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The microRNAs important for ovarian and early embryonic development in cattle

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Abstract: Recent progress in genomic analysis and other biochemical methods has led to the discovery of a large population of microRNAs (miRNAs), which have been demonstrated to play important roles in diseases and a wide range of developmental processes. Characterization of miRNA expression profiles in different stages of ovarian follicular development and early embryogenesis has suggested the potential roles of miRNAs in follicular development, maturation of oocytes, and preimplantation embryonic development. This review focuses on the current studies of miRNAs involved in ovarian and early embryonic development in cattle.

Key words: MicroRNA, cattle, oocyte, follicular development, early embryogenesis

1. Introduction

The main agricultural species of commercial importance suffer from problems related to egg quality and early embryonic development. In the dairy cattle industry, the calving rate is less than 50% among artificially inseminated cows (1). In the in vitro production of bovine embryos, the blastocyst rate is around 30%–50%, and after embryo transfer, the abortion rate is 8% to 13%, with the greatest embryonic loss occurring around day 8 (2–4). The newly fertilized embryos undergo dramatic morphological and genetic changes, mainly characterized as maternal to zygotic transition (MZT) (5). The rapid cell division and development of early embryos before MZT is driven by the maternal transcripts/factors accumulated inside the oocyte. During early development, when transcriptional activity is repressed, these maternal transcripts are translated into functional proteins or stored for later recruitment, leading to embryonic genome activation (6). There is an imperative need to understand the functions of oocyte-derived factors and how their translation and degradation are controlled during early embryogenesis. A growing body of evidence indicates that microRNAs (miRNAs) are major mediators involved in translational control during oocyte development and early embryogenesis (7). The miRNAs are a group of small noncoding RNAs of 18–24 nucleotides that were first discovered in *Caenorhabditis elegans* (8). They can regulate gene expression by recognizing specific sites on the mRNA or promoter region of target genes (9,10). Recent studies have implicated the regulatory functions of miRNAs in ovarian folliculogenesis and early embryonic development. In this review, we will summarize the current knowledge of miRNAs involved in ovarian and early embryonic development in cattle, with an emphasis on the role of miRNAs in the regulation of oocyte-specific maternal effect genes.

2. Biosynthesis of miRNAs

The miRNAs are encoded by the genome and are produced from the intergenic region, introns or exons of protein-coding genes (11). Most miRNAs have their own enhancer and promoters, indicating that their expression could be controlled by transcriptional factors, DNA methylation, or other mechanisms found in protein-coding genes (12–14). The processing pathways of miRNAs are conserved in different species, and miRNAs are transcribed mostly by RNA polymerase II or in some cases RNA polymerase III (15,16). At the beginning of transcription, primary miRNA (pri-miRNA) is transcribed by corresponding RNA polymerase (II or III), which folds into the characteristic hair-pin structure through the complementary regions on the pri-miRNA sequence. While still inside the nucleus, the pri-miRNA is processed by the protein complex RNase III, Dorsha and DiGeorge syndrome critical region 8 (DGCR8), to form precursor miRNAs (pre-miRNAs) of ~70 nucleotides. With the help of exportin 5, a RanGTP-dependent nuclear transporter, pre-miRNA is translocated from the nucleus to the cytoplasm (17). Once in the cytoplasm, the protein complex of RNase III, Dicer, and TEBP, recognizes the pre-miRNA and cleaves the loop, giving rise to a miRNA duplex consisting of a guide strand

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and a passenger strand (18). The guide strand is loaded in the RNA-induced silencing complex and achieves its function through complementary binding to the target sequences (19). Production of some of the miRNAs originating from short intronic regions follows a different pathway, which is Dorsha/DGCR8-independent (20,21).

3. miRNAs in bovine fetal and adult ovaries

The ovary is composed of several cell types, including the oocyte and a number of somatic cells such as granulosal, thecal, luteal, and cumulus cells. Following the signals from the hypothalamus–pituitary–gonad axis, these cells coordinately respond to endocrine and paracrine factors, which eventually work back on the cells and regulate the expression and dynamics of gene networks in order to achieve the ovarian cycle. The development of germ cells is driven by the molecular and cellular changes inside the fetal ovary, which is a prerequisite to the production of mature oocytes capable of fertilization. To identify and study the involvement of miRNAs in germ cell and ovarian development, bovine fetal and adult ovary miRNA libraries were constructed, and over 100 miRNAs were found (22–24). In the bovine fetal ovary, miR-99a and miR-125b are the most abundant miRNAs, while in the adult ovary, the let-7 family miRNAs have the highest expression, which is considered to be required for timing of cell fate determination. The relative abundance of the miRNAs in fetal and adult ovaries indicates their cellular housekeeping roles during ovarian development. Interestingly, miR-10b was found in both fetal and adult ovarian samples and showed an ovary-specific expression pattern. The expression profile of miR-10b during early embryonic development indicates that it might be maternally inherited and plays a role in the maternal to zygotic gene activation (22).

4. miRNAs in bovine follicles

After puberty, follicles start to grow on a cyclic basis, facing 2 possible fates: atresia or full maturation resulting in ovulation of the oocyte. Ovulation occurs only in dominant follicles in each estrous cycle, and most oocytes inside the subordinate follicles are lost through atresia. The follicular selection mechanism is highly dependent upon the functions of granulosal cells. During oocyte development, granulosal cells play important roles in support of oocyte development and prepare the dominant follicle to respond to the LH surge, which triggers massive gene expression change and leads to ovulation. The miRNAs, as one of the molecular cues, play a role in regulation of molecular mechanisms in granulosa cells associated with ovulation and follicular atresia. During the first wave of folliculogenesis (day 3 and day 7), 9 miRNAs (miR-21-3p, miR-221, miR-708, miR-214, miR-335, miR-155, miR-199a-5p, miR-21-5p, and miR-222) were found to be differentially expressed between granulosal cells from dominant follicles and subordinate follicles of Simmental heifers (25). Gene oncology analysis revealed that the differentially expressed miRNAs might be involved in regulation of programmed cell death, cell projection morphogenesis, regulation of cell proliferation, and biosynthesis of macromolecules (25). During the last follicular wave after the dominant preovulatory follicle is selected (day 19), 65 miRNAs were differentially expressed in granulosal cells from dominant versus subordinate follicles. The miRNAs involved in cell death were upregulated, while those involved in inhibition of apoptosis were downregulated. Potential targets of differentially expressed miRNAs were found to be related to cell proliferation and apoptosis mechanisms such as Wnt signaling, MAPK signaling, and TGF-β signaling pathways (26). By comparing miRNA expression profiles between small (4–8 mm) and large (12–17 mm) bovine follicles, several miRNAs including miR-144, miR-202, miR-451, miR-652, and miR-873 were identified to be upregulated in large healthy follicles. Targets of these miRNAs were mapped to signaling pathways involved in follicular cell proliferation, steroidogenesis, prevention of premature luteinization, and oocyte maturation (27). These studies indicated that the stage-specific miRNA profiles in bovine granulosal cells are highly related to follicular selection and development.

Recent studies have implicated the role of extracellular miRNAs in the regulation of bidirectional communication between the oocyte and somatic cells (28,29). Numerous studies in different species have demonstrated that microvesicles and exosomes released from many cell types serve as endocrine and/or paracrine regulatory factors that influence recipient cell phenotypes (30,31). In cattle, exosomal and nonexosomal fractions of extracellular miRNAs in follicular fluid were related to bovine oocyte developmental competence (28). Differentially expressed extracellular miRNAs were identified in both exosomal and nonexosomal fractions between follicles containing mature and immature oocytes. Uptake of exosomes into follicular cells was also observed in primary culture of bovine granulosal cells (28). These studies demonstrate a potential yet important role of extracellular miRNAs in follicular development and oocyte competence.

5. miRNAs in the bovine cumulus–oocyte complex

Cumulus cells surround the oocyte to form the cumulus–oocyte complex (COC). In response to the LH surge, cumulus cells expand and continue to enclose the oocyte even after fertilization until early embryo development. Oocyte secreted factors act on and direct the development and function of cumulus cells. In return, cumulus

cells contribute to oocyte maturation and subsequent developmental potential. To investigate the function of miRNAs in the course of bovine oocyte development, the expression of miRNAs in immature and mature COCs was characterized (32). In total, 59 miRNAs were differentially expressed between immature and mature COCs, of which 31 and 28 miRNAs showed preferential expression in immature and mature COCs, respectively. Additional studies showed that the expressions of miR-205, miR-150, miR-122, miR-96, miR-146a, and miR-146b-5p were decreased dramatically in COCs at different maturation times from 0 h to 22 h (33) and miR-130b was upregulated in immature compared to mature COCs (34). Microinjection knockdown experiments showed that the majority of oocytes injected with anti-miR-130b remained arrested at the telophase I stage and the number of oocytes reaching metaphase II was reduced significantly compared to the control group (34). The general population of miRNAs in bovine COCs has been characterized, and the let-7 miRNA family shows the highest expression in bovine COCs (35). Interestingly, miR-106a expression was found to be significantly higher in oocytes compared to COCs and granulosal cells (35). Target prediction indicated that WEE1A protein kinase is the potential target of miR-106a, and a negatively correlated expression pattern was observed between miR-106a and the predicted target. It was speculated that when the protein kinase activity of WEE1A is suppressed, proper acquisition of meiotic competence in the oocytes is ensured as WEE1A inhibits the maturation-promoting factor.

6. miRNAs in the bovine corpus luteum

The most important functions of the ovary are first to produce viable oocytes resulting in successful fertilization and healthy embryos, and second to form a functional corpus luteum (CL), which is critical in the maintenance of pregnancy (36). After ovulation, the CL, which is transformed from the ovulated follicle, requires intense angiogenesis and produces significant amounts of progesterone, essential for the maintenance of early pregnancy. The formation of the CL also is regulated by miRNAs. In mouse, absence of miR-17-5p and let-7b impaired CL formation (37). When bovine granulosal and thecal cells were cultured in luteinization-promoting media, the expressions of miR-199a-3p, miR-125b, miR-145, and miR-31 decreased significantly, while miR-21, miR-142, and miR-503 showed increased expression during the follicular–luteal transition. Target prediction of downregulated miRNAs revealed multiple genes involved in differentiation of granulosal cells, such as *MYC*, *CDKN1A*, and *LIF*, which are known to be increased in response to human chorionic gonadotropin during ovulation. An upregulated miRNA during the follicular–luteal transition, miR-21, was found to be important in mediating the antiapoptotic effects of the ovulatory LH surge on luteinization of granulosal cells (38). In cattle, comparing the miRNA expression patterns between nonregressed and regressed CL revealed 13 differentially expressed miRNAs. (39). One of them, miR-378, which is known to be associated with apoptosis, was found to be dramatically upregulated in nonregressed CL. The interferon gamma receptor 1 (*IFNGR1*) gene, which potentially plays a role in apoptosis of the luteal cell, was predicted to be the target of miR-378. Western blot analysis showed that miR-378 can repress the expression of IFNGR1 protein but not IFNGR1 mRNA, supporting a role of this miRNA in apoptosis of bovine CL (39).

7. miRNAs in bovine early embryonic development

In conditional DGCR8 knockout mouse, the miRNA biogenesis pathway was specifically blocked; however, normal blastocyst development was not affected by the deficiency of miRNAs (40). Moreover, 3' untranslated regions (UTRs) of mRNAs, which are upregulated in Dicer1 knockout oocytes, were not enriched in the DGCR8 mouse, indicating that the absence of miRNAs does not impact the turnover of mRNAs in early embryos (41). Results of these 2 studies using knockout mouse models downplayed the importance of miRNAs during early embryonic development. However, these studies focused only on 3' UTRs targeting miRNAs generated by canonical Dicer- and DGCR8-dependent pathways, while other miRNAs targeting the open reading frame (ORF) of mRNAs and/or generated by a noncanonical DGCR8-independent pathway were not investigated. miRNAs such as miRNA-430 in zebrafish, miRNA-427 in *Xenopus*, and miRNA-21 in rainbow trout have been shown to be present in early embryos prior to embryonic genome activation, and these miRNAs are responsible for degradation of maternal transcripts (42–45). In bovine early embryos, miR-21 and miR-130a showed significant increases during the 1–8 cell stage (46). Microinjection of bovine zygotes with anti-miR-130b resulted in significantly reduced rates of morulae and blastocysts (34). These studies provided significant evidence supporting a role of miR-130b during bovine early embryonic development. Furthermore, miRNA and mRNA networks have been discovered in bovine blastocysts. Comparison of miRNA expression profiles between unhatched and hatched bovine blastocysts revealed 8 upregulated (miR-127, miR-130a, miR-196a, miR-155, miR-203, miR-29c, miR-28, and miR-376a) and 4 downregulated (miR-135a, miR-218, miR-449b, and miR-335) miRNAs in hatched blastocysts. The direct interactions of 3 miRNAs with developmentally important factors (miR-218 and CDH2, miR-218 and NANOG, and miR-449b and NOTCH1) were confirmed

by multiple biochemical experiments (47). These results indicate that miRNAs are very important during bovine early embryonic development, and the proposed function of miRNAs is to meet the needs for regulation of the maternal mRNAs, which have to be degraded.

8. Bovine oocyte-specific genes and their regulation by miRNAs

The finely orchestrated development and maturation of the oocyte has been the focus of many studies in which essential oocyte-specific genes have been identified (48,49). A number of such genes have been proven to play essential roles in mammalian folliculogenesis and early embryonic development (50). These genes code for a compilation of factors involved in chromatin remodeling, self-renewal proliferation, transcription initiation, and nuclear transportation. Aberrant expression of these essential genes leads to abnormalities in multiple developmental stages, from failure of resumption of oocyte meiosis to disruption of early embryonic development (51–54). Therefore, acute control of the expression of these factors is critical for normal follicular and embryonic development. Recent studies in cattle have demonstrated an important role of specific miRNAs in the regulation of these oocyte-specific factors during MZT.

8.1. Nucleoplasmin 2 (NPM2)

NPM2 is a nuclear chaperone involved in decondensation of sperm chromatin in *Xenopus* oocytes (55). Knockout of NPM2 in mouse led to impaired development in the 2-cell stage and reduced cleavage rate. NPM2-null embryos showed high levels of chromatin abnormalities including loss of heterochromatin and increased acetylated H3 around the nuclei (56). Supplementation of NPM proteins to bovine oocytes after somatic cell nuclear transfer resulted in normal embryos with higher pregnancy rate (57). These results indicate that NPM2 is essential for chromatin reprogramming and normal development of early embryos. In cattle, NPM2 mRNA and protein are most abundant in GV and MII oocytes, but both mRNA and protein decrease sharply in early embryos after embryonic genome activation (58). A specific miRNA, miRNA-181a, was predicted to target bovine NPM2 mRNA. Expression of miR-181a was increased in 4–16 cell stage embryos, which is coincident with the time of embryonic genome activation in cattle when expression of NPM2 decreases. Cotransfection experiments revealed that the expression of bovine NPM2 protein was lower in HeLa cells expressing miR-181a compared to the negative control, indicating a role of miR-181a in translational silencing of NPM2 in bovine embryos (58). miR-181a is also known to be involved in granulosal cell proliferation and embryonic stem cell differentiation (59).

8.2. New born ovary homeobox encoding gene (*NOBOX*)

NOBOX is one of the earliest homeobox genes preferentially expressed in germ cells and is present throughout all stages of folliculogenesis in mice (51,60). NOBOX deficiency in female ovary allowed normal development of primordial follicles; however, further development was arrested at the transition from primordial to primary follicle. NOBOX depletion also reduced expression of a significant number of very important oocyte-specific genes, some of which have been shown to be essential in oogenesis and early embryogenesis. Therefore, NOBOX is a master regulator in follicular development (51). In cattle, NOBOX has been characterized and shown to be important even after fertilization during early embryonic development (22). miR-196a recognizes and binds to the 3' UTR of bovine NOBOX mRNA. Coexpression experiments in cultured HeLa cells showed reduced expression of NOBOX protein in cells expressing miR-196a compared to control cells without miRNA-196a. Specific binding of miR-196a to NOBOX mRNA was confirmed by luciferase assays with constructs containing mutations on the predicted binding site. Furthermore, ectopic expression of miRNA-196a mimic in bovine early embryos reduced NOBOX protein expression. These data indicate that miR-196a mediates the degradation of the untranslated maternal NOBOX mRNA, which is very critical for early embryonic development.

8.3. Factor in germline alpha (FIGLA)

FIGLA is a germ cell-specific transcription factor, which controls expression of oocyte-specific zona pellucida (ZP) genes. Knockout of FIGLA in female mice led to sterility due to impaired meiosis and germ cell apoptosis (52). In cattle, FIGLA expression is restricted to gonadal tissues (61). Bovine FIGLA is expressed abundantly in early embryos up to the 8-cell stage. After MZT, its expression drops to undetectable levels in morula and blastocyst stage embryos (61). A miR-212 binding site was identified in the 3' UTR of bovine FIGLA mRNA, and the seed region appears conserved across several mammalian species. Real-time PCR analysis showed similar expression profiles of miR-212 and FIGLA mRNA during early development, both dropping after embryonic genome activation. Luciferase reporter assays in HeLa cells showed specific interactions between miR-212 and the predicted binding site in the FIGLA 3' UTR. Microinjection of miR-212 mimic into bovine embryos revealed that miRNA-212 mimic could inhibit FIGLA protein expression (61). These studies indicated that FIGLA is a critical factor for follicular and embryonic development and miR-212 is a potential posttranscriptional regulator of FIGLA during MZT in cattle.

8.4. Karyopherin alpha 7 (KPNA7)

KPNA7 is a newly identified member of the KPNA protein family, which is the major player in translocation of macromolecules through an active energy-dependent nuclear import system (62,63). KPNA7 is specifically

expressed in oocytes of several mammalian species (64–66). Knockdown of KPNA7 in early embryos led to arrested embryonic development in cattle and swine (64,66). Both KPNA7 mRNA and protein are abundant in early embryos but almost depleted in embryos after MZT (64,66). To determine if KPNA7 is regulated by miRNA during MZT, a computational prediction was performed to identify miRNA binding sites on the bovine KPNA7 mRNA. Interestingly, 4 binding sites for miR-1296 on the ORF of the gene were identified. Expression of miR-1296 tends to increase in 4-cell and 8-cell stage embryos and declines in morula and blastocyst stage embryos. Cotransfection experiments showed that the expression of bovine KPNA7 protein is reduced in cells expressing miR-1296 compared to the control cells, indicating that translation of KPNA7 is repressed by miR-1296 (67). Luciferase reporter assays using mutant constructs carrying mutations in the predicted binding sites on KPNA7 mRNA revealed that one of the binding sites (1156–1176 bp) is the primary binding site responsible for miR-1296 action (67). These results indicated a potential role of miR-1296 in regulating the expression of KPNA7 during early embryogenesis in cattle.

9. Conclusions

Characterization of expression profiles of numerous miRNAs originated from bovine oocytes, granulosal cells, luteal cells, and early embryos has demonstrated the potential importance of a significant number of miRNAs in the development of follicles, maturation of oocytes, luteal function, and early embryogenesis in cattle. However, very few of these miRNAs have been characterized with regard to their specific target genes and regulatory effects. Therefore, future studies should focus on identifying the specific targets of potentially important miRNAs and understanding the functional roles of these miRNAs in relation to ovarian function and early embryonic development in cattle.

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