The noncoding genome: implications for ruminant reproductive biology

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Advances in the analyses of human and other higher eukaryotic genomes have disclosed a large fraction of the genetic material (ca 98%) which does not code for proteins. Major portion of this non-coding genome is in fact transcribed into an enormous repertoire of functional non coding RNA molecules (ncRNAs) rather than encoding any proteins. Recent fascinating and fast progress in bioinformatic, high-throughput sequencing and other biochemical approaches have fuelled rapid growth in our appreciation of the tremendous number, diversity and biological importance of these ncRNAs in the hidden layer of gene regulation both at transcriptional and post-transcriptional level. Broadly ncRNAs fall into three size classes namely, ~20 nucleotides for the large family of microRNAs (miRNAs), to 25-200 nucleotides for other different families of small RNAs and finally to over thousands of nucleotides for macro ncRNAs involved in eukaryotic gene regulation. Among the ncRNAs that have revolutionized our understanding of eukaryotic gene expression, microRNAs (miRNAs) have recently been emphasized extensively with enormous potential for playing a pivotal role in disease, fertility and development. They are found to be potentially involved in various aspects of physiological regulation of reproductive tissues (testis, ovary, endometrium and oviduct), cells (sperm and oocytes) and embryonic development in addition to other body systems. Here, we review the recent work on miRNAs in details and some other small ncRNAs briefly in animal models focusing on their diverse roles in the physiology of reproductive cells and tissues together with their implications for ruminant reproductive biology.

Introduction

Proteomic analysis of genome sequences in the past highlighted only mRNA-coding genes and non-protein-coding transcripts were often overlooked. Genomic analysis in the last decade however revealed that with an increase in genome complexity, the protein coding fractions of genome is much fewer compared to non-coding portion. It is estimated, that around 98% of the transcriptional outputs of eukaryotic genomes consist of large proportion of RNAs, which do not encode proteins (Adams *et al.* 2000). This vast untranslated fraction of the genome harbors thousands of genes which lead to transcription of a remarkable number of functional non-coding RNAs (Mattick & Makunin 2006). Beside the initial discovery of the ribosomal

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RNAs, small nuclear RNAs and transfer RNAs that are involved in mRNA splicing and translation, many more classes and types of recently discovered ncRNAs are also known to be involved in the regulatory functions namely, but not limited to, transcriptional and post transcriptional gene regulation, chromosome replication, RNA processing, site-specific RNA modification, DNA methylation, telomere synthesis and length differentiation, protein degradation and protein translocation (Storz 2002, Hannon *et al.* 2006). Ongoing identification of new classes of non-coding RNAs (ncRNAs) and new member of existing classes presently underscores the paramount importance of ncRNAs function at many levels essential for gene expression and genome stability.

Types of ncRNAs are emerging from occasional discoveries with varying in size, mechanisms of biosynthesis and their regulatory mechanisms. However, their list is continuously and tremendously increasing and getting appreciation for their functional importance. Broadly, ncRNAs could be differentiated into three classes according to their range in size. Among them, the tiny one known as miRNAs which ranges in size about 20-24 nucleotides (nt) and have been found to modulate development of mammals and engaged in disease development as well as contributing to the fertility of different species through post transcriptional gene regulation. The group of ncRNAs ranging in size 100-200 nt are designated as small RNAs commonly found as translational regulators in bacteria and some other species as well. Lastly, the ncRNAs comprising the majority of longer transcripts to over 10000 nt in size are called macro ncRNAs involved in epigenetic regulation of gene expression in eukaryotes (Hutvagner & Zamore 2002, Storz 2002). In contrast to the uncertainty surrounding the function of most mammalian macro ncRNAs, imprinted macro ncRNAs have clearly been identified as regulator of flanking genes by DNA methylation (Koerner *et al.* 2009). Small non coding RNAs such as miRNAs, short interfering RNAs, piwi-interacting RNAs and short nucleolar RNAs are associated with trans-acting functions, whereas macro ncRNAs are so far only associated to cis-acting functions.

However, as knowledge on the types and the members of each type are still limited, most of these biochemically abundant species of ncRNAs are yet to be discovered. It is likely that there are many more ncRNAs than was ever suspected. Here we review the recent reports on the small non-coding RNAs with particular emphasis on miRNAs in details and some other selected small ncRNAs briefly in animal models focusing on their diverse roles in the physiology of reproductive cells (germ cells) and tissues (testis, ovary, endometrium, oviduct and embryo) together with their implications for ruminant reproductive biology.

Small non-coding RNAs and gene regulation

The notion of the sncRNAs is not new - for example 5S rRNAs, U6 RNA, snoRNAs, BC200 RNA, etc. were discovered long before, but they are only recently highlighted because of growing list of classes and members of sncRNAs which are found to be physiologically important as riboregulators. A short list of several classes of sncRNAs in different species with their potential functions are presented but not limited to Table 1. Among these all ncRNAs, miRNAs and some other small ncRNAs have revolutionized our understanding of a hidden layer of new gene regulation now-a-days. MiRNAs are the well characterized ones getting more attention to the scientific community due to their high level of importance. Diverse expression pattern of miRNAs and high number of their potential target mRNAs suggest their involvement in the regulation of various developmentally related genes at post-transcriptional level (Lau *et al.* 2001, Lai 2003, Ambros 2004, Bartel 2004, Alvarez-Garcia & Miska 2005, Plasterk 2006, Chen & Rajewsky 2007). The tiny (18-24 nt in length) and single-stranded, derived from primary transcripts termed as "pri-miRNAs", having an RNA hairpin structure of 60-120 nt with a mature

Table 1. Classes of small ncRNAs (≤ 200 nt) in different species with their functions

Class of sncRNA	Length (nt)	Potential/probable function	Example species	Reference
Small interfering RNA (siRNA)	19–21	Target mRNA cleavage	Reported in many species	(Fire *et al.* 1998), Reviewed in (Rana 2007)
Trans-acting siRNA (tasiRNA)	21–22	mRNA cleavage	Arabidopsis	(Peragine *et al.* 2004, Vazquez *et al.* 2004)
MicroRNA (miRNA)	19–25	Translational repression	Mammals, Birds, flies, Nematodes, etc. (conserved)	(Lee *et al.* 1993, Bagga *et al.* 2005)
Repeat-associated siRNA (rasiRNA)	24–27	Transposon control, transcriptional silencing	Yeast, plants & flies	(Aravin *et al.* 2001, Reinhart & Bartel 2002, Volpe *et al.* 2002, Grewal & Moazed 2003, Carrington 2005)
Piwi-interacting RNA (piRNA)	26–31	Transposon control in germ cells	Mammals	(O'Donnell & Boeke 2007)
Small-scan RNA (scnRNA)	~28	Histone methylation, DNA elimination	Tetrahymena	(Mochizuki *et al.* 2002)
gCYb gRNA	68	Directs insertion & excision of uridines	*T. brucei*	(Kable *et al.* 1997, Souza *et al.* 1997, Simpson *et al.* 2000)
RyhB sRNA	80	Targets mRNAs for degradation	*E. coli*	(Masse & Gottesman 2002)
DsrA sRNA	87	Preventing formation of an inhibitory mRNA structure	*E. coli*	(Wassarman *et al.* 1999, Altuvia & Wagner 2000)
U18 C/D snoRNA	102	Directs 29-0-ribose methylation of target rRNA	*S. cerevisiae*	(Samarsky & Fournier 1999, Kiss 2001)
OxyS	109	Represses ranslation by occluding ribosome binding	*E. coli*	(Wassarman *et al.* 1999, Altuvia & Wagner 2000)
4.5S RNA	114	Protein translocation	*E. coli*	(Wassarman *et al.* 1999, Keenan *et al.* 2001)
6S RNA	184	Transcription: Modulates promoter use	*E. coli*	(Wassarman *et al.* 1999, Wassarman & Storz 2000)
U2 snRNA	186	RNA processing: Core of spliceosome	Human	(Gu *et al.* 1998, Will & Luhrmann 2001)
BC1 RNA	142–165	Amplification of short interspersed elements	Rodents	(Shen *et al.* 1997)
snR8 H/ACA snoRNA	189	Directs pseudouridylation of target rRNA	*S. cerevisiae*	(Samarsky & Fournier 1999, Kiss 2001)
BC200 RNA	195–205	Encodes a neural small cytoplasmic RNA	Primates, human	(Martignetti & Brosius 1993)

miRNA in one of the two strands (Fig. 1). 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 (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 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 to 7- to 8-nt region of 5´ end of miRNA called seed sequence, which primarily results in inhibition of mRNA translation (Pillai *et al.* 2005) (Fig. 1). Blocking the translation of mRNAs occurs through interaction of RISC with eukaryotic translation initiation factor 6, which prevents assembly of 80S ribosomes (Chendrimada *et al.* 2007), or through inhibition of translation after initiation (Jackson & Standart 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 & Standart 2007) or changes in proteins associated with RISC can cause a shift from translational inhibition to translational enhancement (Vasudevan *et al.* 2007, Orom *et al.* 2008). 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.

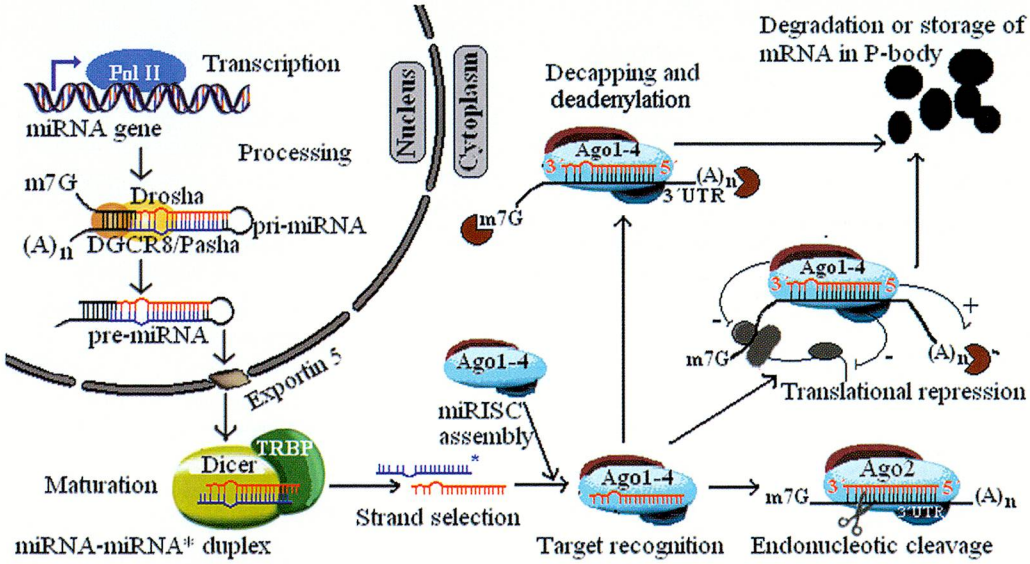

Fig. 1. Biogenesis of miRNAs and their mechanism of gene regulation.
Pri-miRNAs, which are generally synthesized by RNA polymerase II, are trimmed in the nucleus by Drosha to generate a ~60-nt pre-miRNA which is exported to the cytoplasm, where it is further processed by Dicer, in association with one of the four mammalian Argonaute proteins (Ago) and TBRP. Target mRNAs are recognized by miRNAs in the form of ribonucleoprotein complexes (miRNPs) through sequence complementarity, usually between the miRNA and sequences in the 3´-UTR of the mRNA. 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 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. 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). 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 & Jackson 2007).

Despite the fact that animal miRNAs, which are the focus of this review, have a significant importance in the reproductive process, the other types of small noncoding RNA with distinct properties also deserve more attention. Small interfering RNAs (siRNAs) differ from miRNAs mainly in their Origin. They are the products of long, Dicer-processed, double-stranded (ds) RNAs that silence genes by cleaving their target mRNAs (Fig. 2A) [reviewed in Reference (Chu & Rana 2007)]. The RNAi was first discovered by introduction of long ds RNAs into C. elegans (Fire *et al.* 1998). Like endogenous miRNAs, long dsRNAs are processed by the Dicer-TRBP-PACT complex [reviewed in Reference (Chu & Rana 2007, Rana 2007)]. This dsRNA-processing step creates RNA with 2-nt overhangs at their 3′ ends and phosphate groups at their 5′ termini. The anti-sense strand of siRNA, known as the guide strand serves as the template for sequence-specific gene silencing by the RNAi machinery (Fig. 2A). The sense strand is known as the passenger strand. Subsequent to Dicer processing, the 21–23 nt guide strand of duplex siRNA is loaded into Ago2 to form the effector siRISC. Ago2 is the endonuclease responsible for the cleavage activity of siRISC. With perfect base pairing and formation of an A-form helix structure between the siRNA guide strand and its target mRNA, siRISC cleaves its target 10–11 nt from the 5′ end of the guide siRNA strand, and the complex is recycled for the next round of target mRNA cleavage. mRNAs cleaved by siRISC are subsequently degraded by cellular exonucleases, resulting in robust depletion of target genes [reviewed in (Chu & Rana 2007, Rana 2007)].

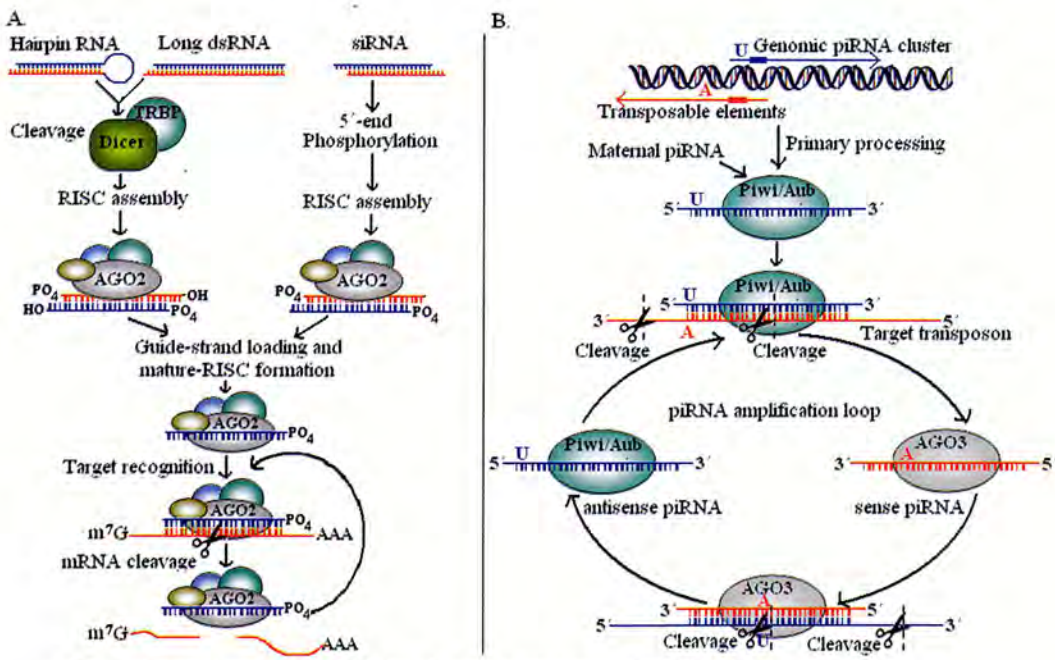

Fig. 2. Gene silencing by siRNAs (A) and biogenesis of piRNAs (B).

Piwi-interacting RNAs (piRNAs) are a third group of small RNAs (24- to 30-nt) generated by a Dicer-independent mechanism and are associated with members of the Piwi family, a subtype of Argonaute proteins with MIWI, MILI, and MIWI2 orthologs (Kuramochi-Miyagawa *et al.* 2001, Aravin *et al.* 2006, Lau *et al.* 2006, Watanabe *et al.* 2006). PiRNAs can be derived from either transposons and other repeated sequence elements or complex DNA sequence elements (Aravin *et al.* 2007, Brennecke *et al.* 2007, Houwing *et al.* 2007). Those piRNAs which are

derived from repeated sequence elements are specifically designated as repeat-associated small interfering RNAs (rasiRNAs). Based on the studies in Drosophila and mouse, piRNA has been found to be produced like ping-pong manner, in which Ago3 bound to sense-strand piRNAs catalyzes antisense-strand cleavage at an A:U base-pair that generates the 5′ end of antisense piRNAs (Fig. 2B) (Aravin *et al.* 2007, Brennecke *et al.* 2007, Gunawardane *et al.* 2007). The 5′ ends of the resulting cleavage products are proposed to be associated with Aub or Piwi, with nucleolytic processing of the 3′ overhangs generating mature 23- to 30-nt antisense piRNAs (Fig. 2B). The mature antisense piRNA Argonaute complexes are then proposed to bind and cleave sense strand RNAs, silencing gene expression and generating the 5′ end of sense-strand piRNA precursors that will be associated with Ago3. Processing of the 3′ overhang produces mature sense-strand piRNAs, completing the cycle (Fig. 2B) reviewed by (Klattenhoff & Theurkauf 2008). Genetic studies in mice, *Drosophila* and zebrafish showed that piRNAs are crucial to germline development (Kuramochi-Miyagawa *et al.* 2004, Carmell *et al.* 2007, Houwing *et al.* 2007) and proteins involved in piRNA production have also been implicated in the control of gene expression in somatic cells (Pal-Bhadra *et al.* 2004, Grimaud *et al.* 2006).

Function 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, Wienholds *et al.* 2003, Giraldez *et al.* 2005) and female infertility in mouse (Otsuka *et al.* 2007, Otsuka *et al.* 2008). 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. Accounting the studies and approaches published so far, the following sub-sections describe the role of miRNAs with respect to reproductive biology.

Function of miRNAs in the female reproduction

Expression and regulation of miRNAs in the mammalian ovarian cells and their function

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 (Ro *et al.* 2007a, Hossain *et al.* 2009). 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), adult bovine ovary (Hossain *et al.* 2009) and new born mouse ovary (Ahn *et al.* 2010) through small RNA library construction and sequencing. Regardless of species these studies showed that let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145 and miR-199b to be most commonly abundant miRNAs in the ovary. The presence of miRNAs and their differential expression can give the primary clue for their potential role in ovarian function. However, further functional characterization of these miRNAs in different cell types of ovary (oocyte, granulosa, theca cells and ovarian stroma) at different follicular stage or at different estrus cycle remains to be elucidated. Although bioinformatic prediction and analysis of ovary specific mRNAs targets for these enriched miRNAs revealed several molecular and cellular pathways and physiological functions important for ovarian follicular development (Hossain *et al.* 2009), atresia, ovulation as well as ovarian dysfunction, the identification of functional target mRNAs remains to be validated by appropriate wet lab experiment.

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 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 approach. For example, the differential expression of miRNAs has been identified during bovine oocyte maturation and preimplantation embryo development in-vitro using the heterologous approach (Tesfaye *et al.* 2009).

The Microarray experiments show that Dicer1 is highly expressed and functionally important in the oocytes during folliculogenesis as well as in the mature oocytes (Su *et al.* 2002, Choi *et al.* 2007, Murchison *et al.* 2007). 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). Moreover, transcriptional analysis through microarray experiments has identified the major portion of the transcripts as misregulated in Dicer1-deficient oocytes. 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). However, Suh *et al.* studied the effect of deletion of another miRNAs processing molecules called Dgcr8 and revealed contrasting conclusion that the effects on the phenotypes in Dicer deficient oocytes are rather due to endogenous siRNAs (Suh *et al.* 2010). Moreover, the expression level of miRNAs in Dgcr8 deficient oocyte found to be reduced as similar to the Dicer deficient oocyte. In addition, there was no effect due to deletion of Dgcr8 allele even from maternal and zygotic genome on the phenotype as well as mRNA profile which were very unlikely for Dicer deficient oocytes. These findings show that miRNA function is globally suppressed during oocyte maturation and preimplantation development.

The progress of the study on miRNAs is higher in case of granulosa cells compared to oocyte and other ovarian cell types. For example, study of expression of miRNAs by Fiedler et al. (2008) in mouse mural granulosa cells collected before and after an ovulatory dose of hCG identified miR-132 and miR-212 as highly upregulated following LH/hCG induction.

Further analysis of these two miRNAs in cultured granulosa cells revealed the roles in the post-transcriptional regulation of CtBP1 gene which is known to be interacting with steroidogenic factor-1 and acts as a co-repressor of nuclear receptor target genes. Recently, studies have been conducted to know the role of miRNAs in human granulosa cells (GC) by transfecting 187 individual synthetic miRNA precursors that mimic endogenous precursor miRNAs representing the majority of human miRNAs (Sirotkin *et al.* 2009a). Interestingly, they have screened 80 miRNAs which control both proliferation and apoptosis in ovarian granulosa cells, as well as they have identified miRNAs which promote and suppress these processes utilizing a genome-wide miRNA screen. Transfection of cultured human granulosa cells with 11 out of 80 tested miRNA constructs resulted in significant increase in percentage of cells containing PCNA a cell proliferation marker. These were mir-108, mir-7, mir-9, mir-105, mir-128, mir-132, mir-141, mir-142, mir-152, mir-188 and mir-191. Eleven out of the 80 miRNAs tested in the same experiment (mir-15a, mir-96, mir-92, mir-124, mir-18, mir-29a, mir-125a, mir-136, mir-147, mir-183 and mir-32) found to promote up to 2-fold accumulation of Bax - proapoptic marker in human primary granulosa cells. However, the detailed regulatory mechanism for regulating such two processes through targeting which mRNAs by the individual miRNAs are unknown and remains to be disclosed in future investigation.

The most recent work highlighted one miRNA (miR-224) in detail for regulation of granulosa cell proliferation and thereafter has shown to affect ovarian estrogen release in mouse (Yao *et al.* 2010). In that experiment miR-224 expression was found to be regulated by TGF-ß/Smads pathway through inhibiting TGF-ß superfamily type I receptors (SB431542) which leads to blockage of phosphorylation of the downstream effectors Smad2/3 in vitro in granulosa cells. The ectopic expression of miR-224 was suggested to enhance TGF- ß 1-induced granulosa cell proliferation through targeting Smad4. This was a good demonstration for the notion that miRNAs could control or promote TGF- ß 1-induced GC proliferation and ovarian estrogen release. However, there are many more miRNAs and their mechanism involved in the function of granulosa cells is still remaining to be elucidated. So, to further clarify the role of miRNAs in oogenesis and folliculogenesis, generation of knockouts or knocking down the individual miRNAs could help to understand their critical roles in ovarian development as well as ovarian cellular functions. 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.

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 first 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 was 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.* 2009b). 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.* 2009b). 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 was also evaluated. 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.* 2009b). However, 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.

Role of miRNAs in other female reproductive tissues and disease conditions

Both physical and functional integrity of the oviduct is responsible for the transport and protection of the oocyte during fertilization and early embryo development through shuttle the oocyte/embryo toward the uterus and secreting necessary proteins. As the activity of this organ largely depends on the level of estrogen and progesterone, it is likely that transcriptional regulation for the cyclic phenotypic changes of the oviduct and uterus could be under control of miRNAs as evidenced in the ovary. But, the expression and regulatory network of miRNAs for the physiology of oviduct is still an open field for investigation. The loss- or gain-of-function studies of Dicer have evidenced primarily the importance of miRNAs for oviductal functions. Conditional inactivation of *Dicer* in the mesenchyme of the developing Müllerian ducts, in ovarian granulosa cells and mesenchyme-derived cells of the oviducts and uterus revealed female sterility in mouse. Several other reproductive defects including decreased ovulation rates, compromised oocyte and embryo integrity, prominent bilateral paratubal (oviductal) cysts, adenomyosis, shorter and hypotrophic oviduct and uterus have been reported in mouse (Hong *et al.* 2008, Nagaraja *et al.* 2008, Gonzalez & Behringer 2009). Thus, findings revealed diverse and critical roles of Dicer and its miRNA products for postnatal differentiation, development and function of the female reproductive tract as well as female fertility. However, expression and functional characterization of individual miRNAs for the physical and functional integrity of oviduct is yet to be investigated.

The uterus, which undergoes cyclic changes throughout the menstrual or estrous cycle during embryo implantation, is also largely dependent on ovarian steriod. 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 this uterine change through regulating or interfering the post transcriptional and translational activity of vast number of genes which are supported by the initial conditional inactivation of Dicer studies. In addition, several studies have reported the regulation of miRNAs in the endometrium by the ovarian steroid (Pan *et al.* 2007, Toloubeydokhti *et al.* 2008, Macias *et al.* 2009). Differential expression of miRNAs in endometrial carcinogenesis and between uterine leiomyoma versus normal myometrium has been studied (Boren *et al.* 2008, Marsh *et al.* 2008). Differential expression of miRNAs in endometrium of women with and without endometriosis has been evidenced and revealed importance of miRNAs in normal endometrial cellular activities, pathogenesis of endometriosis and associated reproductive condition (Pan *et al.* 2007, Teague *et al.* 2009). Additionally, a reduced expression of miR-199a and miR-16 may work synergistically to promote an inflammatory environment by up-regulating COX-2 protein levels, thereby promoting prostaglandin production, neoangiogenesis and estradiol mediated cellular proliferation in endometriotic tissues (Teague *et al.* 2009). So, in addition to the study of conditional inactivation of Dicer, studies on the identification of miRNA expression in the normal & diseased uterus and the characterization of some individual miRNA in the uterus (normal & endometriotic) as well as in the uterine implantation site (discussed in previous section) has shade initial light onto the importance of miRNAs regulating physiological changes of the uterus in response to steroids and pregnancy as well as in pathogenic condition.

In addition to their importance in the regulation of normal ovarian physiology as described in the previous section, recently it has become evident that miRNAs play a major role in ovarian tumorigenesis. Several miRNA expression profiling studies have identified changes in miRNA patterns that take place during ovarian cancer development (Iorio *et al.* 2007, Dahiya *et al.* 2008, Giannakakis *et al.* 2008, Laios *et al.* 2008, Nam *et al.* 2008, Yang *et al.* 2008a, Zhang *et al.* 2008, Wyman *et al.* 2009, Bendoraite *et al.* 2010). Candidate miRNAs which were found to be most commonly altered in ovarian carcinoma compared to normal tissue from different study (observed at least in three experiments) are let-7 family, miR-100, miR-106b, miR-10b, miR-125b, miR-143, miR-145, miR-155, miR-15a, miR-199b, miR-200a, miR-200b, miR-200c, miR-21, miR-22, miR-222, miR-368, miR-424 and miR-99a. The majority of these deregulated miRNAs including miR-15a, miR-34a, miR-34b ; miR-210 and let-7 family were found to be down-regulated in human ovarian cancer, hence suggested to act as tumor suppressor and thereby represent potential targets for therapy (Johnson *et al.* 2005, Giannakakis *et al.* 2008, Kumar *et al.* 2008, Zhang *et al.* 2008). The downregulation of major miRNAs in the epithelial ovarian cancer has been found due to both genomic losses and epigenetic alterations (Zhang *et al.* 2008). Further understanding the underlying mechanisms of how miRNAs are regulated in normal or disease condition together with identification of their specific target genes and their functions might lead to increase reproductive efficiency and the development of preventive or therapeutic strategies by regulating specific target genes associated with such reproductive disorders.

MiRNAs in the male reproduction

Importance of miRNAs in male reproduction has been shown by analyzing the expression and regulation of miRNAs in the testicular cells with their putative functions. MiRNAs were first detected from the testis during establishing the techniques reliable for genome-wide miRNA

profiling (Barad *et al.* 2004, Liu *et al.* 2004). A number of miRNAs differentially expressed during testicular development and bioinformatic identification of several possible male germ cell target mRNAs has been reported (Yu *et al.* 2005). Further analysis revealed mir-122a targeting transition protein 2 (Tnp2) mRNA, a testis-specific and post-transcriptionally regulated mRNA in postmeiotic germ cells first suggested the miRNAs mediated posttranscriptional regulation in the mammalian testis. Small RNAs cDNA library constructed and identified 52 distinct miRNAs as well as other small noncoding RNAs (rasiRNAs and gsRNAs) in the testis (Watanabe *et al.* 2006). The evidence for the potential involvement of the miRNA pathway in the regulation of male germ cell (GC) development were reported by localizing testis-expressed miRNAs (miR-21, let-7a, miR-122a), in the chromatoid body of male GCs and expected to have control in post-meiotic GC differentiation (Kotaja *et al.* 2006). In 2007 Novotny and his coworkers lay out the potential involvement of miRNAs in post-transcriptional regulation in the testis by the miR-17-92 cluster during meiotic recombination (Novotny *et al.* 2007). In the same year several individual efforts were made to clone miRNAs from the testes in a large scale. Through small RNA cloning method Ro et al. (2007a) identified 141 miRNAs from the mouse testis including 29 novel miRNAs and from the pattern of expression they have suggested twenty eight candidate miRNAs which are preferentially (22) or exclusively (6) expressed in the mouse testis for further functional studies. Comparison of miRNAs pattern between immature and mature mouse testes through miRNA microarray (with 892 miRNA probes) identified 19 significantly different miRNAs expression (Yan *et al.* 2007). Future studies ablating specific miRNAs using transgenic technologies or by other suitable approach will help us better understand the role of individual miRNAs in gonadal development. The expression patterns of several members of the miRNA pathway in the testis namely Dicer (Dcr), Drosha, Ago1, Ago2, Ago3 and Ago4 are identified to express in pachytene spermatocytes, round and elongated spermatids and Sertoli cells (Gonzalez-Gonzalez *et al.* 2008). Moreover, miRNAs were found to be localized to XY body of spermatocytes including the nucleolus of Sertoli cells (Marcon *et al.* 2008). The transgenic male mouse lacking Dcr in germ cells were found to be subfertile both due to the defect in the transition from round to elongating spermatids and production of sperm with abnormal motility (Maatouk *et al.* 2008). Recent study has identified that about 86% of X-linked miRNAs actually escape meiotic sex chromosome inactivation (MSCI) during spermatogenesis and transcriptional silencing of genes on X & Y chromosomes was found to occur in mid-to-late pachytene spermatocytes (Song *et al.* 2009). Further more, selective ablation of Dcr in Sertoli cells has led to infertility due to complete absence of spermatozoa and progressive testicular degeneration (Papaioannou *et al.* 2009). In the same study altered expression of several key genes such as Gdnf, Kitl, Man2a2, and Serpina5 which are essential for spermatogenesis, was revealed as a result of the miRNA mediated post-transcriptional control in the Sertoli cells leading to abnormal spermatogenesis. The existence, preferential and temporal differential expression of miRNAs and the involvement of their machinery genes especially Dcr in the mature and immature testis as well as in different testicular cells has evidenced the functional role of miRNAs in the physiology of testis. Despite various studies carried out on comparative expression analysis of hundreds of testicular miRNAs, there is a tremendous research gap in the investigation of exact functional role of specific miRNAs in the development and proliferation of germ cells in testis.

miRNAs regulation of embryonic development process and stem cells maintenance

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 gene 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 suggested 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 gene regulation. While critical roles for miRNAs biogenesis in the early embryonic development are well established, roles for individual miRNAs have only recently been investigated mostly in the mouse.

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. 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). Recent microarray based miRNAs expression profiling in elongated cloned and in vitro-fertilized bovine embryos has suggested that the reprogramming of miRNAs occurred in cloned bovine elongated embryos (Castro *et al.* 2010). 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.

Recent studies 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 & 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.* 2008, Sinkkonen *et al.* 2008) and lineage determination (Kanellopoulou *et al.* 2005, Wang *et al.* 2007, Ivey *et al.* 2008, Tay *et al.* 2008). 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.

miRNAs regulation of epigenetics in reproduction and early development

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. Very little is known about the miRNAs mediated epigenetic processes or epigenetic control of miRNAs expression, which are 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, controversial result were reported by recent studies, where the Dicer deficient stem cells were found to have reduced levels of both de novo DNA methylation and DNA methyltransferases (Dnmts) (Benetti *et al.* 2008, Sinkkonen *et al.* 2008) as well as increased telomere recombination and elongation (Benetti *et al.* 2008). These results supported 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 pluripotency 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 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 (Zhang *et al.* 2005,

Kim *et al.* 2008). 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 could 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.

Implication of sncRNAs for ruminant reproductive biology and challenges

Non-coding RNAs comprise the major part of the mammalian transcriptome and have been suggested to play an important role in the regulation of gene expression. They are important in most epigenetic mechanisms as is exemplified by the role of small RNAs in silencing of transposable elements, miRNAs in gene expression control, large RNAs in X-chromosome inactivation and DNA imprinting and "heritable" RNAs in non-mendelian epigenetic inheritance. Moreover, DNA methylation and histone modifications can be directed by different types of ncRNAs. Among the sncRNAs, 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.

Being an important gene regulator, miRNAs could be an interesting avenue to resolve lot of questions on different regulatory mechanisms of ruminant's reproductive process. Posttranscriptional gene regulation by miRNAs during the periods of ovarian follicular development, atresia and luteolysis could be an interesting field of investigation in ruminants. This is particularly interesting since the ovarian follicle is a complex structure composed of different types of cells that are functionally related and constantly changing and differentiating. Investigations are required but remains to be elucidated for the role of miRNAs in the interaction between granulosa and theca cells which are essential for biosynthesis estrogen. In vitro culture models for a single cell type (primary granulosa), co-cultures of theca and granulosa cells or whole follicle cultures could be utilized for elucidating such miRNAs mediated regulation to overcome the technical difficulties in in-vivo experiment. Identification of the whole set of miRNAs in different ovarian cells in ruminants is paramount important for any functional study. To accomplish this,

miRNAs microarray could be a useful approach either by using arrays from ruminants or by heterologous approaches by using platform from other species like mouse or human. A direct identification through construction of ovarian cell specific small RNAs library can also be an option. In addition to identification, miRNA microarray could be also useful to describe dynamic changes in miRNA transcript levels in closely related to regulatory events of gene expression for successful follicular development to explain how this is all managed by the different ovarian cell types. Although hundreds of genes, which are important for ovarian physiology, are predicted to be potential target of miRNAs (Hossain *et al.* 2009), all these targets should be validated to elucidate key points of such regulation. Thereafter, it might be possible to draw a fine description of the role of miRNAs in the molecular mechanisms of the dynamic processes occurring in these different compartments of ovary during follicular development and might provide insight into how we might be able to enhance reproductive efficiencies.

In the absence of transcription, synthesis of hundreds of new products and disappearance of many proteins during oocyte maturation after germinal vesicle breakdown and early embryogenesis indicating fine regulation of hundreds of transcripts by a mechanism other than transcription. These changes could possibly largely rely on and controlled by miRNAs, but it is still remains to be elucidated. In addition, it has been evidenced that the bidirectional interactions between oocyte and somatic cells control folliculogenesis. In this communication oocyte secretes soluble paracrine factors that act on its adjacent granulosa cells, which in turn regulate oocyte development in bi-directional communication axis (Gilchrist *et al.* 2004). Further investigations are required to know the role of miRNAs in paracrine signaling and gapjuntional exchange and control of regulatory molecules through intercellular communication between oocytes and granulosa cells.

A large number of target genes for a single miRNA and multiple miRNAs targeting the expression of one gene have been recognized as a major challenge in the assessment of the role of specific miRNAs and establishing precise miRNA-target networks. Moreover, the identification of functional targets represents a major hurdle in our understanding of miRNA function for complex phenomena of reproduction in different ruminant species due to lack of complete genomic information, suitable bio-informatic tools and difficulty to carry out in-vivo functional studies. A few number of knockout studies in mice have been carried out to show the involvement of regulatory miRNAs in mammalian reproduction. However, the knowledge on the functions of specific miRNAs from mouse knockout models cannot be systematically applied to ruminants. So, for large ruminant, the production of transgenic animals could help to elucidate miRNAs mediated regulation of reproductive process in vivo. However, the success of such approaches is limited due to technical difficulty, cost of making null miRNA transgenics and extended time frame required to observe the effect in reproductive processes in ruminants.

Presently, our understanding of non-coding RNAs specially miRNAs function in reproductive biology is very limited and much remains to be uncovered in this exciting field of investigation. Better understanding of small non-coding RNAs, especially miRNA-mediated regulatory effects could be potentially used for regulation of ruminant reproductive processes including fertility and for treatment of reproductive and other steroid-dependent disorders in near future and results can be applied in other species due to high level of conservation of miRNAs between species.

Conclusion

Non-coding RNAs comprise the majority of the mammalian transcriptome and have been suggested to play an important role in the regulation of gene expression. In contrast to the uncer-

tainty surrounding the function of most mammalian ncRNAs, imprinted macro ncRNAs have clearly been shown to regulate flanking genes epigenetically and small non-coding RNAs have been shown to have tremendous transcriptional regulation for normal physiology or disease condition of different types of tissues and cells. Among the sncRNAs, miRNAs are the well characterized one which could maintain the delicate balance between normal reproductive biology, system development and tissue maintenance versus deregulated growth and tumor formation. The studies on the role miRNAs in disease development are much extensive than on reproductive biology and furthermore very limited in ruminant species compared to human and mouse. Conditional Dicer1 knockout mice have been used to show the consequences that the lack of miRNA have on ovarian, testicular, oviductal, uterine, oocyte, and embryonic function and development. To date, much of the work on miRNAs has focused on expression profiling rather than their regulation and functional characterization within specific tissues and cells or during the reproductive process. However, this area of research is rapidly moving forward and it is expected that a lot of information regarding miRNA-mediated posttranscriptional gene regulation and their epigenetic regulation in ruminant reproduction biology will be known within the next several years. Studies to identify the specific miRNAs, their target genes and post transcriptional regulatory network will further shed light on the importance of specific miRNA both for the development and function of reproductive tissues as well as disease condition. Once relevant miRNAs and functional targets are identified, possible clinical use for these molecules will represent the next front line and may lead to novel strategies for better enhancing or manipulating reproductive efficiency.

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