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Dynamics and role of MicroRNAs during mammalian follicular development

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Abstract

Ovarian functions, which involve dynamically regulated processes of selection, recruitment and dominance, are known to be regulated by an array of genes, which are expressed in spatiotemporal manner in follicular somatic cells and gametes. This differential expression of genes in mammalian follicular cells is partly regulated by posttranscriptional gene regulators named microRNAs (miRNAs). In addition to the cellular miRNAs, growing number of evidences are available on the potential role of extracellular vesicles mediated transfer of miRNAs in follicular fluid. These extracellular vesicles are shown to be involved in cell-to-cell communication within the follicular environment. The molecular messages carried by the extracellular vesicles released into extracellular space are thought to be reflections of the physiological status of the cells from where they are released. Therefore due to their structural nature and potential to cargo several physiologically relevant molecules, exosomes have a great potential to be used as markers of oocyte developmental competence in follicular environment. Here, we review large sets of literatures to show the dynamic nature of miRNAs during various stages of mammalian follicular development, oocyte growth and the role of some of the miRNAs in ovarian cell functions. Moreover, the presence of microvesicle and exosome-coupled extracellular miRNAs in mammalian follicular fluid and their potential involvement in cell-to-cell communication are briefly discussed.

Keywords: exosomes, extracellular vesicles, folliculogenesis, microRNAs, ovary.

MicroRNAs in mammalian ovary

The mammalian ovary is an organ where series of dynamically regulated processes of follicular recruitment, selection, dominance, ovulation and atresia undergo. These processes are under tight coordination of paracrine and endocrine factors which in turn regulate the expression and interaction of multitude of genes in the ovary (Hunter *et al*., 2004). In line with an attempt to unravel the genetic regulation of ovarian functions, the first functional importance of microRNAs (miRNAs) in female reproduction was evidenced by tissue specific knocking out of the *Dicer1*; an important evolutionary conserved ribonuclease III enzyme involved in miRNAs biogenesis. The conditional inactivation of *Dicer1* in mouse ovarian granulosa, oviductal and uterine cells resulted in female infertility by decreasing the rate of ovulation, shortening the uterine horns and formation of oviductal cysts (Nagaraja *et al*., 2008). This has opened the door for further investigation of miRNAs and their role in mammalian ovaries. Subsequently, in the last decade intensive research on identification and functional analysis of miRNAs was performed in ovaries of various species. Several groups have reported that miRNAs are expressed in mammalian ovarian cells signifying their potential involvement in posttranscriptional regulation of important genes in the ovary. Construction of ovarian small RNAs complementary DNA (srcDNA) libraries revealed that miRNAs are the most abundantly expressed class of small RNAs in mouse ovary (Ro *et al*., 2007). A massive parallel sequencing of small RNA fraction extracted from newborn mouse ovary has identified 398 known and 4 novel microRNAs expressed in newborn mouse ovary (Ahn *et al*., 2010). Similarly, small RNA library constructed from cow ovary identified 50 known and 24 novel miRNAs, among which miRNAs; let-7a, let-7b, let-7c, miR-21, miR-23b, miR-24, miR-27a, miR-126 and miR-143 were found to be the most dominant ones (Hossain *et al*., 2009). The expression pattern of miRNAs in sheep ovaries during anestrus and the breeding season identified 97 known, 369 conserved and 17 predicted novel miRNAs (Di *et al*., 2014). Ovarian samples of uniparous and multiparous goats showed differential expression of miRNAs in which 8 miRNAs of the let-7 family; let-7b, let-7b-5p, let-7-5p, let-7c, let-7c-5p, let-7f-5p, let-7f, let-7 and miR-140, miR-320a were the top 10 abundantly expressed miRNAs in both the uniparous and multiparous goat ovaries (Ling *et al*., 2014). Deep sequencing of miRNAs in porcine ovary also identified the abundance of around 732 mature miRNAs (Li *et al*., 2011). Following the identification of miRNAs in ovaries of various species, studies in the last years have focused on the expression characterization and functional analysis of these regulatory molecules in follicular growth and ovarian function.

Expression dynamics of miRNAs during follicular development

During the course of mammalian folliculogenesis, different miRNAs are expressed in theca cells, granulosa cells (Salilew-Wondim *et al*., 2014; Gebremedhn *et al*., 2015), cumulus-oocyte-complex (Abd El Naby *et al*., 2013), follicular-fluid (Sohel *et al*., 2013) and the corpus luteum (McBride *et al*., 2012; Maalouf *et al*., 2016b). We have previously shown significant differences in the expression of

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miRNAs in bovine granulosa cells derived from subordinate and dominant follicles of early luteal phase (day 3 and 7 of the estrous cycle; Salilew-Wondim *et al*., 2014) and late follicular phase (day 19 of the estrous cycle; Gebremedhn *et al*., 2015). Interestingly, the extent of differences in the relative abundance of miRNAs in granulosa cells of the two follicular categories was minimal at day 3, whereas it was much pronounced at day 7 and 19. However, irrespective of the day of estrous cycle, miR-26a, miR-10b and the let-7 family members were the most abundantly expressed miRNAs in granulosa cells of both dominant and subordinate follicles (Salilew-Wondim *et al*., 2014; Gebremedhn *et al*., 2015) signifying their cellular housekeeping role in bovine follicular development. The dynamic nature of miRNA expression has been also evidenced in ovine species during follicular-luteal transition in which miR-21, miR-125b, let-7a and let-7b were the most abundantly expressed miRNAs during comparative analysis of ovine growing follicles, preovulatory follicles, early and late corpus luteum (McBride *et al*., 2012). Similarly, microarray technology was used to investigate the association of miRNA expression with bovine follicular growth and atresia. For this, genome-wide miRNA expression analysis was performed on tissues samples derived from small growing (4-8 mm), large healthy (12-17 mm) and atretic follicles (Sontakke *et al*., 2014). Among the 17 differentially expressed miRNAs, miR-144, miR-202, miR-451, miR-652, and miR-873 were enriched in larger healthy follicles compared to the small growing follicles. Moreover, 57 miRNAs were differentially expressed between large healthy and large atretic follicles. To determine the involvement of selected miRNAs obtained from in vitro studies; miR-21, miR-23a, miR-145, miR-503, miR-224, miR-383, miR-378, miR-132, and miR-212 in regulating equine follicular development, Donadeu and Schauer (2013) aspirated follicular fluid from dominant follicles during ovulatory and anovulatory seasons. It was shown that the relative levels of miR-21, miR-23b, miR-378 and miR-202 were higher in ovulatory follicles, whereas miR-145 tends to have higher level in anovulatory follicles. The level of miR-21, miR-132, miR-212, and miR-224 was increased in leading follicles, whereas the expression of their predicted target genes; *PTEN*, *RASA1* and *SMAD4* was reduced, indicating their potential involvement in regulating cell survival, differentiation and steroidogenesis during follicular selection in equine ovary (Schauer *et al*., 2013).

Depending on their genomic localization, miRNAs are grouped and termed as clusters (Mathelier and Carbone, 2013). MiRNA expression data analysis revealed an interesting pattern of co-enrichment or degradation of miRNA clusters in specific stages of bovine follicular development. Accordingly, the miR-183-96-182 cluster, miR-132-212 cluster and miR-424-450-542 cluster miRNAs were significantly enriched in granulosa cells of preovulatory dominant follicles while the miR-17-92 cluster miRNAs were enriched in granulosa cells of the subordinate follicles obtained at day 19 of the estrous cycle (Gebremedhn *et al*., 2015).

Potential role of miRNAs in controlling ovarian functions

During the course of follicular development, proliferation and differentiation of granulosa cells are essential for proper follicular recruitment, dominance, maturation, ovulation and atresia. Several studies have indicated the involvement of miRNAs in regulating the proliferation, differentiation, survival, cell cycle transition and/or apoptosis of granulosa cells (Carletti *et al*., 2010; Sirotkin *et al*., 2010; Yin *et al*., 2014; Andreas *et al*., 2016; Gebremedhn *et al*., 2016). Interestingly, in vitro granulosa cell culture models have become most popular and widely used to understand the potential role of miRNAs in ovarian functions. For this reason, majority of the functional studies of miRNAs in granulosa cells are performed through in vitro loss-and-gain of function by modulating the levels of selected miRNAs (Maalouf *et al*., 2016a).

In an attempt to determine the role of human miR-15a in controlling basic granulosa cell functions, Sirotkin *et al.* (2014) transfected primary human granulosa cells with anti-miR-15a and miR-15a precursor followed by evaluation of the expression of marker genes for cell proliferation (*MAPK/ERK1,2* and *PCNA*), apoptosis (*Caspase3* and *Bax*) and hormonal levels (Progesterone, Estradiol and Testosterone) in spent culture media. It was shown that inhibition of the miR-15a increased the protein accumulation of proliferation and apoptosis marker genes, reduction in the release of progesterone and testosterone and promotes the release of estradiol. Contrary to this, overexpression of miR-15a resulted in opposite effect. Evidences showed that multiple extra- and intraovarian factors such as the members of the TGF-β superfamily members (van den Hurk and Zhao, 2005; Knight and Glister, 2006), activin receptor-like kinases (ALK) and the Smads (Miyazawa *et al*., 2002; Florio *et al*., 2010) are implicated in regulation of follicular development. Members of the TGF-β superfamily, which are known to be involved in granulosa cell proliferation and differentiation (Hsueh *et al*., 1984; Hirshfield, 1991), were reported to be posttranscriptionally regulated by miRNAs in granulosa cells (Yao *et al*., 2014). In that study, over expression of miR-224 enhanced TGF-β1 induced proliferation of granulosa cells by targeting *Smad4* gene. Conversely, inhibition of miR-224 suppressed the proliferation of granulosa cells induced by TGF-ß1 treatment. Similarly, miR-181a expression decreased in mouse granulosa cells treated with activin A in dose dependent manner and overexpression of miR-181a inhibited cell proliferation by targeting acvr2a, which in turn resulted in suppression of *CCND2* and *PCNA* (Zhang *et al*., 2013). Recently, we showed that the miR-183-96-182 cluster coordinately target the expression of *FOXO1* and promote proliferation and cell cycle transition of bovine granulosa cells. Overexpression of the miR-183-96-182 cluster miRNAs reduced the proportion of cells under G0/G1 arrest and increased the proportion of cells entering the S-phase of the cell cycle. Interestingly, selective degradation of *FOXO1* using siRNAs also promoted the cell

proliferation and cell cycle transition. This evidence supports the idea of a coordinated role of this miRNA cluster in repressing anti-proliferation genes and their downstream transcripts (Gebremedhn *et al*., 2016). During various stages of ovarian follicular development, majority of the follicles undergo atresia being triggered by granulosa cells apoptosis (Portela *et al*., 2015). Accumulated evidences are available regarding the regulation of miRNAs in granulosa cells apoptosis and determine the follicular cells fate to either ovulation or atresia. In one of the early miRNA functional study, miR-21 as one of the three highly induced miRNAs in murine granulosa cells in response to ovulatory dose of LH administration, induces granulosa cells apoptosis upon its inhibition. In the same study, *in vivo* anti-miR-21 treated ovary resulted in reduced rate of ovulation compared to the untreated contralateral ovary (Carletti *et al*., 2010). The let-7g induces apoptosis in pig granulosa cells by targeting the *TGFBR1* gene (Zhou *et al*., 2015), while porcine atretic follicles are highly enriched with let-7g and its overexpression promoted apoptosis of granulosa cells (Cao *et al*., 2015). MiR-26b promotes porcine granulosa cells apoptosis and induces follicular atresia by targeting *SMAD4* (Liu *et al*., 2014) and *ATM* (Lin *et al*., 2012). MiR-34a promotes granulosa cell apoptosis by targeting *INHBB* in porcine ovary (Tu *et al*., 2014). Similarly, functional analysis of the miR-17-92 cluster, which was found to be enriched in bovine granulosa cells derived from the subordinate follicles of day 19 of the estrous cycle, revealed the regulatory role of this cluster in granulosa cells proliferation, differentiation and steroidogenesis by targeting *PTEN* and *BMPR2* genes. Overexpression of the miRNA cluster promoted cell proliferation, and decreased proportion of differentiating cells (Andreas *et al*., 2016).

MiRNAs in steroidogenesis and their hormonal regulation

According to the two-cell-two-gonadotropin theory, luteinizing hormone stimulates thecal cells to produce androgens, and follicle-stimulating hormone stimulates granulosa cells to produce estrogens from androgens (Barnes *et al*., 2000). Growing evidences indicate that miRNAs act as regulators of steroidogenesis (Schauer *et al*., 2013; Toms *et al*., 2015; Donadeu *et al*., 2016). The effect of miRNAs on the release of major ovarian steroid hormones was determined by transfecting human primary granulosa cells with 80 different human pre-miRNAs constructs, and the release of progesterone was inhibited by 36 miRNAs. Whereas, 10 miRNAs promoted progesterone release (Sirotkin *et al*., 2009). MiR-378 decreases ovarian estradiol production by targeting aromatase. However, overexpression of the aromatase 3'-UTR in granulosa cells enhanced aromatase expression at protein level, possibly mediated by the binding of miR-378 within this region, thereby reducing the binding of this miRNA to the endogenous aromatase 3'-UTR (Xu *et al*., 2011). In a separate study, the progesterone receptor (PGR) was targeted by miR-378-3p which leads to decrease in the protein and mRNA levels and its downstream target genes; *ADAMTS1*, *CTSL1*, and *PPARG*, which are involved in follicular maturation and remodeling (Toms *et al*., 2015). A recent study showed that level of miR-378 in the follicular wall was correlated with estradiol, the estradiol: progesterone ratio and *CYP19A1* (Donadeu *et al*., 2016). MiR-34a and miR-320 inhibit estradiol release from human granulosa cells and murine ovaries, respectively (Sirotkin *et al*., 2009; Yao *et al*., 2014). However, miR-320 could stimulate testosterone and progesterone in murine ovaries. Interestingly, miR-383 has been shown to act as positive regulator of estradiol production in mouse granulosa cells by inhibiting *RBMS1*, which leads to c-Myc inactivation and steroidogenesis (Yin *et al*., 2014). Moreover, miR-132 is involved in the cAMP signaling pathway and promotes estradiol synthesis via the translational repression of *Nurr1* in ovarian granulosa cells and significantly induce expression of *Cyp19a1* through *Nurr1*; a direct target that suppresses *Cyp19a1* expression (Wu *et al*., 2015). Similarly, miR-133b is involved in follicle-stimulating hormone (FSH)-induced estrogen production by down-regulating *Foxl2* and inhibiting the Foxl2-mediated transcriptional repression of *StAR* and *CYP19A1* to promote estradiol production (Dai *et al*., 2013). The overexpression of miR-20a, which is validated to target PTEN and BMPR2 genes, induced the release of progesterone in in vitro bovine granulosa cell culture (Andreas *et al*., 2016)

Recently, we investigated the effect of supra-physiological level of gonadotropins introduction during the process of controlled ovarian hyperstimulation on the level of extracellular miRNAs in bovine follicular fluid and blood plasma (Noferesti *et al*., 2015). It was shown that, a total of 57 and 21 miRNAs were differentially expressed in follicular fluid and blood plasma, respectively, derived from hyper-stimulated heifers compared to the unstimulated control counterpart. Bioinformatics analysis revealed that pathways including the TGF-ß signaling, MAPK signaling and oocyte meiosis are significantly enriched by target genes of the miRNAs selectively upregulated in follicular fluid and blood plasma of hyper-stimulated heifers (Noferesti *et al*., 2015). Despite the fact that the choice of the mechanism of release of miRNAs into extracellular space is not understood well, we have detected circulatory miRNAs in exosomes and Ago2 fraction of follicular fluid and blood plasma of hyperstimulated and unstimulated control heifers. This may evidence the releasing mechanism of miRNAs into extracellular space in both follicular fluid and blood plasma was not affected by super-stimulation treatment.

Extracellular miRNAs in follicular fluid

While the majority of miRNAs are found intracellularly, tremendous numbers of miRNAs have been found to circulate in the extracellular space and biological fluids, including follicular fluid (Donadeu and Schauer, 2013; Sohel *et al*., 2013; Santonocito *et al*., 2014). Mammalian follicular fluid consists of a

complex mixture of nucleic acids, proteins, metabolites and ions, which are known to be secreted by the oocyte, granulosa cells, theca cells and blood plasma components that come to the follicular fluid via theca capillaries (Revelli *et al*., 2009; Rodgers and Irving-Rodgers, 2010). The follicular fluid creates very suitable microenvironment for the growth and development of oocytes and its biochemical composition varies depending on the physiological status of the follicle. As any alteration in the follicular fluid composition can be associated with the oocyte quality, the follicular fluid components may provide useful diagnostic information on oocyte developmental competence and ovarian functions (Revelli *et al*., 2009). Several biochemical components including hormones, growth factors, cytokines and chemokines, which are secreted by follicular cells into follicular fluid, are known to promote oocyte maturation. Evaluation of any of specific components within the follicular fluid at any stage of follicular development revealed the physiological status of the animal in general and the oocyte health or growth status in particular (Sohel *et al*., 2013). In addition to autocrine and paracrine mode of communication inside the ovarian follicle, recently alternative mechanisms come into play. This mechanism in which the oocytes and follicular somatic cells exchange signals is mediated by the so called microvesicles and exosomes carrying RNAs (mRNA, miRNA, lncRNA and other types of RNAs), proteins and DNA fragments.

Extracellular vesicles can be broadly classified into 3 main classes: Microvesicles/ microparticles/ ectosomes, which are produced by outward budding and fusion of the plasma membrane, whereas exosomes are formed within the endosomal network and released upon fusion of multivesicular bodies with the plasma membrane. The release of extracellular vesicles (EVs), including exosomes and microvesicles, is a phenomenon shared by many cell types as a means of communicating with other cells. EVs released into the extracellular space can potentially reach distant tissues via circulation and once up taken by neighboring and/or distant cells, EVs can transfer functional molecules that may alter the status of recipient cells, thereby contributing to both physiological and pathological processes.

Extracellular microvesicles and exosomes in follicular fluid carrying miRNAs as cargo molecules have been detected in bovine (Sohel *et al*., 2013; Noferesti *et al*., 2015; Navakanitworakul *et al*., 2016), equine (da Silveira *et al*., 2012, 2014, 2015) and human (Xiao *et al*., 2016). The presence of EVs (microvesicles or exosomes) in follicular fluid may support the notion that they play a significant role in ovarian functions. The size and concentration of EVs in the follicular fluid between different sized follicles has been investigated (Navakanitworakul *et al*., 2016). In the same study while no significant changes were observed in the size of the extracellular vesicles between small, medium and large follicles, the concentration of extracellular vesicles decreased progressively as the follicle size increases. A microarray or sequencing based expression profiling of exosomes from bovine follicular fluid identified miRNAs enriched in follicular size dependent manner (Navakanitworakul *et al*., 2016) and oocyte developmental competence (Sohel *et al*., 2013). Interestingly, in the former study those miRNAs enriched in exosomes isolated from small follicle were found to be associated with cell proliferation, while miRNAs abundant in large follicle were found to be related to inflammatory response pathways. Similarly, significant number of miRNAs have been identified to be differentially carried by exosomes or Ago2 protein complexes isolated from follicular fluid harboring oocytes with different growth status (Sohel *et al*., 2013). Those differentially expressed miRNAs from both exosomal and non-exosomal fraction of the follicular fluid were found to be involved in molecular pathways (MAPK signaling pathway, focal adhesion and regulation of actin cytoskeleton), which are known to be main regulators of follicular development and oocyte growth.

During the course of bovine follicular development, expansion of cumulus cells is essential for oocyte maturation and release through ovulation for a successful fertilization and early embryo development. LH induced increase in the expression of genes such as prostaglandin-endoperoxide synthase 2 (*Ptgs2*), pentraxin-related protein 3 (*Ptx3*), and tumor necrosis factor alpha-induced protein 6 (*Tnfaip6*) are essential for the expansion of cumulus cells. Recently, co-incubation of bovine COC with extracellular vesicles isolated from follicular fluid aspirated from small and large follicles under in vitro condition resulted in cumulus expansion and increased expression of the aforementioned genes (Hung *et al*., 2015). Moreover, age related differences in exosome mediated miRNA abundance in follicular fluid of mare has been evidenced (da Silveira *et al*., 2015). This differential expression of exosomal miRNAs has been associated with the expression of granulosa cells genes associated with TGF-β signaling pathway, which is the most dominant pathway modulating follicular development and oocyte growth.

Potential role of extracellular miRNAs in cell-to-cell communication

Ovarian follicular development and oocyte maturation are well coordinated processes and mediated by a constant exchange of signals between ovarian somatic cells and the oocytes (Zuccotti *et al*., 2011). These exchanges of signals are made possible through the establishment of gap junctions between the surrounding somatic cells and the oocytes (Cecconi *et al*., 2004; Gilchrist *et al*., 2008). In the follicular environment the physical contact between the surrounding cells especially granulosa and oocytes is mediated by the expression of key molecules mainly connexines (*CX37* and *CX43*) which are also expressed in early stages of mammalian embryo development (Gittens and Kidder 2005; Gittens *et al*., 2005). Mutual communication between the oocyte and cumulus cells is achieved through secretion of both oocyte or cumulus

cells secreted factors and are shown to affect each other in relative abundance of transcripts at mRNA (Regassa *et al*., 2011) and miRNA level (Abd El Naby *et al*., 2013). The oocyte secreted factors which pass through the gap junctions include ions, metabolites and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control oocyte development (Gilchrist *et al*., 2008). In addition to the direct communication, the transfer of signals between the different follicular cell types could be facilitated by extracellular vesicles circulating in the follicular fluid.

The release of EVs, including exosomes and microvesicles, is a cellular characteristics shared by many cell types as a means of communication with other cells and also potentially removing cell contents. This phenomenon is more evident in follicular environment where cell-to-cell communication is vital for the growth of the oocytes. MiRNAs carried by microvesicles and exosomes in follicular fluid are also found to be present in the surrounding follicular cells including theca cells, granulosa cells and cumulus-oocyte-complex (Sohel *et al*., 2013). In addition to the identification of miRNAs carried by exosomes in mammalian follicular fluid, several in vitro experiments have been conducted to simulate the cell-to-cell communication mediated by exosomes (da Silveira *et al*., 2012, 2014; Sohel *et al*., 2013; Di Pietro, 2016). For this, we have previously shown that membrane labeled exosomes isolated from bovine follicular fluid harboring either growing or fully grown oocytes, were co-cultured with granulosa cells in vitro (Sohel *et al*., 2013). The uptake of exosomes by cultured granulosa cells and resulted in subsequent increase in endogenous cellular miRNAs and altered gene expression. Similar experiment in equine has also evidenced the uptake of microvesicles by cultured granulosa cells in vitro and in vivo experiments (da Silveira *et al*., 2012). Exosomes isolated from mid-estrous and preovulatory follicles and known to cargo *ACVR1* and miR-27b, miR-372, and miR-382 were found to alter the ID2 (an *ACVR1* target gene) in preovulatory mare follicle (da Silveira *et al*., 2014).

All in all, the follicular fluid will provide a potential environment for identification of marker molecules associated with follicular development and oocyte competence. Moreover, with the identification of extracellular vesicles as cargo for molecular signals, tremendous opportunities will be opened for therapeutics in the field of fertility treatments.

Conclusion

Mammalian folliculogenesis or ovarian functionality is well coordinated process involving a molecular cross-talk between the various somatic cell types and the developing gamete in the follicular microenvironment to attain successful ovulation, fertilization and development of embryo and to give birth to healthy offspring. Understanding the genetic regulation of these processes paves the way to identify molecular profiles and their regulatory mechanisms associated with ovarian function and facilitates the development of diagnostic markers for oocyte developmental competence. Especially, exploring follicular fluid for the presence of extracellular miRNAs, which could be indicators of ovarian functions and subsequently competence of oocytes, facilitates the development of non-invasive diagnostic tools to address female infertility. Moreover, future researches need to focus in deciphering the exact sources and role extracellular miRNAs in follicular fluid towards the development of therapeutic tools for fertility treatments associated with ovarian disorders.

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