

Commentary

Preventing Granulosa Cell Apoptosis Through the Action of a Single MicroRNA¹

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The development and rupture of an ovulatory follicle is dependent upon the actions of the gonadotropins follicle-stimulating hormone and the midcycle surge of luteinizing hormone (LH), respectively. After rupture, the cellular remnants of the ovulatory follicle differentiate into steroid-secreting luteal cells. Continued survival of the granulosa cells following ovulation and their luteinization is critical for progesterone production and the ability to sustain pregnancy, and several studies [1–3] have documented a prosurvival effect from surge levels of LH on granulosa cells following its binding and signaling via LH/chorionic gonadotropin receptors (LHCGR).

The balance between induction of proapoptotic and antiapoptotic pathways in granulosa cells residing within an ovulatory follicle, however, is complex and not well understood. Factors downstream of LH signaling that determine whether granulosa cell survival or apoptosis is favored have been discovered and investigated individually. For example, progesterone has an antiapoptotic effect on luteinized granulosa cells in most species studied, thereby promoting their continued survival and allowing development of the corpus luteum [4–7]. Other examples of individual factors that play a role in determining granulosa cell survival in the ovulatory follicle include pituitary adenylate cyclase-activating polypeptide (PACAP), epidermal growth factor-like (EGF) ligands, hyaluronan and proteoglycan link protein 1 (HAPLN1), as well as phosphatase and tensin homolog (PTEN) [8–11]. Each of these components regulates distinct pathways that in turn ultimately regulate the induction of apoptosis. A report by Carletti et al. [12] in this issue of *Biology of Reproduction* provides insight regarding a novel mechanism whereby granulosa cell survival is maintained in the ovulatory follicle after LHCGR signaling through the synthesis and action of a specific microRNA (miRNA).

MicroRNA Synthesis and Function

The miRNAs are small (19–25 bp), noncoding RNAs that limit cellular protein expression [13]. In mammals, miRNAs

are first synthesized as RNA transcripts between several tens and hundreds of kilobases long (referred to as primary miRNA [pri-miRNA]) by the DNA-dependent RNA polymerase-II (POLII) [14].

A pri-miRNA typically possesses multiple miRNAs and is cleaved in the nucleus into shorter (~70-bp) precursor miRNAs. This reaction is performed by a protein complex consisting of the enzyme DROSHA and the double-stranded RNA-binding protein PASHA [15]. The precursor miRNA is exported from the nucleus by an EXPORTIN5/RAN complex and cleaved in the cytoplasm by DICER1 into a mature, 19- to 25-nucleotide miRNA duplex. The miRNA strand with lower base pairing stability (the guide strand) is loaded onto the RNA-induced silencing complex (RISC) composed of DICER1, TAR RNA-binding protein isoforms 1 or 2 (TRBP1/2), and the Argonaute protein AGO2 [13, 16]. The RISC complex is guided to its mRNA target by the miRNA strand, where it then prevents protein synthesis via two different mechanisms: inhibition of translation or RNase-mediated destruction of mRNA [17–19]. The former occurs when the imperfect homology between the miRNA and target mRNA interferes with translation, whereas the latter results from a perfect match between the miRNA/mRNA hybrid and the activation of RNase activity that leads to mRNA degradation (Fig. 1).

Recent studies have determined that mRNA degradation grossly affects protein production, whereas inhibition of translation leads to fine-scale adjustments in protein levels [20]. Moreover, as the inhibition of translation occurs with mismatches between the mRNA and the miRNA, numerous genes may be targets of a single miRNA. In fact, the levels of hundreds, and perhaps even thousands, of different proteins can be reduced through the action of a single miRNA [19, 20].

MicroRNA Action in the Ovulatory Follicle

Shortly after the discovery of miRNAs and the realization that they serve as master regulators of posttranscriptional events, a few studies were conducted to determine their presence, and in some cases targets, in the female reproductive tract [21–24]. A critical role for miRNAs in regulating female fertility, however, was firmly established through the generation of conditional *Dicer1* null mutants, wherein its selective deletion in the ovary led to female reproductive effects, including inhibition of antral follicle development, induction of granulosa cell apoptosis, and reduced ovulatory efficiency [25–27].

Fiedler et al. [28] subsequently used a microarray approach to systematically identify differentially expressed miRNAs in granulosa cells before and after LHCGR activation. RNA was

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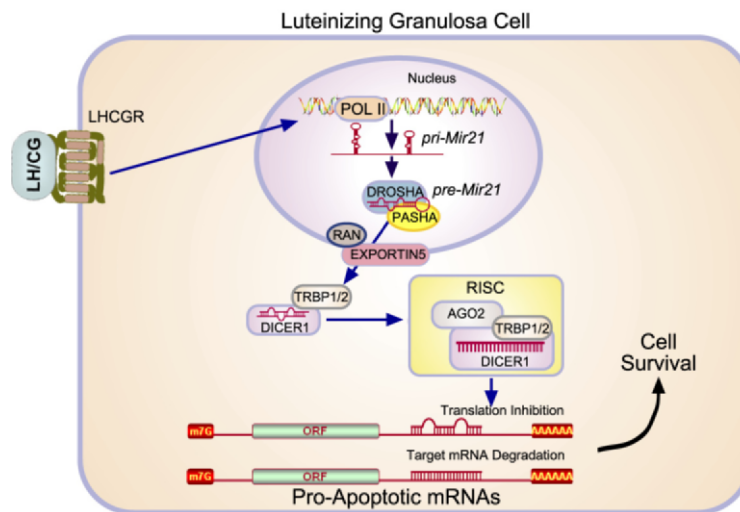
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FIG. 1. Signaling through the LHCGR induces the expression of the prosurvival microRNA *Mir21* in luteinizing granulosa cells. Granulosa cells residing within the periovulatory follicle respond to LH to induce the transcription of primary (pri) *Mir21*, which is subsequently processed by DROSHA and PASHA. Following its export to from the nucleus by EXPORTIN5/RAN, DICER1 converts *Mir21* into its mature form. Following its association with the components (e.g., DICER1, TAR-RNA binding protein [TRBP1/2], and the Argonaute protein AGO2) that form the RISC, *Mir21* binds to target mRNAs and inhibits protein expression through their degradation or interference with translation. In the luteinizing granulosa cells of the ovulatory follicle, such targets include those that otherwise directly or indirectly promote apoptosis.



obtained from mouse granulosa cells before (0 h, nonluteinized cells) and 4 h after human chorionic gonadotropin (hCG) treatment, from which 212 unique miRNA species were identified. Of these, 13 miRNAs were differentially expressed between the 0 and 4 h post-hCG samples, with 10 being downregulated and 3 being upregulated. Two of the latter miRNAs, granulosa cell microRNA-132 (*Mir132*) and granulosa cell microRNA-212 (*Mir212*), were induced 17- and 34-fold, respectively, relative to basal levels (0 h, control) following hCG treatment. Both *Mir132* and *Mir212* are formed from the same pri-miRNA, which, as expected, is also induced in mouse granulosa cells through LHCGR signaling. Furthermore, the study demonstrated that inhibition of mouse granulosa cell *Mir132* action using complementary locked nucleic acid (LNA) oligonucleotides increased C-terminal binding protein-1 (CTBP1) protein levels, which in turn may directly affect granulosa cell steroid production, because CTBP1 has been shown to regulate adrenal steroidogenesis by serving as a corepressor of steroidogenic factor-1 (SF1) activity [29].

The study by Fiedler et al. [28] led to the systematic evaluation of the miRNAs differentially expressed in luteinizing granulosa cells, and it serves as the foundation for the paper by Carletti et al. [12] in this issue of *Biology of Reproduction*. Carletti et al. observed that hCG treatment of equine chorionic gonadotropin (eCG)-primed immature mice led to a 30-fold maximum stimulation of pri-*Mir21* in mural granulosa cells collected 4 h after hCG, whereas mature *Mir21* mural granulosa cell levels increased to a 5.8-fold maximum 6 h after hCG. In contrast, regulation of mature *Mir21* expression in nonluteinized mouse granulosa cells in vitro did not recapitulate what was observed in vivo, in that its abundance in cultured granulosa cells increased 5-fold upon plating, with no change following treatment of cells with hCG or cell-permeable 8-bromo-cAMP. In granulosa cells transfected with a *Mir21*-targeting LNA oligonucleotide (MIR21-LNA), the authors demonstrated that *Mir21* could be depleted to approximately 4% of the levels present in cells treated with a scrambled or control LNA. Reduction of *Mir21* levels to that extent resulted in a marked change in granulosa cell morphology that included their “rounding” and “lifting” off of the culture dish, both of which are hallmarks of apoptosis.

Molecular markers of apoptosis (positive annexin A5 staining determined by flow cytometry; cleaved caspase 3 determined by immunoblotting) increased hours after granulosa cells were transfected with the MIR21-LNA in a dose-

dependent manner. The authors also noted that cleaved caspase 3 in granulosa cells decreased 6 h after administration of hCG in vivo, the time that coincides with increasing levels of *Mir21*. The in vivo effect of *Mir21* on ovarian apoptosis was subsequently established through the intrabursal injection of a *Mir21*-targeting LNA possessing a nuclease-resistant phosphorothioate backbone before hCG administration. Apoptosis was significantly increased in *Mir21*-depleted ovaries compared to the contralateral ovary receiving only saline or scrambled LNA. Moreover, using this same in vivo anti-*Mir21* LNA delivery approach, the number of cumulus-oocyte complexes recovered from the oviduct adjacent to the ovary treated with MIR21-LNA was significantly reduced compared to the ovary treated with scrambled LNA or the saline-treated control ovary. Thus, these findings demonstrate for the first time that a single miRNA (*Mir21*) plays a critical role in maintaining the survival of granulosa cells in the periovulatory follicle in response to LH (Fig. 1). This is also the first study to demonstrate a role for miRNAs, particularly an oncogenic miRNA such as *Mir21*, in the normal physiology of the ovary.

MicroRNAs and Ovarian Function: Future Directions

For studies that shed light on a novel or previously unappreciated biological process, more new questions surface than are answered. The study by Carletti et al. is no different in this respect, with the most prominent question being which mRNAs are posttranscriptionally regulated by *Mir21* that protect luteinizing granulosa cells from apoptosis? Targets previously reported to be under the control of *Mir21* were analyzed by the authors, but none was found to change substantially when granulosa cells were treated with the MIR21-LNA. A systematic genomic and proteomic determination of *Mir21*-regulated targets in granulosa cells from the ovulatory follicle would likely clarify this issue. In the study by Carletti et al., increased expression of *Mir21* was also noted in the granulosa cells obtained following gonadotropin-mediated follicle development (i.e., after eCG injection), raising the question of whether this miRNA exhibits the same antiapoptotic activities during folliculogenesis. This question is particularly relevant in light of the recent published report demonstrating that conditional inactivation of DICER1 in follicular granulosa cells leads to increased primordial follicle numbers and accelerated early follicle recruitment [27]. Lastly,

the mechanisms regulating ovarian *Mir21* expression, or other miRNAs for that matter, are presently unknown and await further investigation.

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