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REVIEW

Characterization and importance of microRNAs in mammalian gonadal functions

M. M. Hossain · M. M. H. Sohel · K. Schellander · D. Tesfaye

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Abstract Recent progress in high throughput sequencing and bioinformatic analysis and other biochemical methods have fuelled our appreciation for the important role of microRNAs (miRNAs) in disease, fertility and development. These tiny RNAs were found to be potentially involved in various aspects of cellular processes of reproductive tissues by posttranscriptional regulation of protein coding genes. Mammalian gonads which exhibit strictly regulated spatio-temporal gene expression patterns are also known to express unique sets of miRNAs and genes involved in the miRNA biogenetic pathway. Studies on miRNAs and their associated processing enzymes have evidenced the contribution of these small regulatory RNAs to germ cell differentiation, post-meiotic male germ cell function and growth, and development and maturation of oocytes through pertaining tightly regulated gene expression. The existence, preferential and temporal expression of miRNAs and their processing machinery genes in different stages of testicular and ovarian cellular development have evidenced the potential role of miRNAs in testicular and ovarian physiology. MiRNAs are also found to be associated with functional regulation of gonadal somatic cells, namely Leydig cells and Sertoli cells in testis and granulosa cells/cumulus cells in the ovary in steroid synthesis. Here, we review the recent works on the involvement and diverse roles of miRNAs in the development and physiology of gonadal cells in mammalian reproduction.

M. M. Hossain
Department of Animal Breeding and Genetics,
Bangladesh Agricultural University,
Mymensingh 2202, Bangladesh

M. M. H. Sohel · K. Schellander · D. Tesfaye (✉)
Institute of Animal Science, Animal Husbandry
and Breeding Group, University of Bonn,
Endenicher Allee,
53115 Bonn, Germany
e-mail: tesfaye@itw.uni-bonn.de

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Introduction

Gonads are the primary reproductive organ in sexual reproduction that produce both gametes and steroid hormones. Successful gametogenesis in the gonads is a prerequisite for mammalian reproduction which involves complex molecular mechanism for cellular functions as well as the interaction of different gonadal cell types, primarily the germ cells that develop into oogonia (primitive eggs) or spermatogonia (stem cell precursors of sperm) and the supporting somatic cells that are either the Sertoli cells and Leydig cells in the male or the granulosa cells and the theca cells in the female. A remarkable aspect of gametogenesis either in the testis or ovary is that germ cells exhibit strictly regulated spatiotemporal gene expression for functional development of sperm or oocytes (Eddy 1998; Grimes 2004). Despite the fact that a large proportion of these transcripts are partially stored in translationally inactive free mRNA ribonucleoprotein particles in mammalian haploid germ cells (Kleene 2003; Schmidt et al. 1999), much evidence has accumulated reporting the expression of miRNAs and their biogenesis pathway genes in germ cells and gonadal somatic cells (Hossain et al. 2009; Kotaja et al. 2006; Ro et al. 2007a, b; Buchold et al. 2010), which suggest the potential involvement of miRNAs in translational control during gametogenesis.

MiRNAs are a class of small non-coding RNAs, which are 18–24 nucleotides long and are considered as one of the posttranscriptional suppressors through binding to their target mRNAs by base-pairing and subsequently inducing either translational repression or mRNA destabilization (Kim 2005). They are estimated to comprise 1–5 % of animal genes, and thousands of miRNAs can be encoded by a genome at a time (Bentwich et al. 2005; Berezikov et

al. 2005). In addition to identification of active regulatory miRNAs in various tissues, several functional studies have shown the role of miRNAs in pathology, fertility and development (Lau et al. 2001; Ambros 2004; Lai 2003; Plasterk 2006). Especially, a large body of evidence is available on the involvement of miRNAs in various aspects of reproductive physiology including folliculogenesis, spermatogenesis and embryo development. Accordingly, functional studies involving inhibition of miRNA biogenesis have shown the occurrence of developmental arrest and female infertility in various species (Bernstein et al. 2003; Wienholds et al. 2003; Otsuka et al. 2008). The following sections give an overview on the expression and the role of miRNAs in mammalian testis and ovary.

MiRNAs and their biogenesis

MiRNAs are the major class of molecules consisting of 20–24 nucleotides and found to play diverse roles in regulating genes posttranscriptionally through sequence complementarities with the 3'UTR of the target mRNAs. The cellular process of miRNA biogenesis involves both nuclear and cytoplasmic processes (Lee et al. 2002). MiRNAs originate from large primary (pri) and precursor (pre) transcripts which undergo successive multi-steps of processing until they reach their mature and functional form (Fig. 1). They are commonly transcribed from intragenic or intergenic chromosomal DNA regions by RNA polymerase II into primary transcripts of various lengths (usually 1–3 kb) named pri-miRNAs (Lee et al. 2004; Borchert et al. 2006; Garzon et al. 2010). The pri-miRNA is then subjected to step-wise processing by two RNase III endonucleases. First, pri-miRNA is endonucleolytically cleaved by an RNase complex composed of the RNase III Drosha and its cofactor, DiGeorge syndrome critical region gene 8 (DGCR8/Pasha), producing a stem-loop hair-pin double-stranded structure (~70 nt long) called precursor miRNA (pre-miRNA) (Lee et al. 2003; Landthaler et al. 2004). The excised pre-miRNA hairpin is then exported to the cytoplasm by Exportin-5 complexed with Ran-GTPase (Kim 2004). In the cytoplasm, this pre-miRNA is further processed to a 20- to 22-nt-long miRNA duplex by a second RNase III enzyme, Dicer, which is coupled with the TRBP and PACT proteins (Lee et al. 2006; Chendrimada et al. 2005). From this miRNA duplex, one strand is designated as mature miRNA, the other being the passenger strand denoted as miRNA* or star strand. The miRNA duplex is unwound in which the miRNA* will be degraded, while the mature miRNA is loaded into the RNA-induced silencing complex (RISC) (Khvorova et al. 2003; Robb and Rana 2007). Here, the functional complex of mature miRNA is formed with the four closely related paralogs of Argonaute proteins (Ago1–4) and integrated into RNA-inducing silencing complex (RISC) (Liu et al.

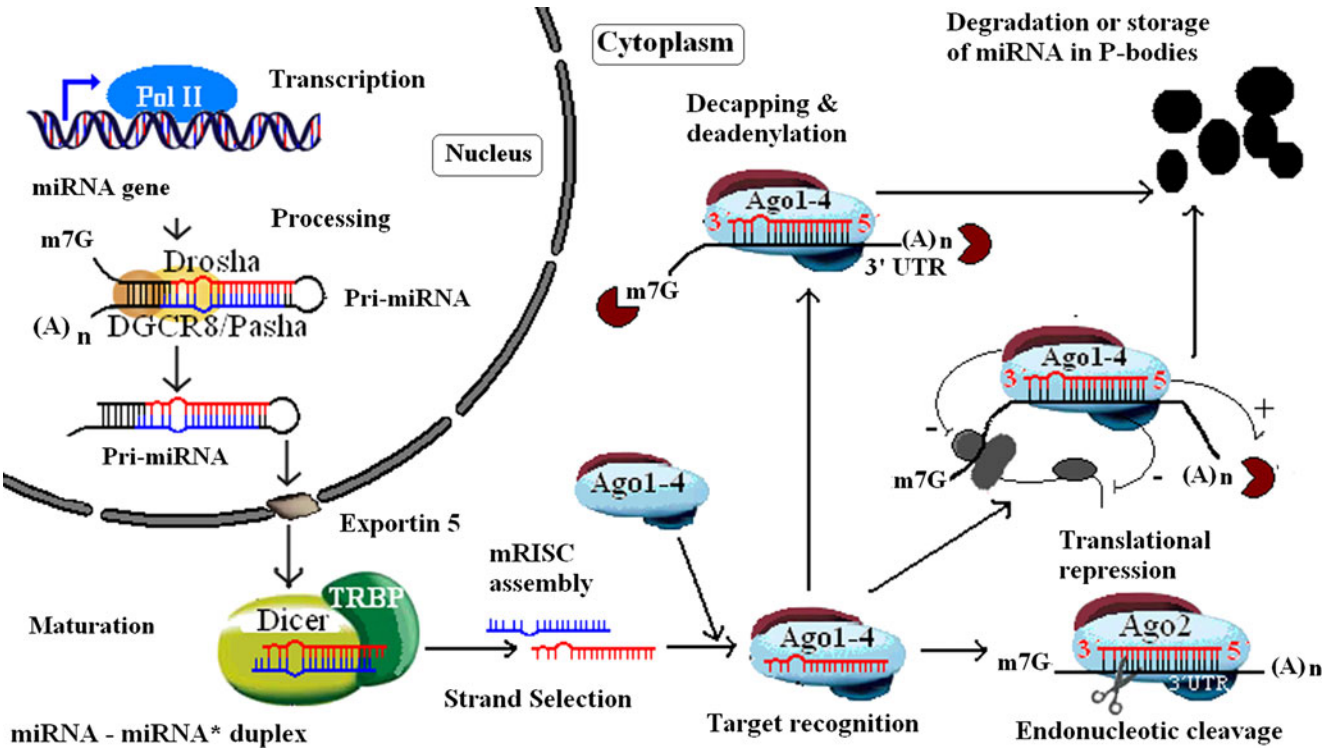

Fig. 1 Biogenesis of miRNAs and their mechanism of gene regulation. Argonaute proteins 1–4 (*Ago1–4*), inhibition of initiation of translation (-), promoting deadenylation (+), 7-methyl-G cap (*m7G*)

2004a). Among the four members, Argonaute2 (Ago2) is known to have an enzymatically competent RNaseH-like PIWI domain to mediate the cleavage of mRNAs (Meister et al. 2004; Fabian et al. 2010). In addition, the 'slicing' activity of Ago2 is also required for Dicer-independent processing of the precursor of some of the evolutionarily conserved miRNAs (Cifuentes et al. 2010). Using the specificity contained within nucleotides 2–7 (5' seed) and 13–16 (anchor) of the guide strand (mature miRNA), the RISC complex targets mRNAs complementary sequences in their 3'UTR either for cleavage or translational repression (Viswanathan et al. 2008; Hayashi et al. 2008). It has been observed that a typical miRNA can regulate hundreds of target genes (Brennecke et al. 2005; Lewis et al. 2005), and altogether it has been postulated that 30 % of the human protein coding genes are under the control of regulatory miRNAs (Lim et al. 2005). Not only can a single miRNA target several hundred genes bu a single gene cant also be targeted by multiple miRNAs. This complex relationship between miRNA and mRNA interaction is one of the biggest challenges for researchers dealing with functional analysis of miRNAs in different biological processes.

MiRNAs in mammalian testicular function

Mammalian spermatogenesis is a very complex process, where primary germ cells develop, differentiate and exit from the mitotic cell cycle with a timely expression of meiotic genes. This is to generate haploid round spermatids, and proceed further to the differentiation process of spermiogenesis that produces elongating spermatids and subsequently mature sperm cells. The timely expression of meiotic genes which is required for initiation of meiotic processes during spermatogenesis and the expression of multitude of testicular genes are known to be under the control of posttranscriptional regulatory mechanisms (Eddy 2002; Papaioannou and Nef 2010; Kimble 2011). MiRNA as posttranscriptional regulators have been shown to be involved in this mechanism in the several studies. The findings of some studies on the role of miRNAs in the testicular function expanding from male germ cells to spermiogenesis are presented in the following subsections.

Role of miRNA in male germ cells development

Due to the potential central role played by Dicer in miRNA biogenesis, it is apparent that most studies which are dealing with functional analysis of miRNAs rely on Dicer knockout model experiments. Conditional Dicer-knockout mice in which Dicer was deleted specifically in the germ cells revealed that Dicer-deleted primordial germ cells (PGCs) and spermatogonia exhibited poor proliferation which might result in late adult infertility due to defective proliferation and/or differentiation of spermatogonia (Hayashi et al. 2008). In Dicer-deleted testis, there is an unexpected suppression of retrotransposon activity in PGCs, but spermatogonia remain unaffected. In contrast to Dicer-deficient testis, spermatogenesis in Argonaute2-deficient testis was indistinguishable from that of the wild-type, suggesting that Dicer knockout abnormalities are independent of Ago2 (Hayashi et al. 2008). In addition to the reduction in size and disruption of spermatogenesis, Dicer1 knockout testes resulted in a reduced number of mature sperm in the epididymis with pronounced morphological abnormalities (Maatouk et al. 2008). Further study demonstrated that Dicer is critical for the normal organization of chromatin and nuclear shaping of elongating spermatids (Korhonen et al. 2011). Selective ablation of Dicer1 at the early onset of male germ cell development leads to infertility, due to multiple cumulative defects at the meiotic and post-meiotic stages culminating with the absence of functional spermatozoa. This finding indicates that Dicer1 is dispensable for spermatogonial stem cell renewal and mitotic proliferation, but is required for germ cell differentiation through meiosis and haploid phases of spermatogenesis (Romero et al. 2011).

Intact and competent Sertoli cells (SCs) are the prerequisite for successful spermatogenesis. Several groups investigate the role of Dicer in mouse Sertoli cell function. Deletion of Dicer in Sertoli cells leads to infertility due to the complete absence of spermatozoa and progressive testicular degeneration. The first morphological alterations appear at early as postnatal day 5 and is associated with a severe impairment of the prepubertal spermatogenic wave due to defective sertoli cell maturation and incapacity to properly support meiosis and spermiogenesis (Papaioannou et al. 2009). In that study, isotope-coded protein labeling was been used to investigate the impact of Sertoli cell-Dicer and subsequent miRNA loss on the testicular proteome. The result shows that a large proportion of proteins (50 out of 130) are upregulated by more than 1.3-fold in testes lacking Sertoli cell-Dicer. This protein upregulation is mild, as it never exceeds a 2-fold change and is not associated with alterations of the corresponding mRNAs (Papaioannou et al. 2011). All these results demonstrate the crucial importance of miRNA machinery mainly Dicer and thereby miRNAs in spermatogenesis and supporting cell lineage in testis.

To find out the role of specific miRNA during spermatogenesis, Bouhallier et al. (2010) tested the miRNA expression repertoire in germ cells, and showed miR-34c to be highly expressed in those cells. Overexpression of miR-34c resulted in downregulation of the TGIF2 gene, an inhibitor of the TGFβ pathway which is crucial for spermatogenesis (Bouhallier et al. 2010). The transcription factor Heat Shock

Factor 2 (HSF2), which plays a vital role in a wide range of developmental processes including embryogenesis and gametogenesis (Inselman et al. 2010), is found to be targeted by miR-18 (a member of Oncomir-1) (Bjork et al. 2010). The inhibition and overexpression of miR-18 in intact seminiferous tubules leads to an increased level of the HSF2 protein and a concentration-dependent decrease in the HSF2 protein, respectively (Bjork et al. 2010). Higher expression of miR-184 in GCs, enriched in the spermatogonial cell population, has been reported compared to somatic cells of day 6 testes (Marcon et al. 2008; Foley et al. 2010). The level of miR-184 has been found to be increased during mouse postnatal testis development, and this overexpression of miR-184 has been associated with the promotion of cell cycle and proliferation of germ cell line, GC-1spg. Overexpression of miR-184 in this cell line resulted in a decrease in the level of the nuclear receptor corepressor 2 (NCOR2) protein indicating that miR-184 regulated the NCOR2 gene posttranscriptionally by translational repression (Wu et al. 2011). MiR-21 is also found to be specially expressed in spermatogonial stem cells and shown to be important in spermatogonial self-renewal, and anti-apoptotic and oncogenic processes (Niu et al. 2011; Zheng et al. 2011a). MiR-21, along with miR-34c, miR-182, miR-183, and miR-146a, are preferentially expressed in the $Thy1^{+}$ spermatogonial stem cell (SSC)-enriched population, compared with $Thy1^{-}$ somatic cells. Transient inhibition of miR-21 in SSC-enriched germ cell cultures increased the number of germ cells undergoing apoptosis and significantly reduced the number of donor-derived colonies of spermatogenesis, indicating that miR-21 plays a crucial role in maintaining the SSC population (Niu et al. 2011).

With advancements in genome-wide expression analysis, in addition to identification, detailed analysis of testicular miRNome showed differential expression of several miRNAs during testicular development (Yu et al. 2005). Subsequently, during recent years, several studies have been carried out to profile the miRNA in mammalian testis by cloning (Ro et al. 2007a, b) or microarrays (Yan et al. 2007). But more recently, with the advent of high throughput technologies, several studies have been carried out using state-of-the-art next generation sequencing to profile miRNAs in mammalian testis and ovary (Li et al. 2011; Huang et al. 2011).

In a recent report, miR-449 was found to be preferentially expressed in the murine testis with the highest levels in meiotic (spermatocytes) and post-meiotic (spermatids) male germ cells (Bao et al. 2012). The miR-449 cluster was mapped to the second intron of *Cdc20b* gene, thereby both were found to exhibit similar expression profiles in mouse multiple tissues and developing testes. In the same study, it was shown that testes-specific transcription factors cAMP responsive element modulator tau (CREMτ) and SOX5 (essential for regulating male germ cell gene expression) mediate meiotic and post-meiotic expression of miR-449 by binding to two highly conserved cis-elements of the *Cdc20b/miR-449* cluster. Similarly, miRNA miR-34c has been shown to be highly enriched in germ cells and regulating germinal phenotypes (Bouhallier et al. 2010). Interestingly, levels of miR-34b/c were significantly upregulated in miR-449-null testes. The revelation of upstream regulators of miR-449 expression and interrelation between miR-449 and miR-34b/c in the control of male germ cell development suggest that the miR-449 cluster and miR-34b/c function redundantly in the regulation of male germ cell development in murine testes (Bao et al. 2012).

Despite the importance of miRNA for germ cell development, compared to global expression studies, miRNA studies focusing on specific stages of germ cell development are limited. A recent study on the expression of miRNA in the isolated the gonocyte and spermatogonia has shown the role of miRNA during the gonocyte to spermatogonial transition (McIver et al. 2012). This study has identified seven miRNA molecules downregulated between postnatal gonocytes and spermatogonia, namely miR-293, 291a-5p, 290-5p, and 294*. These miRNA molecules are located in a miRNA cluster and known to be involved in the maintenance of pluripotency within stem cells (Zheng et al. 2011b). On the other hand, miR-136, MiR-743a, and miR-463* were found to be upregulated in spermatogonia. The predicted target gene analysis of the differentially expressed miRNAs indicated that those target genes are involved in the Wnt/b-catenin (Golestaneh et al. 2009) and the PTEN signaling pathways, which are important signaling pathways for male fertility by maintaining germ cell differentiation and/or pluripotency (McIver et al. 2012).

MiRNAs and spermatogenesis in mammalian testis

Spermatogenesis is initiated in the male testis with the beginning of puberty, where male primary germ cells undergo mitotic division and produce a number of cells termed spermatogonia, from which the primary spermatocytes are derived. Primary spermatocytes undergo successive meiotic division to produce four spermatids, and a metamorphic change (spermiogenesis) occurred in the spermatids to produce spermatozoa. During spermatogenesis, a number of mRNAs associated with the chromatoid body are transcribed and stored for some days in spermatocytes without detectable protein expression (Kleene 2003). Moreover, complexes of miRNAs and their targets as well as Dicer were found to be accumulated in the chromatoid body of spermatids (Kotaja et al. 2006). A dramatic increase in overall miRNAs abundance was observed on postnatal day 14 (P14), when pachytene cells are first present in mouse testis (Kotaja and Sassone-Corsi 2007). Several groups have

identified and described the expression of miRNAs in the adult mouse testis (Ro et al. 2007a; Buchold et al. 2010; Yan et al. 2007), and human testis with azoospermia (Lian et al. 2009). Therefore, the expression and differential changes in miRNA activities may indicate the potential involvement of these RNAs in regulation of translational delay and subsequent control of mammalian spermatogenesis.

MiRNAs were first cloned from the testis along with other body tissue only a few years ago (Barad et al. 2004; Liu et al. 2004b). Shortly after those studies, a detailed analysis of the testicular miRNA species showed that a number of miRNAs are differentially expressed during the transformation/differentiation of the prepubertal testis to the adult testis (Yu et al. 2005). In that study, specific miRNA (miR-122a) was evidenced to target Tp2 genes. Dicer and other components of miRNA pathway (components of micro-ribonucleoprotein complexes, mainly members of the Ago family (Ago2 and Ago3) and testis-expressed miRNAs (miR-21, let-7a, miR-122a) are evidenced to potentially be involved in the regulation of male germ cell (GC) development (Kotaja et al. 2006). A study by Novotny et al. (2007a) indicated that the expression levels of mRNA and corresponding proteins are not always constant in tissue isolated from normal and neoplastic testis, postulating the potential role of miRNA in the control of posttranscriptional gene regulation. The miR-17-92 cluster has been reported to regulate c-Myc expression, which leads to the translational repression of E2F1 thereby preventing apoptosis during meiotic recombination (Novotny et al. 2007b).

Expression profiling either in whole mouse testis using microarray (Yan et al. 2007) or in purified spermatogenic cell (pachytene spermatocytes and round spermatids) through cloning analysis (Ro et al. 2007a) evidenced the differential expression of miRNA during spermatogenesis. The increased levels of miRNA expression during testicular development may result from changes in miRNA levels in germ cells, with the highest expression levels of miRNAs in spermatocytes, which are consistent with the expression data from the developing testes. This suggests that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis (Ro et al. 2007a). The microRNA processing machinery genes including Dicer, Drosha, and the Argonaute family are found to be expressed in pachytene spermatocytes, round and elongated spermatids, and in Sertoli cells (Gonzalez-Gonzalez et al. 2008). Transcriptional silencing of the XY bivalent during mammalian meiosis is a very important event where defects may lead to infertility in males. Due to the fact that miRNAs are localized in the chromosome core, telomeres, and the XY body of spermatocytes and nucleolus of Sertoli cells (Marcon et al. 2008), the potential role of miRNAs in transcriptional regulation during meiosis cannot be ruled out.

Limited information is available on the preferential expression and regulation of androgen biosynthesis in Leydig cell of testis mediated by miRNAs. Gonadotropin-regulated testicular RNA helicase (GRTH2/DDX25), which is present in both leydig and germ cells, has been found to be regulated developmentally by androgen at the transcriptional level (Tsai-Morris et al. 2010). Several miRNAs were found to be highly expressed in purified round spermatids from wild-type compared to GRTH null mice. Among them, miR-469 has been identified as testis-specific with higher expression in germ cells, meiotic spermatocytes, and round spermatids This miRNA has been found to repress the translation of Transition protein 2 (TP2) and Protamine 2 (Prm2) proteins (important for spermatid elongation and completion of spermatogenesis through chromatin remodeling) with no expression at protein level in GRTH null mice (azoospermic due to failure of spermatids elongation) (Sheng et al. 2006). Furthermore, GRTH appears to negatively regulate Drosha/DGCR8 gene expression during miRNA maturation. Therefore, the negative regulation of GRTH via the route of miRNA biogenesis/action and mechanism of miR-469 evidenced their potential role during spermatogenesis (Dai et al. 2011).

The existence, preferential and temporal differential expression of miRNAs and the involvement of their machinery genes especially the Dicer in the development of 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 the exact role of specific miRNAs in the development and proliferation of germ cells and testicular function as a whole.

Role of miRNAs in female gonad

Dynamically regulated sequential recruitment, selection and growth of the follicles, atresia, ovulation, and luteolysis in the ovary are under control of closely coordinated endocrine and paracrine factors which are controlled by tightly regulated expression and interaction of a multitude of genes in the ovary. As one of the major classes of gene regulators, miRNAs are suggested to be involved in the regulation of ovarian genes. The findings of the recent studies expanding from identification and expression profiling to functional analysis of miRNAs in the ovary carried out in different animal species are reviewed in the following sub-sections.

Presence and regulation of miRNAs in the ovary

Primordial germ cells are nurtured, retained and subsequently developed in the ovary through a complex follicular

developmental process in a cyclic basis to end up with a mature oocyte capable of fertilization. The outcome of this end-product is dependent on the precise expression and interaction of several intraovarian gene products in both autocrine and paracrine manner (Albertini and Barrett 2003; Matzuk et al. 2002). As a major regulator of gene expression, miRNAs have been shown to be involved in the ovarian functions. Several attempts have been undertaken to identify the distinct miRNAs in 2-week-old and adult mouse ovary (Ro et al. 2007b), adult mouse ovary and testis (Mishima et al. 2008), ovary and testis of Holstein cattle (Huang et al. 2011), adult bovine ovary (Hossain et al. 2009), bovine fetal ovary (Tripurani et al. 2010), sheep ovaries and testes (Torley et al. 2011), porcine ovary and testis (Li et al. 2011), and new-born mouse ovary (Ahn et al. 2010) through small RNA library construction and sequencing. These studies revealed preferential or exclusive expression of several miRNAs in the ovary of different species, suggesting that they may have an important role in ovarian functions. Regardless of species, let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145, and miR-199b were found to be the most commonly abundant miRNAs populations in the ovary. In addition, sex-biased expression of several predominant miRNAs has been confirmed, thereby indicating the reproductive organ-specific roles of miRNAs (Mishima et al. 2008). Bio-informatic prediction, screening and Gene Ontology analysis of these predominantly expressed miRNAs target genes in the mammalian ovary has identified several biological processes and pathways or molecular networks including cell cycle regulation, cell death, cell to cell signaling, cellular growth, development and proliferation, endocrine system disorder, and different pathways underlying the ovarian functions (Fig. 2) (Hossain et al. 2009).

Studies on the role of miRNAs in ovarian function have been highlighted primarily through Dicer (an essential miRNA processing molecule) knockout in mouse (Gonzalez and Behringer 2009; Lei et al. 2010; Murchison et al. 2007; Nagaraja et al. 2008; Hong et al. 2008). Dicer1/miRNA-mediated posttranscriptional gene regulation has been observed in the reproductive phenotype of mice with loxP insertions in the Dicer1 gene ($Dicer1^{fl/fl}$) by crossing with mice expressing Cre-recombinase driven by the antimüllerian hormone receptor 2 promoter ($Amhr2^{Cre/+}$). This conditional knockdown of Dicer revealed a reduction in ovarian weight and was characterized by lower ovulation rates (Hong et al. 2008). Similarly, Dicer1 cKO mouse [directed by a knocking of the Cre recombinase gene into the anti-Müllerian hormone receptor type 2 (Amhr2) locus] was shown to be infertile due to multiple defects in ovarian functions including abnormal estrous cycles, shorter estrus and longer metestrous, paratubal cyst, and abnormal response to gonadotropin with ultimate problem in ovulation (Nagaraja et al. 2008). Likewise, Dicer1 conditional knockout

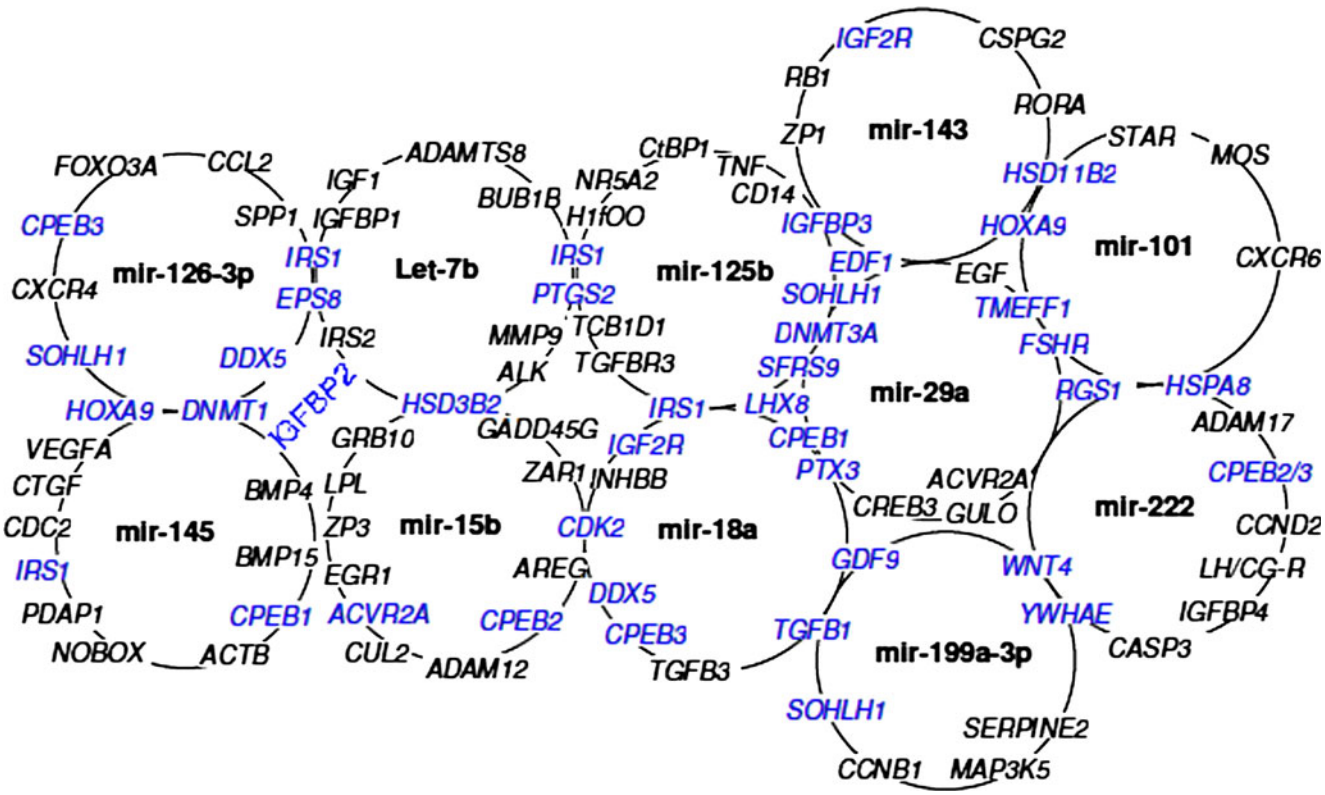

Fig. 2 Eleven ovarian miRNAs with highest number of ovarian function-related genes (Hossain et al. 2009)

mouse driven by similar promoter (Amhr2) in ovarian granulosa cells led to an increased primordial follicle pool endowment, accelerated early follicle recruitment, and more follicle degeneration in the cKO ovaries. The same study has reported significant differences in the expression of some follicle development-related genes between cKO and WT mouse ovaries, such as Amh, Inhba, Cyp17a1, Cyp19a1, Zps, Gdf9, and Bmp15, suggesting the important function of miRNAs in regulating ovarian gene expression (Lei et al. 2010). Deficiency of Dicer1 has been found to result in infertility in female mouse mutant with a hypomorphic Dicer1 allele ($Dicer^{d/d}$), developed through a gene trap method (Otsuka et al. 2008). This infertility was evidenced to be inherent to the ovary of wild-type female mice transplanted with $Dicer1^{d/d}$ and subsequently revealed failures in establishing successful pregnancies. $Dicer^{d/d}$ mice were characterized by the decreased level of serum progesterone concentrations and expression of LH receptor, cytochrome p450 11a1, and prolactin receptor, the genes necessary for progesterone production and corpus luteum functions. Interestingly, impaired ovarian corpus luteum angiogenesis in Dicerd/d mice was associated with the deficiency of miR-17-5p and let-7b, which are involved in regulation of the expression of an antiangiogenic factor tissue inhibitor of metalloproteinase1. This indicates that the physiological process in the development and function of the ovarian corpus luteum requires Dicer1 function and specifically the mechanism of miRNAs-mediated regulation (Otsuka et al. 2008). Interestingly, it has been shown that miRNAs are expressed in the corpus luteum preferentially according to the state of regression. Accordingly, the expression of miR-378 and its target IFNGR1 at specific stages of the corpus luteum may suggest the potential role of this miRNA in induction of apoptosis in the bovine corpus luteum (Ma et al. 2011).

In addition to identification and differential expression studies, further functional characterization of these miRNAs in different cell types of ovary (oocyte, granulosa, theca cells, and ovarian stroma) at different follicular stages or at different stages of the estrous cycle remain to be explored further. Although bioinformatic prediction and analysis of ovary-specific target mRNAs for these enriched miRNAs revealed several molecular and cellular pathways, direct association of this miRNA:mRNA interaction with ovarian follicular development, atresia and ovulation can only be attained by appropriate wet laboratory experiments.

MiRNAs in the granulosa cells and endocrine control

Studies of miRNAs in granulosa cells are relatively advanced compared with other ovarian cell types. For example, the 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 that miR-132 and miR-212 are highly upregulated following LH/hCG induction. Further analysis of these two miRNAs in cultured granulosa cells revealed their role in the posttranscriptional regulation of CtBP1 gene which is known to be interacting with steroidogenic factor-1 and acts as a co-repressor of nuclear receptor target gene. Recently, studies have been conducted to investigate 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. 2009). This transfection of cultured human granulosa cells with selected miRNA constructs (miR-108, miR-7, miR-9, miR-105, miR-128, miR-132, miR-141, miR-142, miR-152, miR-188, and miR-191) resulted in a significant increase in the percentage of cells containing PCNA, a cell proliferation marker. Similarly, miR-15a, miR-96, miR-92, miR-124, miR-18, miR-29a, miR-125a, miR-136, miR-147, miR-183, and miR-32 were found to promote up to 2-fold accumulation of Bax, a proapoptotic marker, in human granulosa cells. The most recent work has highlighted the regulation of granulosa cell proliferation by miR-224 and thereafter its effect on ovarian estrogen release in mouse (Yao et al. 2010). In that experiment, miR-224 expression was found to be regulated by a TGF-β/Smads pathway through inhibiting TGF-β superfamily type I receptors (SB431542), which leads to blockage of phosphorylation of the downstream effectors Smad2/3 in granulosa cells in vitro. The ectopic expression of miR-224 was suggested to enhance TGF-β 1-induced granulosa cell proliferation through targeting Smad4. This was a good demonstration of the notion that miRNAs could control or promote TGF-β 1-induced GC proliferation and ovarian estrogen release. However, there are many more miRNAs involved in the function of granulosa cells but not yet investigated.

Several studies have suggested ovarian steroid-dependent biogenesis and maturation of miRNAs and conversely a set of miRNAs are regulators of 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 cells and glandular epithelial cells in human (Bettegowda et al. 2008). The molecular mechanism by which ovarian steroids regulate the expression of miRNAs was unclear but such a regulatory function has been suggested to alter the expression of their target genes and thereby cellular activities manifested by their products (Bettegowda et al. 2008). It has also been shown that LH/hCG regulates the expression of selected miRNAs, which are involved in posttranscriptional gene regulation within ovarian granulosa cells in the mouse (Fiedler et al. 2008). In that study, miR-21 is identified as one of the three highly LH-induced miRNA in murine granulosa cells, and subsequently the function and temporal

expression of miR-21 within granulosa cells during transition to luteal cells has been examined. Similarly, the depletion of miR-21 activity in vivo in mice treated by a phosphothioate-modified LNA-21 oligonucleotide has led to increased apoptosis in the granulosa cells along with decreased ovulation rates in the treated ovary compared to their contralateral controls (Carletti et al. 2010).

Estrogen was found to suppress the levels of a set of miRNAs expression in mice and human cultured cells through estrogen receptor α (ERα) by interacting with the Drosha complex, thereby preventing the conversion of pri-miRNAs into pre-miRNAs (Macias et al. 2009). In addition, upregulation of a subset of miRNAs in female mice lacking estrogen receptor α and downregulation of some miRNAs in the estrogen target organ (uterus) was observed following estradiol (E2) treatment in ovariectomized female mice (Macias et al. 2009). Altogether the results of that study 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.

In contrast, some miRNAs are also found to play important roles in ovarian steroidogenesis (Sirotkin et al. 2009). Genome-wide screening of miRNAs revealed the involvement of miRNAs in control of the release of the ovarian steroid hormones progesterone, androgen and estrogen in human ovarian cells (Sirotkin et al. 2009). 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. Results revealed 36 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 57 tested miRNAs were found to inhibit testosterone release, only miR-107 enhances the testosterone output. Fifty-one miRNAs suppressed estradiol release, while none of the 80 miRNAs tested were found to stimulate it (Sirotkin et al. 2009). A similar study has shown that miR-378 is spatiotemporally expressed in porcine granulosa cells and taking part in the production of estradiol inversely compared with the expression of aromatase (Xu et al. 2011). Further in vitro overexpression and inhibition experiments in the same study revealed that aromatase expression in granulosa cells is posttranscriptionally downregulated by miR-378, which subsequently affects estradiol production in those cells. However, the complex regulatory mechanisms for controlling miRNAs biogenesis by steroids or vice versa are still unclear. The involvement of miRNAs in such mechanisms as a regulator of several hundreds of genes as potential targets could be much higher than ever speculated.

Association of miRNAs to the regulation and development of ovarian follicles

Follicular development includes morphological and functional changes in different types of follicular cells, mainly increasing the size of oocyte, proliferation and differentiation of their surrounding granulosa and theca cells, and the interaction of these different cell types. Changes in these follicular cell morphology and function are closely controlled by coordinated endocrine and paracrine factors, which are regulated by the expression and interaction of a number of genes in different ovarian cell types to attain oocyte development (Bonnet et al. 2008). Considering the various Dicer knockout and miRNA expression studies, it is evident that these genes are posttranscriptionally regulated by miRNA. The importance of expression of Dicer1 in the oocyte during folliculogenesis has been shown in several studies (Murchison et al. 2007; Su et al. 2002; Choi et al. 2007). Dicer1 conditional knockout (cKO) female mice were found to exhibit abnormal estrous cycles with characteristic increases in the number of atretic follicles compared to the control ovaries (Nagaraja et al. 2008). Dicer-deficient oocytes generated through conditional knockout (cKO) mouse ovarian tissue found to have misregulation of major portion of transcripts and revealed that the Dicer1-regulated miRNA signaling pathway is important for mouse follicular development through the regulation of follicle cell proliferation, differentiation and apoptosis (Lei et al. 2010).

The expression profiles of miRNA during follicular maturation in vitro from the ovary of 12-day-old wild-type and vitamin C-deficient (L-gulonogammalactone oxidase knockout, Gulo$^{-/-}$) C57BL6 mice have been shown to be altered by the hCG supplementation and vitamin C status. So, the impacts of hormone and oxidative stress may have an influence on the expression of miRNAs during follicular maturation in vitro (Kim et al. 2010). Obviously, the expression and Dicer-mediated loss of miRNAs function in the ovarian follicular development give primary clues to the importance of miRNAs, but functional involvement of candidate miRNA in specific cellular processes for successful follicular development have yet to be elucidated.

MiRNAs in the development and maturation of oocyte

Advances in molecular analysis have revealed that miRNA could be involved in different processes of oocyte maturation. The importance of miRNA in the maturation of oocytes has initially been evidenced from Dicer knockout or knockdown studies, and it has become clear that Dicer is required

for meiotic spindle integrity and completion of meiosis I (Murchison et al. 2007). Recent evidence provides initial clues for the role of miRNAs in the oocyte and thereby suggests that a large proportion of the maternal genes are directly or indirectly under the control of miRNAs (Tang et al. 2007). Oocytes lacking Dicer were unable to complete meiosis and arrested with multiple defects on meiotic spindle organization and chromosome congression. Depleting Dicer1 in oocytes resulted in the meiotic failure phenotype and revealed that Dicer1-conditioned ooplasm of primary oocytes contains Dicer1-dependent factors that are crucial for chromosome segregation, and for the progression of metaphase to anaphase during meiotic maturation (Mattiske et al. 2009). More recently, Dicer knockdown in mouse oocytes identified a significant reduction in oocyte maturation with increasing abnormal spindle and chromosomal organization (Liu et al. 2010). Thus, Dicer and miRNA appeared to play important roles during oogenesis and are essential for the completion meiotic division.

In addition to Dicer, conditional deletion of Argonaute2 (Ago2) in the developing mouse oocytes also revealed abnormal spindles and misaligned chromosomes with the ability to develop mature oocytes similar to the phenotype of Dicer-deficient oocytes (Kaneda et al. 2009). The study has suggested that Ago2 plays a key function in the mouse oocyte through global regulation of miRNA stability affecting gene expression in developing oocytes. The importance of miRNAs by showing the effect of loss of Dicer or Ago2 in the oocytes became questionable, while considering that both of these molecules are responsible for the processing of both miRNAs and endogenous-siRNAs (Suh et al. 2010). The result of this study revealed that the effects on the phenotypes in Dicer- or Ago2-deficient oocytes are instead due to endogenous siRNAs (Suh et al. 2010). Moreover, the expression level of miRNAs in Dgcr8-deficient oocytes was found to be reduced in a similar manner as observed in the Dicer-deficient oocytes. In addition, there was no effect due to deletion of the Dgcr8 allele even from maternal and zygotic genomes on the phenotype as well as the mRNA profile which are very unlikely for Dicer-deficient oocytes. Either by reporter assays or using artificial 3'UTR having multiple target sites of target mRNAs, it has been shown that miRNA function is suppressed in fully grown oocytes even though no change in miRNA biosynthesis and the presence of their targets is evidenced (Ma et al. 2010). Altogether, findings from Dicer, Ago2, and DGCR8 knockout studies in mice has shown that miRNA function is globally suppressed during oocyte maturation and preimplantation development (Suh et al. 2010). As supporting evidence, P-bodies were found to be lost during oocyte maturation and reappeared at the blastocyst stage (Flemr et al. 2010; Swetloff et al. 2009). Until recently, it has been clear that the most common findings from the studies on the effect of disrupting global miRNAs and/or endo-siRNAs transcription in the oocyte by deletion or knocking out the processing gene (Dicer1, Dgcr8, or Ago2) include defective spindles and chromosomal configurations. Two recent studies (Abd El Naby et al. 2011; Miles et al. 2012) on miRNAs expression either in the oocytes or cumulus oocyte complex suggest the presence of distinct sets of miRNAs in oocytes and cumulus cells which are dependent on bilateral communication of the two cell types.

Several studies have identified and highlighted the expression and regulation of individual miRNA in oocytes through small RNAs cloning, microarray, or RT-PCR-based miRNAs detection either by homologous or heterologous approaches. Initially, a small number of miRNAs as well as some other small noncoding RNAs (rasiRNAs, gsRNAs) have been identified in mouse oocytes (Watanabe et al. 2006). In addition this to identification study, several other studies have highlighted the expression and regulation of miRNAs in the oocyte during development as well as the maturation process (Abd El Naby et al. 2011; Tesfaye et al. 2009). Investigation of the expression of miRNAs in immature and in vitro matured bovine oocytes using a heterologous approach revealed a differential expression of 59 miRNAs (Tesfaye et al. 2009). Among them, 31 and 28 miRNAs were found to be preferentially expressed in immature and matured oocytes, respectively. The expression of seven miRNAs (miR-496, miR-297, miR-292-3P, miR-99a, miR-410, miR-145, and miR-515-5p) in mature and two miRNAs (miR-512-5p and miR-214) in immature oocytes showed higher abundance by at least 2-fold difference (Tesfaye et al. 2009). The changes of miRNAs expression were also observed in the cumulus cells matured with or without the oocyte cytoplasm (Abd El Naby et al. 2011). Dependency of the two cell types to each other for miRNA expression may be involved in the regulation of genes associated with impaired or normal nuclear maturation of oocytes due to the absence or presence of cumulus cell factors, respectively. This implies that the level of miRNAs transcription or mode of transcriptional regulation of the miRNAs in the oocyte or cumulus granulosa cells is dependent on their secreted factors in a paracrine manner. It is apparent that all the miRNAs or genes are not causal to these defects, but detailed functional analysis of the specific role of candidate miRNA is still lacking. Moreover, the mechanism of miRNA-mediated assembly of meiotic spindles and the establishment of proper meiotic spindle–chromosome interactions involved in various aspects of meiotic process are yet to be uncovered. Although all these efforts provide initial evidence for the role of miRNAs in the oocyte development, in depth functional analysis of individual miRNAs together with the characterization of their targets for the proposed functions and mechanism need to be the focus of future research.

Conclusion

Non-coding RNAs comprise the majority of the mammalian transcriptome, and among them miRNAs are well-characterized ones which could maintain the delicate balance between normal reproductive biology, system development and the diseased condition. Conditional Dicer1 knockout and expression of miRNAs have been used to elucidate the importance of miRNA in ovarian and testicular function and development. To date, much of the work on miRNAs has focused on expression profiling rather than their regulation and functional characterization within gonads. Studies to identify the specific miRNAs, their target genes and posttranscriptional regulatory network will further shed light on the importance of specific miRNA for both the development and function of testis and ovary with the corresponding disease conditions. Once relevant miRNAs and functional targets are identified, possible clinical use of these molecules will represent the next front line and may lead to novel strategies for enhancing or manipulating reproductive efficiency.

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