MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development

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Abstract

The genetic regulation of female fertility (follicular development, oocyte maturation and early preimplantation embryo development) involves the spatio-temporal regulation of those genes that play key roles in various stages of the female reproductive axis.

MicroRNAs (miRNAs), a class of small non-coding RNAs, are known to regulate the expression of a large proportion of such genes. In recent decades, multiple studies have aimed to determine the roles of these non-coding RNAs in mammalian follicular development, oocyte growth and embryo development. These studies have applied a variety of approaches, including conditional knockout of miRNA biogenesis genes, high-throughput sequencing technologies for pattern recognition in miRNA expression and loss- and gain-of-function of miRNAs in various animal models. In addition to the cellular miRNAs, a large variety of RNAs are found in circulation, being coupled with extracellular vesicles, proteins and lipids. Because of their potential as diagnostic markers for abnormal physiologies, there is increasing interest in the identification of extracellular miRNAs in various biological fluids and spent *in vitro* culture media. This review focuses on studies addressing the expression and potential role of cellular and extracellular miRNAs in mammalian follicular cell physiology and subsequent ovarian functionality and oocyte maturation.

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Introduction

The female fertility of mammalian species is a dynamic and well-coordinated process involving the proper function of several tissues and organs, including the hypothalamus, pituitary, ovaries and reproductive tract, which give rise to fertilizable gametes and support early embryo and fetal development. A suitable environment for successful fertilization and embryo development is achieved through regulated, spatio-temporal expression of multiple genes, involving both transcriptional and post-transcriptional (mRNA turnover, processing, storage and translation) regulations (Lim *et al.* 2003, Lasko 2011, Baranyai *et al.* 2015). RNA-binding proteins (RBPs) have long been suggested to be involved in the control of mRNA fate, and initial work had focused on the expression of RBPs in various stages of early embryo development (Bettegowda & Smith 2007). However, the discovery of non-coding RNAs, including microRNAs (miRNAs), has revolutionized the study of post-transcriptional regulation.

The biogenesis of miRNAs is a complex process involving several classes of molecules regulating the transcription and processing of the primary miRNAs (pri-miRNAs) into mature and functional miRNAs. The first event in the activation of miRNAs is

transcription from an miRNA gene, which is mediated by RNA polymerase II (Pol II) or, in some cases, RNA polymerase III (Pol III), to give rise to the pri-miRNA transcript (Lee *et al.* 2003, 2004). From that point onward, miRNA biogenesis can progress by either the canonical or non-canonical pathways. The canonical pathway involves the cleavage of pri-miRNA by a microprocessor complex of RNase II enzyme Drosha and the DiGeorge critical region 8 protein (DGCR8) and gives rise to a 60–70-nucleotide-long miRNA precursor (pre-miRNA) in the nucleus (Gregory *et al.* 2004). The non-canonical pathway skips the Drosha-mediated cleavage of pre-miRNA, and the pre-miRNA is instead generated through the splicing of introns from the mRNA transcript (Ruby *et al.* 2007). Following these nuclear processes, the pre-miRNAs are exported to the cytoplasm by Exportin-5 and Ran-GTP co-factor (Yi *et al.* 2003). Following this, the pre-miRNA is further cleaved by RNase III Dicer coupled with double-stranded TAR RNA-binding protein 2, which generates a 22-nucleotide-long miRNA duplex that binds with the Argonaute protein to be incorporated into the miRNA-induced silencing complex (miRISC) (Hutvagner *et al.* 2001, Hutvagner & Zamore 2002). As shown in Fig. 1, miRISC recognizes

binding sites within the 3'-UTR of the target mRNA, which leads to mRNA degradation or repression of protein translation.

Advances in library preparation and deep sequencing technologies have enabled the identification of several thousand miRNAs in various species, as deposited in the public database, namely miRbase (<http://www.mirbase.org>). However, the roles of miRNAs in various aspects of female fertility have mostly been evidenced by conditional deletion of the different miRNA-processing machinery genes in mouse models (Fig. 1). The importance of canonical miRNA biogenesis for uterine development and fertility in female mice was demonstrated by conditional knockout (cKO) of DGCR8 using the progesterone receptor (PR)-Cre (DGCR8^{d/d}) (Kim *et al.* 2016); adult DGCR8^{d/d} females do not have regular cycles and do not produce offspring. However, normal ovulation could be induced in these mice by administering exogenous gonadotropins. The dicer KO model has been widely used to study the role of miRNAs in female reproduction (Bernstein *et al.* 2003, Murchison *et al.* 2007, Hong *et al.* 2008, Nagaraja *et al.* 2008, Luense *et al.* 2009, Mattiske *et al.* 2009, Lei *et al.* 2010). Dicer is abundant in almost all organisms,

suggesting its evolutionary conservation. Dicer is involved in miRNA biogenesis (both canonical and non-canonical pathways for both miRNAs and other small RNAs in mammals) (Ha & Kim 2014). cKO experiments involving the dicer and DGCR8 genes have revealed the involvement of canonical and non-canonical miRNA pathways in the proper function of the reproductive system in female mice.

While the majority of the miRNAs identified so far are intra-cellular, a tremendous number of miRNAs have been reported to circulate in the extracellular environment in almost all biological fluids, which are referred to as extracellular miRNAs. These extracellular miRNAs are found coupled with extracellular vesicles, high-density lipoproteins and Argonaute (AGO) proteins. Because of their stability under extreme environmental conditions, extracellular miRNAs have the potential as biomarkers for various biological processes, including disease detection and prediction of the developmental competence of oocytes and embryos.

Here we review the literature addressing the role of cellular and extracellular miRNAs in mammalian fertility associated with follicular development, ovarian granulosa cell function, oocyte growth and development.

Figure 1 The microRNA biogenesis pathways and selective knockout of key miRNA machinery genes and the associated phenotypic effects on mammalian female fertility.

miRNAs in various stages of mammalian follicular development

Mammalian follicular development is a highly coordinated and accurately regulated physiological process, which starts with the periodic recruitment of small growing follicles from the primordial follicle reserve, followed by selection of a few follicles for further maturation and dominance that result in ovulation of a developmentally competent oocyte (Fortune 1994, Fortune *et al.* 2000). Thus, the mammalian ovary is a dynamic organ in which a series of tissue remodeling and inflammatory reactions takes place to allow the release of a fertilizable oocyte (Espey 1980, 1994). Follicular recruitment, selection, dominance and ovulation are tightly regulated (Imbar & Eisenberg 2014). Since their discovery as molecular regulators responsible for time-dependent developmental events (Lee *et al.* 1993, Wightman *et al.* 1993), miRNAs have emerged as key post-transcriptional regulatory RNA molecules.

miRNAs in ovarian development and activation of primordial follicles

miRNAs are expressed in almost all mammalian tissues, including the ovarian tissues of humans (Liang *et al.* 2007), mice (Ro *et al.* 2007, Ahn *et al.* 2010), cows (Hossain *et al.* 2009), goats (Ling *et al.* 2014), sheep (Di *et al.* 2014) and pigs (Li *et al.* 2011). Following the successful development of Dicer1 KO mice models (Hong *et al.* 2008, Otsuka *et al.* 2008, Nagaraja *et al.* 2008, Luense *et al.* 2009), multiple studies have reported the involvement of miRNAs in primordial follicle activation and maintenance. A study by Xu *et al.* (2011a) identified the proliferating cell nuclear antigen (PCNA) as a key gene involved in follicular assembly. RNA interference (RNAi) knockdown of PCNA in the neonatal mouse ovary reduced oocyte apoptosis and increased primordial follicle assembly by downregulating pro-apoptotic genes (*BAX*, *TNF* and *TNFR-2*). Following this, post-transcriptional regulation of PCNA by miR-376a in neonatal mouse primordial follicles was reported by Zhang *et al.* (2014). In the same study, overexpression of miR-376a in cultured mouse ovaries increased the number of primordial follicles and decreased oocyte apoptosis, as observed in ovaries transfected with short interfering (siRNA) that selectively targeted PCNA. The involvement of miRNAs in promoting primordial follicle initiation was further substantiated by the enrichment of miR-145 in the neonatal mouse ovary, which alters the expression of zona pellucida sperm-binding protein genes and activates the transforming growth factor (TGF)- β signaling pathway (Yang *et al.* 2013). Also, an inhibitory effect of miRNAs on primordial follicle formation has been reported in which miR-143 was selectively enriched in mouse pre-granulosa cells,

thereby suppressing genes related to cell proliferation and cell cycle (Zhang *et al.* 2013a).

miRNAs during early luteal phase and follicular selection and dominance

As follicles grow in size, the layers of granulosa cells encircling the oocyte increase in number (Braw-Tal & Yossefi 1997) and follicle size variability becomes evident due to the onset of deviation (Ginther *et al.* 1997). Because of this variability, clear differences were observed in the transcriptome profile of granulosa cells derived from small and large follicles (Hatzirodos *et al.* 2014b). With this rationale, Sontakke *et al.* (2014) performed an expression analysis of miRNAs in follicles of variable size and health status and identified an association with follicular selection and dominance. MiR-144, miR-202 and miR-873 were upregulated in large healthy follicles compared to large atretic counterparts. In an attempt to elucidate the involvement of miRNAs in the bovine early luteal phase of the estrous cycle, the expression signature of miRNAs was assessed in granulosa cells of dominant and subordinate follicles derived from days 3 and 7 of the estrous cycle (Salilew-Wondim *et al.* 2014). Here, no difference was observed between early stage (day 3 of the estrous cycle) dominant and subordinate follicles compared to the mid-stage follicles (day 7 of the estrous cycle). This was further observed in the principal component analysis (PCA), which showed no clear separation between dominant and subordinate follicles from day 3 of the estrous cycle. However, dominant and subordinate follicles from the mid-luteal phase showed a clear clustering pattern due to differences in granulosa cell miRNA expression profiles at day 7 of the estrous cycle (Fig. 2). This phenomenon was also observed at the mRNA transcriptome level, where smaller antral follicles tend to be heterogeneous compared to the larger antral follicles (Hatzirodos *et al.* 2014b).

Involvement of miRNAs during the preovulatory stage of follicular development

Understanding the involvement of miRNAs in the later stages of the estrous cycle (follicular phase) could provide useful information on the regulatory mechanisms associated with the luteinization of granulosa cells and ovulation of the preovulatory and degradation of the remaining follicles. Differential expression of miRNAs between dominant vs subordinate and dominant vs luteinized follicles in equine suggests the involvement of miRNAs in follicular selection and ovulation (Schauer *et al.* 2013). Similarly, a significant difference in the expression pattern of miRNAs was observed in granulosa cells of preovulatory dominant and subordinate follicles derived from day 19 of the bovine estrous cycle (Gebremedhn *et al.* 2015). In that

Figure 2 Principal component analysis (PCA) of normalized miRNA expression data derived from granulosa cells of dominant and subordinate follicles from early luteal (days 3 and 7) and late follicular (day 19) phases of the estrous cycle. Each rounded color represents a replicate of the individual follicular category. The dominant follicles of days 7 and 19 are clearly separated from the remaining follicular categories. The normalized miRNA expression data were obtained from [Salilew-Wondim et al. \(2014\)](#) and [Gebremedhn et al. \(2015\)](#).

study, the PCA analysis identified a clear separation of the two follicle populations (Fig. 2). Further *in silico* analysis of the differentially expressed miRNAs, which aimed to characterize the genomic context, revealed an interesting pattern of co-enrichment and repression of specific miRNA clusters. For instance, miR-21 and three miRNA clusters (miR-183-96-182, miR-132-212 and miR-424-450-542) were preferentially enriched in granulosa cells of preovulatory dominant follicles. This could be because these clusters of miRNAs are involved in rescuing granulosa cells of the preovulatory dominant follicles from undergoing apoptosis, as has been described in bovine ([Gebremedhn et al. 2016](#)) and other species ([Carletti et al. 2010](#)).

The dynamics of cellular miRNAs during the estrous cycle

The focus of the majority of the miRNA expression analysis studies has been to identify those miRNAs that are differentially expressed between follicular categories. Nonetheless, determining the highly abundant miRNAs commonly expressed in all follicular categories could shed light on the role of miRNAs in maintaining normal physiological processes of follicular development. For instance, in the above-mentioned studies, among the 20 highly abundant miRNAs, 15 (bta-miR-10b, bta-miR-26a, bta-miR-99b, bta-miR-27b, bta-let-7f, bta-let-7a-5p, bta-let-7i, bta-miR-92a, bta-miR-191, bta-miR-125a, bta-miR-148a, bta-miR-186, bta-miR-143, bta-miR-30d and bta-miR-30a-5p) are expressed irrespective of the follicular categories ([Salilew-Wondim et al. 2014](#), [Gebremedhn et al. 2015](#)). These miRNAs were also reported to be abundantly expressed in ovarian tissues of cow ([Hossain et al. 2009](#)), goat ([Zhang et al. 2013c](#), [An et al. 2016](#)), pig ([Huang et al. 2016a](#)) and other mammalian species, suggesting a housekeeping role for these miRNAs in maintaining the normal physiological processes in mammalian female reproduction ([Zi et al. 2017](#)).

The molecular mechanisms underlying follicular growth, onset of deviation and atresia were assessed by studying the dynamics of gene transcripts in granulosa cells throughout the bovine estrous cycle ([Hatzirodos et al. 2014a,b](#), [Li et al. 2016](#)). Thus, identifying the expression trends of miRNAs in mammalian granulosa cells at different time points of the estrous cycle would likely provide insights into the post-transcriptional gene regulatory mechanisms that orchestrate follicular development. To address this, a meta-analysis was performed on previously generated miRNA expression datasets from dominant and subordinate follicles of days 3, 7 ([Salilew-Wondim et al. 2014](#)) (GOE accession number: GSE55987) and 19 ([Gebremedhn et al. 2015](#)) (GOE accession number: GSE56002) of the bovine estrous cycle. The least square mean for time and type of follicles and a linear regression model for days 3 and 7 and days 7 and 19 were estimated. Finally, the slope and intercept of the trends generated were used to characterize and cluster miRNAs. Based on the sign of the slope between the two time points, four categories ('+', '+', '-', '+', '-' and '-', '-') were identified. For example, '+, +' indicated miRNAs with an increasing trend between days 3 and 7 and days 7 and 19. The genomic contexts of miRNAs in the dominant follicles of each category revealed an interesting pattern, and representative miRNA clusters that belong to each category are indicated in Fig. 3. For example, the miR-132-212 and miR-183-96-182 clusters (transcribed from chromosomes 19 and 4 of the bovine genome, respectively) are included in the '+, +' category, which might be associated with their potential role in promoting survival of granulosa cells in the dominant follicles during the later stages of the estrous cycle. This was validated by a loss-and-gain-of-function experiment in which overexpression of the miRNA cluster promoted cell proliferation and G₁/S cell cycle transition by coordinately targeting the pro-apoptotic *FOXO1* transcription factor and its pro-apoptotic downstream transcript, *FASL* ([Gebremedhn et al.](#)

2016). Similarly, the miR-132-212 cluster miRNAs are associated with ovulation and are elevated after the induction of an ovulatory dose of luteinizing hormone (LH)/human chorionic gonadotropin (hCG) (Fiedler *et al.* 2008), preventing cells from entering into apoptosis. On the other hand, miRNA clusters, including miR-23a-27a-24, miR-222-221 and miR-214-199a (transcribed from chromosomes 7, X and 16 of the bovine genome, respectively), showed increasing expression until the mid-luteal phase but decreasing expression in the dominant follicle granulosa cells during the later follicular phase of the estrous cycle (Fig. 3). MiR-23a and miR-27a have been reported to promote granulosa cell apoptosis by targeting SMAD5 through the FasL-Fas-mediated pathway (Nie *et al.* 2015). This would support the notion that miRNAs are involved in the induction of apoptosis in subordinate follicles, thereby facilitating their destination to atresia.

Functional role of miRNAs in granulosa cells

The proliferation, differentiation and steroidogenic functions of granulosa cells are necessary for key events throughout mammalian follicular development, including recruitment, selection and dominance of follicles and atresia of anovulatory subordinate follicles. The significant role of Dicer in mammalian folliculogenesis has been evidenced using a tissue-specific deletion of Dicer in granulosa cells (Lei *et al.* 2010). In that study, age-specific investigation of

mice ovaries with inactivated Dicer in granulosa cells revealed increased primordial follicle pool endowment, accelerated early follicle recruitment and more degenerate follicles in the cKO ovaries compared to the wild-type counterpart. Although normal ovulation could occur in mice with disrupted dicer in granulosa cells, impaired oocyte development is a known cause of infertility (Nagaraja *et al.* 2008, Gonzalez & Behringer 2009) in those models. Moreover, the defective miRNA pathway in granulosa cells and subsequent effects on oocyte development have been evidenced by a histological analysis of dicer cKO ovaries, showing a higher number of degenerated follicles and impaired oocyte development (Lei *et al.* 2010).

Granulosa cells are relatively easy to isolate and maintain under an *in vitro* cell culture system and, therefore, are a widely studied ovarian cell type. Several molecular genetics studies have used granulosa cells as a model. Granulosa cells are often used in *in vitro* loss- and gain-of-function studies to investigate the role of individual (or clusters of) miRNAs. A large body of studies are available evidencing the regulatory role of miRNAs in granulosa cell proliferation (Sirotkin *et al.* 2010, Yao *et al.* 2010a, Yan *et al.* 2012, Andreas *et al.* 2016, Gebremedhn *et al.* 2016), survival (Carletti *et al.* 2010, Lin *et al.* 2012, Yang *et al.* 2012, Sirotkin *et al.* 2014, Gebremedhn *et al.* 2016), terminal differentiation (Kitahara *et al.* 2013, Andreas *et al.* 2016) and steroidogenesis (Sirotkin *et al.* 2009, Xu *et al.* 2011b, Yin *et al.* 2012, Dai *et al.* 2013). Table 1 summarizes the functionally validated miRNAs of various ovarian tissues, including granulosa and cumulus cells and oocytes.

The proliferation of granulosa cells plays a crucial role in creating a unique microenvironment that nurtures the growing oocyte (Maruo 1995). Extra- and intra-ovarian factors have been reported to regulate follicular development, including TGF- β superfamily members and SMADs (Miyazawa *et al.* 2002, van den Hurk & Zhao 2005). Several members of the TGF- β superfamily are implicated in granulosa cell proliferation (Hsueh *et al.* 1984, Hirshfield 1991). The conserved housekeeping miR-10 family (miR-10a and miR-10b) is expressed at basal levels in granulosa cells. MiR-10 family members have been shown to slow the rate of granulosa cell proliferation and induce apoptosis in human, mouse and rat granulosa cells by suppressing key genes (TGF β 1, Activin A, BMP4 and BMP15) in the TGF- β pathway (Jiajie *et al.* 2017). This suggests the existence of a negative feedback loop between miRNAs and TGF- β pathway. Involvement of miR-15a in the regulation of human granulosa cell proliferation was evaluated by transfecting primary granulosa cells with antisense constructs capable of inhibiting miR-15a, which resulted in increased expression of cell proliferation marker genes (MAPK/ERK1, 2 and PCNA), whereas overexpression of miR-15a leads to an

Figure 3 Temporal miRNA expression dynamics in granulosa cells of dominant follicles during the estrous cycle. The diameter ($\varnothing$) of the dominant follicles increases as the estrous cycle progresses. Representative miRNA clusters belonging to each of the expression dynamics pattern are indicated with different colors. The X- and Y-axes represent the days of the estrous cycle and normalized miRNA expression values, respectively. The analysis was carried out based on the published data from Salilew-Wondim *et al.* (2014) and Gebremedhn *et al.* (2015).

Table 1 Summary of the role miRNAs in different reproductive tissues of various mammalian species.

Biological tissue/ cells/fluid	miRNAs	Associated biological functions	Species	References
Granulosa cells	MiR-143 ¹ , miR-764-3p ² , miR-144 ³ , miR-375 ⁴ , miR- 378-3p ⁵ , miR-15a ⁶ , miR-133b ⁷ , miR-125a ⁸ & miR-455 ⁸	Regulation of steroid synthesis	M ^{1,2,3,7} , P ^{4,5} , H ⁶ , R ⁸	¹ Zhang <i>et al.</i> (2017), ² Wang <i>et al.</i> (2016), ³ Zhou <i>et al.</i> (2017), ⁴ Yu <i>et al.</i> (2016), ⁵ Toms <i>et al.</i> (2015), ⁶ Sirotkin <i>et al.</i> (2014), ⁷ Dai <i>et al.</i> (2013), ⁸ Hu <i>et al.</i> (2012)
Granulosa cells	miR-141-3p ¹ , miR-183-96-182 ² , miR-26b ³ , miR-23a & 27a ⁴ , let-7g ^{5,6,7} , miR-125a-5p ⁸ , miR-146a ⁹ , miR-92a ¹⁰ , miR- 26b ¹¹ , miR-34a ¹² , miR-21 ¹³ ,	Regulation of granulosa cell apoptosis	R ¹ , B ² , P ^{3,7,10,11,12} , H ^{4,5,6,9} , M ^{8,13}	¹ Li <i>et al.</i> (2017), ² Gebremedhn <i>et al.</i> (2016), ³ Liu <i>et al.</i> (2016), ⁴ Nie <i>et al.</i> (2015), ⁵ Cao <i>et al.</i> (2015), ⁶ Zhou <i>et al.</i> (2017), ⁷ Cao <i>et al.</i> (2015), ⁸ Wang <i>et al.</i> (2016), ⁹ Chen <i>et al.</i> (2015), ¹⁰ Liu <i>et al.</i> (2014), ¹¹ Lin <i>et al.</i> (2012), ¹² Tu <i>et al.</i> (2014), ¹³ Carletti <i>et al.</i> (2010)
Granulosa cells	miR-17-92 ¹ , miR-10b ² , miR-181a ³ , miR-145 ⁴ , miR-224 ⁵ , miR-10a, 105 & 182 ⁶ ,	Regulation of granulosa cell proliferation and differentiation	B ¹ , G ² , M ^{3,4,5} , H ⁶	¹ Andreas <i>et al.</i> (2016), ² Peng <i>et al.</i> (2016), ³ Zhang <i>et al.</i> (2013b), ⁴ Yan <i>et al.</i> (2012), ⁵ Yao <i>et al.</i> (2010a), ⁶ Sirotkin <i>et al.</i> (2010)
Cumulus cells	miR-378 ¹ , miR-224 ² , miR-21-5p ³ , miR-509-3p ⁴	Regulate oocyte maturation, embryo development and estradiol secretion	P ^{1,2} , H ³	¹ Pan <i>et al.</i> (2015), ² Li <i>et al.</i> (2017), ³ Karakaya <i>et al.</i> (2015), ⁴ Huang <i>et al.</i> (2016b)
Oocytes and embryos	miR-130b ¹ , miR-125 family (miR-125a-5p, -125b-5p & -351-5p) ² , miR-320 ³ , miR-212 ⁴ , miR-135a ⁵ , miR-196a ⁶ , let-7 ⁷ , miR-106 ⁷	Regulation of oocyte maturation or early embryo development (maternal embryo genome transition)	B ^{1,4,6,7} , M ^{2,3,5}	¹ Sinha <i>et al.</i> (2017), ² Kim <i>et al.</i> (2016), ³ Feng <i>et al.</i> (2015), ⁴ Tripurani <i>et al.</i> (2013), ⁵ Pang <i>et al.</i> (2011), ⁶ Tripurani <i>et al.</i> (2011), ⁷ Miles <i>et al.</i> (2012)
Follicular fluid	miR-654-5p, miR-640, miR-526b and miR-373 ¹ , miR-643, miR-148b, miR-548j, miR-132- 212, miR-486 ² , miR-27b, miR-372, and miR-382 ³ , miR-320 ⁴ miR-21-5p, miR- 190b ⁵ , let-7b, miR-29a, miR-30a, miR-140 and miR-320a ⁶ , miR-24-3p, miR-29a, miR-151-3p, and miR-574-3p, miR-518f-3p ⁷	Indicators of oocyte developmental competence, antral follicle development, follicular maturation	B ^{1,2,3} , MR ⁴ , H ^{5,6,7,8}	¹ Sohel <i>et al.</i> (2013), ² Navakanitwor- akul <i>et al.</i> (2016), ³ da Silveira <i>et al.</i> (2014), ⁴ Feng <i>et al.</i> (2015), ⁵ Diez-Fraile <i>et al.</i> (2014), ⁶ Scalici <i>et al.</i> (2016), ⁷ Sørensen <i>et al.</i> (2016)

B, Bovine; G, Goat; M, mouse; MR, Mare P, pig; R, Rat.

inhibition of these marker genes (Sirotkin *et al.* 2014). The proliferation marker genes *PCNA* and *cyclin D2* have also been reported to be downregulated by the overexpression of miR-181a, which, in turn, suppresses the expression of activin receptor IIA (*acvr2a*), thereby slowing the rate of granulosa cell proliferation (Zhang *et al.* 2013b). Induction of TGF- β 1-dependent granulosa cell proliferation was observed upon overexpression of miR-224 by regulating the expression of SMAD4 (Yao *et al.* 2010a).

It is well known that the majority of the ovarian follicles recruited during follicular development undergo atresia, which is triggered by granulosa cell apoptosis (Hughes & Gorospe 1991). There is accumulating evidence suggesting a role of miRNAs in regulating apoptosis of granulosa cells and determining follicular fates. miR-21 is enriched in murine granulosa cells upon induction of an ovulatory dose of LH (Carletti *et al.* 2010). In the same study, the anti-apoptotic role of miR-21 was verified by an increased abundance of caspase positive cells (an indicator of apoptosis) in the ovarian cortex

upon inhibition of miR-21, which resulted in a reduced rate of ovulation. Similarly, miR-92a prevents porcine granulosa cell apoptosis by targeting the SMAD7 gene (Liu *et al.* 2014). This shows that miR-92a prevents TGF- β -induced apoptosis in granulosa cells by fine-tuning the expression of SMAD7. In a similar study, the enrichment of the miR-183-96-182 cluster in granulosa cells of preovulatory dominant follicle was associated with a downregulation of the pro-apoptotic gene *FASL*, which has been shown to regulate granulosa cell apoptosis in a FOXO-dependent manner (Gebremedhn *et al.* 2016). The expression of Bax and other pro-apoptotic genes was significantly reduced in human granulosa cells due to overexpression of miR-22 (Sirotkin *et al.* 2010). In a separate experiment, it was confirmed that the expression of miR-22 in mice granulosa cells progressively declined as follicles shifted from healthy to atretic follicles, and with a regulatory effect on SIRT1 (Xiong *et al.* 2016a).

In an attempt to determine the effect of miRNAs on ovarian cell steroidogenesis, a genome-wide screening

experiment was performed in human *in vitro* cultured granulosa cells through transfection of 80 different human pre-miRNA constructs. It was shown that 36 miRNA constructs resulted in inhibition of progesterone release, while 10 miRNAs promoted the release of progesterone from granulosa cells (Sirotkin *et al.* 2009). In the same study, the release of estradiol was suppressed by 51 miRNAs, although none of the miRNA precursors stimulated estradiol synthesis. Overexpression of miR-378 in cultured murine granulosa cells downregulated transcription of the PR gene and its downstream transcripts; ADAMTS1, CTSL1 and PPARG (Toms *et al.* 2015). In a separate study, miR-378 suppressed aromatase protein expression and, thereby, estradiol production in porcine granulosa cells (Xu *et al.* 2011b). The release of estradiol from human and murine granulosa cells was inhibited upon overexpression of miR-34a and miR-320, respectively (Sirotkin *et al.* 2009, Yin *et al.* 2014). Estradiol synthesis in mouse granulosa cells was shown to be impaired by the overexpression of miR-764-3p, which suppresses the steroidogenic factor-1 (SF-1) (Wang *et al.* 2016). In the same study, it was reported that miR-764-3p-mediated repression of SF-1 leads to the suppression CYP19A1, which is a downstream target gene of SF-1. Contrary to this, miRNAs are reported to positively affect estradiol production in granulosa cells. A direct link between FoxL2 and miR-133b was reported, and miR-133b was shown to induce estradiol production by inhibiting the FoxL2 binding sites that target the promoter sequences of StAR and CYP19A1 (Dai *et al.* 2013). Moreover, miR-132 promotes estradiol production in mouse granulosa cells via translational repression of Nurr1 in the cAMP signaling pathway, which induces the expression of CYP19A1 (Wu *et al.* 2015). Similarly, miR-383 has been reported to positively regulate estradiol production by inhibiting RBMS1 and inactivating c-MYC (Yin *et al.* 2014).

miRNAs are not only involved in regulating mammalian steroidogenesis, but their expression is also affected by the level and composition of steroids in the follicular environment (Donadeu *et al.* 2016). In 2008, a report by Fiedler *et al.* (2008) demonstrated that treatment of preovulatory mouse mural granulosa cells with hCGs leads to differential expression of 13 miRNAs, and miR-132 and miR-212 were found to be highly upregulated post-hCG treatment. In line with this, hyperstimulation of cyclic heifers with supraphysiological levels of FSH induced changes in the expression of circulating miRNAs of follicular fluid and blood plasma, which could have an association with oocyte development (Noferesti *et al.* 2015). Similarly, treatment of cultured granulosa cells with FSH leads to changes in the expression of a large set of miRNAs, of which miR-29a and miR-30d were suppressed in the short term but upregulated in the long term (Yao *et al.* 2010b).

The expression and role of miRNAs during oocyte maturation

Oocyte maturation is the process by which the oocyte undergoes a morphological, physiological and developmental transformation from the prophase I to metaphase II (MII) stage by sequential meiotic division, chromatin remodeling and cytoplasmic organelle reorganization (Eppig 1996). While the oocyte progresses in growth and development, it acquires maternal stores (mRNAs and proteins) which are essential to support the development of the embryo during the early cleavage stages (Li *et al.* 2010). Global gene expression studies have shown that while about 400 genes are differentially expressed between immature and mature porcine oocytes (Budna *et al.* 2017), as many as 821 gene transcripts can be differentially regulated during the bovine oocyte maturation (Fair *et al.* 2007). Therefore, understanding the mechanisms by which the protein and mRNA are dramatically accumulated or degraded during the different stages of oocyte maturation is the main research focus in the field of female gametogenesis.

In animals, three main classes of small RNAs (miRNAs, siRNA and PIWI-interacting RNAs) are known to post-transcriptionally regulate gene expression through interactions with AGO proteins. Germ-cell-specific dicer knockout models have been used to demonstrate the significant role of Dicer in oocyte meiotic maturation by affecting spindle formation and chromosomal organization (Murchison *et al.* 2007). Similar results were also obtained when the expression of Dicer was depleted using RNAi (Liu *et al.* 2010). Moreover, in mouse oocytes, functional loss of Argonaute 2 (AGO2), which plays a key role in miRNA biogenesis (Diederichs & Haber 2007), caused spindle formation defects and chromosomal arrangement abnormalities, as well as the downregulation of several miRNAs, including let-7a, -b, -c, -d, -f, -g and -7i (Kaneda *et al.* 2009). The expression of genes involved in the RNA microprocessor (RNASEN, DGCR8 and Dicer1), miRNA transportation (RANGAP1, XPO1, XPO4 and XPO5) and components of the RNA-inducing silencing complex (EIF2C1, EIF2C2 and EIF2C3) in immature and mature rhesus monkey oocytes is also an indirect indicator of the presence of mature miRNAs during oocyte maturation (Mtango *et al.* 2009). Furthermore, increasing the level of Drosha, the RNase III which is essential for the first step of miRNA processing in the nucleus (Lee *et al.* 2003), was associated with increased pri-miRNA-processing activity in *Xenopus* oocytes, suggesting the presence of active miRNA biogenesis during oocyte maturation in that species (Muggenheimer *et al.* 2014). Nevertheless, Suh *et al.* (2010) have argued that functional loss of DGCR8 in the mouse oocytes does not affect oocyte maturation and embryo development, despite the significant effect on the miRNA biogenesis, thus suggesting that miRNAs might not be involved in oocyte maturation and embryo

development. Similarly, unlike for Dicer, depletion of Drosha or DGCR8 during porcine oocyte maturation did not affect the maturation rate (Liu *et al.* 2017b). Because Dicer is involved in both RNAi and miRNA biogenesis pathways, Suh *et al.* (2010) claimed (based on Dicer knockout/knockdown models) that the endogenous small interfering pathway is more important than the miRNAs during oocyte maturation. Nevertheless, apart from the canonical pathways, which involves DGCR8, Drosha, Dicer, Argonaute 2 and miRNA-processing genes, evidence indicates that miRNAs are also generated by the non-canonical pathway, which does not involve DGCR8, Dicer, Drosha or Argonaute 2 (Havens *et al.* 2012, Castellano & Stebbing 2013). Therefore, rather than relying on miRNA-processing genes, miRNA-specific knockout/knockdown experiments can be used as an alternative approach to address the roles of miRNAs during mammalian oocyte maturation and subsequent embryo development.

In line with this, specific and global miRNA profiling studies have indicated that changes in the expression pattern of biologically relevant miRNAs occur during the growth and development of mammalian oocytes (Tesfaye *et al.* 2009, Xu *et al.* 2011c, Xiong *et al.* 2016b). For example, miR-21, an miRNA whose target genes potentially participate in fatty acid metabolism and fatty acid biosynthesis, was found to be differentially expressed during oocyte maturation (Song *et al.* 2016). Interestingly, inhibition of miR-21 using an anti-miR21 peptide nucleic acid resulted in a reduced proportion of porcine oocytes reaching the MII stage and further embryo development (Wright *et al.* 2016). Similar results were also obtained by inhibiting the activity of let-7c, miR-27a and miR-322 in mouse oocytes (Kim *et al.* 2013), miR-15/16 in *Xenopus* oocytes (Wilczynska *et al.* 2016) and miR-378 in the porcine oocyte (Pan *et al.* 2015). On the other hand, increasing the expression of miR-224 in cumulus cells reduced the oocyte maturation rate and blastocyst development by regulating PTX3 expression (Li *et al.* 2017). More recently, it was shown that inhibition of miR-130b activity during bovine oocyte *in vitro* maturation reduced the first polar body extrusion, the proportion of oocytes reaching to MII stage and mitochondrial activity in the MII oocytes, indicating the involvement of this miRNA in bovine oocyte maturation (Sinha *et al.* 2017).

Under *in vitro* conditions, the dynamic miRNA profile changes are partly attributed to the *in vitro* maturation environment or ingredients used, while under *in vivo* conditions, the miRNA profile could be affected by the physiological conditions like the age of the animal. For example, in humans, treatment of metaphase I (MI) human oocytes with insulin-like growth factor 1 activates the expression of miR-133a, miR-205-5p and 145 miRNAs and suppresses 200 others, including miR-152 and miR-142-5p (Xiao *et al.* 2014). On the other hand, altered expression of 12 miRNAs (including let-

7b-5p and let-7e-5p) in oocytes derived from older woman compared to young women suggests that the *in vivo* miRNA profile in the oocytes is affected by oocyte aging (Battaglia *et al.* 2016). However, further functional studies are required to gain better insight into the role of those miRNAs whose expression is altered due to suboptimal culture conditions or aging.

The earliest stages of embryogenesis are dependent on the maternal store of transcripts and proteins, which decay during development so that embryonic development becomes dependent on transcripts and proteins from the embryonic genome (Telford *et al.* 1990). This maternal-zygotic transition occurs at the one-two cell stage in mice (Schultz 1993), 4–8 cell stage in humans (Telford *et al.* 1990) and 8–16 cell stage in bovine, with minor transcription at a zygote and 2-cell stages (Memili & First 1999). Like mRNA transcripts, oocytes are also known to be equipped with a large set of maternal miRNAs which are utilized or inherited by the embryo after fertilization and play a significant role in further development (Tang *et al.* 2007). In mice, a large-scale miRNA expression analysis comparing growing and matured oocytes and zygote stage embryos revealed dynamic changes in miRNA expression during oogenesis, whereas matured oocytes and zygotes had similar expression profiles, indicating significant maternal miRNA inheritance before the first cleave and divisions (Tang *et al.* 2007). In the same study, a significant number of these miRNAs (60%) were degraded at the one-two cell stages, suggesting that a significant proportion of the maternally inherited miRNAs are actively degraded during the first cell division. Maternally stored miRNAs might regulate embryonic genome activation and maternal mRNA degradation. This has been evidenced in zebrafish in which zygotic miR-430 targets several maternal mRNAs by accelerating the deadenylation and clearance of maternal mRNAs during early embryonic stages (Giraldez *et al.* 2006). Bovine expression profile studies have revealed that miRNAs are expressed in a stage-specific manner during embryo preimplantation following a typical maternal or embryonic pattern (Mondou *et al.* 2012, Abd El Naby *et al.* 2013).

miRNAs associated with ovarian pathophysiology

The malfunctioning of the ovary due to physiological, endocrine, paracrine or metabolic disorders can result in female infertility. During this time, the expression of some miRNAs could be induced or repressed by the normal ovarian physiology, ultimately leading to the termination of ovarian folliculogenesis. Identification and an in-depth understanding of these dysregulated miRNAs due to the ovarian disorder might identify appropriate molecular strategies to protect against ovarian disorders. For example, miR-18a is believed to be involved in the development of ovarian cancer by inducing cell cycle arrest and enhancing apoptosis by regulating the

expression of the inositol phosphate multikinase gene (Liu *et al.* 2017a). Likewise, while miR-30d suppresses ovarian cancer progression by inhibiting TGF- β 1-induced epithelial–mesenchymal transition (Ye *et al.* 2015), overexpression of miR-1271, miR-223-3p and miR-761 induces the development of ovarian cancer by activating the proliferative activity of the cancerous cells (Liu *et al.* 2015a, Shi & Zhang 2016, Wang *et al.* 2017, Fang *et al.* 2017). Since cancer development in the ovary is unusually caused by cell growth (by promoting the expression of anti-apoptotic genes and masking the role of apoptotic genes), miRNA could aggravate ovarian cancer by suppressing pro-apoptotic genes. Thus, miR-146a-5p is implicated in ovarian cancer development by suppressing the expression of anti-apoptotic genes, such as XIAP, BCL2L2 and BIRC5 (Li *et al.* 2017), and miR-205 has been implicated in aggravating ovarian cancer by suppressing the expression of the tumor suppressor gene TCF21 (Wei *et al.* 2017).

Another pathophysiological disorder that affects female fertility is the occurrence of polycystic ovarian syndrome (PCOS) (Harris & Terry 2016). Dysregulation of the ovarian miRNAs due to PCOS has been evidenced in many instances. Among others, 84 miRNAs, including miR-221, miR-222, let-7d and miR-26b, were found to be altered in the ovarian tissue of a PCOS rat model (Hossain *et al.* 2013). Also, decreased expression of miR-141-3p in the ovaries of rat PCOS models was associated with apoptotic incidences in the granulosa cells by increasing the expression of death-associated protein kinase 1 (Li *et al.* 2017). Other authors have reported that reduced miR-145 expression in the granulosa cells of PCOS women might increase granulosa cell proliferation by activating the MAPK/ERK signaling pathways (Cai *et al.* 2017). Also, downregulation of miR-483 in PCOS ovaries is believed to enhance ovarian cell proliferation and survival (Xiang *et al.* 2016). Interestingly, upregulation of miRNA-509-3p in the cumulus cells of PCOS woman was found to increase estradiol secretion of the granulosa cells by inhibiting the expression of MAP3K8 (Huang *et al.* 2016b).

PCOS also induces miRNA expression alterations in the cumulus and granulosa cells. The downregulation of miRNAs has been implicated in reduced insulin resistance in the cumulus cell of PCOS woman (Shi *et al.* 2015). Furthermore, dysregulation of miRNAs is potentially involved in the Wnt signaling pathway, MAPK signaling pathways and progesterone-mediated oocyte maturation in cumulus cells (Liu *et al.* 2015b, Cai *et al.* 2017). In granulosa cells, activation of miR-93 (Jiang *et al.* 2015) and altered expression of 19 miRNAs (Shi *et al.* 2015) have been detected in the granulosa cells of PCOS woman. The extended effect of PCOS in the preimplantation period is also evidenced by suppression of let-7a, miR-19b, miR-24, miR-92 and miR-93 and induction of let-7b, let-7g and miR-34b in blastocysts derived from PCOS woman (McCallie *et al.*

2010). These findings indicate how the occurrence of PCOS could influence miRNA expression in the ovarian cortex, granulosa cells and even in the oocytes. However, altered expression of miRNAs due to PCOS is not limited to the ovarian tissue; changes in the abundance of the circulatory miRNAs were also observed in the blood plasma (Sathyapalan *et al.* 2015), serum (Jiang *et al.* 2016, Eisenberg *et al.* 2017, Xiong *et al.* 2017) and follicular fluid (Sørensen *et al.* 2016) of PCOS woman. These findings suggest that circulating miRNAs are potential biomarkers for the detection of PCOS.

Extracellular mediated circulation of miRNAs and their role in reproduction

Extracellular vesicles have been identified as important mediators of intercellular communication and are known to regulate a wide range of biological processes. Based on their cellular origin, extracellular vesicles can be designated as ectosomes (vesicles secreted by neutrophils or monocytes), microparticles (vesicles shed from platelets in blood and endothelial cells), prostasomes (vesicles derived from seminal fluid), telerosomes (vesicles isolated from serum of antigen-fed mice) and cardiosomes (vesicles secreted by cardiomyocytes) (reviewed by EL Andaloussi *et al.* (2013)). Depending on their biogenesis and size, extracellular vesicles can be classified as exosomes (30–130 nm), microvesicles (50–1000 nm) and apoptotic bodies (500–2000 nm). Microvesicles are enriched in phosphatidylserine, and their membrane composition reflects that of the parent cell more closely than the membrane composition of exosomes (EL Andaloussi *et al.* 2013).

There is accumulated evidence of the importance of exosome-mediated cell-to-cell communication under pathological conditions. For example, circulating exosome-carried proteins can be used as diagnostic tools for cancer (Melo *et al.* 2015). Extracellular vesicles are present in reproductive tract fluid and potentially support early embryo development (Ng *et al.* 2013b, Ruiz-Gonzalez *et al.* 2015, Alminana *et al.* 2017). Protein characterization of extracellular vesicles from bovine oviductal fluid showed enrichment of proteins known to be involved in sperm–oocyte binding, fertilization and early embryo development (Alminana *et al.* 2017). During maternal embryonic crosstalk, vesicles are released by reproductive tract tissues and the embryo itself (Mellisho *et al.* 2017). In sheep, extracellular vesicles have been shown to have an important role in maternal embryonic crosstalk (Burns *et al.* 2016). This uptake of vesicles by various cell types is evidence of a role for those vesicles in cell-to-cell communication. In the course of this uptake, vesicles transfer their genetic material (e.g., mRNA, miRNAs) to the recipient cells, resulting in an increase in the level of endogenous miRNAs (Sohel *et al.* 2013). Although the molecular cargos of the extracellular vesicles are not characterized,

the supplementation of extracellular vesicles isolated from bovine follicular fluid from 3–6 mm follicles into the maturation and early embryo culture had a significant effect on the mRNA, miRNAs, DNA methylation and hydroxymethylation patterns of the resulting blastocysts (da Silveira *et al.* 2017). Similarly, the co-incubation of extracellular vesicles isolated from the follicular fluid of small (3–5 mm) and large (>9 mm) bovine follicles promoted cumulus expansion with a concomitant induction of cumulus expansion marker genes (Hung *et al.* 2015). Significant effects on cumulus expansion and induction of marker genes were evident during the supplementation of extracellular vesicles from the small follicle. This might be attributed to the differences in the concentration of EVs between follicular fluid derived from small and large follicles (Hung *et al.* 2015) or the higher relative abundance of shuttle RNAs in follicular fluid from follicles harboring a growing oocyte (compared to the fully grown oocyte) (Sohel *et al.* 2013). However, comprehensive molecular analyses (at mRNA, miRNA and protein levels) of the EVs from the biological fluid of interest are needed for a complete understanding of the mechanisms involved.

According to the ExoCarta online exosome database (2017), a large number of proteins (9.769), mRNA (3408), miRNA (2838) and hundreds of lipids (<http://exocarta.org>) have been detected in exosomes (Keerthikumar *et al.* 2016). The number of publications addressing exosomes and their molecular content has increased dramatically in the last five years. A large body of evidence is available for the presence of miRNA-carrying extracellular vesicles in various biological fluids, and these play a significant role in cell-to-cell communication related to male and female reproduction. A panel of miRNAs circulating in blood serum can be used as indicators of various cancers, including prostate, lung and colorectal, with up to 100% specificity (Chen *et al.* 2008, Mitchell *et al.* 2008). Table 1 summarizes the circulatory miRNAs that regulate various physiological activities in multiple species.

Most importantly, miRNAs released into the extracellular environment and carried by vesicles, lipids or proteins are known to be highly stable, withstanding extreme conditions (e.g., frequent freezing and thawing). This property makes these miRNAs potential biomarkers for oocyte and embryo developmental competence. Bovine embryos cultured from day 5–8 *in vitro* release different miRNAs into the culture media depending on their developmental capacity (Kropp *et al.* 2014). In that study, it was shown that certain groups of miRNAs are more abundant in the culture media of degenerated embryos than blastocyst embryos. This is to be expected as degenerated blastocysts are prone to release cellular contents into an extracellular environment, not only through exosomes but also through larger vesicles and apoptotic bodies. In humans, cleaving and morula stage embryos release no miRNAs into spent culture media,

whereas the blastocyst, which is mainly originated from trophectoderm cells, does release miRNAs (Capalbo *et al.* 2016). Retrospective analysis of miRNAs in spent media from unimplanted and implanted blastocysts revealed differential expression of miRNAs; miR-20a and miR-30c are highly abundant in spent media from implanted blastocysts and are predicted to target genes involved in cell-to-cell communication and signaling, cell adhesion and cell growth.

Conclusion

miRNAs play significant roles in regulating various processes in female reproductive axis starting from the primordial follicle activation through early embryo development and implantation for successful reproductive processes. In addition to their tissue-specific expression and role, large sets of miRNAs are also released into the extracellular space as key molecules for cell-to-cell communication or as cellular response to environmental stimuli. Despite the availability of large set of studies on the expression of miRNAs under various physiological conditions, so far, the functional roles of only few miRNAs have been determined in female reproduction. The functional analysis of miRNAs is mainly challenged by the complex interaction between miRNAs and mRNAs showing many-to-one and one-to-many relationships. However, due to their short size, but big impact on female reproductive processes, miRNAs could have a significant potential to be utilized as fertility markers and develop therapeutic tools for fertility-related problems in mammals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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