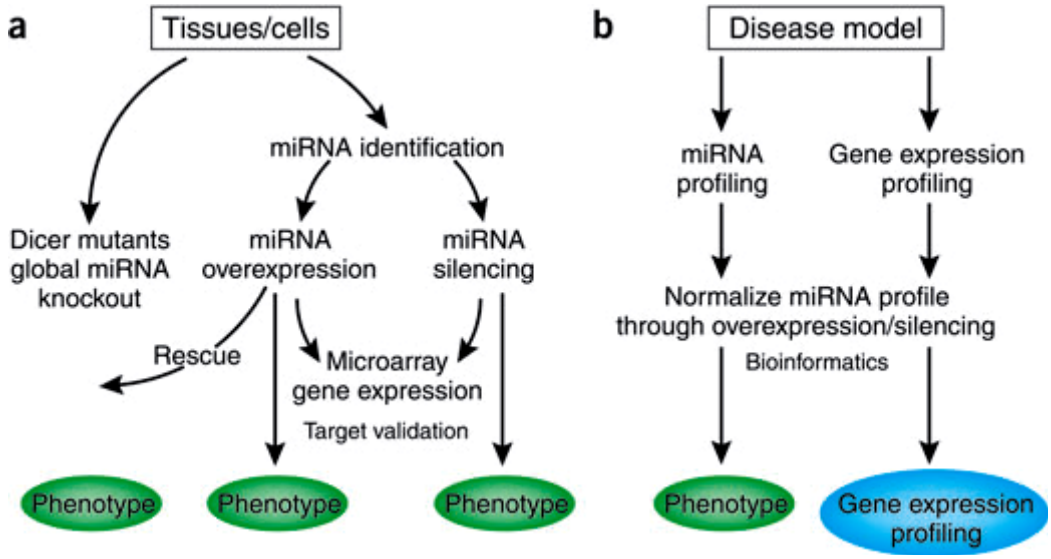


Figure 2.4: Experimental strategies to study miRNA function using molecular, genetic and bioinformatic techniques (adapted from Krützfeldt et al, 2006)

Experimental strategies to study miRNA function using molecular, genetic and bioinformatic techniques are shown in figure 2.4. Identification of miRNAs in the distinct cell types can be achieved by qRT-PCR, microarray expression profiling or random sequencing of miRNAs from any given tissue or cells. The effect of collective silencing of miRNA expression can be studied by inactivation of Dicer by appropriate methods. Alternatively, the expression of specific miRNAs can be manipulated by either over expression or gene silencing. Gene-expression analysis can then be used to validate miRNA targets and analyze phenotypes. Expression profiling can be used to test whether miRNA levels are altered in specific disease states to screen the functions of specific miRNAs. Thereafter, altered miRNA profiles can then be restored *in vivo* by over expression or silencing of specific miRNAs. The effect on the disease phenotype and gene expression at mRNAs or protein level can then be assessed (Krutzfeldt et al. 2006). Aside from direct experimental tests for site function, further indication of the importance of the seed region in miRNA target recognition has been inferred from computational studies showing significant overrepresentation of conservation of matches to miRNA seeds or, in some cases, avoidance of miRNA seed matches (Brennecke et al. 2005, Farh et al. 2005, Krek et al. 2005, Lewis et al. 2005, Stark et al. 2005, Xie et al. 2005). miRNA targets have been predicted using different computational approaches but to validate such miRNA-target interactions there is no high throughput approaches and must be tested one by one in reporter assays. However, from the recent study (Easow et al. 2007, Karginov et al. 2007) it seems that a quite large fraction of miRNA targets are not significantly destabilized at the mRNA level or that the magnitude of repression conferred by the miRNA is small. This could be the general case for miRNA-target relationships in which the miRNA is not absolutely required to reduce the target expression to an inconsequential level, but to buffer it to optimal levels, when it is required for optimum cellular functions.

2.2 Overview of role of miRNAs in reproduction

miRNAs are estimated to comprise 1–5% of animal genes (Bartel 2004, Bentwich et al. 2005, Berezikov et al. 2005) or a given genome could encode nearly thousands of miRNAs (Bentwich et al. 2005). Moreover, a typical miRNA regulates hundreds of target genes (Brennecke et al. 2005, Krek et al. 2005, Lewis et al. 2005, Xie et al. 2005) and altogether they could target a large proportion of genes up to 30% of the genome

(Lim et al. 2005). Changes in the expression of even a single miRNA found to have a significant impact on the outcome of diverse cellular activities. Inhibition of miRNA biogenesis has been found to be resulted in developmental arrest in mouse and fish (Bernstein et al. 2003, Giraldez et al. 2005, Wienholds et al. 2003) and female infertility in mouse (Otsuka et al. 2007, Otsuka et al. 2008). miRNAs seem well suited to maintain the delicate balance between normal reproductive biology, system development and tissue maintenance versus deregulated growth and tumor formation. These small non-coding RNAs have been found to play a central role in various cellular activities, including developmental processes, cell growth, differentiation and apoptosis, cell–cell communication, inflammatory and immune responses through gene expression stability. As many of these processes are an integrated part of gonadal functions, germ cell formation, differentiation, uterine and oviductal cellular activities during different stage of reproduction and steroid synthesis, it is possible to postulate the potential role of miRNAs in regulation of reproductive processes along with other physiological functions. Alteration of the expression of miRNAs in any of these processes could lead to subsequent infertility, reproductive and other steroid-dependent disorders with ultimate failure in reproduction. Investigation on the potential role of miRNA in reproduction up-to-date has been accomplished by the different approach. First, by identifying the population of miRNAs in the germ cells and reproductive tissues through cloning method. Second, by investigating the expression of candidate miRNA or group of miRNAs using microarray platform or RT-PCR approach. Third, by localizing candidate miRNA in the tissue or cell using in-situ hybridization approach. Forth, by knocking down global miRNA expression by creating Dicer1 knockout mice. Finally, by investigating specific miRNA function through using the oligonucleotide inhibitors and/or miRNA mimics or precursors. There are substantial studies by using of the first and second approach to discover and detect the expression of miRNAs in the sperm, oocyte, granulosa cells, and preimplantation embryos during spermatogenesis, fertilization, oocyte and embryonic development providing initial evidence for the potential involvement of miRNAs in reproduction. In addition, the depletion of global miRNAs by knocking out Dicer in various reproductive cells and tissues of mouse supplements and provides the eventual functional importance of miRNAs in reproduction.

2.3 Mammalian ovarian functions and miRNAs

Ovary, the primary gonad is only the source of female germ cells called oocyte which is the pre-requisite for sexual reproduction and female hormones namely estrogen and progesterone for the regulation of sexual behavior and function as well as maintenance of pregnancy. All the processes which are involving in the development of follicles and steroidogenesis in ovary are dependent on complex- co-coordinated cyclic mechanisms from the beginning of development of ovary upto the end of reproductive age. Brief overview of development, structure, functions and particularly follicular development in the ovary are presented in the following sub-sections. In addition, initial evidences for the involvement of miRNAs although it is very limited but discussed later on.

2.3.1 Structural-functional insight in to the ovary

The process of oocyte development involves gradual ovarian follicular development from primordial follicles. Folliculogenesis is the developmental process in which an activated primordial follicle develops to a preovulatory size following the growth and differentiation of the oocyte and its surrounding granulosa cells. During folliculogenesis, a follicle may be classified as primordial, primary, secondary or tertiary (antral, vesicular). This classification is commonly based on the size of oocyte, the morphology of granulosa cells and the number of granulosa cell layers surrounding the oocyte. Following sections describe in brief about the development of ovary, different ovarian cell types and mechanisms of ovarian follicular development as a main ovarian function and their transcriptional regulation.

2.3.1.1 Formation of ovary and different ovarian cell types

Gonadal ridges develop as a thickening of the coelomic epithelium on the medial aspect of the mesonephric kidneys (Dyce et al. 1996) and make connections with mesonephric tissue by rete-ovarii (Byskov and Hoyer 1994). At the gonadal ridge, oogonia become enclosed in germ-cell cords (Byskov and Hoyer 1994), consisted of epithelial cells and oogonia (Hirshfield and DeSanti 1995), which are delineated from surrounding mesenchymal cells by a basal lamina (Byskov and Hoyer 1994). The epithelial cells or the somatic cells are originated from the coelomic epithelium haveing cuboidal or

spherical nuclei and serve as precursors to the granulosa or follicular cells (Hirshfield and DeSanti 1995). The mesenchymal or stromal cells are originated from the stratified medial aspect of the mesonephric kidney having elongated nuclei and give rise to theca cells (Hirshfield and DeSanti 1995). In cattle, the gonadal ridge is transformed to a definitive ovary by Day 40 of gestation. With the disruption of the germ cell cords, the ovary divides into cortical and medullary parts (Smitz and Cortvrindt 2002). Developed ovary consists of stromal tissue containing primordial follicles (homologous to tubules in the testis) and also interstitial glands (homologous to Leydig cells). As in the male, gamete production comprises mitosis, meiosis and maturation. However, in the female the primordial germ cells that entered the embryonic gonad continued their development and proliferated mitotically. They are known as oogonia during this process and they stop once they have entered their first meiotic division which is known as primary oocytes. As they enter meiosis, the oocytes become surrounded by single flatten layer of granulosa cells to form primordial follicles. From puberty, a few primordial follicles recommence growth every day, so a continuous trickle of developing follicles is formed.

2.3.1.2 Bovine ovarian follicular dynamics

Development of follicles within the ovary is a dynamic process which occurs throughout the menstrual/estrous cycle and involves recruitment of follicles into the growing pool, physiologic selection of an ovulatory follicle and ovulation or regression. Although the fate of the vast majority of follicles is atresia, a single follicle (in monovular species) is somehow selected for continued development and eventual ovulation. Sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis, which are reoccurred on a cyclical basis within the ovary are resulting in the development of a number of ovulatory follicles (Hunter et al. 2004). Essentially all of these steady state cyclic changes are controlled by tightly regulated expression and interaction of a multitude of genes and their proteins in different compartments of the ovary (oocyte, cumulus granulosa, mural granulosa, internal and external theca cells) during the follicular programme with the ultimate goal of oocyte development (Bonnet et al. 2008). There is a large pool of resting (primordial) follicles in the mammalian ovary (~100,000 follicles), which are released from the resting pool throughout the life cycle. But, there are a few hundred growing follicles in the bovine

ovary at any one time (Erickson 1966). The ultimate fate for most of the follicles is atresia, which may occur at any stage but the dominant follicle (DF) must survive and become the part of healthy pool of antral follicles.

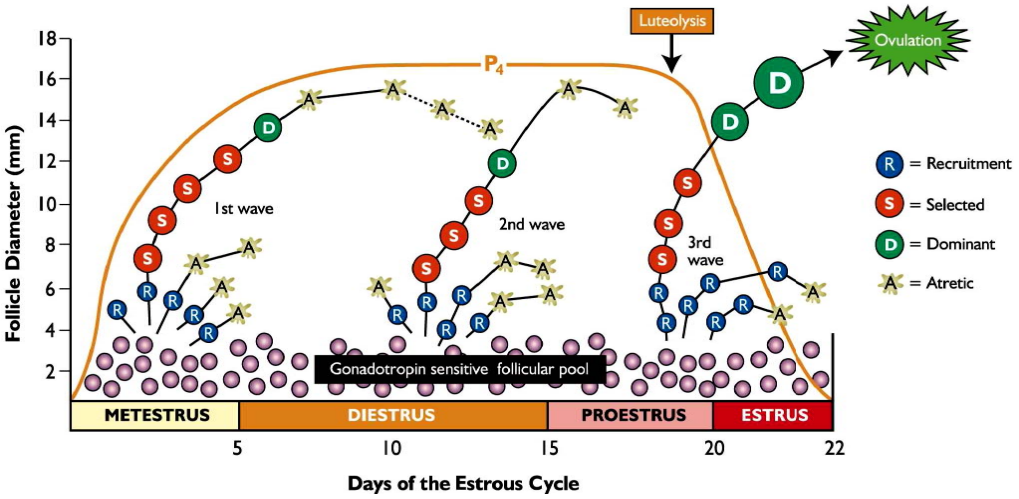


Figure 2.5: Bovine ovarian follicular wave dynamics (adapted from lucy et al, 1992)

Bovine follicular development found to be occurred in a wave like pattern which includes 2 or 3 waves and transient increases in FSH secretion precede the emergence of follicular waves in cattle (Figure 2.5) (Fortune 1994, Webb et al. 1992). Where as, a subsequent decrease in circulating FSH concentrations is temporally associated with selection of the dominant follicle (Adams et al. 1992) as has been observed in primates. Hence, it is assumed that it is the ability of follicles to respond to the endocrine environment on an individual basis that dictates their particular patterns of growth and development (Zeleznik and Hillier 1984). Granulosa cells acquire FSH receptors and theca cells acquire LH receptors before follicular recruitment (wave emergence) and selection of the dominant follicle proceeds (Richards 1994). During the later stages of follicle development, the granulosa cells of large healthy, estrogen-secreting follicles also acquire LH receptors (Ireland and Roche 1982, Ireland and Roche 1983b, Ireland and Roche 1983a, Spicer et al. 1986b, Xu et al. 1995). During the bovine estrous cycle, granulosa cells of large healthy antral follicles have more LH receptors than smaller atretic follicles (Ireland and Roche 1982, Ireland and Roche 1983a, Ireland and Roche 1983b, Spicer et al. 1986a) and levels of messenger RNA (mRNA) for LH receptor in granulosa cells of dominant follicles increase as follicular development progresses (Xu et al. 1995). It has been suggested that the acquisition of LH receptors on granulosa cells plays an important role in the selection of dominant follicles (Jolly et al. 1994, Spicer et al. 1986b, Xu et al. 1995, Zeleznik and Hillier 1984).

2.3.1.3 Transcriptional regulation of ovarian folliculogenesis

Transition from primordial to primary follicles commences the follicular growth, which are regulated by multiple genes. Variety of locally produced growth factors along with gonadotropins have been identified (Bao and Garverick 1998, Gutierrez et al. 2000) by mouse knockout study (Matzuk and Lamb 2002) and reviewed (Knight and Glister 2006, McNatty et al. 2005, Skinner 2005). Most noteworthy among these are members of the TGF-beta superfamily (including the BMP, their receptors and GDF-9), bFGF, EGF (Knight and Glister 2006, McNatty et al. 2005, Webb and Campbell 2007), IGF and IGFBP (Lucy 2000, Spicer 2004, Webb et al. 2003) (Figure 2.6). Oocyte-specific transcriptional regulators (Figla, Nobox, Sohlh1 and Lhx8), oocyte-secreted factors (Gdf9 and Bmp15), as well as genes expressed in the granulosa and other surrounding somatic cells were found to initiate and control follicular growth (Dumesic and Abbott 2008, Matzuk and Lamb 2002). Among the key intra-ovarian factors, the transforming growth factor b (TGFb) family members, including bone morphogenetic protein-4 have been identified as regulators of primordial germ cell generation (van den Hurk and Zhao 2005) (Figure 2.6).

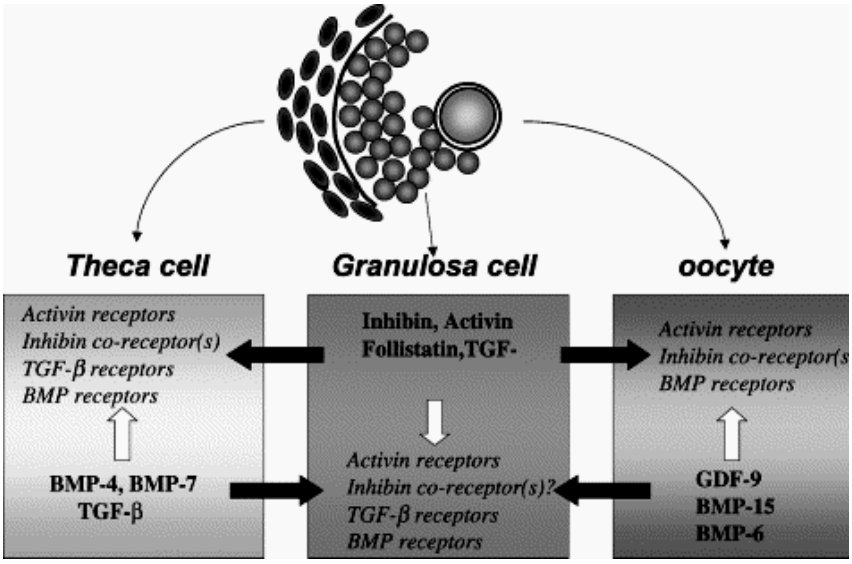
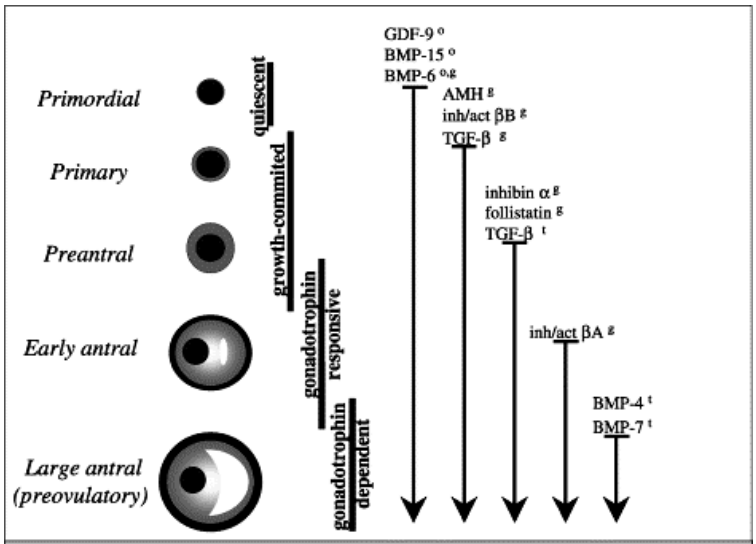


Figure 2.6: TGF-β superfamily members and their associated receptors and binding proteins in the ovarian cells. Members of this family are expressed by theca cells, granulosa cells and oocytes consistent with their proposed roles as intrafollicular autocrine (white arrows) and paracrine (black arrows) signaling molecules (adapted from Knight and Glister 2003).

Participation of follicle pool in recruitment is FSH-dependent, which later on acquires LH receptors in the granulosa and theca cells and becomes dependent on LH (Ireland and Roche 1983a). Once the dominant follicle exposed to LH surge, its fate is ovulation and its cells will differentiate into the corpus luteum, unless and otherwise lead them to be atretic (Valdez et al. 2005). In addition, the dominant follicle is dependent on growth factors for promoting the G1- to S-phase transition of the cell cycle and prevent apoptosis of granulosa cells (Quirk et al. 2004). Even though a persistent follicle with a low-level progestogen treatment have been shown to retain its capacity (Savio et al. 1993, Sirois and Fortune 1990) to trigger an LH surge and ovulate less healthy oocytes by premature activation (Revah and Butler 1996). The LH surge converts the proliferating cells of the follicle into dormant cells that are resistant to apoptosis (Quirk et al. 2004). In response to FSH, the granulosa cell–derived factors such as kit ligand, transforming growth factor α (TGF-α) and epidermal growth factor (EGF) activate the resting follicular growth (Figure 2.7).



Putative roles:	
• GDF-9/BMP-15	oocyte-derived; essential for follicle progression beyond primordial/primary stage; promote granulosa cell proliferation and differentiation
• TGF	modulates granulosa and theca cell proliferation and differentiation; suppresses thecal androgen production
• BMP-4,-6, -7	delays luteinization/atresia of large antral follicles (increases oestradiol and inhibin; decreases progesterone production)
Activin	stimulates granulosa cell proliferation in preantral/early antral follicles and upregulates FSH receptors and FSH-induced aromatase activity; delays luteinization/atresia of large antral follicles; enhances oocyte maturation
• Follistatin	attenuates above actions of activin (and BMPs?); may promote luteinization/atresia of large antral follicles
• Inhibin	enhances LH-induced androgen production in large antral follicles
• AMH	may contribute to control of preantral follicle growth

Figure 2.7: Diagram summarising experimental observations on follicle stage-dependent expression of TGF-β superfamily members in ruminants and their putative roles as intrafollicular autocrine or paracrine signaling molecules. Superscripts (o, g, and t) refer to oocytes, granulosa cells and theca cells, respectively. Adopted from (adapted from Knight and Glister 2003).

The interactions between ovarian germ (oocytes) and somatic cells (granulosa cells) and expression of several intra-ovarian autocrine/paracrine regulators (FSH, estrogen and androgen receptors) are the major contributing factors in the ovary leading to preantral and antral follicles development (Filicori et al. 2003). But, how all these receptors proteins are being regulated in temporal manner rapidly is not yet understood.

2.3.2 miRNAs in the mammalian ovarian functions

Available information on expression and regulation of miRNAs in the mammalian ovary are presented and discussed below.

2.3.2.1 Expression and regulation of miRNAs in the ovarian cells

Dynamically regulated, complex and coordinated ovarian functions include sequential recruitment, selection and growth of the follicles, atresia, ovulation and luteolysis are under control of closely coordinated endocrine and paracrine factors. All these factors are controlled by tightly regulated expression and interaction of a multitude of genes in different compartments of the ovary (Bonnet et al. 2008). As one of the major classes of gene regulators, miRNAs are considered to be involved in the regulation of ovarian genes (Hossain et al. 2009, Ro et al. 2007a). Several studies expanding from identification and expression profiling to functional involvement of miRNAs in the ovary have been carried out in different animal species. Four attempts have led to identify the distinct and major population of miRNAs in 2 weeks old and adult mouse ovary (Ro et al. 2007b), adult mouse ovary and testis (Mishima et al. 2008) and new born mouse ovary through small RNA library construction and sequencing (Ahn et al. 2010). Abundant miRNAs which were observed in newborn and adult mouse ovary are presented in figure 2.8. The presence of miRNAs and their differential expression can give the primary clue for their potential role in ovarian functions. However, discovery and further functional characterization of miRNAs in the bovine ovary along with different ovarian cell types (oocyte, granulosa, theca cells and ovarian stroma) at different follicular stage or at different estrus cycle remains to be elucidated. Several studies highlighted the expression and regulation of some individual miRNAs in different ovarian cells especially in oocyte and granulosa cells. After disclosing the absence or less role of sperm born miRNAs in mammalian fertilization (Amanai et al.

2006), further studies were directed towards these two cell types (oocyte and granulosa). For example, the first attempt was made in 2006 and the study identified small number of miRNAs as well as some other small noncoding RNAs (rasiRNAs, gsRNAs) in mouse oocyte (Watanabe et al. 2006). However, further identification of entire set of miRNAs in oocytes through direct cloning method is still missing rather more initiative has been taken for microarray or RT-PCR based miRNAs detection through homologous or heterologous approaches.

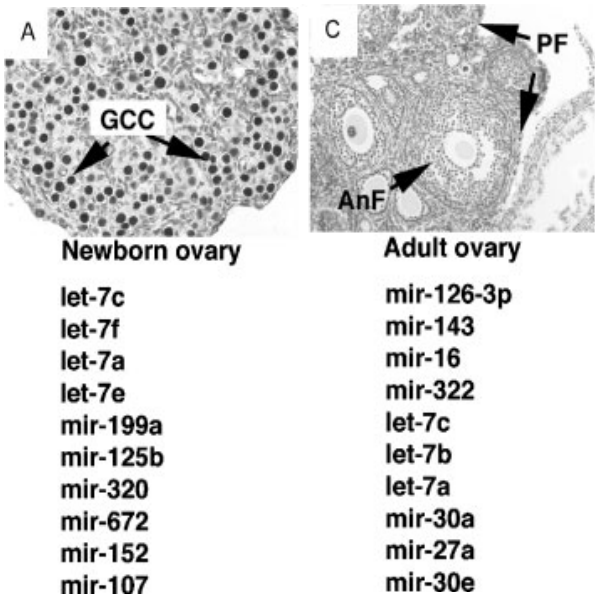


Figure 2.8: Different stages of ovarian development and a subset of abundant miRNAs that are expressed at the newborn ovary and adult stages. (A) Newborn ovary and (C) 7-week (adult) mouse ovary. Germ-cell clusters (GCC), primordial follicles (PF), secondary follicles (SF), and antral follicles (AF) are indicated by appropriate arrows. miRNAs abundantly expressed are listed below the corresponding stage of ovarian development (adapted from Zhao and Rajkovic 2008).

The Microarray experiments have shown that Dicer1 is highly expressed and functionally important in the oocytes during folliculogenesis as well as in the mature oocytes (Choi et al. 2007, Murchison et al. 2007, Su et al. 2002). Conditional knockout of Dicer1 in growing oocytes revealed unaffected oocyte growth and folliculogenesis during the early stage but meiosis I has been found to be arrested with defective spindle organization in oocytes lacking Dicer1 (Murchison et al. 2007). Transcriptional analysis

through microarray experiments has identified the major portion of the transcripts as misregulated in Dicer1-deficient oocytes. Moreover, Dicer knock out ovaries are found to have increased rate of apoptosis (Figure 2.9). These efforts not only provide initial evidence for the role of miRNAs in the oocyte but also suggested that a large proportion of the maternal genes are directly or indirectly under the control of miRNAs (Murchison et al. 2007, Tang et al. 2007). Information on the regulatory role of miRNAs in the ovarian cells of ruminants compared to human and mouse are so limited and these are the open field for the researcher working on ruminant reproductive biology. Currently, the expression and functional evidence of miRNAs in the follicular theca cells in any physiological states of any species remains to be elucidated.

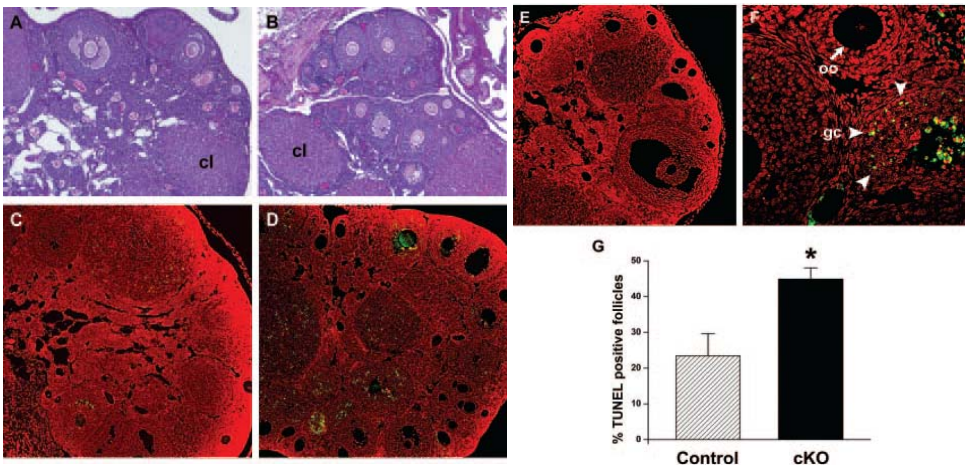


Figure 2.9: Dicer1 knock out ovaries have increased apoptosis. Comparison of ovarian histology from 12-wk-old control (A) and cKO (B) females shows that Dicer1 mutant ovaries contain follicles at all stages of folliculogenesis as well as corpora lutea (cl). Despite these observations, a trend toward increased atretic follicles in cKO ovaries at 6 wk of age. TUNEL staining of 6-wk-old ovaries from control (C) and cKO (D) mice revealed increased apoptotic granulosa cells (green/yellow) in the Dicer1 mutant. Nuclei are counterstained with propidium iodide (red). E, TUNEL-negative control. F, High magnification of TUNEL positive follicles in the Dicer1 cKO ovary. Note that granulosa cells (gc, arrowheads) are TUNEL positive, whereas oocytes (oo, arrow) are negative. G, The proportion of TUNEL-positive follicles was quantified and found to be significantly higher in cKO ovaries (44.9±5.4%) as compared with control ovaries (23.5±10.8%), * P <0.05 (Student's t test), (adapted from Nagaraja et al. 2008).

2.3.2.2 Ovarian steroidogenesis and miRNAs

Recent studies revealed interesting relationship between ovarian steroids and miRNAs. Several studies suggested ovarian steroid dependent biogenesis & maturation of miRNAs and reversely some set of miRNAs could regulate the secretion of ovarian steroid. It has been demonstrated that ovarian steroids influence the expression of some miRNAs (hsa-miR20a, hsa-miR21 and hsa-miR26a) in endometrial stromal cell and glandular epithelial cell in human (Pan et al. 2007). The molecular mechanism by which ovarian steroids regulate the expression of miRNAs is unclear but such regulatory function has been suggested to alter the expression of their target genes and cellular activities manifested by their products thereby (Pan et al. 2007). It has been also shown that LH/hCG regulates the expression of selected miRNAs, which affect posttranscriptional gene regulation in mouse within ovarian granulosa cells (Fiedler et al. 2008). Estrogen was found to suppress the levels of a set of miRNAs in mice and human cultured cells through estrogen receptor α (ERα) by associating with the Drosha complex and preventing the conversion of pri-miRNAs into pre-miRNAs (Yamagata et al. 2009). As down-regulation of miRNAs appeared to stabilize human VEGF mRNA, the posttranscriptional control by estrogen appears to mediate the half-life of estrogen target genes via regulated miRNA maturation (Yamagata et al. 2009). In addition, upregulation of subset of miRNAs in female mice lacking estrogen receptor α and down regulation of some miRNAs in the estrogen target organ (Uterus) was observed following estradiol (E2) treatment in ovariectomized female mice (Macias et al. 2009). Altogether, these studies suggested that ERα bound to E2 inhibits the production of a subset of miRNAs by a mechanism whereby ERα blocks Drosha-mediated processing of a subset of miRNAs by binding to Drosha in a p68/p72-dependent manner and inducing the dissociation of the microprocessor complex from the pri-miRNA (Macias et al. 2009).

In contrast, some miRNAs are also found to play important role in the ovarian steroidogenesis (Sirotkin et al. 2009). Genome-wide screening of miRNAs revealed the involvement of miRNAs in control of release of the ovarian steroid hormones progesterone, androgen and estrogen in human ovarian cells (Sirotkin et al. 2009). They have evaluated the effect of transfection of cultured primary ovarian granulosa cells with gene constructs encoding the majority of identified human pre-miRNAs on release

of progesterone, testosterone and estradiol. These results revealed thirty-six out of 80 tested miRNA constructs inhibiting the progesterone release in granulosa cells and 10 miRNAs have been found to promote progesterone release. Subsequent transfection of cells with antisense constructs to two selected miRNAs (miR-15a and miR-188) revealed induction of progesterone output due to lack of blockage of progesterone release. While fifty-seven tested miRNAs were found to inhibit testosterone release, only one miRNA (miR-107) enhanced testosterone output. Fifty-one miRNAs suppressed estradiol release, while none of the 80 miRNAs tested were found to stimulate it (Sirotkin et al. 2009). The complex regulatory mechanisms for controlling miRNAs biogenesis by the steroids or vice versa are still unclear. The involvement of miRNAs for such mechanisms as regulator of several hundreds of genes as potential target could be much higher than ever speculated. While the investigations on the involvement of miRNAs in ovarian function and steroidogenesis in mouse and human are progressing, no information was available in case of bovine. Even the identity, existence, characteristics, expression pattern, functions and regulatory mechanisms was completely unknown until the work has been carried out as part of this dissertation.

2.4 miRNAs in embryogenesis and early development

Following sections discussed the fundamentals of mammalian embryogenesis from fertilization to preimplantation embryo development association of miRNAs in the relevant processes.

2.4.1 Fertilization, embryogenesis and miRNAs

Following ovulation, the cumulus-oocyte complex (COC) is released into the perionetal or bursal cavity where it is picked up by the infundibulum of the oviduct. Cilia covering the exterior surface of the infundibulum direct the COC into the oviduct (Talbot et al., 2003). Immediately prior to ovulation, elevated estradiol concentrations increase height (Murray, 1996) and ciliation of the luminal epithelial cells of the fimbria and ampulla (Murray, 1995, 1996) to facilitate COC transport to the ampulla, the site of fertilization. Prior to fertilization, sperm deposited into the reproductive tract must undergo a process termed “capacitation”. Capacitation is mediated by cAMP-dependent protein tyrosine phosphorylation (Wassarman, 1999) and involves a series of functional, biochemical

and biophysical modifications of the ejaculated spermatozoa to allow for proper fertilization of the oocyte (Baldi et al., 2000). These modifications of the spermatozoa include increased membrane fluidity due to loss of cholesterol and remodeling of the sperm surface proteins, increased intracellular Ca^{2+} and pH and membrane hyperpolarization (Baldi et al., 2000; Töpfer-Pertersen et al., 2000). These changes of the spermatozoa during capacitation induce hyperactivated motility of the sperm and allow the sperm cell to respond to stimuli that induce the acrosome reaction upon interaction with the oocyte's zona pellucida (Baldi et al., 2000). This reaction facilitates the degradation and sperm penetration of the zona pellucida and subsequently exposes the equatorial segment of the sperm, which allows for fusion of the sperm membrane with the oolemma (Roldan et al., 1994; Baldi et al., 2000). The steroids progesterone and estradiol also mediate the acrosome reaction. Moreover, estradiol modulates that action of progesterone to ensure the appropriate time of the acrosome reaction (Baldi et al., 2000).

Following the acrosome reaction the spermatozoa passes through the zona pellucida, the sperm head crosses the perivitelline space and the equatorial segment of the sperm then fuses to the oocyte plasma membrane (Yanagimachi, 1988). The fusion of the spermatozoa with the oocyte results in oocyte activation characterized by exocytosis of cortical granules and the resumption of meiosis of the oocyte. Prior to activation, the nucleus of the oocyte is in the metaphase stage of the second meiosis, where it was arrested following the transition from the dictyate stage of prophase I and the exclusion of the first polar body following the LH surge (Jones, 2005). The basic steps involved in the mammalian fertilization are presented in figure 2.10.

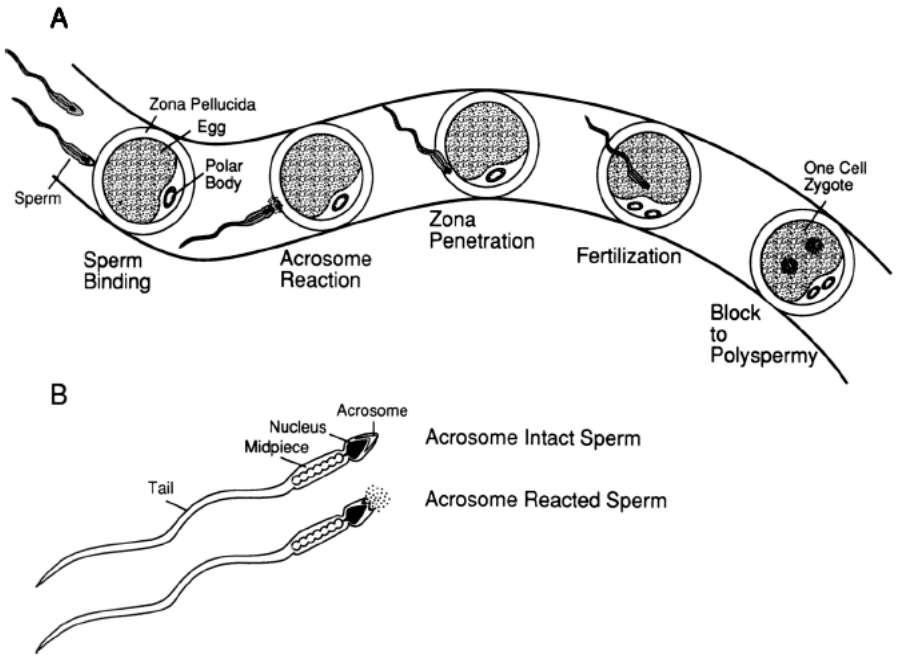


Figure 2.10: Basic steps of mammalian fertilization.

(A) Binding of sperm to the zona pellucida of oocyte induces the sperm acrosome reaction. The release of lytic enzymes from the acrosome and the forward motility of the sperm permit penetration of the zona pellucida. After fusion with the egg's plasma membrane, the sperm enters the cytoplasm and forms the male pronucleus of the one cell zygote. Following fertilization, the zona pellucida is biochemically modified to prevent additional sperm binding (B) The acrosome, a lysosomallike structure on the anterior head of sperm, contains an inner and outer membrane that fuse during the acrosome reaction (adapted from Dean, 1992).

The resumption of meiosis results in cell cycle resumption, extrusion of the second polar body and the formation of the female pronucleus. Likewise, the sperm nucleus decondences and transforms into the male pronucleus. The sperm and egg pronuclei come into close approximation, their nuclear envelopes disintegrate and their chromosomes mingle for the first mitotic division (cleavage). The mingling of the chromosomes can be considered the end of fertilization and the beginning of embryonic development (Yanagimachi, 1988). Regarding molecular aspects of oocyte-sperm membrane interaction, several important molecules were identified as pivotal regulator. As many as two-dozen different sperm proteins have been implicated in species-specific binding of sperm to eggs (Snell and White 1996, Wassarman 1999). These include a variety of enzymes (such as β-galactosyltransferase and α-fucosyltransferase, protein

tyrosine kinase (ZRK) and phospholipase A_2) and lectin-like proteins (such as mannose- and galactose-binding proteins and spermadhesins), as well as several other sperm proteins (such as zonadhesin and sperm protein-56 (SP56)).

In addition, results of recent experiments with homozygous-null mice have implicated two members of the ADAM (so-called because they contain a disintegrin and a metalloprotease domain) family of proteins, sperm β-fertilin and cyritestin, as potential EBPs (Cho et al. 1998, Primakoff and Myles 2000, Shamsadin et al. 1999). Binding of sperm to the egg plasma membrane is thought to be mediated by this member of the ADAM family of transmembrane proteins on sperm and integrin α6β1 receptors on eggs (Snell and White 1996, Wassarman 1999). Two mouse-sperm ADAM proteins in particular, the heterodimer fertilin (-α, ADAM-1; -β, ADAM-2) and cyritestin (ADAM-3), have been studied in some detail and found to interact with integrin in the egg plasma membrane through their disintegrin domains. Results of recent studies indicate that at least two components that are essential for intracellular membrane fusion in somatic cells, Rab3A GTPase and SNAREs, may be present in mammalian sperm and may participate in membrane fusion during the acrosome reaction (Iida et al. 1999, Ramalho-Santos et al. 2000). Results of several investigations indicate that CD9 a member of the tetraspan superfamily of integral plasma-membrane proteins that associate with each other, as well as with a subset of β1 integrins and integrin α6β1 in the egg plasma membrane has a vital function in sperm–egg fusion in mice (Chen et al. 1999).

While considering miRNAs, it has been shown that miRNAs are present in mouse sperm structures that enter the oocyte at fertilization (Amanai et al. 2006). The sperms were found to contain a broad profile of miRNAs and a subset of potential mRNA targets, which were expressed in fertilizable metaphase II (mII) oocytes. Similarly, oocytes were found to have transcripts responsible for miRNAs processing and regulatory mechanisms namely RNA-induced silencing complex (RISC) catalytic subunit, EIF2C3. However, the levels of sperm-borne miRNA (measured by quantitative PCR) were found to be very low relative to those of unfertilized mII oocytes and fertilization did not alter the mII oocyte miRNA repertoire that included the most abundant sperm-borne miRNAs. Coinjection of mII oocytes with sperm heads plus anti-miRNAs to suppress miRNA function did not perturb pronuclear activation or

preimplantation development. In contrast, nuclear transfer by microinjection altered the miRNA profile of enucleated oocytes. These data suggest that sperm-borne prototypical miRNAs play a limited role in mammalian fertilization or early preimplantation development (Amanai et al. 2006).

2.4.2 miRNAs and preimplantation embryo development

Early embryonic development is characterized by the number of nuclei (blastomere) present within the developing embryo. Following fertilization, the cell formed by the union of parental chromosomes is referred to as a zygote, which is then undergoes a number of cleavage and resulting in the development of a 2-, 4-, 8- and 16- cell embryo. Early embryonic developmental events in domestic animals are presented in figure 2.11. As cleavage continues, the blastomeres compact, merging into a single mass of cells called a morula. It is at this point that the embryo moves from the oviduct into the uterus, which occurs between days 4 to 5 of pregnancy in the bovine (El-Banna and Hafez 1970). During this period genes encoding E-cadherin, β-cantenin and ZO-1 are responsible for gap junction assembly and trophectoderm differentiation (Watson et al. 1999). The next distinguishing event is blastocoel formation, which is accomplished by the development of a differentiated polarized epithelium (trophectoderm) that implements a Na/K-ATPase ion transport pump to force fluid into the blactocoel cavity (Biggers et al. 1988). The blastocoel will eventually turn into the cavity of the yolk sac (Schlafer et al. 2000). Cells forming the outer wall of the blastocyst assume specialized function and are known as trophoblast cells. At one pole of the hollow blastocyst, another type of specialized cells called the inner cell mass (ICM) form. This cluster of cells will become the embryo proper. From the ICM, two distinct cell layers develop; the endoderm, which lines the yolk sac and the mesoderm which extends from the ICM to form a layer between the trophectoderm and the endoderm (Schlafer et al. 2000). The initial development of the preimplantation embryo is controlled by maternally inherited molecules in the oocyte but upon the embryonic genome activation which begins at 2-cell stage during this early embryogenesis and eventually acquires developmental control (Maddox-Hyttell et al. 2003). This shift from maternal to zygotic genomic control is referred to as the maternal/zygotic transition (MZT). A unique feature of early embryonic cells is their ability to maintain a full range of developmental capacity, termed totipotency. Blastomeres maintain complete totipotency until the 16-cell stage of

development, afterward increasing differentiation of cell lineages prevents complete totipotency (Pedersen 1988). Lineages differentiation of trophectoderm and ICM results in loss of totipotency of the trophectoderm cells.

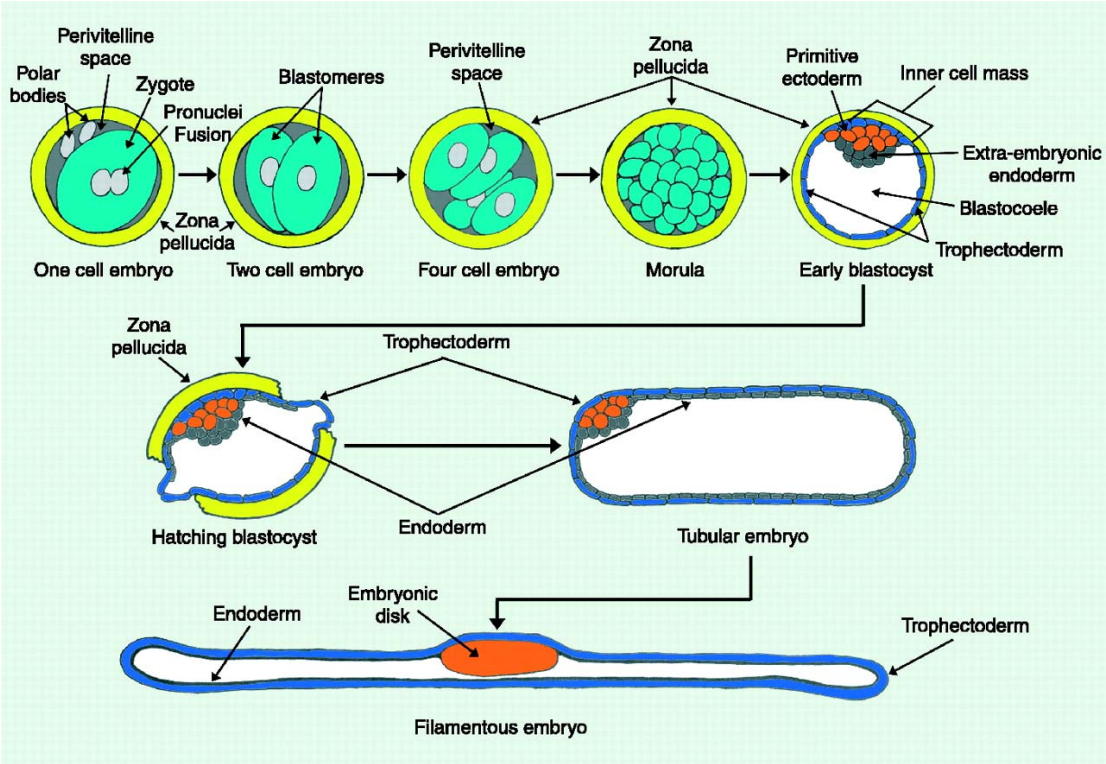


Figure 2.11: Early embryonic developmental events in domestic animals.

Fertilization occurs in the oviduct and morula-stage embryos enter the uterus where they develop into spherical blastocyst and hatch from the zona pellucida by actions of proteases. Thereafter, spherical blastocysts migrate, change to a tubular and then a filamentous form due to rapid elongation of trophectoderm before initiation of implantation (adapted from Bazer et al. 2009).

In mouse, pluripotent cells can be characterized by the expression of specific gene transcripts such as octamer-binding transcription factor-4 (Oct-4), alkaline phsophatase, E-cadherin, stage-specific embryonic antigen-1 (SSEA1), zinc-finger protein-42 (REX1) and Nanog (Boiani and Scholer 2005). In 2-, 4-, 8- and 16-cell bovine embryo, mRNA for Oct-4 has been found to be absent or very low but increased during morula development. Elevated expression of Oct-4 in the ICM and down regulation in the trophectoderm is reflecting the loss of totipotency in trophectoderm cells (Kurosaka et

al. 2004). During the progress of embryonic development, the blastocyst with three concentric cell layers (trophectoderm, mesoderm and endoderm), expands, hatches from the zona pellucida and begins to elongate. Hatching of bovine blastocyst occurs between days 9 and 10 of gestation (Guillomot 1995, Maddox-Hyttel et al. 2003). Following blastocyst hatching there is no definitive cellular contact between the trophectoderm and the endometrial epithelium for several days during embryo elongation in ruminants (Spencer et al., 2004a) and the embryo appears spherical and then becomes ovoidal. The embryo appears tubular in shape and finally assumes a filamentous appearance by approximately day 13 to 14 of gestation as elongation progressed (Guillomot 1995, Maddox-Hyttel et al. 2003, Spencer et al. 2004).

The well-orchestrated expression of genes that are derived from the maternal and/or embryonic genome is required for the onset and maintenance of distinct morphological changes during the embryonic development. Optimum regulation of genes or critical gene regulatory event in favor of early embryonic development have been shown to be directly (individual miRNAs study) or indirectly (disrupting miRNAs biogenesis) under the control of miRNAs. Disruption of Dicer1 - an enzyme important for biogenesis of miRNAs and RNA interference related pathways in mammals was first demonstrated and shown that loss of Dicer1 lead to lethality early in development, where Dicer1-null embryos were found to be depleted of stem cells in mouse (Bernstein et al. 2003). Another report has been published in the same year to show the importance of Dicer1 in vertebrate development through inactivation of the Dicer1 in zebrafish and subsequently observed the early developmental arrest (Wienholds et al. 2003). While defective generation of miRNAs was observed in Dicer-null mouse embryonic stem cells with severe defects in differentiation both in vitro and in vivo, the re-expression of Dicer in the knockout cells has been found to rescue these defective phenotypes (Kanellopoulou et al. 2005). Additionally, maternal miRNAs have been shown to be essential for the earliest stages of mouse embryonic development through the loss of maternal inheritance of miRNAs following specific deletion of Dicer from growing oocytes (Tang et al. 2007). So, these initial reports suggest that miRNAs are essential for embryonic development as the effect of loss of Dicer1 could primarily arise from an inability to process endogenous miRNAs which later on functioning in the gene regulation.

The role of miRNAs has been suggested first for differentiation or maintenance of tissue identity during early embryonic development in zebrafish (Wienholds *et al*. 2005). Several attempts were made to clone miRNAs from the embryo or embryonic tissues to understand the miRNA-mediated regulation of embryonic development. A significant number of miRNAs has been identified at specific stages of mouse embryonic development through massively parallel signature sequencing technology (Mineno *et al*. 2006) and in bovine embryo through small RNAs library construction (Coutinho et al. 2007). The coexistence of dynamic synthesis and degradation of miRNAs has been shown but overall quantity and stage-dependent miRNAs increases as the embryos develop during mouse preimplantation stage embryonic development (Yang et al. 2008b). Even, during the preimplantation stage miRNAs are shown to participate in directing the highly regulated spatiotemporally expressed genetic network as well. The miRNAs profile observed in mouse embryos are provided with a clear insight into the embryonic stage-specific miRNA transcriptome, and facilitated the identification of the primary target for each miRNA and thereby the pathways regulated by embryonic specific mRNAs (Mineno et al. 2006). A dynamic change in miRNA expression during oogenesis in growing and mature mouse oocytes has observed and miRNA levels in mature mouse oocytes and early embryos fall precipitously in the two-cell embryo, but rise again at the four-cell stage, presumably as a result of new transcription from the embryonic genome (Tang et al. 2007). They are also engaged by playing important roles in diverse cellular processes as well. Examination of miRNAs expression in mouse through to the eight-cell stage revealed that dynamic changes of miRNAs during early embryonic development (Tang et al. 2007). Where, the total amount of miRNA was found to be downregulated by 60% between one- and two-cell-stage embryos and then increases, resulting in a 2.2-fold difference between two- and four-cell-stage embryos. Which suggests that a very significant proportion (>90%) of the maternally inherited miRNAs present in the zygote is probably actively degraded during the first cell division, although maternal mRNAs are globally degraded at this time (Hamatani et al. 2004). So, miRNAs may be involved in the regulation of maternal transcripts in oocytes and embryos and whose degradation may be essential for successfully completing meiotic maturation and subsequent development. Study has been also suggested that sperm-borne miRNAs play a limited role, if any, in mammalian fertilization or early preimplantation development (Amanai et al. 2006).

Significant proportion of the maternally inherited miRNAs present in the Zygote is probably actively degraded during the first cell division. The total miRNA in a four-cell-stage embryo has bund to be ~2.2 times higher than the levels in a two-cell stage embryo which suggested that there is de novo expression of miRNAs between the two- and four-cell stages of development (Tang et al. 2007). MicroRNAs belong to the let-7 family are found to be abundant in mouse oocytes and shown dynamic regulation for the period of oogenesis and early embryonic development. In a four-cell-stage embryo the miR-290 clusters (Houbaviy et al. 2003) among the upregulated miRNAs were abundant. Compared with the two cell-stage embryos, they were up-regulated by 15-fold and by 24-fold in four-cell and eight-cell-stage embryos, respectively. Thus, miRNAs from the miR-290 cluster are amongst the earliest to be expressed during early mouse embryonic development. Abundance of miR-17-92 cluster has been shown to be significantly increased during oogenesis and was inherited by the zygote and increased again after the two-cell embryo stage (Tang et al. 2007).

2.4.3 miRNAs in embryonic stem cells development and maintenance

Recent studies have identified a unique set of miRNAs expressed and its functional importance in embryonic stem cells (ES cells). Initial effort has identified that miR-290 through miR-295 (miR-290 cluster) are ES cell-specific and there after suggested that they could potentially participate in early embryonic processes such as the maintenance of pluripotency in mouse (Houbaviy et al. 2003). Similar study in human has also identified some clustered miRNAs (miR-296, miR-301 and miR-302: homologous to the miRNAs reported by Houbaviy et al. in mouse) specifically expressed in human ES cells and not in differentiated embryonic cells or adult tissues (Suh et al. 2004). These clustered miRNA organization is presumably effective for coordinated regulation of their expression and regulation of common targets because a common seed is shared between some miR-290 cluster miRNAs, miR-302a-d and miR-93 (Houbaviy et al. 2003, Houbaviy et al. 2005). The role of miR-290 cluster in embryogenesis has been evidenced in a study, in which the generation of a mouse mutant with a homozygous deletion of the miR-290 cluster resulted in the death of embryos (Ambros and Chen 2007). By the loss- or gain-of-function studies of Dicer, DGCR8 and ES-related miRNA genes such as miR-290-295 cluster have strongly suggested that miRNAs play an important role in ES cell maintenance, differentiation (Benetti et al. 2008b,

Sinkkonen et al. 2008) and lineage determination (Ivey et al. 2008, Kanellopoulou et al. 2005, Tay et al. 2008, Wang et al. 2007). Despite the fact that knowledge on the role of miRNAs in the embryonic development and stem cell maintenance, differentiation and lineage in mouse and human is increasingly building, it is yet to be elucidated for ruminants.

2.5 miRNAs in the embryo produced by assisted reproductive technologies

The advancement of manipulation of reproductive process and application through assisted technology is impressive in the last two decades. Since 1970, artificial insemination (AI) and the associated techniques such as semen cryopreservation and ovulation synchronization dominated the reproductive techniques. The female contribution to genetic progress was achieved with the advent of embryo transfer and the associated techniques such as non-surgical embryo collection, in vitro maturation, fertilization and culturing of bovine oocytes. ET can be accomplished by different approaches. Dams can be super-ovulated, artificially inseminated (AI) and later the resulted fertilized oocytes are collected by flushing. These fertilized oocytes are then cultured till blastocyst stage and are implanted in synchronized heifers. This technique is referred as multiple ovulation and embryo transfer (MOET). Oocytes can also be collected directly from mother's body through ultra-sonography, cultured for maturation and then fertilized with spermatocytes (IVF). If fertilization is successful, similar to MOET, the fertilized ovum after undergoing several cell divisions, is either transferred to the surrogated mother's for normal development in the uterus, or frozen for later implantation. In IVF and in vitro culture procedure, approximately 30 to 40% of the matured oocytes develop to transferable blastocysts (Hagemann et al. 1998, Keskintepe and Brackett 1996). However, IVP bovine embryos display a number of marked differences compared to in vivo one. These differences are evident in gross morphology (color, density, cell number and size), timing of development (Greve et al. 1995), in biochemical features such as buoyant density, chilling sensitivity, zona pellucida stability and resistance to freezing as well as in certain features of embryonic metabolism (Niemann et al. 1993). Another advance technique is somatic cell nuclear transfer (SCNT) with promising implication for the agricultural and biomedical research. Conventional SCNT or cloning involves fusion of a somatic donor cell into an enucleated metaphase II (MII) arrested oocyte. The resulting embryo is cultured to the

blastocyst stage before being transferred to a surrogate cow to produce live offspring. So, cloning procedures adopt to some extent the same procedure as in-vitro fertilization. Additional steps in cloning are enucleating the mature oocyte and thereafter transfer of the somatic nuclei in this enucleated oocyte. Later the cultivation and transfer to recipient heifers follows the same protocol as described for IVF derived foetuses. However, Cloning cattle using Somatic Cell Nuclear Transfer (SCNT) is an inefficient process, where the surviving offspring only represent approximately 5% of the embryos transferred into the surrogate cow (Oback and Wells 2003).

The IVF and SCNT technology has opened the possibilities to manipulate and cultivate the embryo. However, it has also been linked to many abnormalities in embryo development. Well evident abnormalities in foetuses or calves following transfer of in vitro cultivated embryos includes lower pregnancy rate, increased abortion, oversized calves, musculoskeletal deformities and abnormalities of placental development, which are often described as “Large Offspring Syndrome” (LOS). LOS has been described for bovine (Farin et al. 2006), sheep (Sinclair et al. 1999) and mice (Eggan et al. 2001). The abnormalities associated to the IVF, SCNT and in vitro culture of embryo are in principle due to aberrant or alteration of transcriptional activity at sub-cellular level. Several lines of evidence in mouse and cattle indicate that expression patterns of genes from in vitro-produced embryos are not necessarily representative of those of in vivo embryos (as reviewed) (Niemann and Wrenzycki 2000). An important gene that has been found to be expressed by in vivo-derived bovine blastocysts, but not in their in vitro-produced counterparts, is the Connexin-43 that is crucial for maintenance of compaction. The bovine leukemia inhibitory factor (bLIF) and LIF-receptor-R (LR-8) genes were found to be expressed by in vitro produced embryos, but not in their in vivo counterparts. The heat shock protein gene 70.1 (Hsp70.1) has been found upregulated by blastocysts produced in vitro compared to in vivo embryos, while the glucose transporter-l mRNA (Glut-l) is downregulated by morulae produced in vitro as compared to in vivo-derived morulae (Niemann and Wrenzycki 2000). However, such abnormalities are found to be more pronounced in cloned animals. The clones have a high mortality rate due to suffering a number of developmental abnormalities such as higher birth weights, muscular-skeletal problems and incorrect placental formation (Wells et al. 2004). Candidate gene study reveals that the failure of implantation may be due to aberrant expression of genes in the preimplantation cloned embryo, which are

crucial for the early regulation and differentiation of the placenta (Hall et al. 2005). At the cellular level, a higher incidence of apoptosis (Park et al. 2004) and aberrant allocation of inner cell mass (ICM) (Koo et al. 2002) is evident. At the sub-cellular level, aberrant DNA methylation patterns (Bourc'his et al. 2001) and the dysregulation of genes occurs (Humpherys et al. 2002). These abnormalities are thought to mainly be due to epigenetic defects (changes in chromatin structure, not involving a change in DNA base sequence) which occur during cell reprogramming, where the donor cell DNA is reprogrammed by the oocyte cytoplasm to a embryonic state (Schurmann et al. 2006).

Recent global gene expression profiling study has also evidenced aberrant regulation of gene expression either by genetic or epigenetic modification due to manipulation and culture of preimplantation embryos (Aston et al. 2009, Zhou et al. 2008). According to the nature and extend of regulatory mechanisms it could be consider that miRNAs are playing pivotal roles in such aberrant transcriptional processes. Since, miRNA has been appeared as first and foremost epigenomic tool or modifier that regulate gene expression epigenetically at the post-transcriptional or transcriptional level and were found to play important roles during mammalian development (Ambros 2004, Bartel 2004, Kloosterman and Plasterk 2006). They were found to be targeted by epigenetic modification and eventually controlling epigenetics and some imprinted miRNAs found to undergo subsequent epigenetic reprogramming in mouse embryos (Cui et al. 2009, Kircher et al. 2008, Williams et al. 2007). Although few reports until now have been addressed the differential miRNAs regulation in the IVP and SCNT embryo compared to the artificial inseminated one with particular aspects of their function. Among them, one has revealed the disregulated expression of several micro RNAs (miRNAs) in bovine cloned elongated embryos using a heterologous microarray (Castro et al. 2010). miRNAs expression profiling in elongated cloned and in vitro-fertilized bovine embryos has suggested that the different state of reprogramming of miRNAs occurred in cloned bovine elongated embryos (Castro et al. 2010). Among the most notable downregulated miRNAs found in their study were miR-30d and miR-26a. Both of these miRNAs interacted with TKDP, which is involved in maternal recognition of pregnancy in cattle (Lagos-Quintana et al. 2001). However, status of reprogramming error in the extra embryonic tissues (or placenta) has not yet been separated which could be the main reason for the cloned pregnancy loss during the first trimester. Based on these

preliminary data, it can be inferred that aberrant trophoblast gene and miRNAs expression in cloned embryos contributed to pregnancy failures at and beyond implantation. At this moment, much and more information is remaining to elucidate the role of miRNAs in the alteration of important transcripts in the IVP and SCNT embryo and their association to the abnormal embryonic development.

2.6 Maternal recognition of embryo, implantation and miRNAs

A critical need arises early in gestation for the mother to "recognize" that she is pregnant in most species. More specifically, the concentration of progesterone in maternal blood must be sustained at a high level in order that the endometrium be maintained in a state conducive to embryonic survival. This means that the corpus luteum must not die and regress, as it normally does just prior to the onset of the next cycle. The early ruminant embryo secretes copious quantities of a protein called interferon tau. Exposure of the endometrium to this hormone dampens the secretion of PGF, thereby blocking the signal for luteolysis. As a result, the corpus luteum survives and progesterone levels are maintained. During post hatching stage, the mononuclear trophoblast cells of embryo begin to transcribe mRNA for interferon tau (trophoblastin or trophoblast protein-1) at day 12. Highest transcription of mRNA for IFNt occurs on days 15 to 17 and continues until approximately day 25 when the initial adhesion of the conceptus to the luminal epithelium of the uterus halts IFNt gene expression (Figure 2.12) (Bartol et al. 1985, Farin et al. 1990). The expression of IFNt is limited to the trophectoderm and is not transcribed in the endoderm or yolk sac (Farin et al. 1990). The site specific production of IFNt by trophoblast cells may be due to exclusive expression of the blastocyst-specific transcription factor Cdx2 in trophoblast cells regulating IFNt gene transcription (Imakawa et al. 2006) and/or the absence of Oct-4 in these same cells (Ezashi et al. 2001). Uterine produced factors, such as granulocyte-macrophage-colony stimulating factor (Ezashi and Roberts 2004), interleukin 3 and insulin-like growth factors -I and -II (Ko et al. 1991) appear to enhance IFNt secretion by the conceptus by acting via the Ets-2 enhancer region (Ezashi and Roberts 2004). Synthesis and secretion of IFNt by the developing conceptus blocks the luteolytic signal, maintaining the function of the corpus luteum and is thereby the signal for maternal recognition of pregnancy (MRP) in ruminants (Bazer et al. 1989, Bazer et al. 1991, Bazer 1992). In addition to signaling MRP, IFNt is also responsible for inducing

or enhancing the expression of numerous genes referred to as IFNt-stimulated genes (ISGs). These ISGs contribute to the regulation of uterine receptivity and conceptus development at early gestation (Hansen 1998, Spencer et al. 2004).

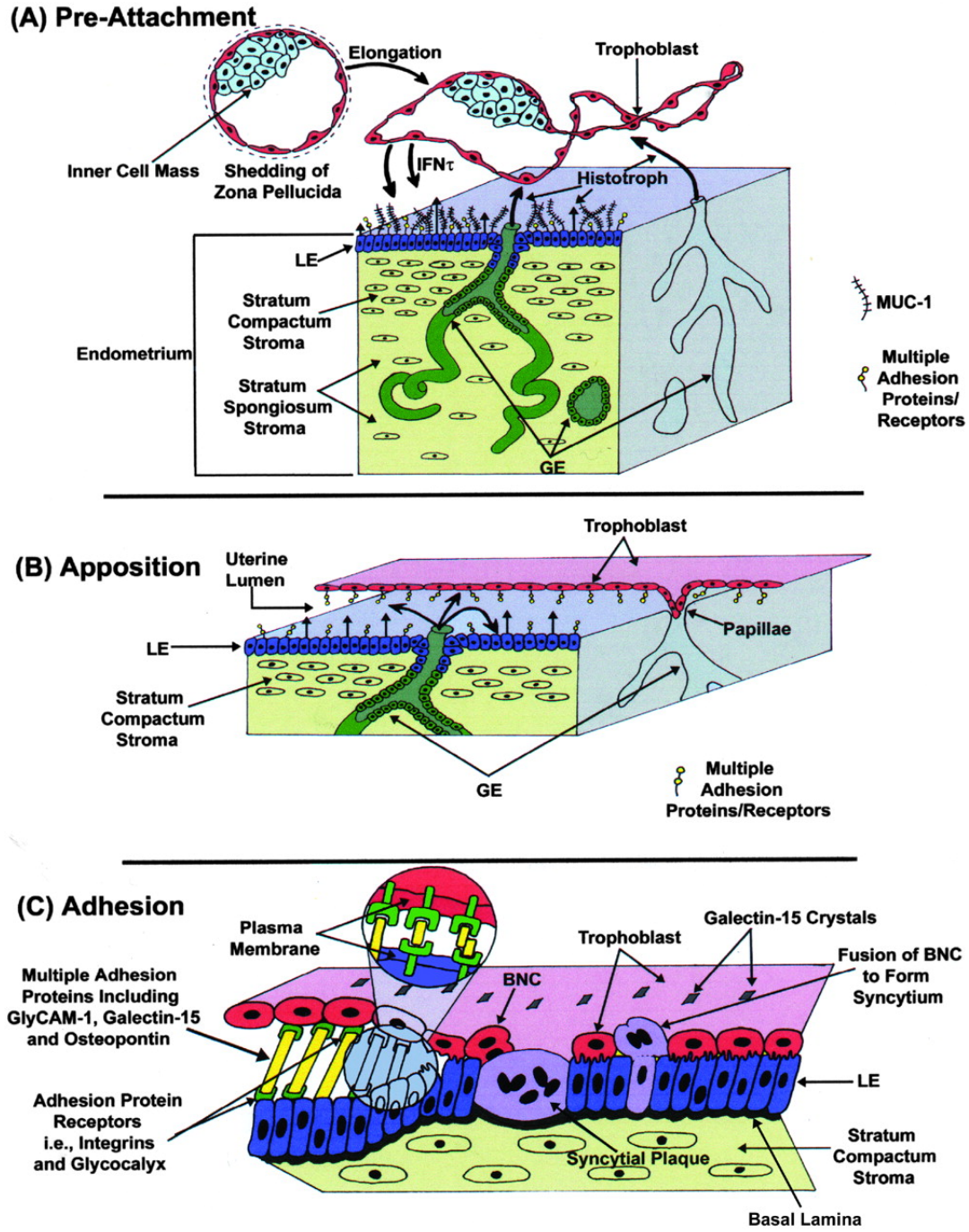


Figure 2.12: Maternal recognition of pregnancy and different phases of blastocyst implantation. (A) Maternal recognition and preattachment, which is involving shedding of the zona pellucida (phase 1) on day 8 and precontact and blastocyst orientation (phase 2) on day 9-11. (B) Apposition and transient attachment (phase 3) after day 11 (C) Adhesion (phase 4) between days 15 and 16 (adapted from Spencer et al. 2004).

Following MRP, the adhesion between trophoblastic cell membranes and the tips of the microvilli of uterine luminal eptithelial cells is evident by days 19 to 20 of gestation (Guillomot 1995). Maternal recognition of pregnancy and different phases of blastocyst implantation are shown in figure 2.12. To facilitate adhesion of the conceptus, remodeling of the uterine endometrial extracellular matrix (ECM) is necessary (MacIntyre et al., 2002; Yamada et al., 2002b). ECM proteins in the uterine endometrium includes several proteins namely types I and IV collagen, laminin and fibronectin (MacIntyre et al. 2002, Yamada et al. 2002). Coordinated production of matrix metalloproteinases (MMPs) by the conceptus and the production of tissue inhibitors of MMPs (TIMPs) by the endometrium are also reported (Salamonsen et al. 1995). Among the several proteins those are involved with adhesion of the trophectoderm to the luminal epithelium of the uterus, the most important are integrins which play a dominant role in the interaction with the ECM to convey cellular signals between the conceptus trophectoderm and uterine epithelial cells (MacIntyre et al. 2002). The adhesion stage is usually characterized by firm adhesion between the conceptus and luminal epithelium, which begins between days 21 to 22 of bovine gestation (Guillomot 1995) and is completed by day 27 (King et al. 1980). Once cellular contact is established between the trophectoderm and luminal epithelium, IFNt gene expression drops immediately down (Guillomot et al. 1990). This attachment is transient and it is replaced within a few days by a more robust adhesion in the glandular intercaruncular region between the trophoblast and luminal epithelium, called an areolae (Guillomot 1995, Spencer et al. 2004). These areolae are specialized areas within the intercaruncular placenta that form openings of gland ducts on the surface of the luminal epithelium (Guillomot 1995). Syncytial plaque formation in the uterine epithelium during implantation is facilitated by the migration and fusion of the trophoblastic binucleate cell (BNC) with uterine cells (Wooding 1983).

Binucleate cells are responsible for the formation of the hybrid feto-maternal syncytia, allowing for successful implantation and subsequent cotyledonary growth of the placentome (Spencer et al. 2007). Binucleate cells- a specialized group of cells in ruminants that are produced by nuclear division of uninucleate trophoblast cell without cytoplasmic division. These cells are responsible for the simultaneous production and paracrine secretion of proteins and growth factors at the materno-fetal interface (Kessler and Schuler 1997). About 20 % of the surface of the trophectoderm comprises

binucleate cells (BNC) (Xie et al. 1997), which migrate through adjacent cell tight junctions and across the microvillar junction to reach the maternal luminal epithelium. They fuse with maternal epithelial cells forming a multicellular complex (Wooding 1983). In the bovine, this migration is maximal at approximately d 24 of gestation, but remains functional throughout gestation (Wooding, 1983). The process of migration and attachment of the BNC is a relatively undefined process but appears to involve such factors as glycoprotein CD9 and fertilin (Xiang and MacLaren 2002). Secretion from BNC includes members of the growth hormone/prolactin family, including: chorionic somatomommotropin 1 (CSH1; also know as placental lactogen), several prolactin-related proteins (PRP; -I, -VII, -VIII and -IX) and members of the aspartic proteinase family called pregnancy associated glycoproteins (PAGs; also known as pregnancy specific proteins B) (Spencer et al. 2007). These factors are placental specific hormones that are not synthesized or secreted by any other endocrine gland (Anthony et al. 1995). In the bovine, mRNA for CSH1 is detected between days 26 to 30 of gestation and increases throughout gestation, however PRP-1 mRNA expression begins between days 20 to 25 of gestation, is of maximal concentration at day 60 of gestation and decreases thereafter (Yamada et al. 2002). Placental CSH1 binds to prolactin homodimer and prolactin/growth hormone heterodimer receptors in the glandular epithelium and stimulates uterine gland growth and/or differentiated functions during pregnancy (Spencer et al. 2007).

Maternal recognition of pregnancy and subsequent implantation is largely dependent on steroidogenic balance and specific transcriptional activities. So there is a strong possibility for the miRNAs to be involved in such mechanism, since miRNAs dependent regulation of steroidogenesis and steroidogenic control of miRNAs biosynthesis has already been evidenced as discussed in the literature on ovary. The receptivity of uterus during blastocyst implantation is achieved through transition from elevated estrogen dependent highly proliferative state to progesterone dependent highly secretory state. miRNAs could also be involved in implantation process via uterine changes through regulating or interfering the post transcriptional and translational activity of vast number of genes. Supported studies suggest that miRNAs participate in regulating dynamic changes in uterine gene expression patterns that occur during the transition from the pre-receptive to the receptive phase and miRNAs mediated regulation of uterine gene expression in the context of implantation is evidenced

(Chakrabarty et al. 2007). Hence, it clear that miRNAs potentially regulate gene expression and participate in directing the highly regulated spatiotemporally expressed genetic network during implantation (Chakrabarty et al. 2007). In addition, several studies have already reported the regulation of miRNAs in the endometrium by the ovarian steroid (Macias et al. 2009, Pan et al. 2007, Toloubeydokhti et al. 2008b). Further, in vitro gain- and loss-of-function experiments showed that the expression of cyclooxygenase-2, a gene critical for implantation, is post-transcriptionally regulated by two miRNAs namely, mmu-miR-101a and mmu-miR-199a* (Chakrabarty et al. 2007). Another study has identified higher expression of miR-21 in the subluminal stromal cells at implantation sites on day 5 of pregnancy but not detected during pseudo-pregnancy or even under delayed implantation (Hu et al. 2008). This revealed that the expression of mmu-miR-21 in the implantation sites regulated by the active blastocysts. Moreover, in the same study, the role of miR-21 in embryo implantation has been suggested due to targeted regulation of the Reck gene (Hu et al. 2008). The topic becomes interesting when it is identified that miRNAs could be released in the extra cellular environment and functioning (Chim et al. 2008, Luo et al. 2009). So, there is a possibility that miRNAs in regulating extra cellular signaling network as like as interferon tau and other associated molecules during the maternal recognition of pregnancy in the pre attachment period. However, relevant study to identify such involvement is still remaining to be elucidated.

2.7 miRNAs in the development and physiology of placenta

The development of the early embryos and placentas in bovine is markedly different from primates and rodents. Human blastocyst gets implanted to the uterus at day 5-7 while in mouse implantation occurs at day 4 of gestation (Paria et al. 2001, Vigano et al. 2003). The bovine placenta is characterized as chorioallantoic type and by structures or by developmental patterns it is different in comparison to human or mouse. Development of the bovine placenta is dependent on proper gastrulation during extended pre-attachment period, which gives rise to the development of the germ layers (endoderm, mesoderm and ectoderm), concomitant trophoblast elongation and the subsequent development and vascularization of the allantois (Maddox-Hyttel et al. 2003). The hypoblast cells or primitive endoderms are formed from the ICM and confluent hypoblast cells are lining the inside of the trophoblast occurs around day-8

and day-10, respectively. Epiblast (ICM at day12) displaces the overlying trophoblast lining (Rauber's layer) and develops the embryonic disk. Later on between days 14 and 16 mesoderm formation is initiated (Maddox-Hyttel et al. 2003). Trophoblast cells together with the somatic mesoderm form the chorion successively. Folds of trophectoderm cells fuse with the somatic mesoderm and form the amnion that create a fluid-filled space and surrounds the embryo proper. During the progress of the embryo to develop into a fetus, an out pocketing of the hindgut extends from the fetus into the loose tissues of the splanchnic mesoderm forming the allantois at around day 20. The allantois is responsible for vascularizing the chorion and amnion. The allantois expands, becomes directly apposed to the chorion and the two tissues fuse to form the chorioallantois. Together with the amnion these tissues form the extra-embryonic fetal membranes, refereed to as the placenta (Schlafer et al. 2000). The early placenta when comes in contact with maternal caruncles, it induces villous processes undergo hypertrophy and hyperplasia and subsequently forms cotyledons. Small villi or papillae develop on the regions of the trophoblast that are apposed to the intercaruncular endometrium and project into the openings of the uterine glands (Wooding et al. 1982). These villi allow for a primary route for absorption of glandular secretions and provide an initial anchor for the conceptus as well. During this period in the luminal surface of the uterine caruncles are found to become wrinkled and concave to allow the ridged surface of the trophoblast to align with the undulated surface of the caruncle (Guillomot 1995). This is the initial development of the placentome, which is the site of fusion of the placental cotyledons with the endometrial caruncles (Spencer et al. 2004). By day 42 fused placental cotyledons with endometrial caruncles are progressed to form larger and complex placentomes (Noden and De Lahunta 1985). Placentomes with extensive villous formation are the primary site of transport for easily diffusible molecules such as amino acids, glucose, oxygen and carbon dioxide, while macromolecules are transported in the inter placentomal areas adjacent to uterine glands (Wooding and Flint 1994).

Regarding involvement of miRNAs in the process of placentogenesis is yet to be discovered in detail. However, complex physiological process and multitude of gene expression in the placentation and function indicates that miRNAs could be involved to play a pivotal role as they are found to be important regulator in many other cellular processes. Recently some sporadic attempts were taken to study miRNAs during

pregnancy, prostaglandin regulation and patho-physiological condition of women placenta. First demonstration was to study the existence of human placental miRNAs in maternal plasma and their stability through out with physical nature by TaqMan MicroRNA Assays (Chim et al. 2008). Interestingly, 4 most abundant placental miRNAs (miR-141, miR-149, miR-299-5p and miR-135b) were detected in the maternal plasma during pregnancy and found to be reduced expression in post-delivery plasma. The plasma concentration of miR-141 has been found to increase as pregnancy progressed into the third trimester. Compared with mRNA encoded by CSH1 [chorionic somatomammotropin hormone 1 (placental lactogen)], miR-141 was stable in maternal plasma (Chim et al. 2008). This initial evidence indicates that miRNAs are involved in the regulation of placental functions and pregnancy. Another study was performed by small RNA library sequencing and miRNA histochemistry using human placental chorionic villi to identify miRNA expression profiles in the human placenta (Luo et al. 2009). The miRNA cluster genes were observed to be differentially expressed in placental development and further revealed that villous trophoblasts express placenta-specific miRNAs. In addition, analysis of small RNA libraries from the blood plasma showed that the placenta-specific miRNAs are abundant in the plasma of pregnant women and the rapid clearance of the placenta-specific miRNAs from the plasma after delivery. It has been also demonstrated that miRNAs are indeed extracellularly released via exosomes while studied trophoblast cell line cultured in vitro (Luo et al. 2009). The study has been suggested that miRNAs are exported from the human placental syncytiotrophoblast into maternal circulation, where they could target maternal tissues. This result interestingly coincides with the previous study (Chim et al. 2008) and placental miRNAs has been identified to enter into maternal circulation. These data provide initial evidence and important insights into miRNA biology of the human placenta. Growth and differentiation of the placenta are fundamental to mammalian reproduction, including humans and functional impairment of this organ by misregulation of miRNAs could lead to severely abnormal pregnancies as well as other associated diseases. For instance, miRNAs were found as differentially regulated in the placenta with severe pre-eclampsia when compared to normal one (Hu et al. 2009). Pre-eclampsia (PE) is usually caused by poor placentation with impaired remodeling of the spiral arteries. It has been found that miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222 were significantly increased in placenta from women with severe PE (Hu et al. 2009). This suggests that different miRNAs may play an important role in

pathogenesis of PE. Investigation to elucidate the importance of miRNAs in regulation of placental development and function is just at the beginning. Whatever involvement identified in human even though it is very few, in case of ruminants it is untouched and could be an interesting field of investigation.

2.8 miRNAs and abnormal placentogenesis in the IVP and SCNT pregnancy

There have been many problems associated with the effect that nuclear transfer has on the normal development of the cloned animal. By far, the most commonly reported theme of deformity is that of placental development. Following sections describe and highlight some commonly observed abnormalities, transcriptional aberration and abnormal epigenetic modification found to arise in the placenta from IVP and SCNT pregnancies together with probable involvement of miRNAs and problem statement.

2.8.1 Abnormal placentogenesis in the SCNT pregnancy

It has been well recognized that animals produced via SCNT method exhibit a wide range of placental abnormalities that result in a substantial loss of live births, the birth of deformed offspring and an inordinate amount of stress on the recipient animal. Abortion throughout gestation and perinatal death of cloned calves has been attributed to such placental abnormalities noted after nuclear transfer. Placental anomalies have been reported in many studies with some of the more common pathologies include, but are not limited to, lack of adventitial placentation, lower number of placentomes, thick and oversized placentomes, edematous membranes, edematous amnion, edematous chorioallantois, intercotyledonary edema (Hill et al. 1999), retarded allantoic growth (Wells et al. 1999), hydroallantois, decreased vascularization, decreased number of cotyledons and binucleate cells, enlarged umbilical vessels (Wells et al. 1999) and increased cotyledonary size and cell numbers (Batchelder et al. 2005, Chavatte-Palmer et al. 2002, Cibelli et al. 1998, Hashizume et al. 2002, Heyman et al. 2002, Hill et al. 2000, Hill et al. 2001, Hoffert et al. 2005, Ravelich et al. 2004a, Stice et al. 1996, Zakhartchenko et al. 1999). Many of the failed pregnancies abort during the middle of the first trimester, around day 40 (Hill et al. 1999, Stice et al. 1996), even though the fetus appears to be developing in a normal manner (Wells et al. 1999). Major causes for the mentioned placental abnormalities are thought to be due to aberrant gene expression,

imprinting loses and defective epigenetic modification resulted from donor cell nuclear reprogramming error by the oocyte cytoplasm. Primary findings related to this field are discussed bellow.

2.8.2 Aberrant gene expression in the placenta of different sources of pregnancies

Abnormal gene expression has been noted in cloned mouse and bovine fetuses and placenta. Dysregulated gene expression has been identified in the placentas or placentomes of cloned bovine fetuses as early as day 25 and throughout gestation to term by several studies (Hashizume et al. 2002, Hill et al. 2002). At the protein level, 60 proteins were found to be differentially expressed in term placentas of cloned calves compared with fertilized controls (Kim et al. 2005). Abnormal expression of over 200 genes was noted in cloned mouse placentas (Humpherys et al. 2002). Further support for dysfunctional placental development in clones comes from the recent study (Jouneau et al. 2006), which showed that the majority of the cloned mouse embryos die before a functional placenta can develop. Elevated interferon tau expression was detected in one study from bovine cloned embryos (Wrenzycki et al. 2001). Major histocompatibility complex I expression has been detected abnormally early in cloned bovine embryos and fetuses and has indicated immunologic rejection by the recipient as a possible cause of early embryonic death (Hill et al. 2002, Pfister-Genskow et al. 2005). Insulin-like growth factor binding proteins 2 and 3 showed increased expression in the extraembryonic membranes of bovine nuclear transfer fetuses (Ravelich et al. 2004a). Examination of gene expression patterns in bovine placentomes collected from SCNT, IVF and AI pregnancies demonstrates gross abnormalities in gene expression associated with SCNT cloning (Everts et al. 2008, Oishi et al. 2006). Finally, when gene expression in cloned mice derived from nuclei of different cell types was examined (Humpherys et al. 2002), some cell type-specific effects were seen, but most of the abnormalities in cloned mice were independent of donor cell type and seemed to be a consequence of the nuclear transfer procedure. Moreover, a comparison of the effects in placenta and liver of cloned pups demonstrated that the placental effect is especially pronounced, with at least 4% of genes expressed in the placenta showing dysregulation. Aberrant placental lactogen, pregnancy-associated glycoprotein, leptin levels and heparanase levels were noted in the placental tissues derived from cloned bovine pregnancies (Hashizume et al. 2002, Ravelich et al. 2004b). These studies have

indicated widespread abnormalities in gene expression from both fetal and placental tissues of cloned offspring. The common occurrence of aberrant gene expression in early SCNT cotyledons compared to cotyledons from artificial insemination derived pregnancy (Aston et al. 2009). In particular, most aberrant expression was found for the imprinted transcripts in SCNT placenta compared to AI placenta. These issues are discussed detail in the following section.

2.8.3 Aberrant epigenetic reprogramming and genomic imprinting in the placenta from SCNT pregnancies

Epigenetic process is the heritable differences in gene function and expression leading to phenotypic differences which cannot be explained by the DNA sequence itself (Smith and Murphy 2004, Wolffe and Matzke 1999, Wu and Morris 2001). Cellular development and function may be affected by changes in epigenetic processes altering gene expression (Smith and Murphy 2004). Abnormal epigenetic mechanisms including aberrant DNA methylation, X-chromosome inactivation and histone modification have been shown to be associated with cloned placenta. It is obvious that gene expression patterns affected by epigenetic processes may not be correctly reestablished after nuclear transfer. After SCNT chromatin structure and transcriptional activity of donor nuclei must revert to that of an early one-cell embryo after nuclear transfer in order to direct embryonic development. This process is known as nuclear reprogramming and essentially involves both morphological and biochemical changes (Di Berardino 1997, Kikyo and Wolffe 2000, Sun and Moor 1995). In mammals, nuclear remodeling after nuclear transfer has been recognized as chromatin condensation, nuclear envelope breakdown and the formation of a pronuclear-like structure that undergoes swelling and transferred nucleus tends to behave more like that of early stage embryos (Adenot et al. 1997, Baran et al. 2002, Collas and Robl 1991, Czolowska et al. 1984, Hyttel et al. 2001, Kanka et al. 1991, Prather et al. 1990, Stice and Robl 1988, Szollosi et al. 1988). Transcription from the donor nucleus is normally ceased after nuclear transfer during nuclear remodeling, but reappeared later on during genomic activation at 8- to 16- cell stage (Kanka et al. 1991, Kim et al. 2002). Protein and lamin expression have been reported to change after nuclear transfer in mammalian studies (Kubiak et al. 1991, Prather et al. 1989, Prather et al. 1991, Prather and Rickords 1992). Nuclear transfer bovine embryos were found to be absent of immunoreactive somatic histone H1 after

nuclear transfer and then reappeared at the time of genomic activation in bovine embryos (Bordignon et al. 1999). Epigenetic alterations of the genome are found to be occurred primarily by histone modification and DNA methylation (Smith and Murphy 2004) and affecting critical developmental processes, namely genomic imprinting, X-chromosome inactivation and gene expression.

DNA methylation involves the addition of a methyl group to the 5th position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring (cytosine and adenine are two of the four bases of DNA with the specific effect of reducing gene expression. Changes in embryo-wide DNA methylation patterns and global DNA methylation levels were also reported in association with adverse preimplantation development (Cezar et al. 2003, Hiendleder et al. 2004). In cloned mice, individual clones showed differing aberrant methylation patterns of CpG islands in placental tissues (Ohgane et al. 2001). Elevated methylation levels were found in cloned bovine embryos (Dean et al. 2001, Santos et al. 2003). It has been found that an initial demethylation of the donor nucleus takes place immediately after transfer but no further demethylation during early embryogenesis followed by precocious de novo methylation at the 8-cell stage (Dean et al. 2001). Demethylated euchromatin and hypermethylated heterochromatin in cloned bovine embryos has also been reported (Bourc'his et al. 2001). The methylation of the ninth lysine residue on histone H3 has been shown to correlate with the pattern of DNA methylation in cloned bovine embryos with abnormal elevation and altered histone acetylation levels in many of the cloned embryos and placenta (Enright et al. 2003, Santos et al. 2003).

X-inactivation is a process by which one of the two copies of the X chromosome present in female mammals is inactivated. The inactive X chromosome is silenced by packaging into transcriptionally inactive heterochromatin. X-inactivation occurs so that the female, with two X chromosomes, does not have twice as many X chromosome gene products as the male, which only possess a single copy of the X chromosome. In cattle, X-chromosome inactivation was found to be abnormal (Xue et al. 2002). Random X-inactivation in the placental tissues cloned calves and abnormal methylation as well as altered expression patterns of X-linked genes were observed (Gutierrez-Adan et al. 2000, Niemann et al. 2002, Wrenzycki and Niemann 2003, Wrenzycki et al. 2005). In contrast, surviving cloned calves were found to have normal X-linked gene expression

patterns (Xue et al. 2002). Just as X-chromosome dosage compensation can be impaired in cloned offspring, histone modification patterns may be abnormal in cloned embryos. Genomic imprinting is a genetic phenomenon by which certain genes are expressed in a parent-of-origin-specific manner. It is an inheritance process independent of the classical Mendelian inheritance. Imprinted genes are either expressed only from the allele inherited from the mother (e.g. H19 or CDKN1C), or in other instances from the allele inherited from the father (e.g. IGF-2) through DNA methylation and histone modifications in order to achieve monoallelic gene expression without altering the genetic sequence. Nuclear transfer and inadequate in vitro culture conditions leading to improper genetic reprogramming which results in improper establishment of imprinting at the regulatory imprinting centers during the critical period of pre implantation and later on, such errors are affecting placental development. Sequences associated to the control of the imprinted genes H19 and Snprn have been found to be demethylated in cloned mouse embryos (Mann et al. 2003). Investigations on imprinted gene expression in bovine and ovine fetuses derived from IVP and SCNT have been suggested that improper genomic imprinting. Examination of gene expression patterns in the placentas of cattle, sheep and mice produced using in vitro culture procedures demonstrated altered expression of imprinted (Bertolini et al. 2002, Blondin et al. 2000, Doherty et al. 2000, Wrenzycki et al. 2004, Young et al. 2001) and autosomal non-imprinted genes (Ravelich et al. 2004a, Ravelich et al. 2006, Wrenzycki et al. 2004). Adult cloned animals were generally considered to be normal when analyzed for epigenetic changes, suggesting that perhaps only the most epigenetically normal embryos survive to adulthood (Cezar et al. 2003, Ohgane et al. 2001). These studies all indicate that widespread chromatin remodeling, modification of DNA methylation and histone modification occurs during nuclear reprogramming which could be the primary reason for abnormal placentogenesis leading to the loss of cloned embryos.

2.8.4 miRNAs in the placenta from SCNT pregnancy in relation to its aberrant genetic and epigenetic modification

According to the aforesaid reviews, it is completely evident that the placental deformities are a major hindrance in the production of nuclear transfer animals. So, studying the important factor which is involved in regulating aberrant genetic and epigenetic gene regulation, reprogramming errors, aberrant genomic imprinting and in a

word the genetic or epigenetic modification associated to the abnormal placentogenesis in somatic cell nuclear transfer pregnancies is paramount important. As mentioned, key mechanisms underlying this epigenetic reprogramming are DNA methylation, histone remodeling and telomere maintenance, which are involved in the control of gene expression, X chromosome inactivation and genomic imprinting (Holmes and Soloway 2006, Li et al. 1993, Perecin et al. 2009). Interestingly, all mechanisms are not contributing equally to the embryonic and extra-embryonic lineage (McGrath and Solter 1984, Oudejans et al. 1997, Surani et al. 1984, Wagschal and Feil 2006). Specially, genomic imprinting has been shown to be less or not dependent on DNA methylation in the placenta for its somatic maintenance than in the embryo. Rather, placenta-specific imprinting involves repressive histone modifications and non-coding RNAs (Brockdorff 2002, Higashimoto et al. 2002, Lewis et al. 2004, Li et al. 1993, Sado et al. 2000, Sado and Ferguson-Smith 2005, Yatsuki et al. 2004). When considering non-coding RNAs, then miRNAs a recently discovered class of small RNAs appeared as first and foremost epigenomic tool or modifier that regulate gene expression epigenetically at the post-transcriptional or transcriptional level and were found to play important roles including, but not restricted to, cell proliferation, apoptosis, diseases and differentiation during mammalian development (Ambros 2004, Bartel 2004, Kloosterman and Plasterk 2006). So, miRNA could be consider as an important regulatory factor directly link to mentioned aberrant molecular mechanisms for genetic and epigenetic modification. They were found to be targeted by epigenetic modification and eventually controlling epigenetics and some imprinted miRNAs found to undergo subsequent epigenetic reprogramming in mouse embryos (Cui et al. 2009, Kircher et al. 2008, Williams et al. 2007). Many of the miRNAs have been predicted and found to have pivotal roles in controlling DNA methylation, regulating chromatin structure and controlling telomere recombination (Benetti et al. 2008a, Benetti et al. 2008b, Fabbri et al. 2007, Guil and Esteller 2009, Lewis et al. 2005, Lujambio and Esteller 2007, Ting et al. 2008, Valeri et al. 2009). Interestingly, they could be imprinted like genes and many of X-linked microRNAs escape meiotic sex chromosome inactivation (Kanellopoulou et al. 2009, Song et al. 2009).

Recent epigenetic regulation by the miRNAs has opened up a new dimension of mode of regulation from translational suppression and classic RNAi degradation. In addition to regulation of gene expression at the posttranscriptional level in the cytoplasm, recent

findings suggest additional roles for miRNAs in the nucleus. MiRNAs which are encoded within the promoter region of genes could be involved in silencing such genes at transcription level epigenetically. Such cis-regulatory roles of miRNAs have been observed in transcriptional silencing of POLR3D expression and endothelial nitric oxide synthase (eNOS) promoter activity (Kim et al. 2008, Zhang et al. 2005). Recently, aberrant epigenetic reprogramming of imprinted miR-127 in cloned murine embryos has been reported in relation to the aberrant epigenetic reprogramming of the mouse retrotransposon-like gene Rtl1 (a key gene in placental formation) (Cui et al. 2009). Moreover, the miRNA has been recognized as an important regulator of genetic and epigenetic modifier but study on their role in the well recognized aberrant genetic and epigenetic mechanism associated to malformation or abnormal development of placenta in somatic cell nuclear transfer pregnancies is missing. Thereby, very little or no information are available about the specific miRNA and their targets to regulate epigenetic machinery or epigenetic regulation of specific miRNAs that are required for normal physiological condition or for any phenotypic effects of placenta derived from SCNT pregnancies. Considering this important facts, part of the present study has been conducted to identify the expression, regulation or deregulation if there is, in the placenta derived from in vitro, SCNT pregnancies compared to that in vivo pregnancies as well other associated molecular mechanisms.

3 Part I: miRNAs in the bovine ovary

Follicular development begins with the establishment of a finite pool of primordial follicles, which culminates in either by the atretic degradation or release of a mature oocyte from the follicle. Entire process is governed by numerous intra-ovarian factors through complex mechanisms including transcriptional regulation which could be regulated by recently identified new class of non-coding small RNAs of ~22nt i.e. miRNAs. Most miRNAs in animals are thought to function through the inhibition of effective mRNA translation of target genes through base pairing with the 3´-untranslated region. They are already proved as one of the key transcriptional regulators in different biological processes for disease, development and fertility. But their presence & expression in bovine ovary has not yet determined. The present study was conducted with a view to discover distinct miRNAs in bovine ovary as well as to find out their pattern of expression and characteristics.

To identify miRNAs in bovine ovary, small RNA-cDNA library was constructed. For this purpose, total RNA enriched with small RNA was isolated from ovary and size fractionated (18-24 nt) by denaturing PAGE using 21nt size marker. Once the enriched small RNA fraction has been recovered from the acrylamide gel slice, the small RNAs were ligated with a 3´ linker and containing a Ban-I restriction site, which upon purification from dPAGE again, were subjected to reverse transcription. At this point an exonuclease digestion was carried out and then a second 3´ ligation was done using a different linker sequence. Linkered product (60 nt) was purified from dPAGE and subsequently, the amplification of the RT product was done. Then the amplicon was subjected to Ban I digestion, concatemerization, end filling and non-templated adenosine addition for serially ligated fragments. Concatemers were later on cloned in to PCR cloning vector and transformed into TOP 10 chemically competent cells. Colonies were picked and sequenced. Bioinformatic analysis was done according to the published criteria's for the small RNAs by means of publicly available web tools. In addition, bioinformatic characteristics, target prediction, molecular mechanisms including different pathways and functions of the predicted targets and expression patterns of the identified miRNAs in other reproductive tissues and cells were studied. Detail materials and methods used for this study and the findings obtained thereafter in the study are presented below.

3.1 Material and methods

3.1.1 Materials and tools

3.1.1.1 List of chemicals, kits, biological and other materials

List of Chemicals, kits and other materials	Manufacturer/Supplier
1-STEP NBT/BCIP	Thermo Fisher Scientific Inc. IL, USA
2- Propanol	Roth, Karlsruhe, Germany
5-Bromo-4-chloro-3-inolyl-phosphate (BCIP)	AppliChem GmbH, Darmstadt, Germany
Acetic acid	Roth, Karlsruhe, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ammonium acetate	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ammonium peroxide sulphate	Roth, Karlsruhe, Germany
Ampicillin	Roth, Karlsruhe, Germany
Anti-Digoxigenin-AP	Roche Diagnostics GmbH, Mannheim, Germany
Ban I restriction endonuclease	New England Biolabs, MA, USA
BioThermD™ Taq DNA Pol	Ares Bioscience GmbH, Cologne, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, Germany
Bromophenol blue	Roth, Karlsruhe, Germany
Chloroform	Roth, Karlsruhe, Germany
dNTPs	Roth, Karlsruhe, Germany
Dye terminator cycle sequencing	Beckman Coulter, Krefeld, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid	Roth, Karlsruhe, Germany
ExoSAP-IT	USB, Ohio, USA
Fast Red Substrate System	Dako Deutschland GmbH, Hamburg, Germany
Fish sperm DNA	Roche Diagnostics GmbH, Mannheim, Germany
Formaldehyde	Sigma-Aldrich Chemie GmbH, Munich, Germany
GelStar® Nucleic Acid Stain	Lonza Bioscience, Köln, Germany
GenElute™ plasmid Miniprep kit	Sigma-Aldrich Chemie GmbH, Munich, Germany

Glycogen	MBI Fermentas GmbH, Leon-Rot, Germany
Glycogen for sequencing	Beckman Coulter, Krefeld, Germany
Heparin	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isopropyl β-D-thiogalactoside IPTG	Roth, Karlsruhe, Germany
Levamisol	Sigma-Aldrich Chemie GmbH, Munich, Germany
Methylsalicylate	Sigma-Aldrich Chemie GmbH, Munich, Germany
MiniElute™ reaction cleanup kit	Qiagen, Hiden, Germany
miRCat-33™ Conversion Oligo	Integrated DNA Technologies, Munich, Germany
miRCat™ Small RNA Cloning Kit	Integrated DNA Technologies, Munich, Germany
miRCURY™ LNA Detection probe	Exiqon, Vedbaek, Denmark
mirVana miRNA isolation kit	Applied Biosystems, Foster City, CA, USA
miSPIKE internal RNA control	Integrated DNA Technologies, Munich, Germany
Oligonucleotide primers	Eurofins MWG Operon, Ebersberg, Germany
One Shot® TOP10 E. coli	Invitrogen, Carlsbad, CA,USA
Penicillin	Sigma-Aldrich Chemie GmbH, Munich, Germany
Performa® DTR Gel Filtration	Edge BioSystems, Maryland, USA
Permount* Mounting Medium	Fisher Scientific GmbH, Schwerte, Germany
Phenol:Chl:Isoamyl Alcohol	Invitrogen, Carlsbad, CA, USA
Poly(A) Tailing Kit	Ambion Inc, Austin, TX, USA
QIAquick Gel Extraction Kit	Qiagen, Hiden, Germany
QIAquick PCR purification kit	Qiagen, Hiden, Germany
Ribo-nuclease inhibitor (RNasin)	Promega, Mannheim, Germany
RNA later	Sigma-Aldrich, MI, USA
RNAse-OUT™	Invitrogen, Carlsbad, CA, USA
RNeasy mini kit	Qiagen, Hiden, Germany
Sample loading solution (SLS)	Beckman Coulter, Krefeld, Germany
Sequagel XR Sequencing Gel	Beckman Coulter, Krefeld, Germany
Sheep serum	Sigma-Aldrich Chemie GmbH, Munich, Germany
Sodium acetate	Roth , Karlsruhe, Germany
Sodium pyruvate	Sigma-Aldrich Chemie GmbH, Munich, Germany
SuperScript™ III RT	Invitrogen, Carlsbad, CA, USA
T4 DNA ligase	Promega, Mannheim, Germany
TOPO TA Cloning® vector	Invitrogen, Carlsbad, CA, USA
Tris	Roth , Karlsruhe, Germany

Triton X-100	Roche Diagnostics GmbH, Mannheim, Germany
Vectashield Mounting Medium	Vector Laboratories, Inc. Burlingame, CA USA
Vector Methyl green nuclear	Vector Laboratories, Inc. Burlingame, CA USA
X-Gal (5-bromo-4-chloro-3-indolylbeta-D-galactopyranoside)	Roth, Karlsruhe, Germany
Yeast tRNA	Invitrogen, Carlsbad, CA, USA

3.1.1.2 List of equipments

ABI PRISM® 7000 SDS	Applied Biosystems, Foster City, CA, USA
Agilent 2100 bioanalyzer	Agilent Technologies , CA, USA
ApoTome microscope	Carl Zeiss MicroImaging, Germany
Centrifuge	Hermel, Wehing
CEQ 8000 genetic analysis apparatus	Beckman Coulter, Brea, CA, USA
Confocal laser scanning microscope-510	Carl Zeiss, Germany
Electrofusion machine, CFA 400	Kruess, Hamburg, Germany
Electrophoresis unit (for agarose gels)	BioRad, Munich, Germany
Fluorescence microscope (DM-IRB)	Leica, Bensheim, Germany
Inverted fluorescence microscope DM IRB	Leica, Bensheim, Germany
Millipore apparatus	Millipore Corporation, USA
My Cycler Thermal cycler	Bio-RadLaboratories, CA, USA
NanoDrop 8000 spectrophotometer	NanoDrop, Wilmington, Delaware, USA
Power supply PAC 3000	Biorad, Munich, Germany
PTC-100 thermal cyclers	BioRad, Munich, Germany
Savant SpeedVac	GMI, Inc. Minnesota, USA
SHKE6000-8CE refrigerated Shaker	Thermoscinentific, IWA, USA
Spectrophotometer, Ultrospec™ 2100 *pro*	Amersham Bioscience, Munich, Germany
Stereomicroscope SMZ 645	Nikon, Japan
Tuttnauer autoclave	Conn. unlimited, Wettenberg, Germany
Ultra low freezer (-85 °C)	Labotect GmbH, Gottingen, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.1.3 List of softwares

BLAST cow sequences	http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/
Blast ncRNA database	http://ncrnadb.trna.ibch.poznan.pl/blast.html
ENSEMBL genome browser	http://www.ensembl.org/index.html
Entrez Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Gene Ontology	http://www.geneontology.org
Genomic tRNA database	http://lowelab.ucsc.edu/GtRNAdb/
Mfold web server v 3.2	http://frontend.bioinfo.rpi.edu/applications/mfold/
miRBase Targets Version 5	http://microrna.sanger.ac.uk/targets/v5/
miRBase_12.0	http://microrna.sanger.ac.uk/sequences/
Multiple Sequence Alignment	http://searchlauncher.bcm.tmc.edu/
Primer Express 2.0	Applied Biosystems, Foster City, CA, USA
Ribosomal RNA BLAST	http://bioinformatics.psb.ugent.be/webtools/rRNA/
RNAdb	http://research.imb.uq.edu.au/rnadb/default.aspx
tRNAscan-SE	http://lowelab.ucsc.edu/tRNAscan-SE

3.1.1.4 Reagents and media preparation

All solutions used in this investigation were prepared with deionised and demineralised (Millipore) and where necessary the pH was adjusted with Sodium hydroxide or hydrochloric acid. In addition, the solutions or buffers were subsequently filtered through 0.2 µ filter and autoclaved at 120°C for 20 minutes where it is necessary. Prepared solutions were aliquot in to small volume and stores at desired temperature according to the recommendation of suppliers.

Name of the medium/buffer		Constituents	Amount/ volume
10X PBS	:	NaCl	8.77 g
		Na_2HPO_4	1.50g
		NaH_2PO_4	2.04g
		Water upto	1000.0 ml
1X PBS	:	10X PBS	100.0 ml
		DEPC upto	900.0 ml

10x TBE buffer	:	Tris base	218.0 g
		Boric Acid	105.0 g
		EDTA solution (pH.8.0)	80.0 ml
		Water upto	2000.0 ml
Ammonium Persulfate (APS) 10%	:	APS	0.12 g
		H_2O	1.2 ml
40% acrylamide	:	Acrylamide	76.0 g
		bis-acrylamide	4.0 g
		Water to final volume of	200.0 ml
SDS (10%)	:	Sodium dodecil sulphate	5.0 g
		Water	100.0 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)	480.0 µl
		ddH_2O upto	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 upto	800.0 ml
DEPC-treated water (1000 ml)	:	DEPC	1.0 ml
		Water upto	1000.0 ml
Lysis buffer (100 µl)	:	Igepal (0.8%)	0.8 µl
		RNasin	5.0 µl
		DTT	5.0 µl
		Water upto	100.0 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 upto	1000.0 ml
TE (1x) buffer	:	Tris (1 M)	10.0 ml
		EDTA (0.5 M)	2.0 ml

X-gal	:	ddH_2O upto	1000.0 ml
		X-gal	50.0 mg
PBS + PVA (50 ml)	:	Polyvinyl alcohol (PVA)	300.0 mg
		PBS upto	50.0 ml
Physiological saline solution	:	Sodium chloride	9.0 g
		Water upto	1000.0 ml
Agarose loading buffer	:	Bromophenol blue	0.0625 g
		Xylencyanol	0.0625 g
		Glycerol	7.5 ml
		ddH_2O upto	25.0 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 upto	400.0 µl
IPTG solution	:	IPTG	1.2 g
		ddH_2O upto	10.0 ml
3M Sodium Acetate, pH 5.2	:	Sodium Acetate	123.1 g
		ddH_2O upto	500.0 ml
1M EDTA, pH 8.0	:	EDTA	37.3 g
		ddH_2O upto	1000.0 ml
1x PBS-Tween (PBST)	:	1x PBS	999.50 ml
		Tween®20	0.50 ml
SSC (20x)	:	NaCl	87.65 g
		Sodium citrate	44.1 g
		Water upto	500.0 ml
4% paraformaldehyde (pH7.3)	:	Paraformaldehyde	4.0 g
		1X PBS	100.0 ml
0.5M Sucrose/PBS (30% sucrose)	:	Sucrose	85.57 gm
		1X PBS upto	500.0 ml
Acetylation solution	:	triethanolamine	2.33 ml
		acetic anhydride	500.0 µl
		DEPC water upto	200.0 ml
Yeast tRNA (10 mg/ml)	:	Yeast tRNA	25.0 mg

		DEPC-treated H_2O	2.50 ml
Hybridization solution	:	Formamide -65%	32.25 ml
		20X SSC -5X	12.5 ml
		Tn-20- 0.1%	50.0 µl
		1M citric acid	460.0 µl
		Heparin 50 µg/ml	2.5 mg
		10mg/ml tRNA-500µg/ml	2.5 ml
		DEPC water upto	50.0 ml
Hybridization wash solution	:	Formamide -65%	65.0 ml
		20X SSC -5X	25.0 ml
		Tn-20- 0.1%	100.0 µl
		1M citric acid	1.2 µl
		DEPC water upto	100.0 ml
50% Formamide/SSC	:	Formamide	1000.0 ml
		1X SSC	1000.0 ml
50% formamide/Tn-20/SSC	:	Formamide, 50%	500.0 ml
		Tween-20, 0.1%	1.0 ml
		1X SSC	499.0 ml
5X SSC	:	20X SSC	250.0 ml
		DEPC water	750.0 ml
2X SSC	:	20X SSC	100.0 ml
		DEPC water	900.0 ml
1X SSC	:	20X SSC	100.0 ml
		DEPC water	1900.0 ml
0.2X SSC	:	20X SSC	10.0 ml
		DEPC water	990.0 ml
1X PBST	:	1X PBS	999.0 ml
		Tween-20	1.0 ml
Blocking solution	:	0.5% blocking powder	0.2 g
		10% inac. Sheep serum	4.0 ml
		0.1% tween-20	40.0 µl
		1X PBS upto	40.0 ml
		dd H2O up to	1000.0 ml
Stop solution	:	EDTA 1mM	14.61 mg

		PBS pH 5.5 upto	50.0 ml
10N NaOH	:	NaOH	40 gm
		dd H_2O upto	100.0 ml

3.1.2 Methods

3.1.2.1 Isolation of Small RNAs and subsequent miRNAs fractionation

Bovine ovaries were obtained from a cyclic heifer with the age of 30 months at a stage of mid cycle with a visible mature corpus luteum including normally distributed different types of follicles. Small RNA samples from different bovine tissues and cells were isolated using mirVana miRNA isolation kit (Applied Biosystems Inc, Foster City, CA) according to the manufacturer's instructions. For cloning, 10 µg of the ovarian small RNA was loaded into 12% denaturing poly acrylamide gel electrophoresis with size markers miSPIKE (Integrated DNA Technologies, Inc., Iowa, USA) and fractions of 18-26 nt were recovered using DTR gel filtration cartridge (Edge BioSystems, Maryland, USA).

3.1.2.2 Cloning of small RNAs

For cloning the small RNAs, "5´ Ligation independent Cloning" was followed to ensure complete recovery of conventional small RNAs as well as small RNAs with 5´ modifications or non-standard 5´ ends. All the linkers and primers were obtained from Integrated DNA Technologies, Inc., Iowa, USA. List and sequence of linkers and primers are given in table 3.1. Briefly, once the enriched small RNA fraction has been recovered from the acrylamide gel slice, the small RNAs were ligated with a 3´ linker - adenylated oligos, modified with a 3´-terminal dideoxy-C (ddC) containing Ban-I restriction site (Lau et al. 2001). The ligated products were loaded on dPAGE for purification and reverse transcription was performed. An exonuclease digestion was carried out after first strand cDNA synthesis and then a second 3´ ligation was done using a different linker sequences. The second 3´ linkered product (60nt) was purified from dPAGE to remove free linkers. Subsequently, the amplification of the RT-PCR product was done using linker specific primer set with the thermocycler program of 95.0ºC for 10 minutes, 35 cycles of (95.0 ºC for 30 seconds, 52.0 ºC for 30 seconds 72.0

ºC for 30 seconds) and followed by incubation at 72.0ºC for 5 minutes. Then, the amplicon was subjected to Ban I digestion, concatemerization and end filling with non-template adenosine followed by cloning into TOPO TA Cloning® vector (Invitrogen, Carlsbad, CA). Concatemer clones were picked up, cultured and colony PCR was performed for screening the insert size. Plasmid DNA preparation and DNA sequencing was performed for screened clones and small RNAs which were separated by well defined linker units with the reconstituted Ban I site.

3.1.2.3 Bioinformatic analysis of small RNA sequences

The small RNA sequences were first compared with the sequences in miRBase (Ambros et al. 2003a, Griffiths-Jones 2004, Griffiths-Jones et al. 2006, Luciano et al. 2005). Small RNAs completely or partially matched by less than two mismatches to any registered miRNA in miRBase were considered putative bovine miRNA. The remaining sequences were compared to the bovine nucleotide collection (nr/nt) and the expressed sequence tags (EST) database in NCBI (Bettegowda et al. 2008) and different noncoding RNA databases (Babiarz et al. 2008, Gonzalez and Behringer 2009, Toloubeydokhti et al. 2008a, Watanabe et al. 2005, Yang et al. 2008b). Sequences, which were matched 100% to any mRNA, rRNA or tRNA were excluded from further evaluation to generate novel miRNA candidates. All the remaining sequences and the putative bovine miRNA sequences were submitted to BLAST-search in the Ensembl 52: bovine genome assembly (Btau_4.0) (De La Fuente and Eppig 2001) and the 75 bp genomic flanking sequence upstream from the 3´ end or downstream from the 5´ end of the miRNA was considered putative precursor of the matching miRNA.

All the putative precursor sequences were analyzed for hairpin structure using the mfold Web server (version 3.2) (Goud et al. 1998) to evaluate the ability to form thermodynamically stable hairpin structures (Zuker 2003) based on other criteria described elsewhere (Ambros et al. 2003a). Chromosome locations, orientation and genomic features of the predicted miRNA precursors as well as other small RNAs sequences (not meeting miRNAs criteria) and whether they were located in intragenic or intergenic genomic regions were determined using ensembl. Other small RNAs were categorized according to published research articles (Ambros et al. 2003b, Aravin et al. 2003, Aravin and Tuschl 2005).

3.1.2.4 Detection of miRNAs expression by semi-quantitative RT-PCR

Small RNA samples isolated from the 11 different tissues and cells, such as ovarian cortex, fetal ovary at about six month of pregnancy, cumulus cells, matured corpus luteum, oviduct (entire), uterus (horn), placenta, heart, liver, lung and spleen were used for the detection of miRNAs by PCR method according to Ro et al (Ro et al. 2006) with some modifications. In this, the poly (A)-tailed small RNA was purified by acid phenol: chloroform: iso-amyl alcohol and ethanol precipitation method. All small RNA-cDNA samples were diluted to the same concentration of 6 ng/µl (which was the lowest amount obtained from cumulus cells). Three microliters of cDNA was used as template for conventional PCR and the products were analyzed on a 2% agarose gel. List of primers and oligos used are shown in the table 3.1. Some representative RT-PCR products were cloned into PGEM-T easy vector (Promega Corporation, Wisconsin, USA) and transformed to E. coli and sequenced to verify the specificity of PCR amplification.

Table 3.1: List of oligos and primers used for cloning and detection of miRNAs

Name[A]	Sequence (5´-3´)	Usage
3´ Linker	rAppCTGTAGGCACCATCAAT/3ddC	Library
RT Primer	GATTGATGGTGCCTACAG	Library
2nd 3´Linker	rAppTGGAATTCTCGGGTGCCAAGGT/ddC	Library
PCR Primer	CCTTGGCACCCGAGAATT	Library
M13 Forward	GTAAAACGACGGCCAG	Sequencing
M13 reverse	CAGGAAACAGCTATGAC	Sequencing
RTQ1	CGAATTCTAGAGCTCGAGGCAGGCGACATGGCT GGCTAGTTAAGCTTGGTACCGAGCTCGGATCCAC TAGTCC$(T)_{25}^{VN}$	RT PCR
RTQ2	GAATTCTAGAGCTCGAGGCAGGCGACATG$(T)_{25}^{VN}$	RT PCR
RTQ-UNI	CGAATTCTAGAGCTCGAGGCAGG	RT PCR
Bta-Let7b	TGAGGTAGTAGGTTGTGTGGTT	RT PCR
Bta-miR-15b	GTAAACCATGATGTGCTGCTA	RT PCR
Bta-miR-18a	ATCTGCACTAGATGCACCT	RT PCR
Bta-miR-29a	AACCGATTTCAGATGGTGCTA	RT PCR
Bta-miR-101	TTCAGTTATCACAGTACTGTA	RT PCR
Bta-miR-125b	TCACAAGTTAGGGTCTCAGGGA	RT PCR
Bta-miR-126	CGCATTATTACTCACGGTACG	RT PCR
Bta-miR-145	GTCCAGTTTTCCCAGGAATCC	RT PCR
Bta-miR-199a	TAACCAATGTGCAGACTACTGT	RT PCR
Bta-miR-222	ACCCAGTAGCCAGATGTAGCT	RT PCR

Bomir-22/22*	ACAGTTCTTCAACTGGCAGCTT	RT PCR
Bomir-140/140*	CAGTGGTTTTACCCTATGGTAG	RT PCR
Bomir-143:	TGAGATGAAGCACTGTAGCTC	RT PCR
Bomir-152	CCAAGTTCTGTCATGCACTGA	RT PCR
Bomir-193a	GGGACTTTGTAGGCCAGTT	RT PCR
Bomir-378	CTGGACTTGGAGTCAGAAGGC	RT PCR
Bomir-382	GAATCCACCACGAACAACTTC	RT PCR
Bomir-409	AGGGGTTCACCGAGCAACAT	RT PCR
Bomir-424	CAAAACGTGAGGCGCTGCTA	RT PCR
Bomir-503	TGCAGTACTGTTCCCGCTGCTA	RT PCR
Bomir-542	TCTCGTGACATGATGATCCCCGA	RT PCR
Bomir-578	TGTGGGTGTGTGCATGTGCGTG	RT PCR
Bomir-652	CACAACCCTAGTGGCGCCATT	RT PCR
Bomir-940	GCAGGGCCCCCGCTCCCC	RT PCR
Bomir-A4052	GGGAGCCTCGGTTGGCCTCGG	RT PCR
Bomir-A3341	GTGGCTGTCCCTGGAGGTGGG	RT PCR
Bomir-C2841	GCCCCGGCCGCTCCCGGCC	RT PCR
Bomir-E2664	AGGGCGGGCGGCGACTGGAA	RT PCR
Bomir-G2511	AGGCGGGCCGGGGTTGGAAGG	RT PCR
Bomir-F2531	TGGTGGAGATGCCGGGGACGT	RT PCR
Bomir-A2143	CGGCAGATGAAGTCCATCGG	RT PCR
Bomir-C1931	CCTGCTGATCTCACATTAATT	RT PCR
Bomir-F1821	AGCCCTGGCCCTGCCATCGTG	RT PCR
Bomir-C1511	GTGGAGGAGAATGCCCGGGG	RT PCR
Bomir-D1431	GGCGACGGAGGCGCGACCCCC	RT PCR
Bomir-F1353	ATCTTTGGGCTAGGTTAGTTC	RT PCR
Bomir-F1351	GCCCCGGCCGCTCCCGGCCTT	RT PCR
Bomir-A3711	TTCCGCGCTCTACGCCAGC	RT PCR
Bomir-F0131	GGGGCGGGGGGGCGGGTG	RT PCR
Bomir-F0132	AGCCCGGGCCCCTCCCCTG	RT PCR
Bomir-H0121	ACTTCCCGTGTGTTGAGCC	RT PCR
Bomir-F0244	GCTACTACCGATTGGATGG	RT PCR
Bomir-H0222	CGGCGGCAGCGCCGGGGC	RT PCR
Bomir-A0321	AGCGCCGCCGGCCGCACC	RT PCR
Bomir-C0533	CGGGACCGGGGTCCGGTGC	RT PCR
Bomir-F0522	GGTGGGGTGGGGGGGTTGG	RT PCR
Bomir-B0821	GTCCCCGGGGCTCCCGCC	RT PCR
Bomir-F2422	GGTGGGAGGGTCCCACCGAG	RT PCR
Bomir-D3011	CCGAGTGCTCCCGCGAGCGCT	RT PCR

[VN]: Two variable nucleotides, where V is A, G, or C; N is A, G, C, or T

[A]: Name started with Bta and Bomir denotes annotated and new miRNAs bovine miRNAs, respectively.

3.1.2.5 In situ hybridization of miRNAs in ovarian cryo-sections and whole mount cumulus oocyte complexes

For in-situ hybridization of miRNAs, bovine ovary (21/0) days of estrus cycle was fixed in 4% PFA overnight at 4°C followed by overnight incubation in PBS with 30% sucrose at 4°C and frozen in Tissue-Tek OCT reagent (Sakura Finetek, Zoeterwoude, NL). Cryo-sections (10 µm) preparation, post-fixation, acetylation and proteinase K treatment was done as described previously (Obernosterer et al. 2007). Two hours of pre-hybridization was performed at 52°C in hybridization solution (50% formamide, 5 × sodium chloride/sodium citrate [SSC; pH 6.0], 0.1% Tween-20, 50 µg/ml heparin, and 500 mg/ml yeast tRNA). Ovarian sections were incubated overnight at 52°C with 3'-Digoxigenin (DIG) labeled LNA-modified oligonucleotide probes (1pM) for miR-29a, U6 RNA and scrambled miR (Exiqon, Vedbaek, Denmark) in hybridization buffer in a humidified chamber. Blocking, incubation with anti-DIG-AP antibody, washing and color development using Fast Red reaction was performed as described previously (Obernosterer et al. 2007). The slides were mounted with VectaShield containing DAPI (Vector laboratories, Burlingame, CA) and analyzed by confocal laser scanning microscope (CLSM LSM-510, Carl Zeiss, Germany). For whole mount in-situ hybridization, cumulus oocyte complexes were aspirated from more than 8 mm of ovarian follicles. Pre-fixation, processing, digestion with Proteinase K, pre-hybridization, hybridization, post-hybridization washing was performed in 4-well embryo culture dishes according to the high-resolution whole mount in situ hybridization protocol from Exiqon. The rest of the procedures were similar to cryo-section hybridization protocol.

3.1.2.6 Prediction and analysis of ovarian miRNA targets

For this purpose, initially a raw list of all genes found to be targeted by cloned miRNA was generated using MIRANDA algorithm, miRBase target version 5 (Hong et al. 2008). Subsequently, about 800 distinct important genes related to mammalian reproductive system development, function and disorders were extracted from Ingenuity knowledge base (IPA 7.0) by key word search. Then, two filtration steps were applied to generate a comprehensive list of target genes. Firstly, raw target set and genes set extracted from database were cross matched and common genes were extracted. Secondly, the condition of multiple genes targeted by multiple miRNAs from the

common target list was cosidered. From these screened target sets, 11 miRNAs having the highest number as well as overlapping target genes were enlisted. Then, the Gene Ontology (GO) analysis of the screened and sub sets of miRNAs target genes were performed in order to predict the possible biological processes and functions that were most likely to be affected by miRNAs using web delivered tools of Ingenuity Pathway Analysis (Redwood City, California). Top significant GO categories, biological functions and different canonical pathways were analyzed for miRNA specific targets as well as for all screened targets based on significant over-representation of genes using a selected threshold for p-values ≤ 0.05 of hypergeometric distribution (Delfour et al. 2007).

3.2 Results

3.2.1 Description of the bovine ovarian small RNA library

To identify miRNAs in the ovary, RNAs of 18 to 26 nt in length from bovine ovarian small RNAs (~ 200 nt) were purified, cloned, sequenced and analyzed. About 233 concatemer clones were sequenced to generate 479 sequences (after discarding non-quality and self ligated linker sequences). Of these 80 small RNA-cDNA sequences were beyond the expected range of nucleotides (18-26nt) in length. Only sequences of 18 nt or more in length were subjected to detail analysis. Distribution of different lengths of nucleotide sequences found in this library is presented in figure 3.1. All identified sequences were categorized according to their properties as determined by in-silico analysis based on the criteria reported elsewhere for different types of small RNAs (Ambros et al. 2003a, Ambros et al. 2003b, Aravin et al. 2003, Aravin and Tuschl 2005). The 479 sequences identified in the library represented 41% miRNAs, 12% mRNA, 12% rRNA, 6.3% tRNA, 6.0% repeat associated siRNA, 2.7% small antisense RNA, 3.5% tiny noncoding RNA, 1% small nuclear RNA and 15.2% sequences that did not match to bovine genome (Figure 3.2).

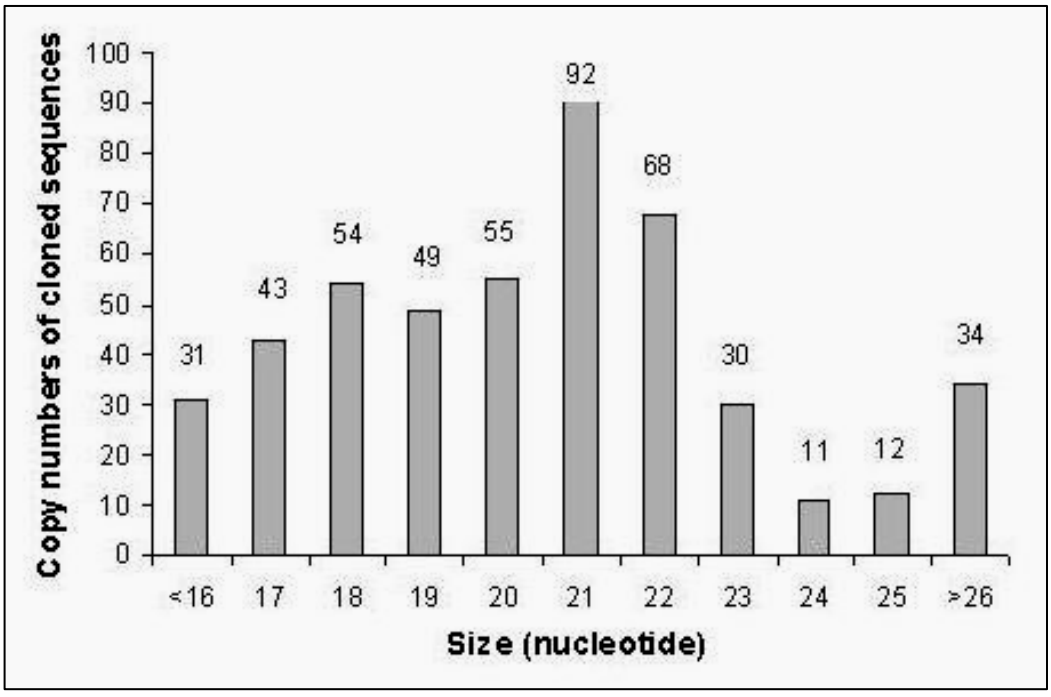


Figure 3.1: Size distribution of 479 small RNAs sequences cloned from the bovine ovary

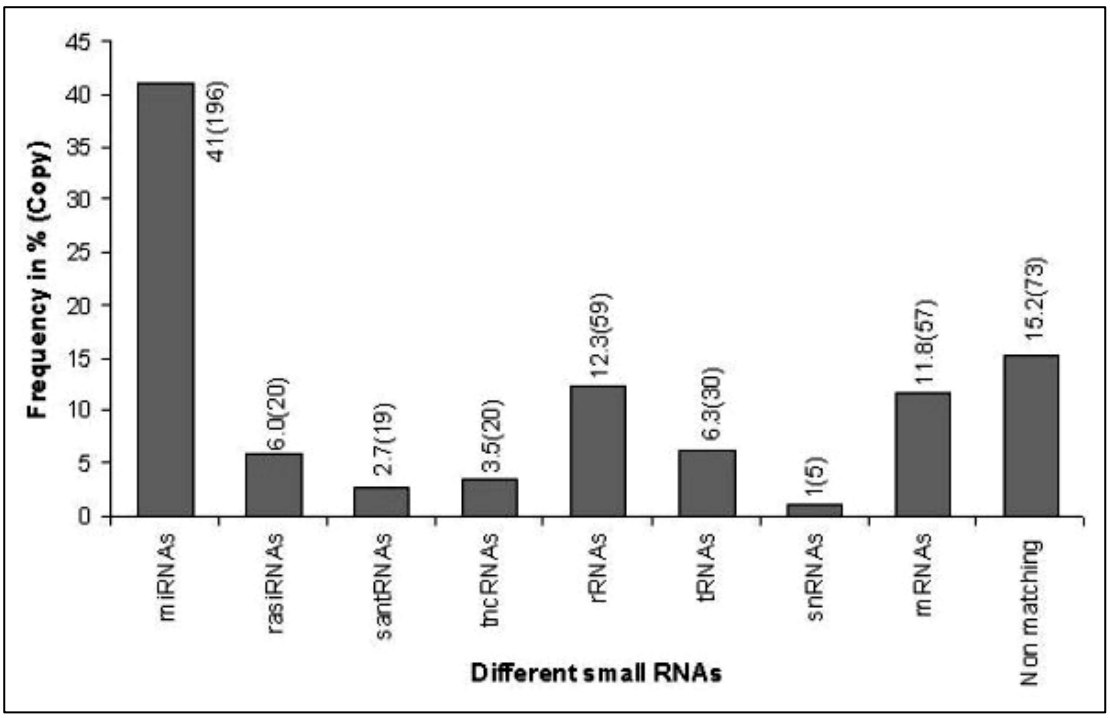


Figure 3.2: Frequency (%) of different types of RNA represented in the library

3.2.2 Distinct miRNAs identified in the bovine ovary

In cDNA library a total of 196 sequences were found to be miRNA like molecules, of which 74 revealed distinct miRNAs (Table 3.2). Out of these 74 miRNAs, 36 were found to be reported in miRBase 12.0 for different species including bovine, 14 are registered only for other species and 24 were completely new. Of these 38 new bovine miRNAs, 15 miRNAs were identical or differed by only one or two nucleotides from known mammalian miRNAs. All the new miRNAs were denoted as starting with prefix 'bomir' followed by their homologue miRNA number or by clone name in case of no sequence homology. Already annotated miRNAs were named as they were stated in miRBase.

Two miRNAs, namely: mir-22/22* and 140/140* which are cloned from 5′ fold back arm of the hairpin precursor, have shown exact match to human miRNAs but not to bovine as annotated in miRBase. So, previously annotated bta-miR-22 and 140 seem to be miR-22* and miR-140*, respectively. The number of times that each miRNA cloned in the library ranged from 28 clones for let-7b to a single clone (singleton) for 39 of the 73 miRNAs. All in all, 22 of the 73 miRNAs were cloned for three or more times (Figure 3.3).

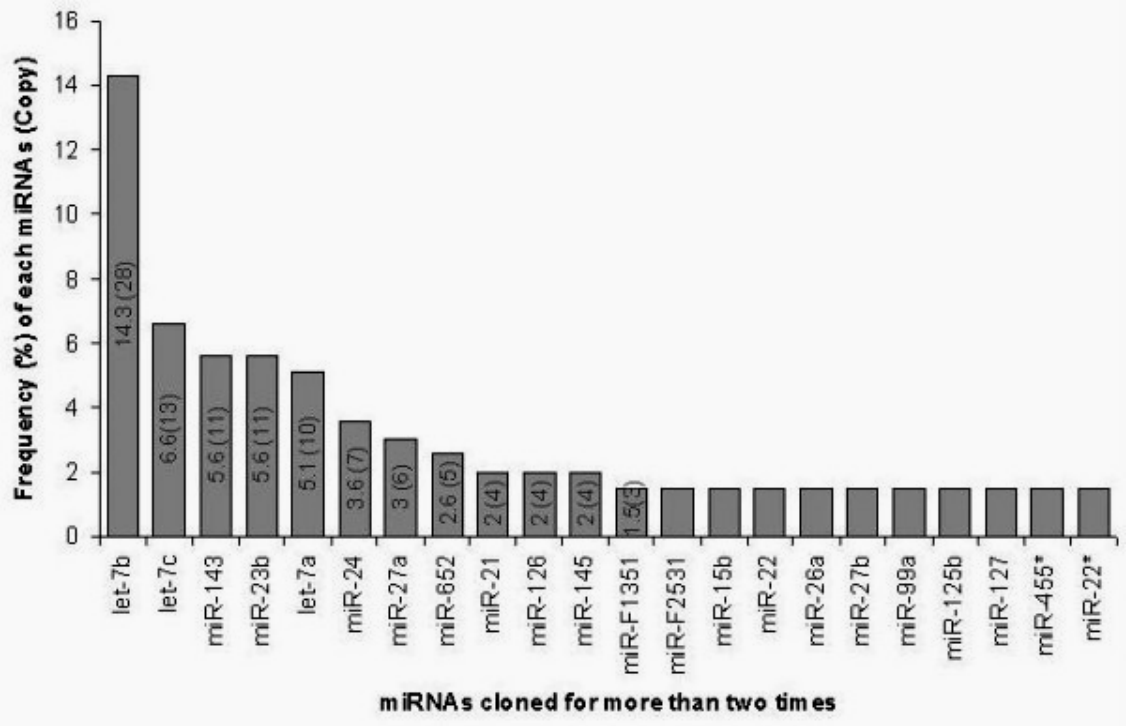


Figure 3.3: Frequency (%) of cloned miRNAs along with their copy number

The corresponding bovine genomic sequences and their locations were identified for each miRNA. The 5′ or 3′ flanking genomic sequences were then tested for the ability to fold into canonical ~70-nt miRNA precursor hairpin structures by using the MFOLD web server (Eppig 1991). Small RNA clones with proper positioning within an arm of the hairpin suggest that they have been excised during dicer processing in the cells. Nearly in all of those cases, sequences were found to be conserved in different species including the predicted precursors (Table 3.2 and Table 3.3). The Bomir-652, which could not be located in bovine genome, was found to be cloned for five times in the library and share sequence homology with already identified miRNA in other species.

Table 3.2: List of new miRNAs cloned from bovine ovary

miR ID	L	Homolog	C	S	Sequence	Genomic Location [e]	T
bomir-22*/22-5p [a]	22	hsa-miR-22	3	+/-	ACAGUUCUUCAACUGGCAGCUU	19:22901905:22901926:1[f]	mt
bomir140*/140-5p[b]	22	hsa-miR-140	1	+	CAGUGGUUUUACCCUAUGGUAG	18:35987052:35987073:1[f]	mt
bomir-143-3p	22	ggo-miR-143	11	+/-	UGAGAUGAAGCACUGUAGCUCG	7:60268857:60268878:1[f]	Ie
bomir-152-5p	21	hsa-miR-152	1	-	CCAAGUUCUGUCAUGCACUGA	19:39650399:39650419:-1[f]	It
bomir-193a-2-3p[c]	19	bta-miR193a	1	-	GGGACUUUGUAGGCCAGUU	14:889828:889846:-1[f]	In
bomir-378-1-3p	21	hsa-miR-378	1	+	CUGGACUUGGAGUCAGAAGGC	7:60536513:60536533:1[f]	In
bomir-378-2-5p	21	hsa-miR-378	-	+	CUGGACUUGGAGUCAGAAGGC	4:11116898:11116918:1[h]	In
bomir-382-3p	22	hsa-miR-382	1	-	GAAUCCACCACGAACAACUUC	21:66031757:66031777:-1[f]	In
bomir-409-5p	22	hsa-miR-409	2	-	GGGGUUCACCGAGCAACAUUC	21:66042162:66042182:-1[f]	In
bomir-424-3p	22	hsa-miR-424*	1	-	CAAAACGUGAGGCGCUGCUAU	Un.04.53:446874:446894:-1[f]	In
bomir-503-3p	23	mmu-miR-503	1	+	UGCAGUACUGUUCCCGCUGCUA	Un.004.53:446563:446584:1[f]	Ie
bomir-542-3p	23	hsa-miR-542	1	+	UCUCGUGACAUGAUGAUCCCCGA	Un.004.53:441604:441626:1[f]	Ie
bomir-574-5p	22	hsa-miR-574	1	-	UGUGGGUGUGUGCAUGUGCGUG	16:59370677:59370698:-1[f]	Ie
bomir-652-3p[d]	21	hsa-miR-652	5	+	CACAACCCTAGTGGCGCCATT	(from *H. sap.*)	--
bomir-940-5p	18	hsa-miR-940	1	-	GCAGGGCCCCCGCUCCCC	20:75274475:75274492:-1[h]	Ie
bomir-F0131-5p	18	mmu-miR-667	1	+	GGGGCGGGGGGGCGGGUG	7:10905965:10905982:1[h]	Ie
bomir-F0132-5p	19	hsa-miR-1469	1	+	AGCCCGGGCCCCUCCCCUG	7:13891718:13891736:1[h]	It
bomir-H0121-3p	19	hsa-miR-1471	1	+	CUUCCCGUGUGUUGAGCC	18:7202610:7202627:1[h]	Ie
bomir-F0244-5p	19	osa-miR1423	1	-	GCUACUACCGAUUGGAUGG	12:45758300:45758318:-1[g]	Ie
bomir-H0222-3p	22	cre-miR1172.1	1	-	GGACGGCGGCAGCGCCGGGGCG	29:41706141:41706159:-1[f]	Ie
bomir-A0321-3p	18	mml-miR-638	1	+	AGCGCCGCCGGCCGCACC	19:39110507:39110524:1[g]	In
bomir-C0533-5p	20	oan-miR-1418*	1	+	CGGGACCGGGGUCCGGUGCG	18:59928733:59928752:1[f]	Ie
						21:52041918:52041937:-1[f]	Ie
bomir-F0522-1-3p	19	hsa-miR-1234	1	+	GGUGGGGUGGGGGGGUUGG	21:35870379:35870397:1[h]	Ie
						22:59347395:59347413:1[h]	In
bomir-B0821-5p	21	oan-miR-1394	1	-	GUCCCCGGGGCUCCCGCCGGC	20:19373746:19373766:-1[h]	Ie
bomir-F1351-3p	20	gga-miR-1607	3	+	GCCCCGGCCGCUCCCGGCCU	25:41129497:41129516:1[h]	Ie
bomir-F1353-5p	20	dre-miR-430c	1	+	AUCUUUGGGCUAGGUUAGUU	28:27885036:27885055:1[h]	In
bomir-D1431-5p	22	pta-miR1310	2	-	GGCGACGGAGGCGCGACCCCCC	12:75102030:75102051:-1[g]	Ie
bomir-C1511-5p	20	hsa-miR-877	1	+	GUGGAGGAGAAUGCCCGGGG	Un.04.1059:20639:20658:1[h]	In
bomir-F1821-3p	21	hsa-miR-631	1	+	AGCCCUGGCCCUGCCAUCGUG	Un.04.152:123191:123211:1[h]	In
bomir-C1931-5p	23	gma-miR1523	1	+	CCUGCUGAUCUCACAUUAAUUCA	26:12405838:12405860:1[h]	Ie
bomir-A2143-3p	18	oan-miR-181c*	1	+	CGGCAGAUGAAGUCCAUC	16:47801336:47801353:1[h]	In
bomir-F2422-5p	20	hsa-miR-659	1	+	GGUGGGAGGGUCCCACCGAG	18:53584142:53584161:1[h]	It
bomir-F2531-3p	18	ppt-miR1030i	3	+	UGGUGGAGAUGCCGGGGA	8:77307661:77307678:1[g]	Ie
bomir-G2511-3p	18	bmo-miR-92	1	+	AGGCGGGCCGGGGUUGGA	18:41190536:41190553:1[h]	Ie
bomir-E2664-3p	20	mml-miR-638	1	-	AGGGCGGGCGGCGACUGGAA	18:64361001:64361020:-1[h]	It
bomir-D3011-3p	21	mml-miR-650b	1	+	CCGAGUGCUCCCGCGAGCGCU	18:39424938:39424958:1[g]	It
bomir-A3341-1-3p	22	bta-miR-487a	1	+	GUGGCUGUCCCUGGAGGUGGG	3:124988008:124988028:1[h]	Ie
						Un.04.4799:1335:1355:1[h]	Ie
bomir-A3711-5p	19	hsa-miR-937	2	+	UUCCGCGCUCUACGCCAGC	9:63475804:63475822:1[g]	Ie
bomir-A4052-1-5p	19	hsa-miR-615	1	+	GGGAGCCUCGGUUGGCCUC	18:59928630:59928648:1[f]	Ie
						21:52042022:52042040:-1[f]	It
						Un.04.2732:16069:16087:-1[f]	Ie

L:length, C: copy number, S: strand, In: intronic, It: intragenic, Ie: intergenic, mt: miR transcript, [a]: Cloned sequence is homolog to has-miR 22 but not to bta-miR-22, may bta-miR-22 presented in miRBase v. 12 is bta-miR-22*, [b]: Cloned sequence is homologue to has-miR-140 but not to bta-miR-140, may bta-miR-140 presented in

miRBase v. 12 is bta-miR-140*, [c]: Sequence is smaller than bta-miR-193a and has different genomic locus. [d]: Sequence does not match to bovine genome, [e]: Genomic location presenting chromosome number with start and end position along with sense/antisense orientation by 1/-1 of cloned mature sequence. Conservation pattern of the predicted precursor sequences from flanking bovine genome sequence is indicated by- [f]: found in more than 6 mammalian species, [g]: present at least in 2 species, [h]: only in bovine.

3.2.3 Genomic distribution, properties and clustering of new miRNAs

Genomic locations and properties of the new miRNAs are shown in table 3.2. All newly identified bovine miRNAs (except bomir-652) are corresponded to 43 distinct loci. Putative precursor hairpin structures have been predicted for all these 43 loci using genomic sequences flanked from candidate miRNAs (Table 3.3). Thirty three of these are found to be encoded by single copy miRNA genes, whereas the other five (bomir-378, bomir-C0533-5p, bomir-F0522-3p, bomir-A3341-3p and bomir-A4052-5p) have multiple loci in the bovine genome (Table 3.2). The analysis of the genomic positions of 61 sequences corresponding to 38 distinct new miRNA genes showed that the majority (23 out of 44 loci) are localized to intergenic regions and the rest corresponded to the intragenic regions in either sense or antisense orientation (Table 3.2). However, 11 sequences are found to be exclusively from known intronic region.

Table 3.3: Predicted secondary structure of new miRNAs

1. >bomir-22*/22-5p:Btau_4.0:19:22901896:22901976:1(10-31) dG = -31.40 kcal/mol
CAGAGGGCA**acaguucuucaacuggcagcuu**UAGCUGGGUCAGGACAuaaagcuugccacugaagaacuACUGCGGCUCAG
CA G- ac ac - GC GGU
GAG GCA aguucuuca uggca gcuuUA UG C
||| ||| ||||||||| ||||| |||||| || |
CUC CGU ucaagaagu accgu cgaaau AC A
GA GG CA c- u -- AGG

2. >bomir-140*/140-5p:Btau_4.0:18:35987036:35987127:1(17-38) dG = -51.10 kcal/mol
UCUCUCUGUGUCCUGC**caguggguuuacccuaugguag**GUUACGUCAUGCUGUUCuaccacaggguagaaccacggACAGG
AUACCGGGGCA
UC U - a a UU UC
UCUC GUGUCCUG Cc gugguuuuacccu ugguagG ACG A
|||| |||||||| || |||||||||||||| ||||||| ||| |
GGGG CAUAGGAC gg caccaagauggga accauCU UGU U
AC C A - c -- CG

3. >bomir-143-3p:Btau_4.0:7:60268810:60268888:1(57-78) dG = -48.50 kcal/mol
UCUCCCAGCCUGAGGUGCAGUGCUGCAUCUCUGGUCAGUUGGGAGUCugagaugaagcacuguagcucgGGAAGGGAGA

```
          AG      G         G      U  -  AG
     UCUCCC  CCUGAG UGCAGUGCU CAUCUC GG UC  U
     ||||||  |||||| ||||||||| |||||| || ||  |
     AGAGgg  gggcuc augucacga guagag CU AG  U
          aa      g         a      u  G  GG
```

4. >bomir-152-5p:Btau_4.0:19:39650354:39650424:-1(6-26) dG = -38.00 kcal/mol
CGGGCccaaguucugucaugcacugaCUGCUCCAGAGCCCGAGUCGGAGUGUAUCACAGAACCUGGGCCGG

```
     CG      a       c       --    ---   C
       GGCcca guucugu augcacu  gaCU   GCU C
       |||||| ||||||| |||||||  ||||   ||| |
       CCGGGU CAAGACA UAUGUGA  CUGA   CGA A
     GG      C       C       GG    GCC   G
```

5. >bomir-193a-2-3p:Btau_4.0:14:889826:889878:-1(32-50) dG = -17.80 kcal/mol
AUGGCUGCCUCACAAGGUUUGGAGCUGUGCCUgggacuuuguaggccaguuCA

```
     AU    -    C       - UG A  U
       GGCU GCCU ACAAGGU U  G GC \
       |||| |||| ||||||| |  | ||  G
       uuga cgga uguuuca g  C CG /
     AC    c    -       g gU -  U
```

6. >bomir-378-1-3p:Btau_4.0:7:60536464:60536541:1(49-69) dG = -47.70 kcal/mol
CCACCCAGGGCUCCUGACUCCAGGUCCUGUGUGUUACCUCGAAAUAGCAcuggacuuggagucagaaggcCUGAGUGG

```
          C    G  C               UGU      CCU
     CCAC CAGG CU CUGACUCCAGGUCC   GUGUUA   C
     |||| |||| || ||||||||||||||   ||||||   |
     GGUG GUCc ga gacugagguucagg   cACGAU   G
         A    g  a               u--      AAA
```

7. > bomir-378-2-5p:Btau_4.0:4:11116890:11116965:1(9-29) dG = -14.80 kcal/mol
GGAGAGCAcuggacuuggagucagaaggcUGGAGCUUACAGGGCAGCACCGUCAUCUACUGGUGGAGAACUACGCC

```
       AG-  CA  gg   ugg   c   a   U  A   UAC
     GG   AG  cu  acu   agu aga ggc GG GCU   A
     ||   ||  ||  |||   ||| ||| ||| || |||   |
     CC   UC  GA  UGG   UCA UCU CUG CC CGA   G
       GCA  AA  GG   ---   -   A   -  A   CGG
```

8. >bomir-382-3p:Btau_4.0:21:66031742:66031827:-1 (51-71)dG = -28.30 kcal/mol
UUUGGUACUGAAAAAAGUGUUGUCCGUGAAUGAUUCGUCAUAAGUAAAGCgaauccaccacgaacaacuucUCUUCAAGUA
CCACA

```
     UU      -    AAAAGU     C    AAU       CAUA
       UGGUAC UGAA       GUUGU CGUG   GAUUCGU    A
       |||||| ||||       ||||| ||||   |||||||    |
       ACCAUG ACUU       caaca gcac   cuaagCG    G
     AC      A    CUcuu-     a    cac       AAAU
```

9. >bomir-409-5p:Btau_4.0:21:66042116:66042194:-1(13-33) dG = -33.60 kcal/mol
UGAUACCGAAAAgggguucaccgagcaacauucGUCGUCCAGAUGCAAAGUUGCUCGGGUAACCUCUCCCCGCGUACCA

```
     U A   -  AAA       ca         a  -    GU
      G UAC CG   Agggguu  ccgagcaac uu cGUC  \
      | ||| ||   |||||||  ||||||||| || ||||  C
      C AUG GC   UCUCCAA  GGCUCGUUG AA GUAG  /
     A C   C  CCC       UG         A  C    AC
```

10. >bomir-424-3p:Btau_4.0:Un.004.53:446853:446952:-1(59-79) dG = -46.20 kcal/mol
UUCGUUGACUCCGAGGGGAUGCAGCAGCAAUUCAUGUUUUGAAGUGCUUUAAACGGUUcaaaacgugaggcgcugcuauACCC
CCUUGCGAGGAAGUAGG

```
     U CG  GA   -       A  C      AA             G  CUU
      U  UU  CUC CGAGGGG UG AGCAGC  UUCAUGUUUUGAA UG   \
      |  ||  ||| ||||||| || ||||||  |||||||||||||| ||   U
      G  GA  GAG GUUCCCC Au ucgucg  gagugcaaaacUU GC   /
     G AU  AG   C       C  a      cg             G  AAA
```

12. >bomir-542-3p:Btau_4.0:Un.004.53:441560:441637:1(45-67) dG = -25.80 kcal/mol
CCCAGACCUUUCAGUUAUCAAUCUGUCACAAGUGCACAGUGGUAucucgugacaugaugaucccgaGAUGUCUGAGG

```
       -     C    A---       AUC      A  -   AC
     CC CAGAC UUUC    GUUAUCA   UGUCAC AG UGC  A
     || ||||| ||||    |||||||   |||||| || |||  |
     GG GUCUG AGag    uaguagu   acagug uc AUG  G
       A     U    cccc       ---      c  u   GU
```

13. >bomir-574-5p:Btau_4.0:16:59370641:59370704:-1(7-28) dG = -25.20 kcal/mol
AGAGUgugggugugugcaugugcgugUGUGCACAUGCAUAUGUGUGUGGCUAUCUUAGCUGU

```
     AG   Gu--    g  ug             G
       AGU    gugg ug  ugcaugugcgugU U
       |||    |||| ||  ||||||||||||  |
       UCG    UAUC GU  GUGUAUACGUACA G
     UG   AUUC    G  GU             C
```

14. >bomir-652-3p: **hsa**:X:109185213-109185310:-1, dG = -50.10 kcal/mol
CACAUCCAACACGUGACguguuggggaucaccgcgguaaGUUAAUAUCAGAUACACUUACCGUGGGAGAGGAUCCCAACACGUC
ACGUAUCGGUAAGCA

```
   CACAU-  AAC                  a----           UAAUAU
         CC   ACGUGACguguuggggauc     ccgcgguaaGU      \
         ||   ||||||||||||||||||     ||||||||||||      C
         GG   UGCACUGCACAACCCUAG     GGUGCCAUUCA       /
   ACGAAU  CUA                  GAGAG           CAUAGA
```

15. >bomir-940-5p:Btau_4.0:20:75274429:75274494:-1(3-20) dG = -39.80 kcal/mol
CCgcagggcccccgcuccccGUGGCCACCAGAGUGCCCUGUGGGGAGGCGGGGGAGCCACCUGGGG

```
       g    g----      -       UG C-   C
     CC cagg     cccccg cuccccG  G  CAC A
     || ||||     |||||| |||||||  |  ||| |
     GG GUCC     GGGGGC GAGGGGU  C  GUG G
       G    ACCGA      G       GU CC   A
```

16. >bomir-F0131-5p:Btau_4.0:7:10905950:10906020:1(16-33) dG = -29.40 kcal/mol
UGUGUUUGGGAGUUGggggcgggggggcgggugGAUUCCAGCUGUCUUCCCCUCGUGGACACUGUGACAUA

```
          UG  A   Gg-   c          g   A
     UGUGUU  GG GUU   ggg gggggggcgg ugG \
     ||||||  || |||   ||| |||||||||| ||| U
     AUACAG  UC CAG   CUC CCCUUCUGUC ACC /
          UG  A   GUG   -          G   U
```

17. >bomir_F0132-5p:Btau_4.0:7:13891717:13891774:1(2-20) dG = -23.40 kcal/mol
CagcccgggccccucccugGCUCCGCCCACAGCAUCAUGGGACCCAGCCCAGGGCCU

```
     Ca    -    ccc-    c  --   CCG
       gccc gggc    uccc ug  GCU   C
       |||| ||||    |||| ||  |||   |
       CGGG CCCG    AGGG AC  CGA   C
     UC    A    ACCC    U  UA   CAC
```

18 >bomir-H0121-3p:Btau_4.0:18:7202568:7202632:1(43-60) dG = -27.60 kcal/mol
GAGGCGGCCAGACCAGCAGGGCGCAGGGGACCAUGUGUCCUCcuucccguguguugagccGCGUG

```
     GAG     CA   CA   -   CGC   -    CA
        GCGGC  GAC  GCA GGG   AGG GGAC  \
        |||||  |||  ||| |||   ||| ||||  U
        CGccg  uug  ugu ccc   ucC CCUG  /
     GUG     ag   --   g   u--   U    UG
```

19. >bomir-F0244-5p:Btau_4.0:12:45758242:45758332:-1(15-33) dG = -20.10 kcal/mol
GCCACUGCCCCAUUgcuacuaccgauuggauggUUUAGUGUUGCCCUUGGAUAAUCCAAUAAGUAGAAUUAAUAGGUGAAAA
GUUGUGAGC

```
       -   U  ------  C    cua    cg       g      UGU
     GC CAC GC      CC AUUg   cuac  auuggau gUUUAG   U
     || ||| ||      || ||||   ||||  ||||||| ||||||   |
     CG GUG UG      GG UAAU   GAUG  UAACCUA UAGGUU   G
       A   U  AAAAGU  A    UAA    AA       A      CCC
```

20 >bomir-H0222-3p:Btau_4.0:29:41706131:41706188:-1(30-48) dG = -31.50 kcal/mol
UCUGGGGCGCCCCGCACCGGCGGGCAGGAcggcggcagcgccggggcgCCGUCUCGGA

```
          ---        -  A---   G  GGC
     UCUGG   GGCGCCCC GC    CCG CG   \
     |||||   |||||||| ||    ||| ||    A
     AGGCU   CCgcgggg cg    ggc gc   /
          CUG        c  cgac   g  AGG
```

21. >bomir-A0321-3p:Btau_4.0:19:39110468:39110542:1(40-57) dG = -20.40 kcal/mol
CCGGCCUACUUGGGGCUCCGAGCCGCUGCCGCAUACAUUagcgccgccggccgcaccGCCGUCACCUUCAGCGCU

```
     CC   -  ACU--   -   UC--  A    C  C   AUA
       GGC CU     UGG GGC    CG GCCG UG CGC   C
       ||| ||     ||| |||    || |||| || |||   |
       UCG GA     ACU CCG    gc cggc gc gcg   A
     --   C  CUUCC   G   ccac  -    c  c   aUU
```

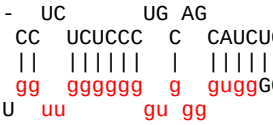
22. >bomir-C0533-1-5p:Btau_4.0:18:59928730:59928786:1(4-23) dG = -26.70 kcal/mol
CGUcgggaccgggguccggugcgGAGAGCCCUUCGUCCCGGGACACGGGGCGCGGCC

```
     C    gga-   ggg     ug     A
      GUcg    ccg   uccgg  cgGAG G
      ||||    |||   |||||  ||||| |
      CGGC    GGC   GGGCC  GCUUC C
     C    GCGG   ACA     CU     C
```

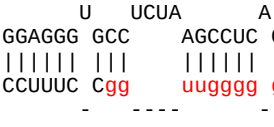
23. >bomir-C0533-2-5p:Btau_4.0:21:52041884:52041940:-1(4-23) dG = -26.70 kcal/mol
CGUcgggaccgggguccggugcgGAGAGCCCUUCGUCCCGGGACACGGGGCGCGGCC

```
     C    gga-   ggg     ug     A
      GUcg    ccg   uccgg  cgGAG G
      ||||    |||   |||||  ||||| |
      CGGC    GGC   GGGCC  GCUUC C
     C    GCGG   ACA     CU     C
```

24. >bomir-F0522-1-3p:Btau_4.0:21:35870333:35870398:1(47-65) dG = -27.40 kcal/mol
CCUCUCUCCCUGCAGCAUCUCAGGGCACUGUGUGACUUGGGUCGGGgugggguggggggguuggU



25. >bomir-F0522-2-3p:Btau_4.0:22:59347353:59347420:1(43-61) dG = -34.40 kcal/mol
GGAGGGUGCCUCUAAGCCUCACUUGUCCUGCCUGUCAAAUGGgugggguggggggguuggCCUUUCC

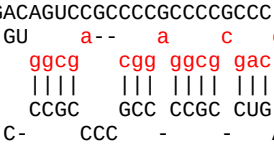


26. >bomir-B0821-5p:Btau_4.0:20:19373708:19373768:-1(3-23) dG = -32.60 kcal/mol
GCgucccggggcucccgccggcGGAGUUCGGUUUGCACCGGGGAGGGGGCCGGGGAUCGG

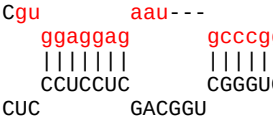
27. >bomir-F1351-3p:Btau_4.0:25:41129445:41129520:1(53-72) dG = -44.20 kcal/mol
CGGCGGCCGGGAAAGGUGGGCUGGGCUCCGGGGCCCCUCCCCGCCCGGUGCUgccccggccgcucccggccuUGUG

28. >bomir-F1353-5p:Btau_4.0:28:27885032:27885119:1(5-24) dG = -18.30 kcal/mol
AAAAaucuuugggcuagguuaguuCUAUUUUAUGAUCUGUUAUGAAAUGGGUUAAGGAAAAGGAUCCAUGUGGAAGA
AAGAUCAGAGA

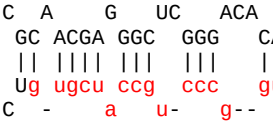
29. >bomir-D1431-5p:Btau_4.0:12:75101950:75102053:-1(3-24) dG = -44.60 kcal/mol
GUggcgacggaggcgcgacccccccCCCCCCCCCCCCCCCCCCCCGCCCCGGUCCGACGGUGCGACCCGCCAGGGGCGCUC
UGGGGACAGUCCGCCCCGCCCCGCCC



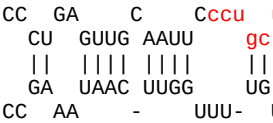
30. >bomir-C1511-5p:Btau_4.0:Un.004.1059:20638:20698:1(2-21) dG = -32.00 kcal/mol
CguggaggagaaugcccggggcgugcuggggcgguGCGGGCUGGGCUGGCAGCUCCUCCCUC



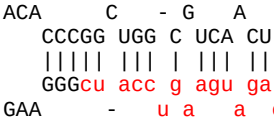
31. >bomir-F1821-3p:Btau_4.0:Un.004.152:123132:123213:1(60-80) dG = -31.60 kcal/mol
CGCAACGAGGGCUCGGGACACAGCCCUGCUUCCUGGCCCACGUCACCCGUCGUGGCCAGagcccuggcccugccaucgugUC



32. >bomir-C1931-5p:Btau_4.0:26:12405822:12405907:1(17-39) dG = -11.60 kcal/mol
CCCUGAGUUGCAAUUCccugcugaucucacauuaauucaUCUUUGCUAGAGAAAUAUCUGAGUGUCUGUUUUGGUUCAAU
AAAGCC



33. >bomir-A2143-3p:Btau_4.0:16:47801303:47801359:1(34-51) dG = -18.50 kcal/mol
ACACCCGGCUGGCGUCAACUCUGAACAGAUUCUcggcagaugaaguccaucGGGAAG



34. >bomir-F2422-5p:Btau_4.0:18:53584142:53584210:1(1-20) dG = -35.50 kcal/mol
ggugggaggguccc accgagCACCCUGUCGGCUGGCAGCCCCGGCCCAGGUAGUGGGUGCUCUGCCACA
g g - cgagCAC UC CA
gugg agggu cccac CCUG GGCUGG \
|||| ||||| ||||| |||| |||||| G
CACC UCUCG GGGUG GGAC CCGGCC /
A G U AU----- -- CC

35. >bomir-F2531-3p:Btau_4.0:8:77307612:77307688:1(50-67) dG = -13.80 kcal/mol
GGUUUAAUGCUCUGCUGUCAGCGCUUUGAAAUUCUUACUAAUCUUUUUUugguggagaugccggggaCGUAAUAAUU
GGUUUA- - CU AG UU UUCUUA
AUG CUCUG GUC CGCU GAAA \
||| ||||| ||| |||| |||| C
UGC ggggc uag gugg UUUU /
UUAAUAA a cg ag uU UCUAAU

36. >bomir-G2511-3p:Btau_4.0:18:41190497:41190564:1(40-57) dG = -21.10 kcal/mol
AGGGGGAGAGUUGCGGCUCAUGAACUGGGUCCAGGUGGAaggcgggccgggguuggaAGGUUCCAGCU
AGGG AG G A AA GG- A
GGAG UU CGGCUC UG CUG UCC G
|||| || |||||| || ||| ||| |
CCUU Aa guuggg gc ggc AGG G
UCGA GG g - cg gga U

37. >bomir-E2664-3p:Btau_4.0:18:64360999:64361052:-1(33-52) dG = -21.10 kcal/mol
AGUCAAGUGAGCGGCCGCACCGGCUCUCCCGGagggcgggcggcgacuggaaAG
AG- A GAGCG A G C
UC AGU GCCGC CCG CUCUC \
|| ||| ||||| ||| ||||| C
ag uca cggcg ggc ggga G /
GAa g g---- - - G

38. >bomir-D3011-3p:Btau_4.0:18:39424877:39424959:1(62-82) dG = -47.50 kcal/mol
ACGCGCUUCUCGGCGCUCACUCGGCGGCACCACUGCCGCAAAUGCGGCUUCGUGGUCUGCGccgagugcucccgcgagcgcuU
AC U U CGCU GC- U-- A
GCGC UC CGG CACUCGGCG ACCAC GCCGC A
|||| || ||| ||||||||| ||||| ||||| |
cgcg ag gcc gugagccGC UGGUG CGGCG A
Uu - c cuc- GUC CUU U

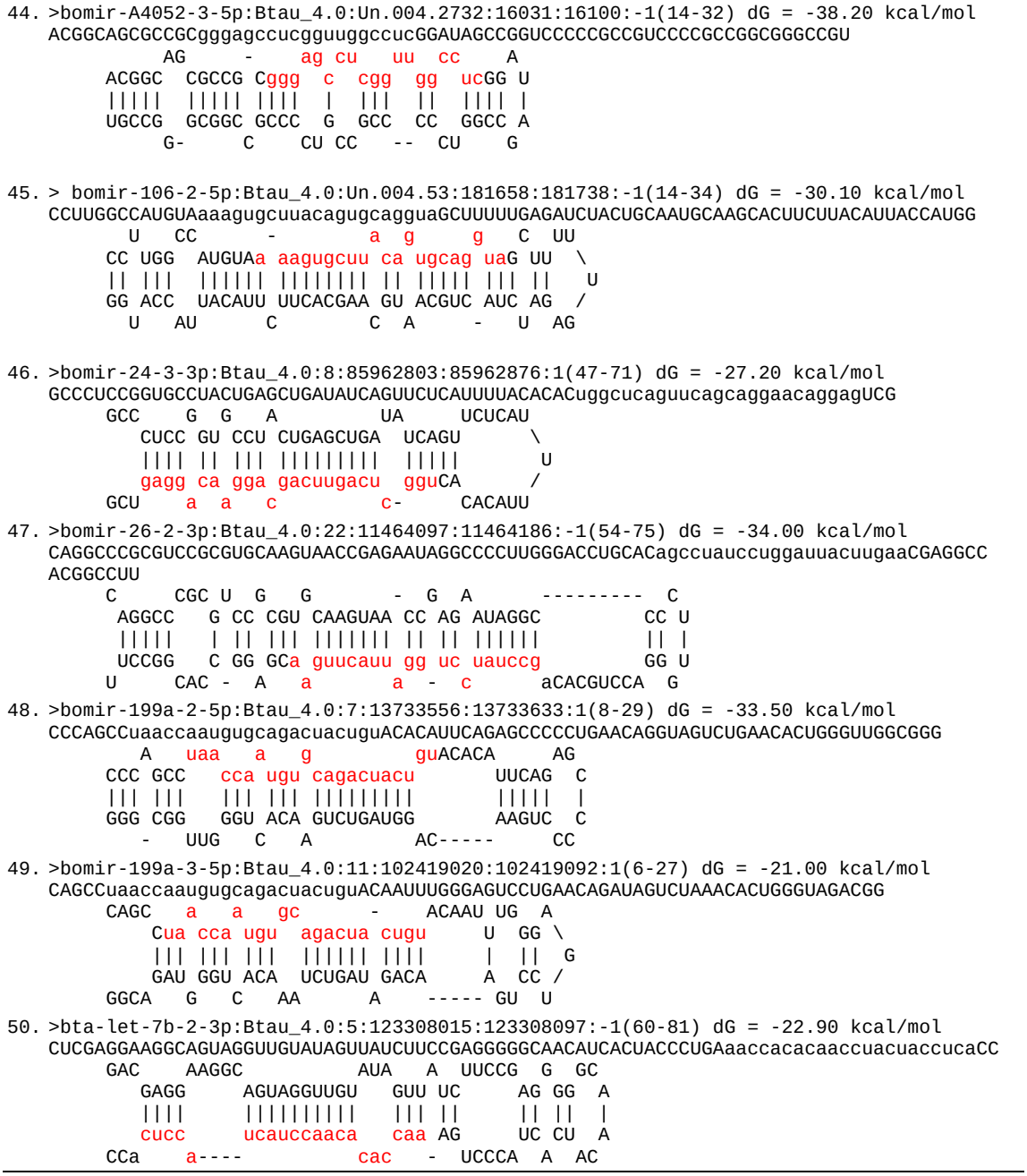
39. >bomir-A3341-1-3p:Btau_4.0:3:124987968:124988032:1(41-61) dG = -28.00 kcal/mol
UCACCCCAGUCUCUAGUGAGAGAGCCAUGGCGGCAGGGGCguggcugucccuggaggugggACUG
UCAC G UGA G G GGC
CCCA UCUCUAG GA AGCCAUG C \
|||| ||||||| || ||||||| | A
gggu ggagguc cu ucggugC G /
GUCA - c-- g - GGG

40. >bomir-A3341-2-3p:Btau_4.0:Un.004.4799:1295:1359:1(41-61) dG = -28.00 kcal/mol
UCACCCCAGUCUCUAGUGAGAGAGCCAUGGCGGCAGGGGCguggcugucccuggaggugggACUG
UCAC G UGA G G GGC
CCCA UCUCUAG GA AGCCAUG C \
|||| ||||||| || ||||||| | A
gggu ggagguc cu ucggugC G /
GUCA - c-- g - GGG

41. >bomir-A3711-5p:Btau_4.0:9:63475787:63475874:1(18-36) dG = -64.40 kcal/mol
CGCCGCUCCGCUCCCGGuuccgcgcucuacgccagcCCGCUGCCUGCCGGGCUGGUGCAGGCCGCGGAGCCGGGCGGCCCGG
CAGGCA
C - CC - cu a - UG
GCC GCU GCU CCCGGuuccgcg cu cgccagcCC GC \
||| ||| ||| |||||||||||| || ||||||||| || C
CGG CGG CGG GGGCCGAGGCGC GA GUGGUCGGG CG /
A A CC C CG C C UC

42. >bomir-A4052-1-5p:Btau_4.0:18:59928617:59928686:1(14-32) dG = -38.20 kcal/mol
ACGGCAGCGCCGCgggagccucgguuggccucGGAUAGCCGGUCCCCCGCCGUCCCCGCCGGCGGGCCGU
AG - ag cu uu cc A
ACGGC CGCCG Cggg c cgg gg ucGG U
||||| ||||| |||| | ||| || |||| |
UGCCG GCGGC GCCC G GCC CC GGCC A
G- C CU CC -- CU G

43. >bomir-A4052-2-5p:Btau_4.0:21:52041984:52042053:-1(14-32) dG = -38.20 kcal/mol
ACGGCAGCGCCGCgggagccucgguuggccucGGAUAGCCGGUCCCCCGCCGUCCCCGCCGGCGGGCCGU
AG - ag cu uu cc A
ACGGC CGCCG Cggg c cgg gg ucGG U
||||| ||||| |||| | ||| || |||| |
UGCCG GCGGC GCCC G GCC CC GGCC A
G- C CU CC -- CU G



Secondary structure for new miRNAs and new loci of annoated miRNAs predicted by mfold web server. First line started with the name of the miRNA followed by database (Ensemble 52; Btau_4.0), genomic location (chromosome number: start: end: sense or antisense orientationand orientation, (position of the mature sequence in the precursor) and minimum free energy. Position of the mature sequence in the hairpin precursor is indicated as lower case and by the red color.

Characterization of miRNAs was done based on the annotation in the bovine genome data base Ensembl 52: Btau_4.0 (De La Fuente and Eppig 2001). Bomir-F0522-3p and bomir-A4052-5p were mapped to both intergenic and intronic locations. Bomir-F0132-

5p (sense), bomir-E2664-3p (antisense) and bomir-A4052-5p (antisense) are originated from the exons of protein-coding genes. While searching the genomic location for all miRNAs, six new genomic locations for annotated miRNAs like bta-mir-106, 24, 26, 199a and let-7b were found.

All the 50 new genomic loci were found to be distributed in 19 chromosomes (Chr.) namely: Chr. 3, 4, 5, 7, 8, 9, 11, 12, 14, 16, 18, 19, 20, 21, 22, 25, 26, 28 and 29. However, eight loci were found to be mapped to unknown chromosome in the Ensembl 52: Btau_4.0 (end note). Among all newly identified loci, eight miRNA genes were found to be located on Chr. 18 and five miRNAs found on Chr. 7 and 21. Further analysis of the already annotated miRNAs and the newly predicted loci has revealed six miRNAs gene clusters which were mapped within < 10 kb. This clusters are i) bta-miR-10a and bomiR-A0321 on Chr. 19; ii) bta-miR-23b, bta-miR-27b and bta-miR-24-3 on Chr. 8; iii) bta-let-7a-3 and let 7b-2-3P on Chr. 5; iv) bomiR-A4052-1 and bomiR-C0533 on Chr. 18; v) bta-miR-487a, bta-miR-487-b, bomiR-382 and bomiR-409 on Chr. 21; vi) bomiR-C0533-2 and bomiR-A4052-2 on Chr. 21.

To determine whether new miRNAs are conserved among closely related species, homology for precursor sequence in the ENSEMBL genome databases have been searched. Results revealed that 17 precursor loci (out of 43 loci for 38 new bovine miRNAs) were found to be conserved in at least six species. While five miRNAs (bomiR-F0244, bomiR-A0321, bomiR-F2531, bomiR-D3011 and bomiR-A3711) were found to be conserved in at least two species, 21 miRNA loci were specific to bovine. All of the newly cloned miRNAs were found to be conserved as mature sequences in the genome of different species. Thermo-dynamically stable hairpin structures have been found for those conserved and new miRNAs as shown in table 3.3.

3.2.4 Other small RNAs and their genomic properties found in the library

Analysis of small RNA library in the present study has enabled us to identify 57 different endogenous siRNAs. They were categorized broadly into two groups, namely: 29 sequences composed of 27 distinct RNAs derived from genomic repetitive region as repeat associated small interfering RNAs (rasiRNAs) and other 30 RNAs associated to non repetitive regions as non-repeat associated small interfering RNAs (nasiRNAs).

According to their sequence properties 13 out of 30 nasiRNAs were found to be natural antisense transcripts with ~20 nt in length. Therefore, since they seem to be endogenous siRNAs, they were denoted as small antisense RNAs (santRNAs) and the rest 17 as tiny non-coding RNAs (tncRNAs). Size ranges for rasiRNAs were 18-28 nt with mean ± SD 21.5 ± 3.1 nt, which do not reveal a sharp size distribution characteristic. However, for the santRNAs and tncRNAs the size distribution was 19.6 ± 1.9 and 19.5 ± 1.1 nt, respectively. Cloned rasiRNAs were found to be distributed on various chromosomes and mapped to repeat sequences mostly corresponding to retrotransposons in both sense and antisense orientation. Total numbers of hits for 27 rasiRNAs were 581 (ranging from 4 to100). Seventy five percent of the rasiRNAs were found to have preference for uridine and adenine residues in either 3′ or 5′ end position. While seven of the santRNAs were precisely mapped to intergenic region, six fitted to intronic region. All the 13 santRNAs were cloned as antisense orientation to the genome or intron of the protein coding genes on 12 different chromosomes.

Secondary structure analysis of all santRNAs revealed no characteristic hairpin as found for the miRNAs. While eleven tncRNAs were mapped to intergenic region, five were mapped to intronic and two to exonic regions. Two of the seventeen tncRNAs are predicted to form potential fold back structures like the miRNAs. However, these putative tncRNA precursor structures deviate significantly from the miRNA hairpins in key features and they were found to be poorly conserved in closely related species.

3.2.5 Detection and expression of miRNAs in the ovary and other bovine tissues

The expression of all new miRNAs including nine annotated miRNAs (let-7b, mir-15b, mir-18a, mir-29a, mir-125b, mir-126, mir-145, mir-199a and mir-222) in 11 different bovine tissues were analyzed using semi-quantitative RT-PCR (details in figure 3.4, table 3.4). As small RNAs cloned in the library derived from all compartments of the ovary, samples from ovarian cortex, cumulus cells and matured corpus luteum were used to determine the sub-cellular expression profile of the new miRNA using RT-PCR (Table 3.4). This is because of two facts: firstly, the bovine ovary is continuously changing throughout the process of folliculogenesis and secondly, the distinct nature of function of intra-ovarian cells and tissues compartments in the ovary.

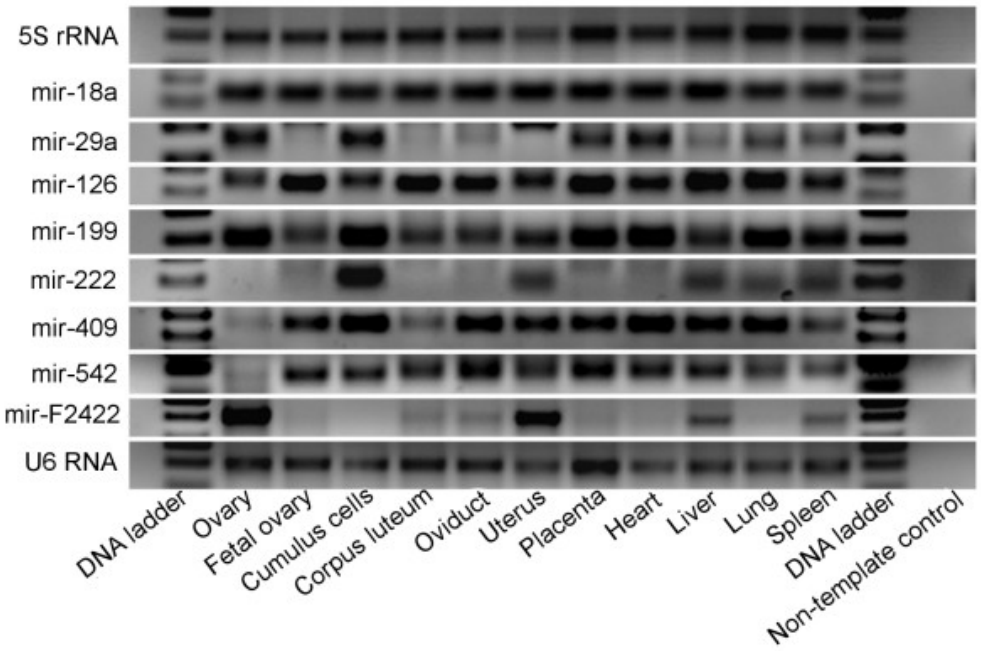


Figure 3.4: Detection and expression analysis of selected miRNAs in multiple tissues. Expression profiles of some representative miRNAs (out of detected 44 miRNAs) in multiple tissues by PCR approach. The expressions for all miRNAs are summarized in the table 3.4. Amplicons were analyzed on 2% agarose gel. 5S rRNAs and U6 RNA were used as a loading control. A DNA ladder (M) indicating the size of the fragments (50-100-150 nt) on each side. Ovary denotes only the ovarian cortex without corpus luteum.

Table 3.4: Detection and expression of selected miRNAs in multiple tissues

miRNAs	Ovary[a]	Fetal Ovary[b]	Cumulus cells	Corpus luteum[c]	Oviduct	Uterus	Placenta	Heart	Liver	Lung	Spleen
5s rRNA	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
U6 RNA	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
bta-let7b	++	+++	+++	+++	++	+	+++	++	+	++	+++
bta-mir-15b	+	+++	+++	+++	+++	+++	+++	+++	+++	+	+++
bta-mir-18a	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
bomir-22*/22-5p	+	+	+	-	-	-	-	+	-	+	+
bta-mir-29a	+++	-	+++	-	-	-	+++	+++	+	++	++
bta-mir-125b	-	++	+++	++	++	+	++	++	-	+++	+
bta-mir-126	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
bomir140*/140-5p	+++	+++	+++	++	++	++	++	++	++	-	++
bomir-143-3p	++	+++	+	++	+	+	+++	++	+	+	++
bta-mir-145	++	+++	-	++	-	-	+++	++	++	-	+++
bomir-152-5p	++	++	-	++	+++	+++	++	++	+++	+++	+++
bomir-193a-2-3p	+	++	++	-	++	++	++	+	+	++	++
Bta-mir-199	+++	++	+++	++	++	++	+++	+++	++	+++	+++
bta-mir-222	-	-	+++	-	-	++	-	-	+	+	+
bomir-378-3p	+++	+++	-	-	++	+++	+	++	+	+	-
bomir-382-3p	+	-	-	-	+++	+++	+++	+++	-	-	+++
bomir-409-5p	+	+++	+++	++	+++	+++	+++	+++	+++	+++	++
bomir-424-3p	++	-	-	-	+	++	-	-	++	-	+
bomir-503-3p	++	+++	+++	++	+	+	++	++	+	++	++
bomir-542-3p	-	++	++	++	+++	++	+++	++	++	+	+
bomir-574-5p	+	+	-	+	+	++	++	++	++	++	++
bomir-652-3p	+	+++	-	+++	++	+++	-	-	++	-	-
bomir-940-5p	+	-	-	-	+++	+	-	+	+++	++	-
bomir-F0132-5p	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
bomir-F0244-5p	+	++	+++	++	+	++	++	+	++	+	+
bomir-H0222-3p	-	++	-	+++	+	+	++	++	-	+	+
bomir-A0321-3p	++	++	++	++	++	++	++	++	++	+	++
bomir-C0533-5p	+	+++	++	+	-	+	+	+	-	-	-
bomir-F0522-3p	-	-	++	++	-	-	-	+	-	-	-
bomir-F1351-3p	++	-	-	+++	++	+++	+	+++	+++	++	+++
bomir-F1353-5p	++	++	-	++	+	+++	+++	+	+++	-	+
bomir-D1431-5p	++	+++	++	+++	++	+	++	+++	-	+	-
bomir-C1511-5p	+	+	-	+	++	++	+	++	++	+	+
bomir-F1821-3p	++	++	++	++	++	++	++	++	++	++	++
bomir-C1931-5p	+	++	-	+++	+++	+++	+++	+++	+++	-	+++
bomir-A2143-3p	-	++	-	+++	+++	+++	++	++	++	++	+++
bomir-F2422-5p	+++	-	-	+	+	+++	-	-	++	-	+
bomir-F2531-3p	+	-	-	+	++	++	+	+++	++	+	-
bomir-G2511-3p	+	-	-	+	+	+++	+	+++	++	++	-
bomir-E2664-3p	++	++	++	++	+	++	++	+	++	+	++
bomir-D3011-3p	+	+	++	+	+	+	+	+	+	++	+
bomir-A3341-3p	+	+	+	+	+	+	+	+	+	+	+
bomir-A3711-5p	+	+	-	+	+	+	+++	+	+	+	+
bomir-A4052-5p	+	+	-	++	++	++	++	++	+++	++	++

Expression profiles of 44 miRNAs including all new miRNAs in multiple tissues by PCR approach. Amplicons were analyzed on 2% agarose gel. 5S rRNAs and U6 RNA were used as a loading control. Relative band intensity was categorized

into four groups like '+++' for Strong signal, '++' for Medium signal, '+' for Weak signal or smear like product and '-' for not detected by comparing the expression signal of each miRNA to the expression level of 5S rRNA and U6 RNA. [a]: Ovarian cortex with no visible corpus luteum, [b]: Ovary from fetus at about 5th month of pregnancy, [c]: Mature corpus luteum from the same Ovary.

Of all 47 miRNAs (38 new and 9 already annotated miRNAs) 44 were detected in both ovarian cells and multiple tissues. Five miRNAs (bta-mir-126, bomir-F0132, bomir-A0321 and bomir-F1821) were found to be expressed at similar level in all experimental tissues. Seven miRNAs (bta-mir-18a, bta-mir-29a, bomir-140, bta-mir-199, bomir-378, bomir-F0132 and bomir-F2422) were found to be expressed at relatively higher levels in ovarian cortical portion (Table 2). On the other hand, all undetected or less expressed miRNAs in ovarian cortex have been shown to be highly expressed in cumulus cells or corpus luteum. Most of the miRNAs were found to be differentially expressed between adult ovarian tissues and fetal ovary. Among them bta-mir-15b, bomir-409, bomir-652, bomir-C0533 and bomir-D1431 were highly expressed in the fetal ovary compared to that of adult ovarian cortex. However, bta-mir-29a, bta-mir-199 and bomir-F2422 were found to be expressed at higher level in the adult ovarian cortex than that of the fetal ovary (Table 2). Bta-mir-125b, bta-mir-222, bomir-542, bomir-652, bomir-H0222, bomir-F0522, bomir-C1931 and bomir-A2143 were found to be expressed at very low level or not detected at all in the ovarian cortex. However, their abundance was higher in the cumulus cells and matured corpus luteum. The expression of bta-mir-222 was detected exclusively in the cumulus cells. In addition, higher expression of bta-mir-125b, bomir-409, bomir-503 and bomir-F0244 was also observed in the cumulus cells. The expression of bomir-652, bomir-H0222, bomir-C1931 and bomir-A2143 was higher in the corpus luteum.

Moreover, higher expression level of different miRNAs in various reproductive tissues was also observed. This includes bomir-940 in the oviduct; bta-mir-222, bomir-F2422 and bomir-G2511 in the uterus; and bta-mir-29a, bomir-143, bta-mir-145, bta-mir-199, bomir-542 in the placenta. All these investigated miRNAs were detected at least in one of the non-ovarian somatic tissues including heart, liver, lung and spleen (Table 3.4). The RT-PCR analysis did not confirm the expression of three novel miRNAs (bomir-F0131, bomir-H0121 and bomir-B0821) in any of the tissues under investigation (image not shown).

In order to elucidate the cellular localization of one miRNA, bta-miR-29a was selected due to its differential expression between adult and fetal ovary, which are distinct in their functional activity.

Accordingly, in-situ localization of this miRNA in the sections of bovine ovarian follicle revealed its expression in the different intra-ovarian cells (theca, mural granulosa, cumulus granulosa and oocyte) of different stages of development including primordial, primary, growing and matured/tertiary follicles (Figure 3.5). Stable expression was detected in the whole mount cumulus-oocyte-complexes derived from the follicles of more than 8 mm in diameter. In the semi-quantitative RT-PCR data, expression of this miRNA was found in the cortex region of the adult ovary where follicles with cumulus cells are residing. Moreover, the expression of this miRNA was detected further until early stage of corpus luteum (Figure 3.5), but very low or no expression in the matured corpus luteum (Figure 3.4).

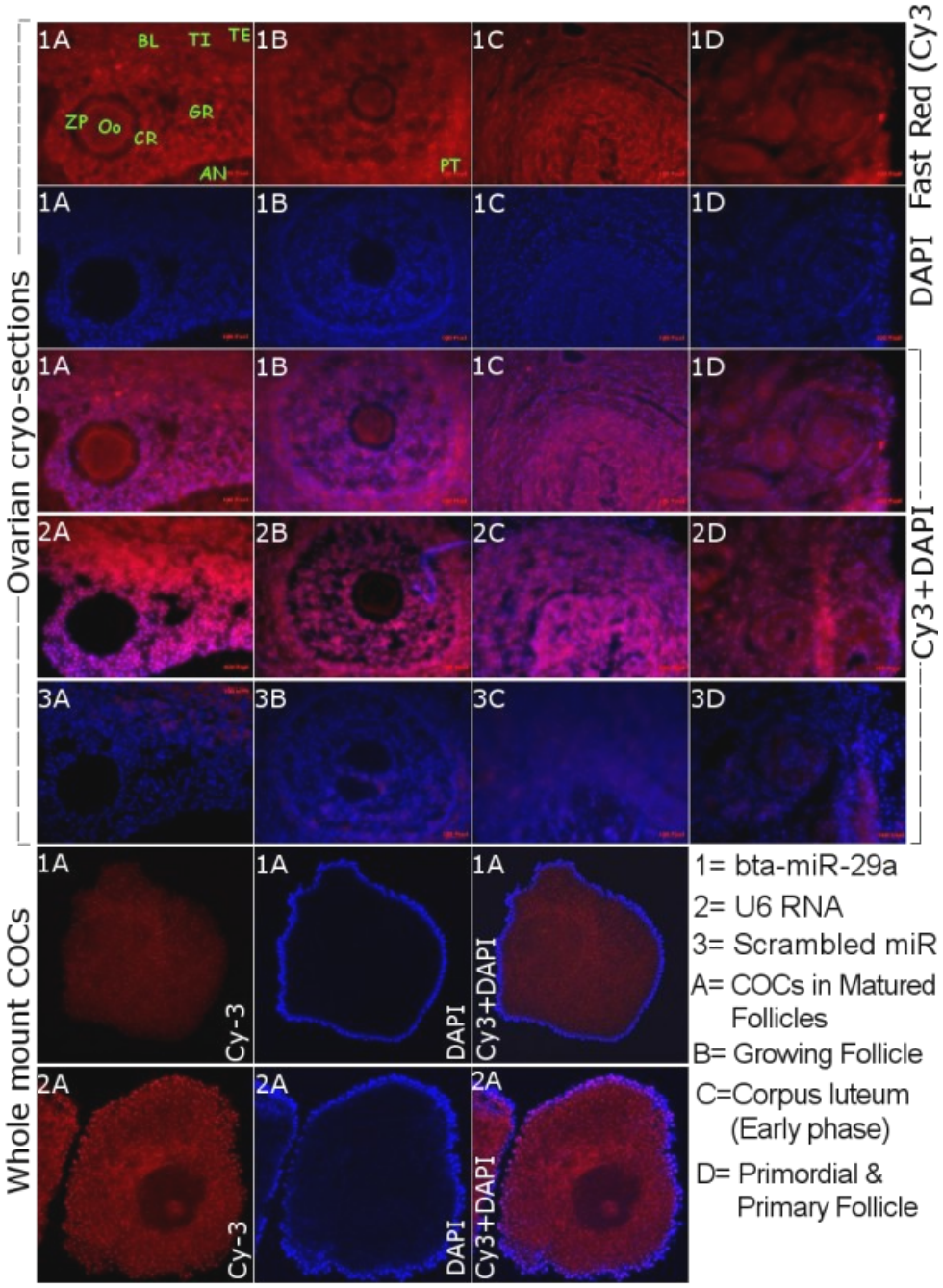


Figure 3.5: In-situ detection of mir-29a in the ovarian sections and whole mount COCs. Bovine ovarian cryo-sections and whole mount cumulus-oocyte complexes were in situ hybridized with 3'-digoxigenin labeled locked nucleic acid (LNA) microRNA probes for miR-29a (1), U6 RNA (2) and scrambled miRNA (3). BL- Basement Laminae, TI- Theca Interna, TE- Theca Externa, GR- Multiple layers of Granulosa, ZP-Zona Pellucida, OO-Oocyte, CR-Corona Radiata, AN-Antrum of the follicle, PT- Presumptive theca in the growing follicle.

3.2.6 Prediction and functional categorization of cloned miRNA targets

The goal of this prediction and analysis was to find the major biological processes and signaling pathways in the ovary that are most likely affected by a group of miRNAs. Even though there were many potential target genes predicted for the cloned miRNAs, several filtering and screening procedures (see materials and methods) have enabled us to generate a comprehensive target list consisting of 115 potential genes from all the predicted targets. From this screened target set, let-7b, mir-15b, mir-18a, mir-29a, mir-101, mir-125b, mir-126, mir-143, mir-145, mir-199a and mir-222 found to have the highest number and overlapping targets (Figure 3.6). Interestingly, all of these targeting miRNAs were represented at higher frequency in the constructed library.

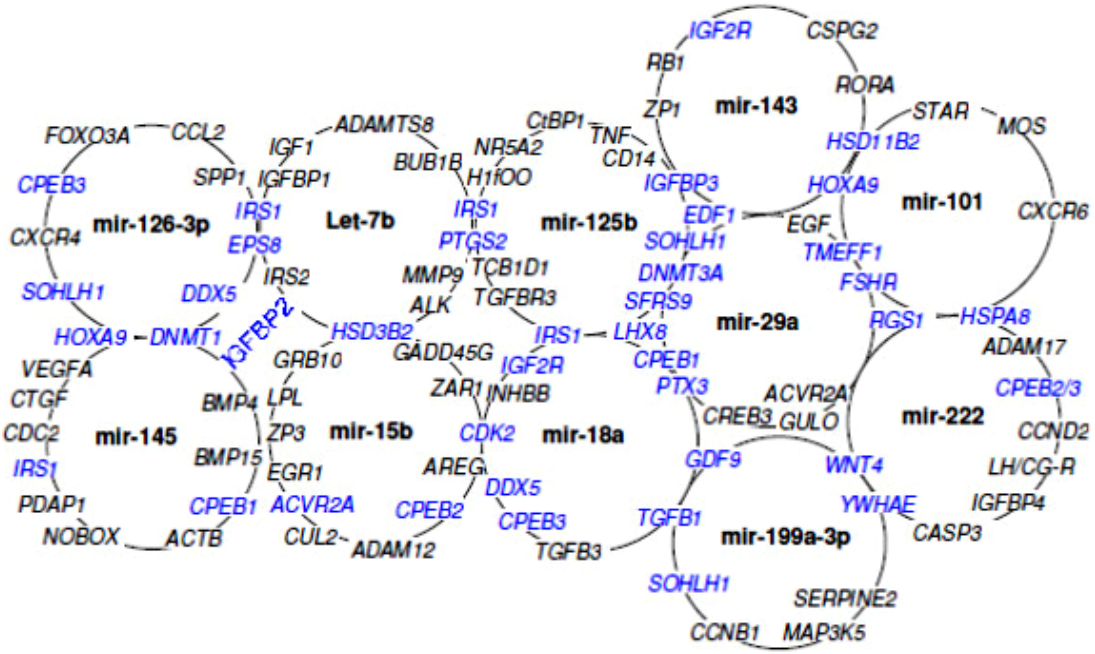


Figure 3.6: Eleven miRNAs with highest number of screened target genes (sub-set miRNAs targets). Each circle representing one miRNAs and the surrounding genes are targeted by that miRNA. Genes shared by the different circles highlighted as blue (overlapping genes between miRNAs), which are commonly targeted by the corresponding miRNAs.

Table 3.5: Ingenuity analysis of the genes targeted by top eleven screened miRNAs

miRNAs	Functions and disease categories enriched with the selected miRNA targets	Canonical Pathways enriched with the selected miRNA targets
Let-7b	Tissue morphology, cellular growth and proliferation, endocrine system disorders	IGF-1 signaling, hepatic fibrosis/hepatic stellate cell activation
mir-15b	cell death, connective tissue development and function, cell cycle	p53 signaling, PPARα/RXR activation
mir-18a	Cell cycles, cellular function, endocrine system development	Cell cycle: G1/S checkpoint regulation, TGF-β signaling
mir-29a	Reproductive system development and function, organ development, endocrine system development	Ephrin receptor signaling Aminophosphonate metabolism
mir-101	Endocrine system development, lipid metabolism, small molecule biochemistry	C21-steroid hormone metabolism, Androgen and estrogen metabolism
mir-125b	Inflammatory response, cell cycle, cellular function and maintenance	LPS/IL-1 Mediated inhibition of RXR function, LXR/RXR activation
mir-126	Cellular movement, Endocrine system disorders, cell mediated immune response	Pro-apoptosis, PXR/PXR activation
mir-143	Cellular growth and proliferation, DNA replication, recombination and repair, gene expression	G1/S transition of the cell cycle, p53 signaling
mir-145	Reproductive system diseases, reproductive system development and function, cell death	BMP signaling pathway, VEGF signaling
mir-199a	Cellular development, cell death, cellular growth and proliferation	Cell cycle: G2/M DNA damage checkpoint regulation, p38 MAPK signaling
mir-222	Cellular development, reproductive system development and function, cell death	IGF-1 signaling, Axonal guidance signaling

Eleven top miRNAs targeting highest number of genes from the screened and filtered all predicted targets and their top Gene Ontology categories and pathways based on Fisher' Exact P-value (<0.05) are presented in the table.

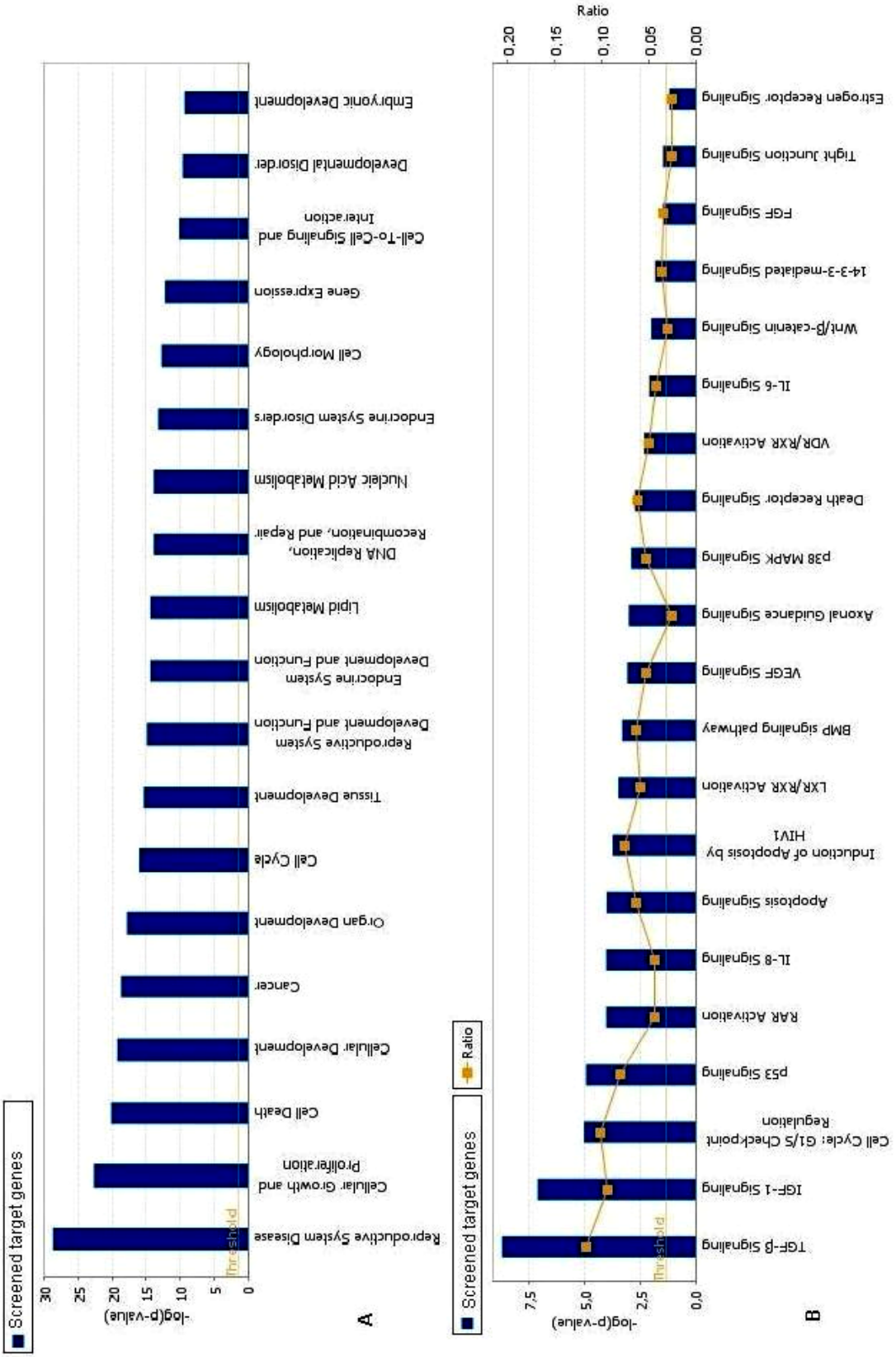


Figure 3.7: Top biological function, disease categories and pathways enriched with predicted and screened miRNA target genes. A. Top biological functions and disease categories and B. pathways enriched with predicted and

screened miRNA target genes. Ratio is the number of affected genes to total number of genes in the pathway. Threshold $p < 0.05$ is shown as yellow line. Bars that are above the line indicate significant enrichment of a functional category or pathway.

Detailed Gene Ontology (GO) analysis of the screened and sub-sets of miRNAs target genes were found to be associated with reproductive system development, function and disorders. These include cell cycle, morphology, cell death, cell to cell signaling, cellular growth, development and proliferation, DNA replication, recombination & repair, endocrine system disorder and different pathways underlying the ovarian functions. To further elucidate the specific functions of these genes, a detailed pathway analysis was performed using Ingenuity Pathway Analysis (Redwood City, California) for all target sets (Figure 3.7) as well as for the sub-set of genes (Table 3.5).

3.3 Discussion

3.3.1 Identification of small RNAs

MicroRNAs play an integral part of animal gene regulatory networks as one of the most abundant classes of gene regulators. They are estimated to comprise 1–5% of animal genes (Bartel 2004, Bentwich et al. 2005, Berezikov et al. 2005) or a given genome could encode nearly thousands of miRNAs (Bentwich et al. 2005). Moreover, a typical miRNA regulates hundreds of target genes (Brennecke et al. 2005, Krek et al. 2005, Lewis et al. 2005, Xie et al. 2005) and altogether they could target a large proportion of genes up to 30% of the genome (Lim et al. 2005). Changes in the expression of even a single miRNA could have a significant impact on the outcome of diverse cellular activities regulated by the product of these genes. Beyond the strict conservation of miRNAs across different species, some miRNAs appears to be species specific (Ambros et al. 2003a, Bentwich et al. 2005, Stark et al. 2007). Compared with computational or heterologus approaches, direct cloning has the advantage of identifying non-conserved and new miRNAs.

Cloning and expression analysis led to the identification of 74 miRNAs out of which 38 are new in bovine. Mature sequences were found to be conserved in closely related species, but when considering precursor sequence only 51% was found to be conserved in human, mouse, rat, dog, horse and also in other non-mammalian vertebrates. However, in the present study 17 miRNA precursors corresponding to 21 genomic loci were found to be not conserved (Table 1). This could be either due to the lack of sequences in draft genome assembly or these miRNAs are bovine specific. The genomic properties of identified new miRNAs showed that they are derived from exon, intron and intergenic region. This may suggest that these miRNAs can be transcribed in parallel with their host transcripts. In addition, two different transcription classes of miRNAs (‘exonic’ and ‘intronic’) recognized here may require somewhat different mechanisms of bio-genesis as stated previously (Rodriguez et al. 2004). Discovery of six clusters composed of 15 miRNA genes on six chromosomes showed that these closely located host genes may share the same cis-regulatory elements and the miRNAs within the clusters might be expressed in the same tissues or at the same developmental or physiological stage.

The representation of many known and novel miRNAs in this single library indicates the presence of potential miRNAs, which are not yet discovered. Both in-depth analysis of the existing library and cell-type-specific analysis of individual miRNAs will give insight into the functional mechanisms and pathways involved in ovarian folliculogenesis in particular and female fertility in general. All the sequences for new and known miRNAs were submitted to the miRBase for official annotation and recognition. The accession number and annotated name for all novel miRNAs were assigned by the miRBase in version 15.0 according to the universal criteria specific to the miRNAs, which are presented in table 3.6.

Table 3.6: Annotated novel miRNAs with their accession number provided by the miRBase organization

Original clone ID	Sequence (5′-3′)	Annotated new name	Assigned accession
bomir-424-3p	CAAAACGUGAGGCGCUGCUAU	bta-mir-424*	MI0012212
bomir-193a-2-3p	GGGACUUUGUAGGCCAGUU	bta-mir-193a-2	MI0013051
bomir-378-1-3p	ACUGGACUUGGAGUCAGAAGGC	bta-mir-378-2	MI0013052
bomir-574-5p	UGUGGGUGUGUGCAUGUGCGUG	bta-mir-669	MI0013053
bomir-F0131-5p	GGGGCGGGGGGGCGGGUG	bta-mir-2881	MI0013054
Bomir-F0132-5p	AGCCCGGGCCCCUCCCCUG	bta-mir-2882	MI0013055
bomir-H0121-3p	CUUCCCGUGUGUUGAGCC	bta-mir-2883	MI0013056
bomir-F0244-5p	GCUACUACCGAUUGGAUGG	bta-mir-2884	MI0013057
bomir-H0222-3p	CGGCGGCAGCGCCGGGGCG	bta-mir-2885	MI0013058
bomir-A0321-3p	AGCGCCGCCGGCCGCACC	bta-mir-2886	MI0013059
bomir-C0533-1-5p	CGGGACCGGGGUCCGGUGCG	bta-mir-2887-1	MI0013060
bomir-C0533-2-5p	CGGGACCGGGGUCCGGUGCG	bta-mir-2887-2	MI0013061
bomir-F0522-1-3p	GGUGGGGUGGGGGGGUUGG	bta-mir-2888-1	MI0013062
bomir-F0522-2-3p	GGUGGGGUGGGGGGGUUGG	bta-mir-2888-2	MI0013063
bomir-B0821-5p	GUCCCCGGGGCUCCCGCCGGC	bta-mir-2889	MI0013064
bomir-F1351-3p	GCCCCGGCCGCUCCCGGCCU	bta-mir-2890	MI0013065
bomir-F1353-5p	AUCUUUGGGCUAGGUUAGUU	bta-mir-2891	MI0013066
bomir-D1431-5p	GGCGACGGAGGCGCGACCCCCC	bta-mir-2892	MI0013067
bomir-C1511-5p	GUGGAGGAGAAUGCCCGGGG	bta-mir-2893	MI0013068
bomir-F1821-3p	AGCCCUGGCCCUGCCAUCGUG	bta-mir-2894	MI0013069
bomir-C1931-5p	CCUGCUGAUCUCACAUUAAUUCA	bta-mir-2895	MI0013070
bomir-A2143-3p	CGGCAGAUGAAGUCCAUC	bta-mir-2896	MI0013071
bomir-F2422-5p	GGUGGGAGGGUCCCACCGAG	bta-mir-2897	MI0013072
bomir-F2531-3p	UGGUGGAGAUGCCGGGGA	bta-mir-2898	MI0013073
bomir-G2511-3p	AGGCGGGCCGGGGUUGGA	bta-mir-2899	MI0013074
bomir-E2664-3p	AGGGCGGGCGGCGACUGGAA	bta-mir-2900	MI0013075
bomir-D3011-3p	CCGAGUGCUCCCGCGAGCGCU	bta-mir-2901	MI0013076
bomir-A3341-1-3p	GUGGCUGUCCCUGGAGGUGGG	bta-mir-2902-1	MI0013077
bomir-A3341-1-3p	GUGGCUGUCCCUGGAGGUGGG	bta-mir-2902-2	MI0013078
bomir-A3711-5p	UUCCGCGCUCUACGCCAGC	bta-mir-2903	MI0013079
bomir-A4052-1-5p	GGGAGCCUCGGUUGGCCUC	bta-mir-2904-1	MI0013080
bomir-A4052-1-5p	GGGAGCCUCGGUUGGCCUC	bta-mir-2904-2	MI0013081
bomir-A4052-1-5p	GGGAGCCUCGGUUGGCCUC	bta-mir-2904-3	MI0013082

bomir-22*/22-5p	ACAGUUCUUCAACUGGCAGCUU	bta-mir-3600	MI0015943
bomir-152-5p	CCAAGUUCUGUCAUGCACUGA	bta-mir-2957	MI0015944
bomir-382-3p	GAAUCCACCACGAACAACUUC	bta-mir-3578	MI0015945
bomir-409-5p	GGGGUUCACCGAGCAACAUUC	bta-mir-3581	MI0015946
bomir-542-3p	UCUCGUGACAUGAUGAUCCCCGA	bta-mir-3601	MI0015947
bomir-652-3p	GUGUUGGGAUCACCGCGGUAA	bta-mir-3602	MI0015948
bomir-26-2-3p	AGCCUAUCCUGGAUUACUUGAA	bta-mir-3603	MI0015949
bomir-199a-2-5p	UAACCAAUGUGCAGACUACUGU	bta-mir-3604-1	MI0015950
bomir-199a-2-5p	UAACCAAUGUGCAGACUACUGU	bta-mir-3604-2	MI0015951
bta-let-7b-2-3p	AACCACACAACCUACUACCUCA	bta-mir-3596	MI0015952

In the present study several types of endogenous small interfering RNAs were identified along with the miRNAs. Among them, 27 distinct rasiRNAs represented the frequent class of small RNAs. Thirteen RNAs were classified as small antisense RNAs, while 17 small RNAs were tiny non-coding RNAs. The small RNA cloning and profiling from another study revealed less representation of that group of rasiRNAs compared to the miRNAs (Aravin and Tuschl 2005). The properties identified for rasiRNAs here support the notion that they are presumably emerged from dsRNA produced by annealing of sense and antisense transcripts that contain repeat sequences related to transposable elements (Aravin et al. 2003). These rasiRNAs are known to repress the repeat sequences at the transcriptional or post-transcriptional level and maintain a centromeric heterochromatic structure (Lippman and Martienssen 2004). Identity and properties of new types of small RNAs in the present study showed the presence of diverse modes of small RNA-mediated gene regulation in bovine ovary, as reported in other species (Ambros et al. 2003b). Therefore, identification and characterization of other small RNAs and their expression patterns are important for elucidating detailed gene regulatory networks involved in the ovary. So, all these endogenous small interfering RNAs need to be further characterized to elucidate their cellular functions.

3.3.2 Expression of miRNAs in diverse tissue types

Expression analysis of 44 miRNAs in different ovarian cells and tissues types has enabled us to determine their site of action in terms of tissue specific abundance as well as functional regulation (Table 2). Nearly all of these miRNAs in at least one part of the entire ovary and other somatic tissues have been detected. In the present study, some miRNAs appear to be extremely tissue specific. For example; bomir-C0533 and bomir-F0522 were found to be exclusively expressed in ovarian tissues suggesting their potential role in ovary-specific miRNA-dependent regulatory processes. Five miRNAs

(miR-29a, miR-125b, bomir-409, bomir-503 and bomir-F0244) were found to be highly abundant in the cumulus cells and four (bomir-652, bomir-H0222, bomir-C1931 and bomir-A2143) in corpus luteum. These cumulus enriched miRNAs in the present study may represent those miRNAs with potential association with the regulation of cumulus secreted factors, which are important for cumulus-oocyte communication and subsequent oocyte development. Similar study in mouse showed hormonal regulation of miRNAs expression in preovulatory mural granulosa cell (Fiedler et al. 2008).

The expression of various ovary related genes was reported to be differentially regulated between ovaries from fetal, new born and adult animals (Baillet et al. 2008, Herrera et al. 2005, Olesen et al. 2007, Vaskivuo et al. 2001). Furthermore, alteration in expression of small RNAs has been addressed at different stages of mouse ovary (Ro et al. 2007b). Similarly, in the present study, differential expression of mir-29a, bomir-140, mir-199, mir-378, bomir-F0132 and bomir-F2422 in the ovarian cortical portion between fetal and adult cows. This may indicate their possible involvement in regulating follicular development in the adult cyclic ovarian function. This notion was further supported by higher detection of miR-29a in different follicular cells (theca, cumulus-granulosa, and oocyte) of adult ovary by in situ hybridization (Figure 5) and higher expression in cumulus cells by RT-PCR but no detection in the fetal ovary. The expression of miRNAs in ovarian cells is reported to be regulated by FSH and LH / hCG (Fiedler et al. 2008, Yao et al. 2009) which functions in the cyclic ovary but not in fetal ovary (Abel et al. 2000). Moreover, most of the targets predicted for this miRNA (Figure 6) are known to be involved in various cyclic adult ovarian functions.

Noticeable expression level of miR-29a was found in different phases of corpus luteum (CL) development. According to RT-PCR and in situ hybridization results, expression of miR-29a was detected in the early phase CL but not in mid phase (matured) CL. These two phases of CL development are known to vary in multiple aspects of luteal physiology, angiogenesis and sensitivity to luteolytic actions, which are accompanied by differential expression of multiple genes (Copelin et al. 1988, Goravanahally et al. 2009, Watts and Fuquay 1985, Wiltbank et al. 1995). Bovine corpus luteum is reported to be resistant to luteolysis by exogenous PGF2α in early stage of CL (before Day 5) due to differential expression of genes associated with the PGF2α receptor (Goravanahally et al. 2009). Considering these facts and restricted expression of miR-

29a in early phase of CL in the present study, it is possible to suggest that miR-29a is involved in gene regulatory action during early phase of CL. All in all, present results on miR-29a may elucidate the potential involvement of this regulatory miRNA in growth and differentiation of cumulus cells, endocrine regulation of theca cells and early luteinisation in cyclic ovary.

Cloning, determining potential secondary structures and expression analysis of all new miRNAs in multiple tissues indicate their tissue specific existence and regulation of gene expression. Only 7.8% of the new miRNAs could not be detected by the RT-PCR procedure in various reproductive tissues. This may be due to the fact that these transcripts were cloned at lower frequency (only once) showing their lower abundance and subsequent difficulty to detect them (Gu et al. 2007). In general, the expression profiling analysis in the present study revealed that cloned miRNAs were either ubiquitously expressed in multiple tissues or preferentially expressed in a few tissues including the intra-ovarian cells and tissues.

3.3.3 Features of predicted target genes

Multiple genes contributing to mammalian folliculogenesis have been identified in mouse knockout study (Matzuk and Lamb 2002). Primarily, oocyte-specific transcriptional regulators like Figla, Nobox, Sohlh1 and Lhx8, oocyte-secreted factors such as Gdf9 and Bmp15, as well as genes expressed in the granulosa and cumulus cells (FSHR and PTX3) were found to initiate and control follicular growth (Dumesic and Abbott 2008, Matzuk and Lamb 2002). Among the key intra-ovarian factors, the transforming growth factor b (TGFb) family members, of which bone morphogenetic protein-4 have been identified as regulators of primordial germ cell generation (van den Hurk and Zhao 2005).

In response to FSH, the granulosa cell–derived factors such as kit ligand, transforming growth factor alfa (TGF-α) and epidermal growth factor (EGF) activate the resting follicular growth. The interactions between ovarian germ and somatic cells (granulosa cells and the oocytes) and expression of several intra-ovarian autocrine/paracrine regulators (FSH, estrogen and androgen receptors) are the major contributing factors in the ovary leading to preantral and antral follicles development (Filicori et al. 2003).

During follicle growth, IGF system works in synergy with gonadotrophins (follicle-stimulating hormone and luteinising hormone) to regulate proliferation and differentiation of granulosa and theca cells (Campbell et al. 1998, Gutierrez et al. 1997). In addition, it has been shown that the processes of follicular dynamics (Recruitment, selection, dominance and ovulation) are associated with temporal changes of peripheral gonadotropins concentration and IGF system (Austin et al. 2001, Fortune et al. 2001, Ginther et al. 2002, Ireland et al. 2000, Mihm and Evans 2008, Quintal-Franco et al. 1999, Schams et al. 1999). All the above-mentioned genes are represented in the predicted and analyzed targets. Altogether 115 genes were among potential target genes of identified miRNA. These target genes are already experimentally validated for potential ovary related functions in different mammalian species. Interestingly, several well-known target genes including IRS1, IGFBP3, DNMT3A, HOXA9, TNF, etc. which are identified by new screening approach, are already validated in wet lab experiments and reported as targets of multiple miRNAs (miR-145, miR-125b, miR-126 and miR-29) (Fabbri et al. 2007, Shen et al. 2008, Shi et al. 2007a, Shi et al. 2007b, Tili et al. 2007). Accordingly these studies have elucidated the potential involvement of these miRNAs in broad class of functions related to apoptosis, differentiation signal, cell differentiation, tumorogenesis, DNA methylation and innate immune responses.

Gonadotropins, intra-ovarian mediators and their receptors which are identified as target genes for identified miRNAs might mediate important intracellular actions necessary for normal follicular development and other ovarian functions. Alterations in the expression of these mediators by miRNAs will result in various ovarian dysfunctions causing infertility, polycystic ovary syndrome and tumorigenesis. Recent evidences also support the hypothesis, where at least six of 11 top ranked ovarian miRNAs were found to be related to cancer or tumors in the ovary. For example, miR-199a, miR-145, miR-125b and let-7 clusters were found to be the most differentially regulated miRNAs in human ovarian cancer (Iorio et al. 2007, Yang et al. 2008a). While miR-145 (Iorio et al. 2005) and mir-199a (Murakami et al. 2006) have recently been shown to be down-modulated in the tumor cells, the miR-222 is reported to be down-regulated in ovarian epithelial carcinomas (Iorio et al. 2007). Furthermore, higher expression of miR-18a and lower expression of let-7b and miR-199a were shown to be correlated with serous ovarian carcinoma (Nam et al. 2008). In another study, miR17-5p and let-7b were found to be involved in the regulation of development and function of the ovarian corpus

luteum specially angiogenesis of corpus luteum (Otsuka et al. 2008). Interestingly, nearly all of these 11 selected miRNAs (Figure 6) in the present study are reported to be differentially regulated in endometrium of women with and without endometriosis (Pan et al. 2007). Altogether present findings and other evidences support the relevance of these 11 miRNAs to ovarian physiology and may be the most important regulatory miRNA group in ovary, as their predicted and analyzed target genes are involved in a broad range of signaling cascades and pathways of the ovarian function.

4 Part II: miRNAs in the placentas derived from in vivo, in vitro and somatic cell nuclear transfer pregnancies

Somatic Cell Nuclear Transfer is a break-through technology but it is hindered by very low rates of live birth due to high incidence of placental abnormalities. The major source of these abnormalities is thought to be due to genetic and epigenetic modifications arise from improper reprogramming of the donor cell after nuclear transfer, which leads to post-implantation lethality to embryo with ultimate placental defects. miRNAs have been evidenced as an important modifier that regulates gene expression. However, the role of miRNAs in placenta derived from SCNT is unknown. The study aimed to elucidate the difference in expression profile of miRNAs in the placenta (at day 50) derived from artificial insemination (AI), in vitro production (IVP), SCNT pregnancy and in the donor cells (fibroblast from the same bull used to generate AI & IVP derived pregnancies) by quantifying 377 miRNAs. For this purpose, in vitro fertilization and SCNT was performed using semen and fibroblast, respectively from the same bull to generate embryo in vitro. Then the both group of embryos were cultured in vitro up to day-7. Some blastocysts were fixed and freezed for in situ hybridization and expression profiling, respectively. The rest of the embryos were transferred to the synchronized recipients to maintain pregnancy. On the other hand artificial insemination was performed to generate control embryos which were recovered at day 7 by flushing and the rest of the heifers were used to maintain pregnancy up to slaughter stage to recover control placentas. Animals were slaughtered gradually at day 16, day 50 and day 225 of pregnancy from all three groups (AI, IVP and NT) to recover elongated embryos, placenta and placentomes, respectively. Total RNAs from the frozen placenta (20 mg) of different sources and fibroblast cells were isolated and real time qRT PCR of miRNAs was performed using 384-well miRNAs arrays comprised of 377 individual miRNAs. Furthermore, cell specific expression of some selected miRNAs in the expanded blastocysts of different sources by whole mount in situ hybridization was performed. Temporal expression of selected miRNAs in fibroblast, blastocyst, expanded blastocyst, day-16 elongated embryo, day-50 placenta and day 225 placenta (AI, IVP and SCNT pregnancies) by real time qRT-PCR. In addition, global DNA methylation in the day 16 elongated embryos and day 50 placenta of different sources of pregnancies were performed. The detail materials and method followed and the obtained findings are presented in the following sections.

4.1 Materials and methods

Following sections describe the detail materials which were utilized and brief methods followed in this experiment to study the pattern of miRNA expression and their regulation in the placenta and embryos from the pregnancy derived by nuclear transfer and in vitro produced embryo comapared to that from artificial insemination pregnancy. List of some materials which have also been used in the part I and mentioned in the related previous section are not listed here.

4.1.1 Materials and tools

4.1.1.1 List of chemicals, kits, biological and other materials

List of Chemicals	Manufacturer/Supplier
2-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
5 α DH *E. coli* competent cells	Stratagene, Amsterdam, The Neatherlands
6-dimethylaminopyridine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Digoxigenin-AP	Roche Diagnostics GmbH, Mannheim, Germany
BioThermD™ Taq DNA Pol	Ares Bioscience GmbH, Cologne, Germany
Bovine serum ablbumin (BSA)	Promega, Mannheim, Germany
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe, Germany
DNA/RNA/Protein purification kit	Norgen Biotek corporation, Thorold, Canada
dNTPs	Roth, Karlsruhe, Germany
DMEM-F12 Ham	Sigma-Aldrich Chemie GmbH, Munich, Germany
Dulbecco's phos buff saline D-PBS	Sigma-Aldrich Chemie GmbH, Munich, Germany
Epinephrine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Essential amino acids (BME)	Gibco BRL, life technologies, Karlsruhe
EZ DNA Methylation direct Kit	Zymo Research, Orange, CA, USA
Fast Red Substrate System	Dako Deutschland GmbH, Hamburg, Germany
Fast Red Substrate System	Dako Deutschland GmbH, Hamburg, Germany
Fertal Bovine Serum (FBS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Fetal calf serum (FCS)	Gibco, Karlsruhe, Germany
FSH	Sigma-Aldrich Chemie GmbH, Munich, Germany

GenElute™ plasmid Miniprep kit	Sigma-Aldrich Chemie GmbH, Munich, Germany
Goat anti-EIF2C2 antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Goat anti-GAPDH antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Heparin sodium salt	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hepes	Sigma-Aldrich Chemie GmbH, Munich, Germany
Penicillin G sodium salt	Sigma-Aldrich Chemie GmbH, Munich, Germany
Streptomycin sulfate salt	Sigma-Aldrich Chemie GmbH, Munich, Germany
Hoechst 33342	Invitrogen, Carlsbad, CA, USA
Ionomycin	Sigma-Aldrich Chemie GmbH, Munich, Germany
iTaq SYBR Green Supermix	Bio-Rad laboratories, Munich, Germany
ROX Reference Dye	Roche Diagnostics GmbH, Mannheim, Germany
L-Glutamine	Sigma-Aldrich Chemie GmbH, Munich, Germany
LNA™ PCR primer set, UniRT	Exiqon, Vedbaek, Denmark
Medium 199	Sigma-Aldrich Chemie GmbH, Munich, Germany
Methylamp Global DNA Meth kit	Epigentek, Brooklyn, NY, USA
Mineral oil	Sigma-Aldrich Chemie GmbH, Munich, Germany
miRCURY™ LNA Detection probe	Exiqon, Vedbaek, Denmark
miRNeasy mini kit	QIAGEN GmbH, Hilden, Germany
Mouse anti-goat IgG-HRP	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Nitrocellulose membrane	Protran®, Schleicher & Schuell Bioscience
Penicillin	Sigma-Aldrich Chemie GmbH, Munich, Germany
pGEM-T vector and related reagents	Promega, Mannheim, Germany
pGEM-T vector and related reagents	Promega, Mannheim, Germany
Phenol red solution (5% in D-PBS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
QIAquick Gel Extraction Kit	Qiagen, Hiden, Germany
QIAquick PCR purification kit	QIAGEN GmbH, Hilden, Germany
Random primer	Promega, Mannheim, Germany
Ribo-nuclease inhibitor (RNasin)	Promega, Mannheim, Germany
Rigid thinwall 96X0.2ml microplate	STARLAB GmbH (Ahrensburg)
RNA later	Ambion Inc, Austin, TX, USA
RT^2 miRNA first strand kit	SABioscienecs, Frederick, MD, USA
RT^2 qPCR-Grade miRNA isolation	SABioscienecs, Frederick, MD, USA
RT^2 Sybr green master mix	SABioscienecs, Frederick, MD, USA
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH, Munich, Germany

Streptomycin	Sigma-Aldrich Chemie GmbH, Munich, Germany
Streptomycin sulfate	Sigma-Aldrich Chemie GmbH, Munich, Germany
SuperScript^{TM} II cDNA syn kit	Invitrogen, Carlsbad, CA, USA
SYBR Green master mix, Uni-RT	Exiqon, Vedbaek, Denmark
Trypsin-EDTA	Sigma-Aldrich Chemie GmbH, Munich, Germany
Universal cDNA synthesis kit	Exiqon, Vedbaek, Denmark

4.1.1.2 List of equipments

ABI 7900 HT real time PCR system	Applied Biosystems, CA, USA
ABI PRISM® 7000 sequence detection sys	Applied Biosystems, CA, USA
Agilent 2100 bioanalyzer	Agilent Technologies , CA, USA
Biomek® NX^{P}, Multichannel laboratory automation workstation	Beckman Coulter, Krefeld, Germany
Centrifuge	Hermel, Wehing
CEQ 8000 genetic analysis apparatus	Beckman Coulter, Brea, CA, USA
CO2-incubator (MCO-17AI)	Sanyo, Japan
Confocal laser scanning microscope-510	Carl Zeiss, Germany
ECL plus western blotting detection system	GE Healthcare, Freiburg, Germany
Electrofusion machine, CFA 400	Kruess, Hamburg, Germany
Electrophoresis unit (for agarose gels)	BioRad, Munich, Germany
Embryo flushing catheter	CH15, Wörrlein, Ansbach, Germany
Embryo transfer syringe and sheath	IMV, L'Aigle, France
Fluorescence microscope (DM-IRB)	Leica, Bensheim, Germany
Four well dishes	Thermo Fisher Sc, Roskilde, Denmark
Four-well culture dishes	Nunc, Roskilde, Denmark
Inverted fluorescence microscope DM IRB	Leica, Bensheim, Germany
Memmert CO2 incubator	Fisher Scientific, Leicestershire, UK
Millipore apparatus	Millipore Corporation, USA
My Cycler Thermal cycler	Bio-RadLaboratories, CA, USA
NanoDrop 8000 spectrophotometer	NanoDrop, Wilmington, Delaware, USA
PTC-100 thermal cyclers	BioRad, Munich, Germany
SHKE6000-8CE refrigerated Shaker	Thermoscinentific, IWA, USA
Stereomicroscope SMZ 645	Nikon, Japan

ThermoMax microplate reader	Molecular Devices, CA, USA
Ultra low freezer (-85 °C)	Labotect GmbH, Gottingen, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

4.1.1.3 List of softwares

BDGP Search tools	http://www.fruitfly.org/seq_tools/promoter.html
BLAST cow sequences	http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/
ENSEMBL genome browser	http://www.ensembl.org/index.html
Entrez Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Gene Ontology	http://www.geneontology.org
Genomatix software suite 2.0	http://www.genomatix.de
MethPrimer	http://www.urogene.org
Methyl Primer Express® v1.0	Applied biosystem, Foster City, CA
miRBase_12.0	http://microrna.sanger.ac.uk/sequences/
PCR array data analysis portal	http://www.sabiosciences.com/pcr/arrayanalysis.php
Primer Express 2.0	Applied Biosystems, Foster City, CA, USA
Promoter 2.0 Prediction Server	http://www.cbs.dtu.dk
SAS (version 8.02)	SAS Institute Inc., NC, USA

4.1.1.4 Reagents and media preparation

All solutions used in this investigation were prepared with deionised and demineralised (Millipore) and where necessary the pH was adjusted with Sodium hydroxide or hydrochloric acid. In addition, the solutions or buffers were subsequently filtered through 0.2 µ filter and autoclaved at 120°C for 20 minutes where it is necessary. Prepared solutions were aliquot in to small volume and stores at desired temperature according to the recommendation of suppliers.

Name of the medium/buffer		Constituents	Amount/ volume
10X PBS	:	NaCl	8.77 g
		Na_2HPO_4	1.50g
		NaH_2PO_4	2.04g

		Water upto	1000.0 ml
1X PBS	:	10X PBS	100.0 ml
		DEPC upto	900.0 ml
10x TBE buffer	:	Tris base	218.0 g
		Boric Acid	105.0 g
		EDTA solution (pH.8.0)	80.0 ml
		Water upto	2000.0 ml
Capacitation medium (50 ml)	:	Sodium chloride	0.2900 g
		Potassium chloride	0.0115 g
		Sodium hydrogen carbonate	0.1050 g
		Sodium dihydrogen phosphate	0.0017 g
		Hepes	0.1190 g
		Magnesium chloride hexahydrate	0.0155 g
		Calcium chloride dihydrate	0.0145 g
		Sodium lactate solution	60% 184 µl
		Phenol red solution (5% in D-PBS)	100.0 µl
		Water upto	50.0 ml
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	100.0 µl
		Sodium hydrogen carbonate	0.080 g
Epinephrine solution	:	Sodium hydrogen sulfite	0.04 g
		Sodium lactate solution (60%)	100.0 µl
		Epinephrine	0.00183 g
Fertilization medium	:	Sodium chloride	0.3300 g
		Potassium chloride	0.0117 g
		Sodium hydrogen carbonate	0.1050 g

		Sodium dihydrogen phosphate	0.0021 g
		Penicillin	0.0032 g
		Magnesium chloride hexahydrate	0.0050 g
		Calcium chloride dihydrate	0.0150 g
		Sodium lactate solution (60%)	93.0 µl
		Phenol red solution (5% in D-PBS)	100.0 µl
		Water upto	50.0 ml
Modified parker medium	:	HEPES	0.140 g
		Sodium pyruvate	0.025 g
		L-Glutamin	0.010 g
		Gentamicin	500.0 µl
		Medium 199	99.0 ml
		Hemi calcium lactate	0.06 g
		Water upto	110.0 ml
Ammonium Persulfate (APS)10%:		APS	0.12 g
		H_2O	1.2 ml
40% acrylamide	:	Acrylamide	76.0 g
		bis-acrylamide	4.0 g
		Water to final volume of	200.0 ml
SDS (10%)	:	Sodium dodecil sulphate	5.0 g
		Water	100.0 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)	480.0 µl
		ddH_2O upto	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 upto	800.0 ml
DEPC-treated water (1000 ml)	:	DEPC	1.0 ml

		Water upto	1000.0 ml
Lysis buffer (100 µl)	:	Igepal (0.8%)	0.8 µl
		RNasin	5.0 µl
		DTT	5.0 µl
		Water upto	100.0 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 upto	1000.0 ml
TE (1x) buffer	:	Tris (1 M)	10.0 ml
		EDTA (0.5 M)	2.0 ml
X-gal	:	ddH_2O upto	1000.0 ml
		X-gal	50.0 mg
PBS + PVA (50 ml)	:	Polyvinyl alcohol (PVA)	300.0 mg
		PBS upto	50.0 ml
Permeabilizing solution (10 ml)	:	Triton X-100	5.0 µl
		Glycine + PBS added	10.0 ml
Physiological saline solution	:	Sodium chloride	9.0 g
		Water upto	1000.0 ml
Agarose loading buffer	:	Bromophenol blue	0.0625 g
		Xylencyanol	0.0625 g
		Glycerol	7.5 ml
		ddH_2O upto	25.0 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 upto	400.0 µl
IPTG solution	:	IPTG	1.2 g
		ddH_2O upto	10.0 ml
3M Sodium Acetate, pH 5.2	:	Sodium Acetate	123.1 g
		ddH_2O upto	500.0 ml
1M EDTA, pH 8.0	:	EDTA	37.3 g
		ddH_2O upto	1000.0 ml

0.2% Triton-X100	:	Triton	2.0 ml
		10x PBS upto	1000.0ml
0.3% BSA in PBS	:	BSA	3.0 g
		10x PBS upto	1000.0 ml
3% BSA in PBS	:	BSA	30.0 g
		10x PBS upto	1000.0 ml
1x PBS-Tween (PBST)	:	1x PBS	999.50 ml
		Tween®20	0.50 ml
SSC (20x)	:	NaCl	87.65 g
		Sodium citrate	44.1 g
		Water upto	500.0 ml
4% paraformaldehyde (pH7.3)	:	Paraformaldehyde	4.0 g
		1X PBS	100.0 ml
0.5M Sucrose/PBS (30% sucrose):		Sucrose	85.57 gm
		1X PBS upto	500.0 ml
Acetylation solution	:	triethanolamine	2.33 ml
		acetic anhydride	500.0 µl
		DEPC water upto	200.0 ml
Yeast tRNA (10 mg/ml)	:	Yeast tRNA	25.0 mg
		DEPC-treated H_2O	2.50 ml
Hybridization solution	:	Formamide -65%	32.25 ml
		20X SSC -5X	12.5 ml
		Tn-20- 0.1%	50.0 µl
		1M citric acid	460.0 µl
		Heparin 50 µg/ml	2.5 mg
		10mg/ml tRNA-500µg/ml	2.5 ml
		DEPC water upto	50.0 ml
Hybridization wash solution	:	Formamide -65%	65.0 ml
		20X SSC -5X	25.0 ml
		Tn-20- 0.1%	100.0 µl
		1M citric acid	1.2 µl
		DEPC water upto	100.0 ml
50% Formamide/SSC	:	Formamide	1000.0 ml
		1X SSC	1000.0 ml

50% formamide/Tn-20/SSC	:	Formamide, 50%	500.0 ml
		Tween-20, 0.1%	1.0 ml
		1X SSC	499.0 ml
5X SSC	:	20X SSC	250.0 ml
		DEPC water	750.0 ml
2X SSC	:	20X SSC	100.0 ml
		DEPC water	900.0 ml
1X SSC	:	20X SSC	100.0 ml
		DEPC water	1900.0 ml
0.2X SSC	:	20X SSC	10.0 ml
		DEPC water	990.0 ml
1X PBST	:	1X PBS	999.0 ml
		Tween-20	1.0 ml
Blocking Solution	:	FCS	2.0 ml
		B1 solution	18.0 ml
B1 solution	:	1 M Tris pH 7.5	100.0 ml
		5 M NaCL	30.0 ml
		dd H2O up to	1000.0 ml
Stop solution	:	EDTA 1mM	14.61 mg
		PBS pH 5.5 upto	50.0 ml
10N NaOH	:	NaOH	40 gm
		dd H_2O upto	100.0 ml

4.1.2 Methods

This section describes the detail material used and methods followed to carried out the present study. All experimental animals were heifers of the same breed (Simmental), age (15-20 months) and body weight (380-500 kg) and were housed within one farm under identical conditions. All experiments have been carried out according to the existing animal protection law of Germany. Semen for artificial insemination, in vitro insemination and donor cells for nuclear transfer were originated from the same sire. The detail materials and methods are outlined below.

4.1.2.1 In vitro production (IVP) and processing of blastocysts

Bovine ovaries were collected from local abattoir and transported to the laboratory in a thermosflask (35°C) containing physiological NaCl solution (0.9% NaCl supplemented with 50 µl/100 ml Streptocombin (Albrecht GmbH, Germany). Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm-diameter follicles using a 10-ml syringe loaded with an 18- gauge needle upon washing the ovaries once with 70% ethanol and twice with physiological saline. Quality of COCs has been assessed under stereomicroscope and those with multiple cumulus layers including evenly granulated cytoplasm were selected, washed three times in pre-warmed maturation (MPM supplemented with 12 % heat-inactivated estrous cow serum, 10 µg/ml FSH, 0.73 mg/ml of sodium bicarbonate, 50 µg/ml of gentamicin, 0.23 mg/ml of sodium pyruvate, 1.27 mg/ml HEPES and 0.55 mg/ml calcium lactate). Selected COCs were subsequently transferred in groups of 50 to each well of four-well dishes (Nunc, Roskilde, Denmark) containing 400 µ1 maturation medium without being covered with mineral oil. Maturation was carried out at 39°C in a humidified atmosphere with 5% CO_2 for 22 h. Sperm cells were separated by ‘‘swim up’’ technique for in vitro fertilization (IVF) according to (Parrish et al. 1988), where 50 matured oocytes in a well were co-cultured for 18 hours with 1 X 10^6 spermatozoa/ml at the same condition followed for maturation step. Following IVF, presumed zygotes were gently vortexed to separate them from the surrounding cumulus cells and attached or dead spermatozoa. Cumulus free zygotes were washed three times in CR-1aa culture medium (Rosenkrans & First 1994) supplemented with 10% oestrus cow serum, 10 µl/ml basal medium Eagle (BME-essential amino acids) and 10 µl/ml minimum essential medium (MEM-non essential amino acids) and were transferred into the well containing 400 µl culture medium covered with mineral oil. Embryos were cultured in vitro for 8 days. At day 7 (blastocyst) and day 8 (fully expanded blastocyst) of culture, 5-8 embryos (washed in PBS) were freezed in liquid nitrogen in triplicates with 2 µl of lysis buffer [(5mM DTT, Promega, P1171), 0.8% Igepal (Sigma, I 3021), 1U/ µl RNasin (Promega, N 2511)]. A group of 20 embryos from each developmental stage were fixed in 4% parafomaldehyde overnight at 4°C for whole mount in situ hybridization. In addition, in vitro derived day-7 blastocysts (n=20) were transferred singly to the recipients by nonsurgical standard procedures to generate day-16, day-50 and day-225 pregnancies.

4.1.2.2 In vivo embryo production and establishing control (artificial insemination) pregnancy

To collect in vivo derived blastocysts, six Simmental heifers were synchronized by intra muscular injection of cloprostenol ($PGF_2\alpha$, Estrumate; Essex Tierarznei, Munich, Germany) twice within 11 days and subsequently superovulation was performed by injection of FSH (Stimufol, Ulg FMV, Belgium) starting at day 11 after onset of estrus. Frozen–thawed semen was used to inseminate all heifers. The blastocysts were flushed out with 500 ml D-PBS at day 7.5 post inseminations by embryo flushing catheter (CH15, Wörrlein, Ansbach, Germany) fixed in the uterine horn. All flushed blastocysts were assessed under stereo microscope for their quality and stages. Only morphologically good-quality early blastocyst and expended blastocysts (5 embryos per pool in triplicate for both stages) were snap frozen for the isolation of RNA as mentioned before. Another group of 20 expanded blastocysts were fixed overnight in 4% paraformaldehyde at 4°C for whole mount in situ hybridization. Heifers (n=15) synchronized with a single dose of $PGF_2\alpha$ followed by estrus check were artificially inseminated (AI) using frozen semen of the same sire (except the sire used to generate day 225 pregnancies) after 10 hours of standing estrus and pregnancy were maintained to provide control placenta tissue at day-50 (n=3) and day-225 (n=4).

4.1.2.3 Donor cell preparation and nuclear transfer

Preparation of donor cells, nuclear transfer and culture of reconstructed embryos has been performed according to the protocol described elsewhere with some modification (Hölker et al. 2005). Briefly, a primary cell line was established from ear skin biopsy of the bull used also production of in vivo and in vitro derived embryos and placentas. The biopsy was minced, washed, dispersed in T25 cell culture flask and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Sigma, Deisenhofen, Germany) containing 10% fetal calf serum (FCS) (Gibco, Karlsruhe, Germany) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Outgrowing cells were trypsinized (0.05% Trypsin/0.53 mM EDTA; Gibco) and replated to allow proliferation to 90% confluence. The harvested cells were reconstituted at a concentration of 1x 10^6 cells/ml and then either frozen in 10%

dimethyl sulfoxide (Sigma, Deisenhofen, Germany) in DMEM until later use or returned to culture. The fibroblasts used for nuclear transfer were from passages 4-5 and were induced to enter a period of quiescence (presumptive G0) by serum starvation for 7 days (0.5% fetal calf serum).

Oocytes were placed in hepes-buffered TCM-199 (25mM Hepes, 5mM $NaHCO_3$) medium containing 1 µg/ml Hoechst 33342 (Sigma, B-2261) and 7.5 µg/ml cytochalasin B (Sigma, C-6762) for ~ 10 min prior to enucleation. Metaphase II oocytes were enucleated by removal of the polar body and the attached cytoplasm with the metaphase plate utilizing a 25 µm beveled glass pipette under the microscope. The absence of the metaphase plate or enucleation was confirmed by a brief exposure of the karyoplast to ultraviolet light. Successful enucleation was also indicated by the typical blue fluorescence of Hoechst 33342 within the pipette. Cytoplasts derived from enucleated oocytes were maintained in TCM-air for up to 2 h and nuclear transfer was conducted in the same medium as enucleation, but without Hoechst 33342 stain.. Immediately before donor cell transfer, a suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in TCM-air and remained in this medium until injection. A single cell was sucked into a 30-µm (outer diameter) bevelled glass pipette and carefully transferred into the perivitelline space of the recipient oocyte in close contact with the oocyte membrane. Reconstructed embryos were electrically fused at 26 h after onset of maturation. Fusion of donor cell and oocyte was induced with a single electrical pulse of 25 V DC for 45 µsec between two electrodes with a spacing of 150 µm by electrofusion machine (CFA 400; Kruess, Hamburg, Germany). Fusion was assessed approximately 45 min later by light microscopy. At 28-29 hours after onset of maturation, the reconstructed embryos were chemically activated by incubation in 5 µM ionomycin (Sigma) in hepes-buffered TCM 199 for 4 min followed by a 3.5 hours incubation in 2 mM 6-dimethylaminopyridine (6-DMAP; Sigma) in CR-1aa culture medium at 39°C.

In vitro culture of activated reconstructed complexes has been performed as described for the production of in vitro blastocyst. Developmental rates were assessed periodically up to day 7.5 of culture. Produced embryos were used for three purposes in this study. First, 5 triplets of both early and expanded blastocysts in were freezed as described before. In addition, another group of embryos were fixed overnight in 4%

paraformaldehyde for whole mount in situ hybridization. Finally, rest of the blastocysts were transferred singly to the synchronous recipients (n=30) by nonsurgical standard procedures as described below.

4.1.2.4 Recipient preparation and embryo transfer

Estrous synchronization and transfer of embryos to the recipients has been carried out according to the previous report (El-Sayed et al. 2006). Briefly, estrous cycles in normal cycling heifers were synchronized by intra-muscular injection of prostaglandin F2a (2 ml Estrumate; Fa. Essex, Germany) followed by a second administration 11 days later. Standing estrus was monitored and embryos were matched with synchronous recipients of no more than ~12 h asynchrony. Single NT (n=30) and IVP (n = 20) embryos that were of good or excellent quality (Grades 1 or 2) were transferred into the uterine horn ipsilateral to the corpus luteum of recipients, respectively.

4.1.2.5 Pregnancy monitoring and retrieval of experimental material

All recipients were monitored for coming back to estrus at day 21. Heifers that returned to estrus at day 21 were considered as non-pregnant. Pregnancy diagnosis was performed at gestation days 28 and 42 by transrectal ultrasonography (Pie Medical, 5 MHz) and by rectal palpation at day 42 and 56. A viable pregnancy was defined as the presence of fetus with a detectable heartbeat. Beginning on Day 120, recipients underwent repeated transabdominal ultrasonography (Pie Medical, 3,5 MHz) every 2–3 wk until day 220. Recipients of IVP embryos, NT embryos and artificial insemination were slaughtered at day 16 (IVP-N=5, AI-N=5, NT-N=5), day 50 (IVP-N=3, AI-N=3, NT-N=3) and day 225 (IVP-N=4, AI-N=4, NT-N=4) of pregnancy. Assessment of the morphometric quality or any abnormalities in the embryos, fetus and placenta were noted accordingly. On day 16, the entire conceptus was weighed together with measurement of length; on Day 50 the fetus and placenta were weighed separately; on Day 225 the fetus was dissected and the major organs (liver, heart) were weighed and the weight of the fetal membranes and number of cotyledons was recorded.

Morphologically similar elongated embryos at day 16, chorioallantois with early cotyledon (placentomes) at day 50 and placentomes at day 225 of pregnancy were

collected, washed twice in PBS, cut into reasonable pieces if required and stored in RNA later (Ambion Inc, Austin, TX, USA) for later use. Three samples of apparently same morphological quality from each group were used for the present study.

4.1.2.6 Extraction and purification of small RNAs from placenta

Total RNAs from the three individual frozen placentas (15 mg) of from each group of pregnancy (IVP, NT and AI) and fibroblast cells ($4x10^6$) were isolated using miRNeasy mini kit (QIAGEN GmbH, Hilden, Germany). Large (>200 nt) and small RNAs (<200 nt)) were separated using special silica membrane spin column and chemicals of RT^2 qPCR-Grade miRNA isolation kit (SABioscienecs, Frederick, MD, USA) according to manufacturer's instructions. For every case, the quality and the concentration of the small RNAs and large RNAs were assessed by NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, Delaware, USA). Isolated small RNAs were used for the study of expression profiling of 377 individual miRNAs and large RNAs originated from the same sample were used for analysis of regulatory miRNAs processing transcripts in different groups of placenta.

4.1.2.7 Genomic DNA, total RNA and protein extraction from different stages of placenta and embryos

To study the global methylation pattern and quantification of selected transcript at mRNA and protein level, genomic DNA, total RNAs and proteins were isolated from the same sample. Whole individual elongated embryo at day 16, placenta at day 50 (15 mg) and placentomes at day 225 (15 mg) of pregnancy from IVP, NT and AI (at least three of each) were used for isolation using DNA/RNA/Protein purification kit (Norgen Biotek corporation, Thorold, Canada) according to methods recommended by the manufacturer. In addition, 5 early blastocysts, 5 expanded blastocysts (from each IVP, NT and AI method), donor fibroblast cells with serum starvation and without starvation (each in triplicate) were also used to isolate total RNAs using the same procedures. For every cases the quality and the concentration of the nucleic acid was assessed by NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, Delaware, USA) and subsequent analysis was performed using Agilent, 2100 Bioanalyzer (AgilentTechnologies, Santa Clara, CA). Protein quantity was assessed by NanoDrop

using the absorbance at 280 nm. All these nucleic acids and protein were stored at -80°C in aliquots for the downstream experiments.

4.1.2.8 Large scale expression profiling of miRNAs by real-time quantitative PCR

Total of 166 ng small RNAs from 3 placentas derived from every group of pregnancy at day 50 (IVP, NT and AI) and donor cells (in triplicate) were synthesized individually into first strand cDNAs using RT^2 miRNA first strand kit (SABiosciences). Real time qPCR of miRNAs was performed using 384-well miRNAs primed PCR plate (SABiosciences) comprised of 377 individual miRNAs (most of them are conserved in human, mouse and bovine), 4 endogenous controls (U6, Snord44, Snord47 and Snord48), 2 reverse transcription controls and 2 positive PCR controls according to the protocols provided by the manufacturer. The assays were performed in ABI 7900 HT real time PCR system (Applied Biosystems, Foster City, CA, USA) with sybr green technology (SABiosciences). Synthesized cDNAs were diluted 10 times, mixed with 2 ml of 2 X RT^2 Sybr green PCR master mixes (SABiosciences) and 1.9 ml of ddH_2O. Mixed cocktail (10 µl per well of 384 well plate) was added and thermal cycling was performed as 95°C for 10 min, 40x of (95°C for 15 sec, 60°C for 40 sec and 72°C for 30 sec). Multichannel laboratory automation workstation was used pipette the mix into 384 well plates precisely (Biomek® NX^P, Beckman Coulter, Krefeld, Germany). Each individual sample was applied to one 384 well PCR plate and quality of the assay was assessed by the result of control wells and melting curve analysis as recommended. Instrument was set to automatic baseline but threshold value was adjusted manually to 0.045 (above the background signal but within the lower half to one-third of the linear phase of the amplification plot) for all assays performed in the study. Data were analysed by $\Delta\Delta C_t$ method and normalization was performed by geometric mean of four endogenous controls through SAbiosciences's PCR array data analysis on-line web-based analysis portal, which is provided with t test (http://www.sabiosciences.com/pcr/arrayanalysis.php). Expression levels were compared in multiple ways for different group of placenta to find out fold regulation and a fold regulation 2 or more with the value of P less than 0.05 were considered as significant different expression.

4.1.2.9 Whole mount blastocyst in situ hybridization of miRNAs

Whole mount in situ hybridization of miRNAs in the in vitro produced expanded blastocysts was performed as described elsewhere (Hossain *et al.* 2009). At least 3 embryos were used for the hybridization of each miRNA. According to the expression patterns miRNAs in IVP embryos, selected candidate trophoblast and inner cell mass specific and imprinted miRNAs were localized to the expanded blastocysts derived from AI and nuclear transfer. For hybridization, embryos were rehydrated in series of methanol/PBS, post-fixation (4% paraformaldehyde for 10 minutes), acetylation (2.33 ml triethanolamine, 500 µl acetic anhydride, H_2O up to 200 ml, readily prepared and treated for 10 minutes) and proteinase K treatment (10 µg/ml, 10 minutes) were carried out, where each step was followed by a 3 times brief wash (10 minutes) in PBS. Two hours of pre-hybridization was performed at 55-59°C in hybridization solution (50% formamide, 5× SSC, 0.1% Tween-20, 50 µg/ml heparin, and 500 mg/ml yeast tRNA). Embryos were incubated overnight with 3'-Digoxigenin (DIG) labeled LNA-modified oligonucleotide probes (1 pM) for mir-31, -96, -127, -215, -222, -223, -299, -320a, -302b,- 431, -450, -544 and let-7d, together with U6 RNAs (Exiqon, Vedbaek, Denmark) in hybridization buffer in a humidified chamber at the temperature 20°C below the Tm of probes. After overnight incubation, embryos were washed briefly in wash buffer (similar to hybridization buffer but without tRNA) and serial wash in 2XSSC/wash buffer (each time 10 minutes) to final three washes in 0.2X SSC each for 30 minutes at hybridization temperature was performed. Blocking, incubation with anti-DIG-AP antibody, washing and color development (Fast Red substrate reaction) was performed as described previously (Obernosterer et al. 2007). Embryos were mounted individually with VectaShield containing DAPI (Vector laboratories, Burlingame, CA) and analyzed by confocal laser scanning microscope (CLSM LSM-510, Carl Zeiss, Germany).

4.1.2.10 Reverse transcription and SYBR green qPCR for selected miRNAs

Temporal expression of selected miRNAs has been examined in blastocyst, expanded blastocyst, day-16 elongated embryo, day-50 placenta and day 225 placentome (from AI, IVP and SCNT). All the reagents and kits used for this purpose were obtained from Exiqon (Exiqon, Vedbaek, Denmark). A 36 ng total RNA from each sample was applied to synthesize first strand cDNA using Universal cDNA synthesis kit. Real time

qPCR was performed using LNA™ PCR primer set for mir- 21, -24, -127-3p, -135b, -299-5p, -302, -376a, -431, and mir-544a with universal RT primers using SYBR Green master mix in ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Dilution of cDNA, preparation of mix and thermal cycling condition was performed as recommended by the manufacturer. Data were analyzed as mentioned before except, normalization was performed using the mean Ct value of U6 RNA and 5S ribosomal RNA.

4.1.2.11 Reverse transcription and SYBR green qPCR for miRNA processing genes

Important candidate genes involved in transcription, processing and generating mature miRNAs were quantified in day 50 placenta derived from IVP, AI and NT pregnancy by qPCR. Primers for qRT-PCR analysis (Table 4.1) were designed using the Primer Express 2.0 software program (Applied Biosystems, Foster City, CA) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). The sequences of PCR primers are listed in Table 4.1. Reverse transcription of 600 ng total RNA from each sample was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in combination with random primers (Invitrogen, Carlsbad, CA) and oligo $(DT)_{23}$(Sigma). The cDNA was stored at -20°C until use. All primers utilized were designed and optimized in order to ensure optimum reaction efficiencies both for target and housekeeping reference genes (GAPDH, Histone). Triplicate reactions were performed for each gene by standard PCR protocol with a 20 µl reaction volume consisting of 10 µl of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), forward and reverse primers at 200-300 nM final concentration and 2 µl diluted template cDNA. A universal thermal cycling parameter specified for the instrument was 50°C for 10 sec, 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec and at 60°C for 1 min. In addition, at the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60˚C and taking measurements every 7 sec interval until the temperature reached 95˚C. The same PCR protocol was used for all primers and Data was normalized using ΔCt (average Ct for the housekeeping gene minus Ct for the gene of interest) and subsequent analysis was performed using ΔΔCt methods (Livak and Schmittgen 2001). One-way analysis of variance with tukey test was performed to compare expression of each gene in different types of placenta. A probability of $P \leq 0.05$ was considered to be significant differentially expressed.

Table 4.1: List of miRNA processing genes and primers used in this study

Gene			Sequences (5´-3´)	T_A (°C)
GAPDH	:	Forward	AATGGAAAGGCCATCACCATC	57
		Reverse	GTGGTTCACGCCCATCACA	
Histone	:	Forward	GCCGTATTCATCGACACCTGA	55
		Reverse	CTCCACGAATAGCAAGTTGCAA	
ADAR1	:	Forward	AATGGCTTTGCTGCAGAGTT	55
		Reverse	GCGCTCTGCTTTCTCTGTTT	
DGCR8	:	Forward	GGAAGCTGGCAAACAAGATCC	55
		Reverse	GGTTGGTTTCATGTGCTCGAA	
EIF2C1/AGO1	:	Forward	AGAGTGGAGTATGCAGTGCTCG	55
		Reverse	GGGCATCAACATCGTTGTCA	
EIF2C2/AGO2	:	Forward	AGCGCTGCATTAAGAAGCTGA	55
		Reverse	CCGTCATGTCATCCTTCACCTT	
EIF2C3/AGO3	:	Forward	TTCCACACGGGCATTGAGAT	55
		Reverse	TATTTACAGAAGCATGGCTGGC	
EIF2C4/AGO4	:	Forward	CAACACCAAGCCACGGAGTAT	55
		Reverse	GAAATCTTCCGCAGCTGGTCT	
FMR1	:	Forward	TAGTGGCAGGACAGCGATGTA	55
		Reverse	TTTAAGGTATGGGTCAGGGCC	
GEMIN4	:	Forward	TCCCAACAAACCTGCCACA	55
		Reverse	TCACTGATGGACAAAACCACG	
GEMIN6	:	Forward	TGGTGGGAAATGATGAAGCAC	55
		Reverse	GCATGGTTGGACACACATCTG	
GEMIN7	:	Forward	AGAGCTGAGTGGGTTTGAGCA	55
		Reverse	TGTTACGCTGACCGCTTTGTA	
POLR2A	:	Forward	ACCTGGACGTGGCCAATTT	54
		Reverse	AACATATGGAGGCCTGGGAGA	
POLR2G	:	Forward	AACCTGTGCAGGAAACATGA	54
		Reverse	CTTGGAAGAGTCCACAAGCA	
RNASEN	:	Forward	GATGATTACCTGGGGCTTGT	55
		Reverse	GGCTGCTAAGCCATAGGAAG	
RANGAP1	:	Forward	AGGGTCTTCCCATCGATTCT	55
		Reverse	GCTTGCTCCCTTAAGCAATG	
SIP1	:	Forward	GGCACAATTTTCGACTGTTCG	55
		Reverse	AGCCCCTTCAGCACATAACCT	
XPO1	:	Forward	TCCGACTTGCTCCAACAATGT	55
		Reverse	CAAGGAACCAATGTGAAGGGA	
XPO4	:	Forward	CAAGGTACACACGGTCCAAAGA	55
		Reverse	GGCTCCAAATGTACAAGCCAA	
XPO5	:	Forward	TCTTTGTGAAGCCTCTGGTG	55
		Reverse	TGTTCCTCCAGCATCTCTTG	

4.1.2.12 Quantification of global DNA methylation

Genomic DNA isolated from the 3 elongated day 16 embryos and 3 day 50 placenta from each IVP, AI and SCNT pregnancy was used to quantify the global methylation status using Methylamp Global DNA Methylation Quantification Ultra kit (Epigentek, Brooklyn, NY) according to user instruction provided by the manufacturer. Briefly 200 ng of genomic DNA from each sample was immobilized to the strip well specifically coated with DNA affinity substance. The methylated fraction of DNA was recognized by 5-methylcytosine antibody and quantified through an ELISA-like reaction. Serial dilution of positive control (synthesized polynucleotide methylated at every 5-cytosine) in 6 points (0.4, 1, 2, 5, 10 and 20 ng/well) was used to generate a standard curve. Color was developed and absorbance read was performed in ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Slope was determined as OD/ng by plotting OD value versus amount of positive control. DNA methylation (%) was calculated by using the formula [x 100%] where, 41.7 is the GC content in bovine genomic DNA and Methyl DNA (ng) = {Sample OD- Negative control OD)/slope}.

4.1.2.13 Western blotting

Total proteins extracted from three Day-50 placentas from each group (IVP, AI and SCNT) were pooled equally (30 µg) and separated by SDS-PAGE (gradient 4-18%) and transferred onto a nitrocellulose membrane (Amersham Biosciences) and blocking was performed in buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% polyvinylpyrolidone) at room temperature for 1 hour. The membrane was then incubated with goat anti-eIF2C2 polyclonal antibody **(**Santa Cruz Biotechnology, Santa Cruz, CA) in the blocking medium overnight at 4°C. Non-specific binding of antibody was washed off with six changes of 0.1% PBST. The HRP-conjugated mouse anti-goat IgG (Santa Cruz) was used as the secondary antibody. The membrane was incubated for 1 h at room temperature with secondary antibody, followed by washed with six changes of 0.1% PBST. The chemiluminescence was detected by using the ECL plus western blotting detection system (Amersham Biosciences) and visualized by using Kodak BioMax XAR film. GAPDH antibody (Santa Cruz) was used as a loading control. The membrane was stripped by incubation in 2% SDS, 100 mM Tris-HCl and 0.1% beta-mercaptoethanol for 30 min at 60°C and re-probed with GAPDH antibody.

4.2 Results

4.2.1 Differential miRNA expression in Day-50 placenta of different sources of pregnancy

The miRNAs expression profiles of the Day 50 NT placenta (n=3) were compared either to that of AI (n=3) or IVP (n=3) placenta. In addition, the level of miRNAs expression in the IVP placenta has subsequently been compared to that of AI placenta. The magnitude and number of differentially regulated miRNAs in different comparisons are presented in figure 4.1 and 4.2, respectively.

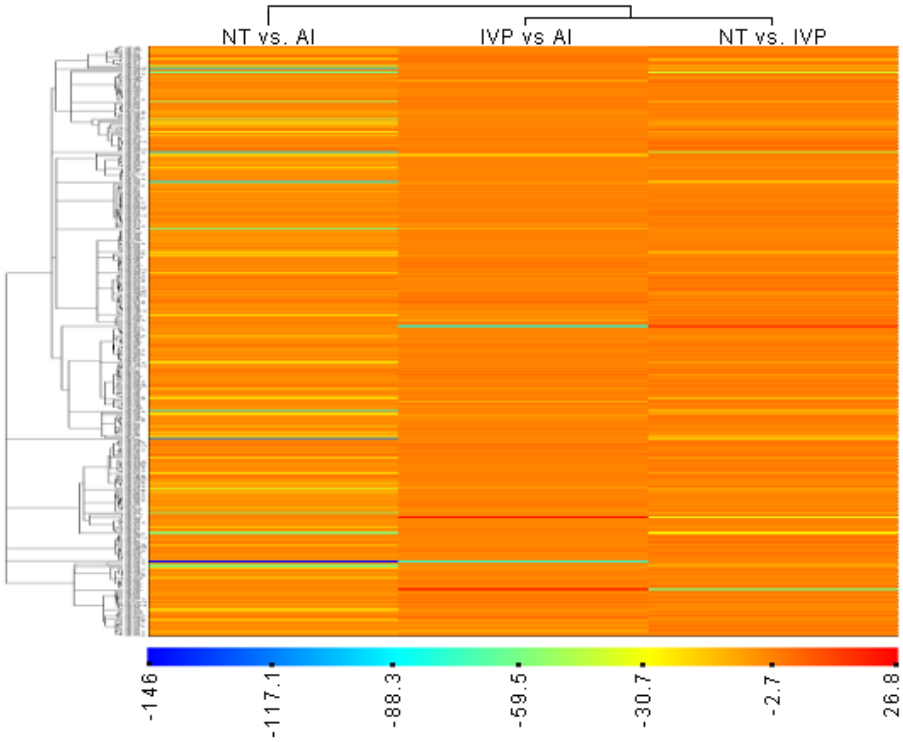


Figure 4.1: Hierarchical cluster of NT, IVP and AI Day 50 placenta compared to each other and characterization of differentially expressed miRNAs. The log2 fold change value of 377 miRNAs from each comparison is cluster arranged to highlight global differential miRNA expression.

Of the 377 miRNAs used in the comparison of their expression between Day 50 NT and AI placenta, 320 miRNAs were found to be downregulated in NT placenta with 2 or more fold change (278 miRNAs with P-value ≤ 0.05), while only 5 miRNAs (miR-527, miR-608, miR-637, miR-649 and miR-938) were found as upregulated by 2 or more

fold change in NT placenta compared to the placenta of AI group (Figure 4.2). Out of these 5 miRNAs miR-608 was uniquely upregulated in NT placenta compared either to AI or IVP placenta. On the other side, out of 320 downregulated miRNAs 116 miRNAs were uniquely downregulated in the NT placenta compared either to AI or IVP placenta.

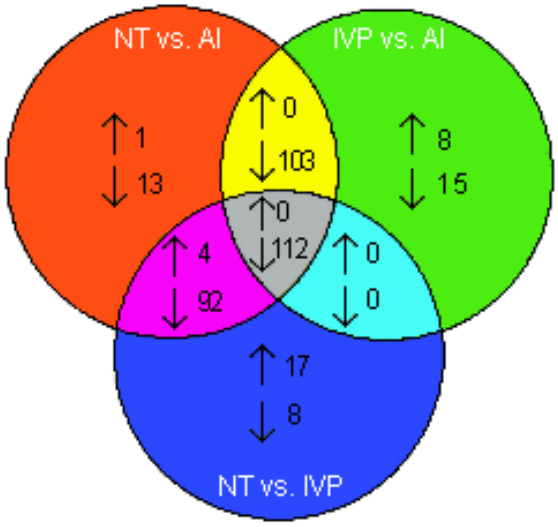


Figure 4.2: Venn diagram of the distribution and the number of differential miRNA expression between and specific to different types of Day 50 placenta. Each circle represents the number of differentially expressed miRNAs between two placenta types out of 377 analyzed miRNAs. For example, NT vs. AI represents the number of differentially regulated miRNAs (↓ denotes down regulation and ↑ denotes upregulation) in the NT placenta compared to that of AI. Similarly IVP vs. AI and NT vs. IVP represent the number of differentially regulated miRNAs in IVP compared to AI and the number of differentially regulated miRNAs in NT compared to IVP placenta. The circle (upper left) shows a total of 325 miRNAs that are differentially expressed between AI and NT placenta where 5 are upregulated and the rest are downregulated; 1 miRNA (red) are uniquely overexpressed in the comparison of NT vs. AI, 4 miRNAs (pink) are overexpressed commonly in the comparison between NT vs. AI and NT vs IVP and no miRNA (yellow) are commonly upregulated in NT vs. AI and IVP vs. AI placenta. Number in the center (gray) shows the common differential miRNAs in any of the three ways of comparison.

Similarly, the expression profiles in the NT placenta were compared to that of IVP placenta. In this comparison, 233 miRNAs were found to be differentially expressed in the NT placenta (Figure 4.2). Among them only 21 miRNAs were upregulated (4 common when compared to AI as mentioned before) and rest (212) were found to be downregulated. The 17 miRNAs which are uniquely upregulated in NT placenta compared to that of IVP placenta were miR-17*, miR-19a, miR-106b,, miR-219-2-3p, miR-296-5p, miR-372, miR-450a, miR-502-5p, miR-520d-5p, miR-548d-3p,, miR-549, miR-564, miR-566, miR-612, miR-616*, miR-619 and miR-937. Among the 212 downregulated miRNAs, 204 were found to be commonly downregulated in NT placenta compared to that of AI. The rest (miR-124, miR-302b, miR-302c, miR-525-3p, miR-526b and miR-590-3p) were down regulated uniquely in NT placenta compared to IVP placenta. Overall, about 62 % miRNAs were found to be differentially regulated in the NT Day 50 placenta compared to that of IVP placenta, where 90% of them were found to be downregulated.

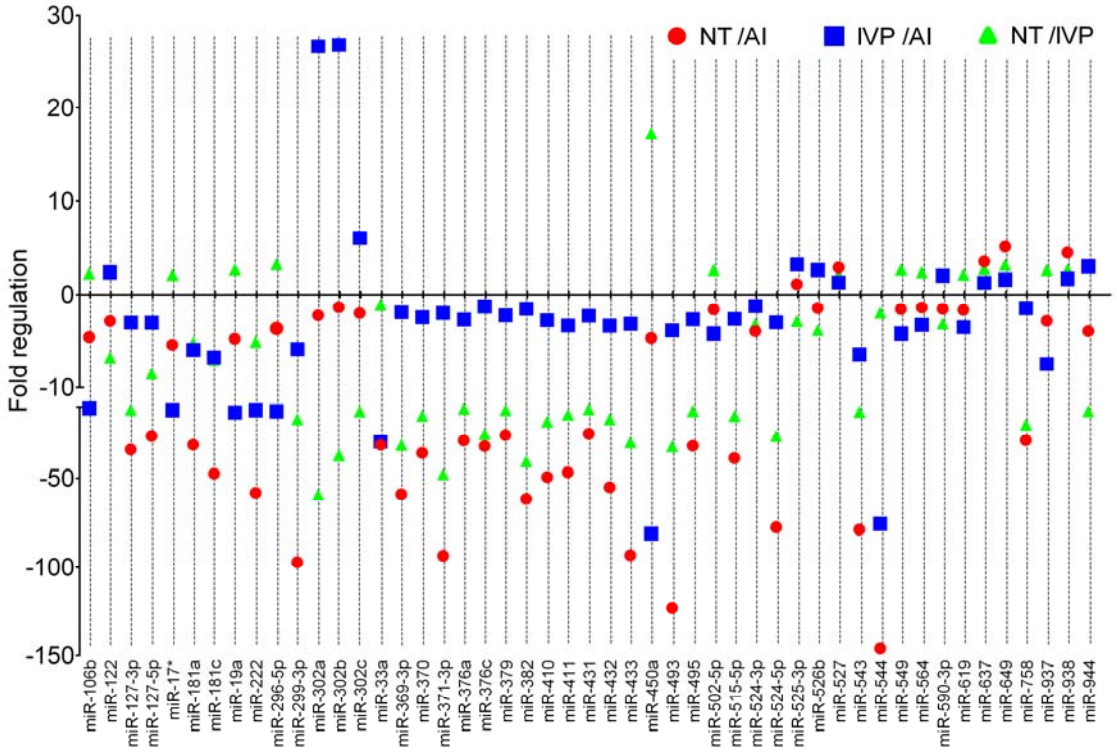


Figure 4.3: Plot diagram of the magnitude of fold regulation of most differentially regulated miRNAs in Day 50 placenta of different sources of pregnancies. The diagram shows the degree of expression difference of 49 miRNAs in Day 50 NT placenta compared to AI (red circle), in Day 50 IVP placenta compared to that of AI (blue rectangle) and in Day 50 NT placenta compared to that of IVP (green triangle).

Additional comparison of miRNAs expression between IVP placenta and AI placenta has been performed. Analysis revealed differential expression of 238 out of 377 tested miRNAs (Figure 4.2). Of the 238 differentially miRNAs, 230 were found to be downregulated and 8 miRNAs (miR-122, miR-302a, miR-302b, miR-302c, miR-525-3p, miR-526b, miR-590-3p and miR-944) were upregulated in IVP placenta compared to their expression in AI placenta. Among the 230 downregulated miRNAs, 215 miRNAs were also downregulated in the comparison of NT vs. AI and 112 miRNAs of them were commonly downregulated in the comparison of NT vs. IVP. So, the rest 15 downregulated miRNAs (miR-219-2-3p, miR-346, miR-372, miR-502-5p, miR-516a-3p, miR-520e, miR-549, miR-564, miR-616*, miR-619, miR-632, miR-647, miR-766, miR-875-3p and miR-940) were exclusively differential in the comparison between IVP and AI placenta and not appeared in any other way of comparison.

4.2.2 miRNA expression profile comparison of donor cells and NT Day-50 placenta

To investigate any source of origin specific expression of miRNAs in the Day-50 NT placenta being reflecting ultimate differential expression compared to that AI placenta, expression profiling of same 377 miRNAs in the fibroblast donor cells (serum starved condition at exactly before nuclear transfer stage condition) has been performed. The expression profiles of Day-50 NT placenta were compared to that of fibroblast donor cells. Analysis revealed 244 miRNAs differentially expressed in the Day 50 NT placenta compared to fibroblast with 2 or more fold change (209 miRNAs with P value $\leq$0.05 and 2 or more fold change). Among the 209 miRNAs, 185 and only 24 miRNAs were found to be up and down regulated in NT placenta compared to fibroblast. Out of the 24 downregulated miRNAs, miR-486-5p, miR-508-3p, miR-519b-5p, miR-519e*, miR-548b-3p and miR-885-5p were also found to be down regulated in the Day 50 NT placenta compared to that of AI. miR-302b and miR-432 were found to be down regulated in NT placenta compared to that of IVP placenta. Among the miRNAs which are not differentially regulated (less than 2 fold regulation with P value $\geq$ 0.05) in the Day 50 NT placenta compared to fibroblast, 18% of them were found also not to be differentially regulated in NT placenta when compared to either that of AI or IVP placenta. Whereas, 22% of them were different to AI and collectively 39% of them were different when compared to both AI and IVP placenta.

4.2.3 Genomic patterns of deregulated miRNAs in NT and IVP placenta

Majority of the identified deregulated miRNAs in the Day 50 NT and IVP placenta compared to placenta from artificial insemination were further characterized bio-informatically. Chromosomal location of selected all miRNAs were retrieved from miRBase v 14 and ENSEMBL genome browser has been used to find out the features of genomic regions of interest.

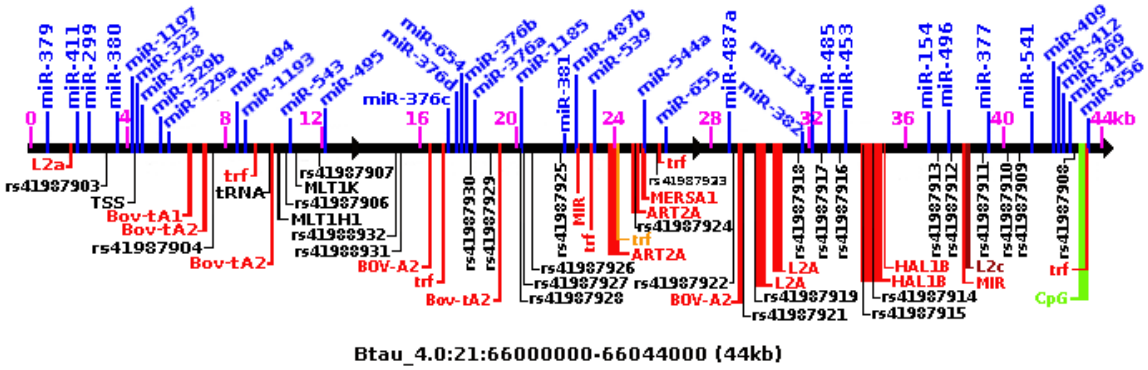


Figure 4.4: Genomic region (44kb) of bovine chromosome 21 harboring at least 3 big clusters of miRNAs which are down regulated in Day 50 NT and IVP placenta compared to that of AI. Text in blue above the black line (forward genomic strand) represents the residing miRNAs; number in pink represents range and scale of the region in kilo bases (kb) , number in black starting with rs- denotes dbSNPs, others are different types of transposone (red or orange) namely type I line/SINE, type II, tandem repeats (trf) and pseudo transfer RNA (tRNA).

Interestingly, different deregulated miRNAs in the NT and IVP placenta were found to be affected in similar patterns and they are located in the chromosome as polycistronic clusters. For example, one such genomic region in bovine chromosome 21 is presented in figure 4.4 representing 44 kilo bases (66000000-66044000 bases). This region found to be harboring at least 3 clusters of miRNAs comprised of more than 38 miRNAs. Most of these clustered miRNAs tested were found to be downregulated in NT and some in IVP placenta compared to the placenta of artificially inseminated pregnancy. Most of the genomic region of the miRNAs cluster were found to be absent of any protein coding gene but abundant in a number of other genomic variable region. These include different types of transposone elements, (type I LINE, type I SINE, type II), tandem repeats (TRF), long terminal repeats (LTRs) and numerous SNPs. However, most of these elements were found to be residing out side and no SNPs were present in

side the miRNA precursors. So, any transcriptional disturbance or lack of optimum transcription process could lead to the deregulation of all miRNAs belong to the same cluster.

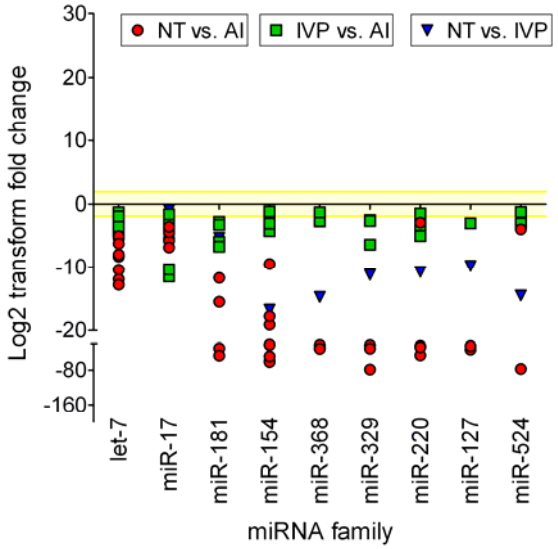


Figure 4.5: Family wise differential expression of miRNAs in different sources of Day 50 placenta. Log2 fold change of 43 miRNAs belong to 9 miRNA families which were deregulated in different sources of placenta were plotted to visualize the family wise expression differences in the placenta of different sources of pregnancy.

In addition to cluster wise regulation, deregulated miRNAs in the NT and IVP placenta compared to that of AI were found to be affected family wise (Figure 5). Forty three top deregulated miRNAs were in either three ways of comparison between three sources of placenta were found to belong 9 distinct miRNA families. All the 9 miRNA families were found to be downregulated in the NT and IVP Day 50 placentas compared to the placentas from AI. Similarly most of the miRNAs having highly similar sequence between them (denoted and distinguish by a, b, c, etc at the end of miRNA name) were also found to be deregulated in the placenta in an identical manner.

4.2.4 Localization of selected miRNAs in expanded blastocyst of NT, IVP and AI origin

Whole mount in situ hybridization of 10 miRNAs in in vitro, in vivo and NT expanded blastocyst was performed to identify specific expression pattern either in the trophectoderm or inner cell mass. The study has identified several cell specific miRNAs in embryo from AI and subsequently compared their expression pattern to the embryo

of IVP and NT. Among the localized miRNAs, miR-24, and miR-299 were found to be intensively localized to the trophectoderm and miR-203b were inner cell mass specific in the expanded blastocyst from AI group (Figure 4.6). Tested other miRNAs were found to be generally expressed both in trophectoderm and inner cell mass but when their expression patterns in AI blastocyst were compared to that in NT or IVP group, the extent of their expression were found to be different.

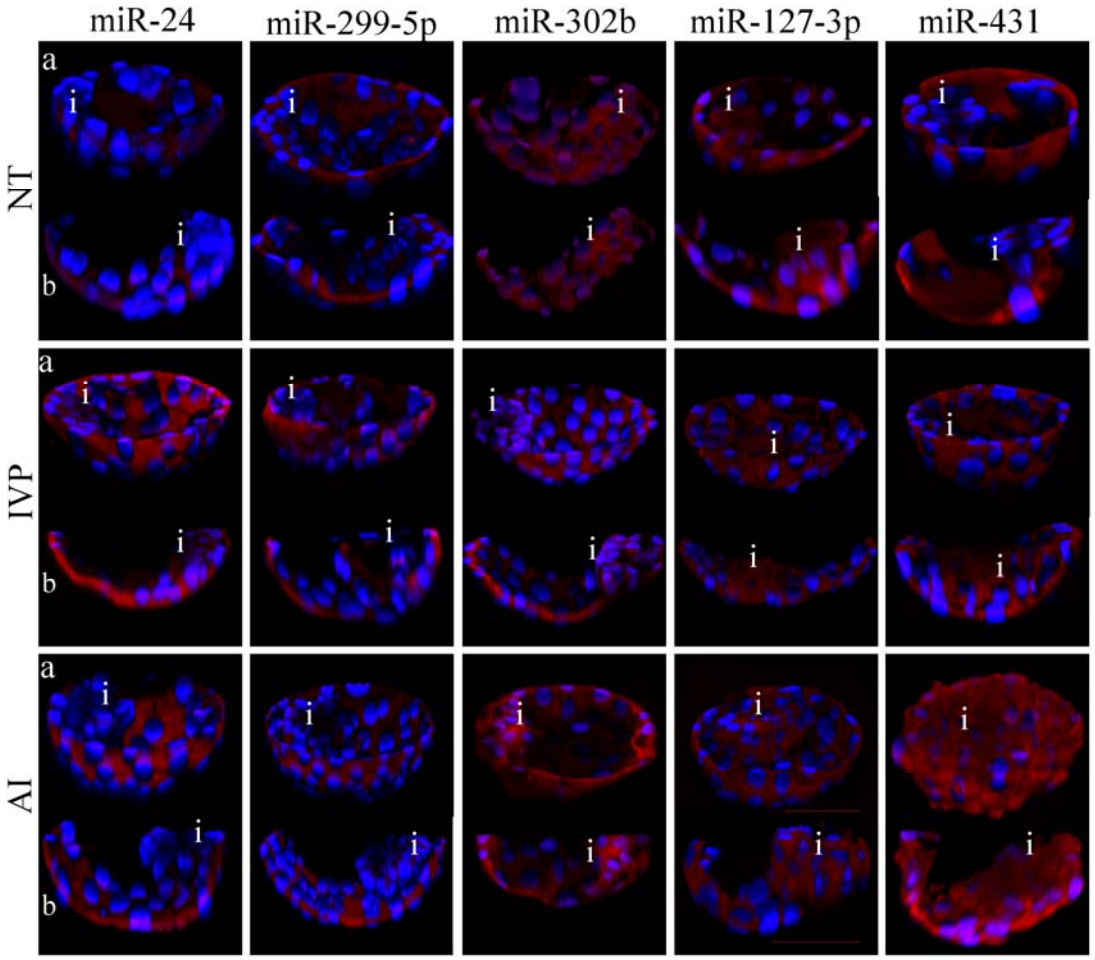


Figure 4.6: Whole-mount in situ hybridization of miRNAs in in vivo, in vitro and NT expanded blastocysts (miRNAs are stained red and blue represents nuclear stain by DAPI). About half of the blastocyst comprising approximately middle of the inner cell mass and corresponding trophectoderm has been visualized in the upper image (a) in each group as obtained by scanning the blastocyst at every 2 µm interval using laser scanning confocal microscope and projected in three dimensions (3D). Similarly lower image (b) for every group of embryos represents a 3D transverse region of upper and same blastocyst to visualize inner cell mass and trophectoderm clearly. Scale bar represents 50 µm and 'i' indicates the region of inner cell mass.

Expressions of trophectoderm specific miRNAs were entirely different in the NT blastocyst which can be characterized as very low compared to that in AI blastocysts. Whereas, their expressions in the IVP blastocysts were similar or in some blastocysts slightly decreased compared to AI blastocysts. In case of miR-302b, the expression pattern has been identified not to be largely different in the NT or IVP blastocysts compared to the blastocyst from artificial insemination. In addition, two other miRNAs (miR-127 and miR-431), which are originated from the large imprinted chromosomal region were also localized in the NT, IVP and AI blastocysts (Figure 4.6). Interestingly, they were found to be similarly expressed either in the inner cell mass or trophectoderm of the AI blastocysts but aberrantly expressed in NT blastocyst. In the NT blastocysts, miR-127 shows almost depletion of its expression in different part of the trophectoderm with no change in the inner cell mass and this pattern was not noticeable in AI or IVP blastocysts. On the other side, miR-431 shows opposite pattern in the NT blastocysts, where the expression was found to be depleted from the inner cell mass but such anomalies was not evident in case of AI or IVP blastocysts. Result of in situ hybridization of miRNAs has revealed more aberrant or deregulated sate miRNAs expression in the trophectoderm and inner cell mass of NT expanded blastocyst and which was less in the IVP blastocysts.

4.2.5 Temporal differences in the expression of selected miRNAs in different sources of placenta

Later on expression profiling of the candidate trophectoderm, embryonic specific and imprinted miRNAs in the fibroblast, day-7 blastocyst, expanded blastocyst, day-16 elongated embryo, and day-225 placenta derived from in vivo, in vitro and NT placenta has identified that the major deregulations are likely to happen in the NT placenta at day-50 of the pregnancy when compared to the in vivo placenta (Figure 4.7).

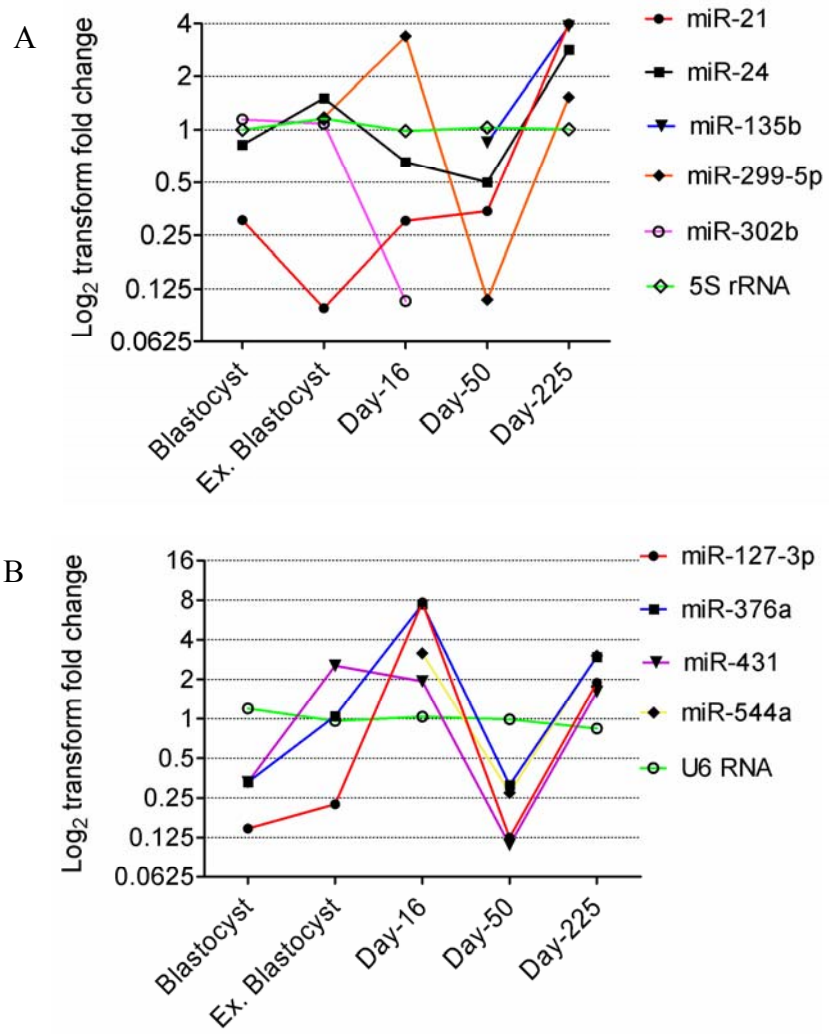


Figure 4.7: Expression pattern (in fold change) of the candidate trophectoderm/placenta specific (A) and imprinted miRNAs (B) in blastocyst, expanded blastocyst, Day-16 elongated embryo, Day-50 placenta and Day-225 placentome derived from NT pregnancy compared to that of AI pregnancy

However, the extent of this deregulation in IVP placenta at day 50 was not evident and showed very less difference compared to the placenta of AI at day 50 (Figure 4.8). Moreover the result reveals that deregulation of miRNAs started after construction of nuclear transfer embryo due to improper reprogramming and it is progressing to the later stage specially in the placenta around day-50 has most aberrant miRNAs expression when redifferentiation for placentogenesis is happening. Later stage, (around day-225) NT placenta were found likely to recover some anomalies of miRNAs expression.

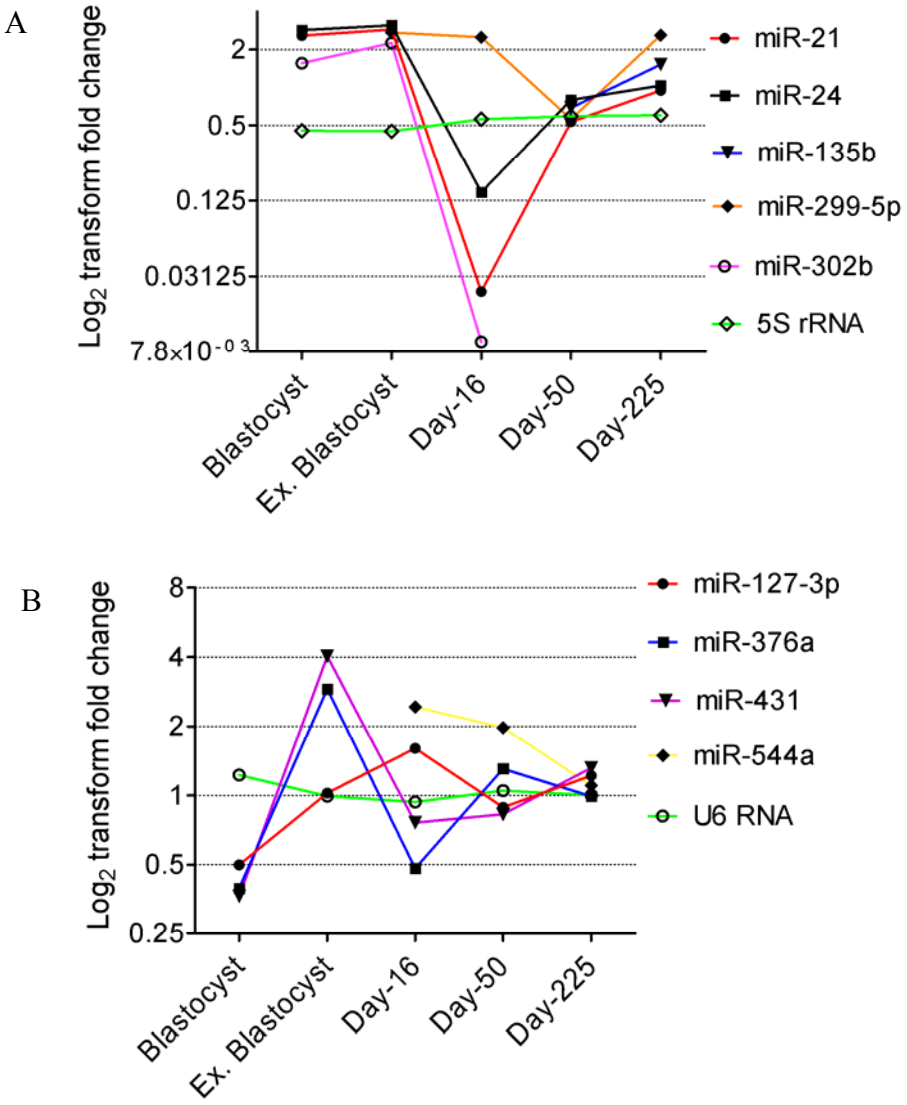


Figure 4.8: Expression pattern (in fold change) of the candidate trophectoderm/placenta specific (A) and imprinted miRNAs (B) in blastocyst, expanded blastocyst, Day-16 elongated embryo, Day-50 placenta and Day-225 placentome derived from IVP pregnancy compared to that of AI pregnancy.

4.2.6 Global DNA methylation status in different sources of elongated embryos and placentas

Global DNA methylation has been quantified in the elongated embryos at day 16 and placenta at day 50 derived from NT, IVP and AI pregnancies. Level of DNA methylation was expressed in percentage compared to the positive control (synthetic oligos where every 5 cytosin were methylated). Analysis revealed a global hypomethylation of DNA either in three groups (NT, IVP and AI) across two stages of

development (Elongated embryo and placenta). Status of DNA methylation is presented in figure 4.9. Study has identified that there is no significant differences in global DNA methylation in the elongated embryos at Day 50 as well as same in the Day-50 placentas of NT, IVP and AI pregnancies.

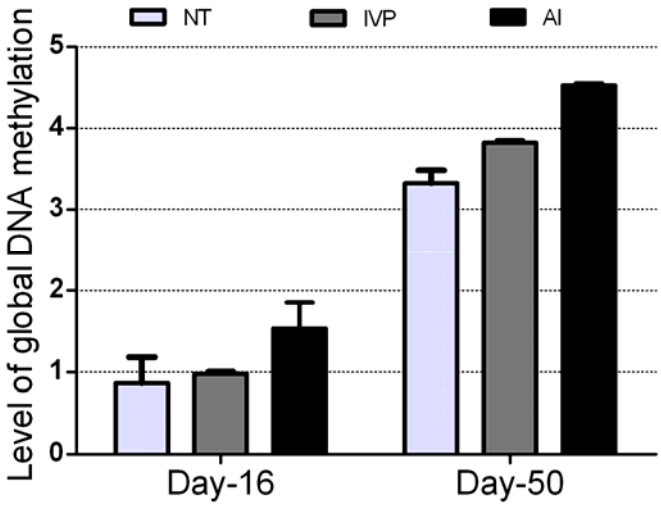


Figure 4.9: Global DNA methylation (%) in Day-16 elongated embryos and Day-50 placenta of different sources of pregnancy

4.2.7 Aberrant regulation of miRNAs processing genes in IVP and NT placentas

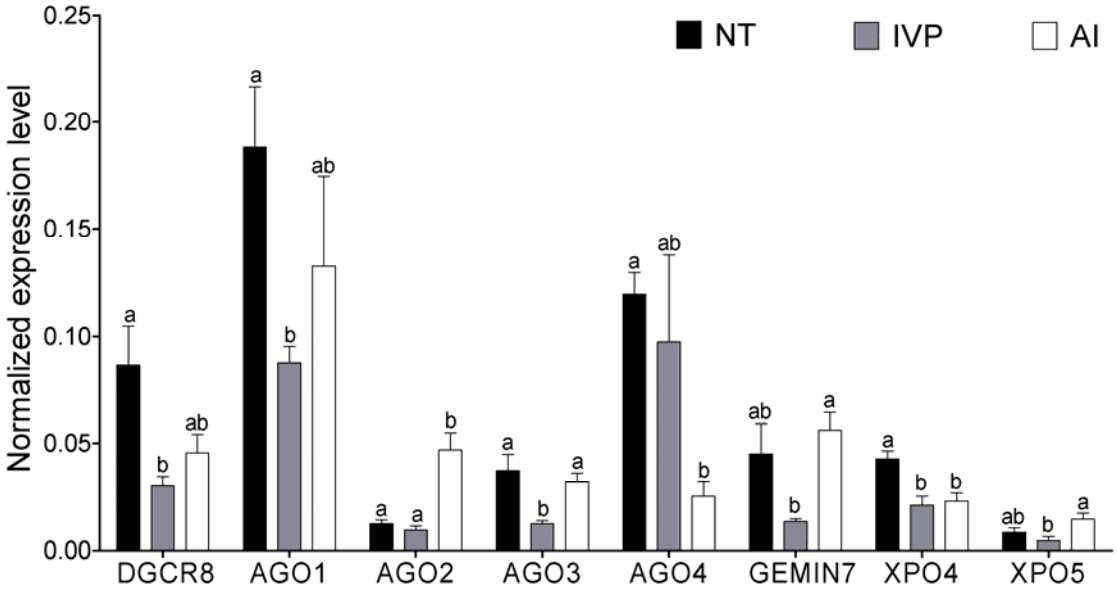


Figure 4.10: Normalized expression level of significantly differentially regulated miRNAs processing machinery genes in Day-50 placenta derived from NT, IVP and AI pregnancy. Bars denoted by different alphabet (a, b) were found to be significantly different ($P \leq 0.05$).

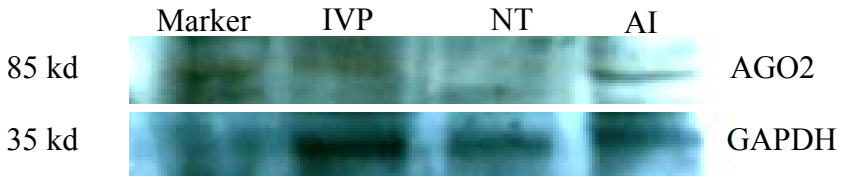


Figure 4.11: Expression of AGO2 protein in Day-50 placenta derived from NT, IVP and AI pregnancies. GAPDH was used as loading control

Expression profiling of 18 miRNA processing machinery genes (ADAR1, DGCR8, AGO1, AGO2, AGO3, AGO4, FMR1, GEMIN4, GEMIN6, GEMIN7, POLR2A, POLR2G, RNASEN, RANGAP1, SIP1, XPO1, XPO4 and XPO5), in Day-50 placentas from NT and IVP pregnancies was performed and compared to that of AI (Figure 4.10). Analysis revealed that most of them except AGO2 are well reprogrammed in NT compared to AI palcentas. Expression of the miRNAs processing molecules were found to be similar or in some cases upregulation in NT placenta. Whereas, it was not the case in IVP placenta, where more machinery genes were found to be deregulated compared to AI (Figure 4.10). Among them especially AGO3, GEMIN7, XPO4 including AGO2 were noticeable. In both NT and IVP Day-50 placentas, AGO2 or EIF2C2 was found to be down regulated compared to that of AI. The reduced expression of AGO2 protein in the NT and IVP placentas was also observed (Figure 4.11).

4.2 Discussion

4.2.1 Day-50 placentas from nuclear transfer and IVP pregnancy

Nuclear transfer and in vitro embryo production are the promising technology with potential applications in agricultural as well as biomedical research. However, only 2% of NT embryos result in live birth and frequent abnormalities are observed in IVP pregnancy. The losses or abnormalities being mostly due to improper placenta formation due to nuclear reprogramming error after nuclear transfer and in vitro culture (Yang et al. 2007). The normal bovine placenta progressively attaches to the endometrium throughout the first trimester and the initial contact with a maternal caruncle induces villous processes to undergo hypertrophy and hyperplasia to form cotyledons that progress to form large and complex placentomes by Day 42 (King et al. 1979). Poor viability of somatic cell cloned fetus during Days 35-60 (period of placentome development) has been found to be associated with either rudimentary or marginal chorioallantoic development (Hill et al. 2000). Due to the critical importance of this period, present study has been conducted on Day-50 as a baseline stage of placenta from different sources of pregnancies. Placentas from all NT Day-50 pregnancies were characterized with reduced by half the number of barely visible cotyledons having sporadic hemorrhagic areas compared to those in AI placentas. Where as, the placentas from Day-50 IVP pregnancies were apparently normal in size and similar number of cotyledons compared to those in AI.

4.2.2 miRNAs are deregulated in Day-50 NT and IVP placentas

The aberrant genetic or epigenetic modifications in the NT placenta from nuclear reprogramming error have been evidenced due to aberrant non-coding RNA expression, imprinting problem, DNA methylation and histone/chromatin modification. Among the non-coding RNAs, miRNAs were found to be aberrantly reprogrammed in cloned mouse blastocyst (Cui et al. 2009) and bovine Day-17 elongated embryos (Castro et al. 2010). In addition, miRNAs were also evidenced to be regulated by and/or regulate epigenetic processes (Kircher et al. 2008, Williams et al. 2007). Present study has identified that most of the miRNAs studied being reprogrammed but a large number among them are not reprogrammed correctly in the NT (68% miRNA) and IVP (36% miRNA) placentas. Relative expression reveals a massive down regulation of miRNAs

in Day-50 NT and IVP placenta compared to the Day-50 placenta from AI pregnancy. This difference in expression of miRNAs could be due to the cumulative effects of embryos constructed by somatic cell nuclear transfer and in vitro culture of embryos which were absent in control AI pregnancies. At least 62% of the miRNA studied in Day-50 NT placenta were deregulated compared to IVP placenta, where 90% of them were found to be downregulated. This difference could be due to effect of embryos constructed by nuclear transfer and transferred to the recipients for establishing the NT pregnancy. Overall 36% miRNAs of 377 miRNAs tested were differentially regulated between IVP and AI placentas, where about 96% of these differentially regulated miRNAs were downregulated in IVP placentas. This difference in the expression of miRNAs could be attributed due to the effect of the transfer of embryos which were fertilized and cultured in vitro. The study has identified that most of the miRNAs are reprogrammed but may not be in proper way or any error in the regulatory molecule for regulation of miRNAs expression are responsible for massive deregulation of miRNAs in NT or IVP placentas compared to that of AI. These massive deregulations could be entailed to the reported radically altered gene expression in cloned placenta (Aston et al. 2009, Everts et al. 2008, Hall et al. 2005, Oishi et al. 2006) associated to poor placentomes development in NT pregnancy in first trimester leading to pregnancy loss.

4.2.3 Major deregulation of miRNAs in NT or IVP happened during Day-50 of pregnancy

Reprogramming error in the NT or IVP has been postulated to be happened as a multistep process and the effect could be different in different stage of development (Jouneau et al. 2006). It has been suggested that the commonly observed low developmental efficiency of NT embryos may not be largely due to nuclear reprogramming during early embryo development but may be potentially caused by abnormal gene reprogramming during postimplantation feto-placental development (Smith et al. 2005). Where, NT blastocyst closely resemble to in vivo, but not to in vitro fertilized embryos in terms of global gene expression (Smith et al. 2005) but later stage, trophoblast lineage was found to be affected more by the reprogramming error arised from epigenetic modifications after the blastocyst stage (Jouneau et al. 2006). However, differences between trophectoderm and inner cell mass could be different in NT blastocyst compared to AI blastocyst which should be exploited to conclude precisely

that they are similarly reprogrammed also in cell specific manner. Comparison of either expression of miRNAs as a major modifier of genetic/epigenetic gene regulation or global gene expression between different stage of the embryo and placental development through out the NT or IVP pregnancy has not been carried out. Present study has identified that the major deregulation of miRNAs in the NT placenta are appearing at around Day-50 of pregnancy. Although the differences in their expression are evident during expansion of blastocyst and at elongation stage, but the deregulation are more profound at around Day-50 of pregnancy in NT and less in IVP compared to that of AI. In addition, trophectoderm specific miRNAs were found to be aberrantly expressed in the NT and IVP expanded blastocysts compared to that of AI. Where as, inner cell mass specific miRNAs were less or not aberrantly expressed in either NT or IVP expanded blastocyst compared to the expanded blastocyst from AI. In case of miRNAs which are located within or nearby imprinted region of the chromosome showed a clear abnormality in their expression in NT and IVP expanded blastocyst when compared to their expression in the expanded blastocyst from AI. Results of this study indicate that trophoblast specific and imprinted miRNAs are aberrantly expressed in the NT blastocysts which were not the case for inner cell mass or embryonic stem cell specific miRNA. In addition to this result, reported changes of miRNAs during trophectoderm specification (Viswanathan et al. 2009) and aberrant epigenetic reprogramming and expression of imprinted miRNAs (e. g. miR-127, miR-136) in cloned mouse embryo (Cui et al. 2009) suggest that deregulation of miRNAs expression due to reprogramming error is the primary cause of early improper placentation and that is magnified during Day-50 of the NT pregnancy while the complex placentomes are thought to be developed. This is more evident in elongated Day-16 embryo and Day-50 placenta from cloned pregnancy. In the elongated embryos, the deregulation of miRNAs could be attributed also due to their cell specific aberrant expression, but this could not be elucidated because the elongated embryos were comprised of embryonic and extra-embryonic tissues. However, at day 50 the differences of miRNAs expression characterized by their down regulation in NT placentas compared to that of AI were more clear which was not noticeable in IVP placenta. Interestingly, the degree of deregulation of miRNAs at day 225 of pregnancy either in NT or IVP compared to AI were found to be very less which shows almost optimum or some increased expression. This is possible that those cloned fetuses that survive up to term could recover the aberrant expression of miRNAs in the cloned placenta. At approximately Day 50, those

fetuses with subnormal placentomes formation and with more deregulated miRNAs expression could be slowly starve to death but the fetus that progress beyond this stage have better placentome development as studied previously (Hill et al. 2000).

4.2.4 Major causes of miRNAs deregulation in NT and IVP placenta

Major sources of aberrant gene expression in the NT placentas have been found to be due to abnormal epigenetic and genetic processes. These include non-coding RNA mediated regulation, aberrant DNA methylation, genomic imprinting and chromatin remodeling. Maintenance of imprinting has been shown to be less dependent on DNA methylation in the placenta than in the embryo, with involvement of repressive histone methylation rather than DNA methylation (Wagschal and Feil 2006). Present findings revealed that there is no significant difference in global DNA methylation in the Day-16 elongated embryos and placentas at day 50 between NT or IVP and AI. There is no correlation between identified global hypomethylation of DNA to the massive down regulation of miRNAs in the NT placenta compared to that of AI. So, it is possible that other genetic or epigenetic processes (aberrant regional chromatin remodeling) rather than DNA methylation are responsible for massive deregulation of miRNAs in the NT or IVP placenta.

Bioinformatic analysis of the deregulated miRNAs reveals that the miRNAs are oftenly deregulated as cluster and shows similarities in their deregulation between the members of the same miRNAs family. In addition to the own promoters as independent entities or as polycistrons, they were found to be transcribed in a large transcription units of coding or non-coding genes (Kim 2005). So, any transcriptional or processing disturbance during the earlier stage of cloned embryo and extra embryonic lineage differentiation due to improper reprogramming of specific regulatory molecules which are responsible for global transcription, processing and maturation of miRNAs could be the possible reason for global down regulation of miRNAs in the NT or IVP placentas. To elucidate these possibilities, present study has profiled 18 well known miRNA processing machinery molecules in different sources of placentas under investigation. Results indicate that several of such molecules are not well reprogrammed in the NT or IVP placentas when compared to that of AI or donor fibroblast cells. Among them, eukaryotic transcription initiation factor 2C2 (EIF2C2) or AGO2 has been found to be

down regulated in the NT or IVP placenta compared to that of AI, which could be linked to massive down regulation of miRNAs as also reported previously (Cifuentes et al. 2010, Zhang et al. 2009). Expression of AGO2 has been shown to be important in many biological processes and development including mouse oogenesis (Kaneda et al. 2009), early development (Morita et al. 2007) and maternal-zygotic transition (Lykke-Andersen et al. 2008). Mouse embryonic fibroblasts and hematopoietic cells from Ago2 knockout showed subsequent reduction of all mature miRNAs (Diederichs and Haber 2007, O'Carroll et al. 2007). So, according to the previous reports and our findings, it could be postulated that global down regulation of miRNAs in the NT or IVP Day-50 placenta compared to that of AI is due to down regulation of AGO2 and aberrant reprogramming of other factors if there is any.

5 General summary

MicroRNAs are the major class of gene-regulating molecules playing diverse roles through sequence complementarity to target mRNAs at post-transcriptional level. Identification of entire set of miRNAs and study of their expression patterns are the fundamental step towards understanding miRNA-guided gene regulation in different biological functions. Tightly regulated expression and interaction of a multitude of genes for ovarian folliculogenesis and their aberrant expression due to genetic or epigenetic modification widely addressed in feto-placental development by different assisted reproductive biotechnology could be regulated by these miRNAs. Despite increasing efforts in miRNAs identification across various species and diverse tissue types, little is known about miRNAs in bovine reproductive tissues specially ovary or placenta. For the elucidation of this research gap, two subsequent studies have been carried out.

First study hypothesized that tightly regulated expression and interaction of a multitude of genes for ovarian folliculogenesis are regulated by miRNAs and thereby aimed to identify and characterize them in bovine ovary through cloning, expression analysis and target prediction. For this purpose, RNAs of 18 to 26 nt in length from bovine ovarian small RNAs (< 200 nt) were purified, small RNA library constructed, sequenced and analyzed. A total of 233 concatemer clones were sequenced to generate 479 sequences. The 479 sequences identified in the library represented 41% miRNAs, 12% mRNA, 12% rRNA, 6.3% tRNA, 6.0% repeat associated siRNA, 2.7% small antisense RNA, 3.5% tiny noncoding RNA, 1% small nuclear RNA and 15.2% sequences that did not match to bovine genome. A total of 196 sequences were found to be miRNA like molecules, of which 74 revealed distinct miRNAs. Out of these 74 miRNAs, 36 were found to be reported in miRBase 12.0 for different species including bovine, 14 were registered only in other species and 24 were completely new. All in all, 22 of the 74 miRNAs were cloned for three or more times where, let-7a, let-7b, let-7c, miR-21, miR-23b, miR-24, miR-27a, miR-126 and miR-143 were cloned 10, 28, 13, 4, 11, 7, 6, 4 and 11 times, respectively. This multiple cloning reflects the abundant expression in the ovary and potential involvement in ovarian functions. In addition to miRNAs, small RNA library has enabled us to identify 57 different endogenous siRNAs.

The expressions of 47 miRNAs were analyzed in 11 different bovine tissues using semi-quantitative RT-PCR. Among these, 44 were detected in both ovarian cells and multiple tissues. Seven miRNAs (bta-mir-18a, bta-mir-29a, bomir-140, bta-mir-199, bomir-378, bomir-F0132 and bomir-F2422) were found to be expressed at relatively higher levels in ovarian cortical portion. On the other hand, all undetected or less expressed miRNAs in ovarian cortex have been shown to be highly expressed in cumulus cells or corpus luteum. Bta-mir-15b, bomir-409, bomir-652, bomir-C0533 and bomir-D1431 were highly expressed in the fetal ovary compared to that of adult ovarian cortex. However, bta-mir-29a, bta-mir-199 and bomir-F2422 were found to be expressed at higher level in the adult ovarian cortex than that of the fetal ovary. Bta-mir-125b, bta-mir-222, bomir-542, bomir-652, bomir-H0222, bomir-F0522, bomir-C1931 and bomir-A2143 were found to be expressed at very low level, but as abundant in the cumulus cells and matured corpus luteum. All these investigated miRNAs were detected at least in one of the non-ovarian somatic tissue. Bta-miR-29a was localized in ovarian section by in situ hybridization and found to be expressed in different intra-ovarian cells of different stages of development.

miRNA target gene prediction and analysis by in silico method has identified the major biological processes and signaling pathways in the ovary that are most likely affected by a group of miRNAs. From the screened target genes, let-7b, mir-15b, mir-18a, mir-29a, mir-101, mir-125b, mir-126, mir-143, mir-145, mir-199a and mir-222 were found to have the highest number and overlapping targets. Gene Ontology analyses of the miRNAs target genes were found to be associated with reproductive system development, function and disorders. These include cell cycle, morphology, cell death, cell to cell signaling, cellular growth, development and proliferation, DNA replication, recombination & repair, endocrine system disorder and different pathways underlying the ovarian functions. So, the results of the first study suggest the presence of miRNAs in the bovine ovary, thereby elucidate their potential role in regulating diverse molecular and physiological pathways underlying the ovarian functionality.

The second study carried out to identify the expression pattern of miRNAs in the placenta at day 50 derived from SCNT, IVP and AI pregnancy. The reasons or background behind this study was the abnormal placentogenesis in SCNT and IVP which were widely admitted due to genetic and epigenetic modifications. Since,

miRNAs have been evidenced as one of important modifiers of genetic and epigenetic gene regulation so, this study was aimed to elucidate the differences in expression profile of miRNAs in the placenta of different sources of pregnancies by quantifying 377 miRNAs using qRT-PCR. The miRNAs expression profiles of the Day 50 NT placenta (n=3) were compared either to that of AI (n=3) or IVP (n=3) placenta. In addition, level of miRNAs expression in the IVP placenta has subsequently been compared to that of AI placenta. Of the 377 miRNAs used in the comparison of their expression between Day 50 NT placenta and AI placenta, 320 miRNAs were found to be downregulated in NT placenta with 2 or more fold change, while only 5 miRNAs were found to be upregulated by 2 or more fold change in NT placenta compared to the placenta of AI group. The comparison revealed that about 68% of the miRNAs tested in this analysis were differentially regulated in Day 50 NT placenta compared to that of AI, where 98% of them were found to be downregulated. This difference in expression of miRNAs could be due to the cumulative effect of embryos which were constructed by somatic cell nuclear transfer, treatment, in vitro culture and transfer to the recipients.

Similarly, about 62 % miRNAs were found to be differentially regulated in the NT Day 50 placenta compared to that of IVP placenta, where 90% of them were found to be downregulated. This difference could be due to effect of embryos constructed by nuclear transfer and transferred to the recipients for establishing the NT pregnancy. On the other way, 36% miRNAs were differentially regulated between IVP and AI placenta, where about 96% of these differentially regulated miRNAs were downregulated in IVP placenta. This difference in the expression of miRNAs could be attributed to the effect on the transfer of embryos which were fertilized and culture in vitro.

Additionally, the expression profiles of Day 50 NT placenta were compared to that of fibroblast donor cells which revealed 209 differentially regulated miRNAs Among the 209 miRNAs, 185 and only 24 miRNAs were found to be up and down regulated, respectively in the NT placenta compared to fibroblast. So it could be postulated that most of the miRNAs were reprogrammed in NT placenta. However, but due to their lower expression compared to that of AI it is evident that they were not reprogrammed in a correct manner. Most of the miRNAs were found be deregulated and affected as large chromosomal cluster and miRNA families. Further, cell specific localization of

miRNAs in the expanded blastocysts and expression profiling in different developmental stages of embryos and placenta derived from AI, IVP and SCNT by real time qRT-PCR identified that the major difference in miRNAs expression arises at day 50 of pregnancy. Analysis of global DNA methylation revealed common hypomethylation in the Day-16 elongated embryos and Day-50 placentas from NT, IVP and AI pregnancy with no significant differences in level among them. So, deregulation of miRNAs were found to be less dependent on global DNA methylation, rather aberrant miRNA processing molecules were evident as one of the major causes. Among the aberrant expression of miRNAs processing regulatory molecules Eukaryotic transcription initiation factor 2C2 (EIF2C2) or AGO2 were found to be downregulated both in NT and IVP Day-50 placenta compared to that of AI. Hence, deregulation of AGO2 could be a reason for global down regulation of miRNAs in the NT or IVP placenta, as AGO2 knock out animals evident with such deregulation of miRNAs.

Taken together, present findings suggest that the genome wide aberrant expression of miRNAs due to reprogramming error of miRNA processing regulatory molecules for example AGO2 or aberrant regional chromatin remodeling or imprinting problem or other mechanism if there is any in the NT placenta may result abnormal transcriptional regulation leading to the early stage of pregnancy loss.

6 Conclusion and future perspectives

Results of the first study revealed the presence of different classes of miRNAs in the bovine ovary, thereby elucidate their potential role in regulating diverse molecular and physiological pathways underlying the ovarian functionality. The presence of distinct miRNAs and other small RNAs, with different expression patterns and various target genes in bovine ovary suggest the potential role of such miRNAs in follicular development in particular and female fertility in general. Further functional characterization of some selected miRNAs including expression profiling and in situ localization in the ovarian follicles at different cyclic stages will supplement the results of this study and help to get insight into their specific roles in the ovarian function. Moreover, the results of this study will help to identify candidate miRNAs targeting specific molecular and cellular pathways important for ovarian follicular development, atresia, ovulation as well as ovarian dysfunction. The results of the second experiment revealed a massive deregulation of miRNAs in the placenta from cloned and IVP pregnancies. Most of the miRNAs were found as poorly reprogrammed and affected as large chromosomal cluster and miRNA families. Taken together, present findings suggest that the genome wide aberrant expression of miRNAs due to reprogramming error of miRNA processing regulatory molecules for example AGO2 or aberrant regional chromatin remodeling or imprinting problem in the placenta from nuclear transfer pregnancy, which may result abnormal transcriptional regulation leading to pregnancy loss. Moreover, the result of this study will help to move one step closer towards the development of a genetic screen to select healthier genetic profiles in cloned embryos and will enable to improve the pregnancy outcome from nuclear transfer embryos. Additional research is needed to address whether the low efficiency and abnormal placentogenesis in clone pregnancies are caused by the deregulation of miRNAs during the first trimester.

Collectively, the present study has discovered miRNAs in the bovine ovary and elucidated the pattern of expression of miRNAs along with their regulatory mechanism in the placenta derived from pregnancies of various origins. Being an important gene regulator, miRNAs could be an interesting avenue to resolve lot of questions on different regulatory mechanisms of ovarian folliculogenesis and aberrant genetic or epigenetic modification in the placenta from NT or IVP pregnancies along with ruminant's other reproductive processes.

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Acknowledgements

First, I would like to express my earnest gratitude to Professor Dr. Karl Schellander, the director of the Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn for providing me with the opportunity to study with a position of scientific research fellow in his Institute and for supervising me to pursue the proposed Doctoral degree. Moreover, I am grateful to him for teaching me clarity of valuable thought in scientific discussions and for giving me lots of extra curricular supports, being consistently helpful and encouraging during my whole study period which was essential for the completion of this work.

I would like to thank Prof. Dr. rer. nat. Brigitte Schmitz, Institute für Tierwissenschaften Abteilung Biochemie, University of Bonn for her willingness, kind eavaluation and assistance as co-supervisor of this work.

I am very grateful to Dr. Dawit Tesfaye for supervising my lab work, for tutoring, providing me with necessary things whatever needed, helping to solve the problem whenever I encounter, for always being a ready listener, for the critical reading of my all documents and thesis, for helping me in solving any problems, for his extra supports for either oriented to lab or not and encouragement from beginning to end of my study. His technical expertise and knowledge made my easier fruitful throughout these years. Their scientific and technical advices were essential for the successful completion of this work.

I want to acknowledge Dr. Michael Hölker along with Ms. Franca Rings and other stuffs in the Frankenfrost research station for important ideas on experimental design, valuable disscussion, providing valuable experimental sample and encouragement. I feel thankful to Prof. Dr. Christian Looft, Dr. Ernst Tholen and Dr. Chrawath Phatsara for continuous encouragement and stimulating comments during my study. I would like to thank all the members of the administration, Institute of Animal Science, University of Bonn, especially Ms. Bianca Peters and Ms. Ulrike Schröter for their kindly helping me with all documents, accomplishing necessary formalities including Mr. Peter Müller for advice on computer. I would like to thank Ms. Christiane Neohoff and Ms. Maren Julia Pröll for helping me with the German version of the part of my dissertation. Moreover, I thank with all my heart the people who have been there for me in the good times.

Many thanks go to all technical assistant especially Ms. Nadine Leyer, Ms. Jessica Gonyer, Ms. Helga Brodeßer, Ms. Birgit Koch-Fabritius, Ms. Steffi Heußner, Ms. Katrin Bauch and Mr. Stefan Knauf for their technical help, for answering numerous questions, supporting lab assistance, providing good working environment and for sharing wonderful and interesting events as well. I would like to recognize my previous fellows Dr. Nasser Ganem, Dr. Alemu Reggasa Hunde, Dr. Parinya Wilaiphan, Dr. Saowaluck Yammuen-art and Dr. Dessie Salilew Wondim for their good contribution on my hands on training for learning laboratory techniques, solving research oriented problem, valuable suggestion and for sharing woderful moments.

In addition, I am so grateful to my wonderful friends, Mr. Mehmet Ulas Cinar, Dr. Abdullah Mohammadi-Sangchesmeh, Ms. Anke Brings, Mr. Dagnachew Hailemariam, Mrs. Simret Weldenegodguad, Mrs. Pritam Bala Sinha, Mrs. Walaa Abd-El-Naby, Ms. Autchara Kayan, Mr. Watchara Laenoi, Ms. Kanokwan Kaewmala, Mr. Ahmed Yehia Gad, Mr. Ijaz Ahmad, Mr. Huitao Fan, Ms. Christine Große-Brinkhaus, Ms. Hanna Heidt, Mr. Luc Frieden and Ms. Eva Held for the good time we had together to share our experiences, fun and problems. My especial thank goes to Mr. Muhammad Jasim Uddin, Mr. Ariful Islam, Dr. Abdul Gaffer Miah, Dr. Umme Salma, Mr. Md. Mahmodul Hasan Sohel as friends, as seniors and being Bangladeshi for making friendly environment just like home in abroad.

I am indebted to all the people in the lab not mentioned here by name for providing a warm and friendly environment during my study and for their patience and helping for my poor German language.

Last, and most heartfelt, I would like to thank my beloved parents, family members, my wife and my son for their endless love and infinite support during the whole study period in abroad without which it could not be possible to finish my study successfully.

Above all, many thanks to almighty Allah for providing me with everything for my life and without whom nothing is possible in any cases.