Involvement of miRNAs in ovarian follicular and luteal development

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Abstract

Although much progress has been made in the genetic dissection of biological networks involved in follicular/luteal development in the mammalian ovary, the gene regulation mechanisms involved are still poorly understood. Over the last 10 years, miRNAs have emerged as master regulators of tissue growth and differentiation in animals. However, compared with other body tissues, little is still known about the functional involvement of miRNAs in the ovary. Several studies have identified miRNA populations specifically associated with the development of follicles and corpora lutea, particularly in relation to the follicular–luteal transition, and the functional involvement of some of these miRNAs has been characterised in vitro and/or in vivo. Specifically, three different miRNAs, miR–224, miR–378 and miR–383, have shown to be involved in regulating aromatase expression during follicle development. In addition, miR–21 has been identified as promoting follicular cell survival during ovulation, and pro-angiogenic miR–17–5p and let–7b were shown to be necessary for normal development of the corpus luteum. Experimental evidence for the involvement of several other miRNAs in different aspects of follicle/luteal development has also been obtained. In addition, many of these studies exemplify the challenges associated with identifying physiologically relevant targets of ovarian miRNAs. Continuous advances in this field will be considerably facilitated by progress in understanding miRNA physiology in other body systems and will eventually lead to a much better understanding of the control of follicular/luteal development. In turn, through the potential offered by miRNA diagnostics and miRNA therapeutics, this new knowledge should bring considerable benefits to reproductive medicine.


Introduction

The mammalian ovary is an extremely dynamic organ within which sequential waves of follicular growth and regression, rupture of mature follicles and the adjacent ovarian wall during ovulation, repair of the ovulation wound and the formation of fully functional corpora lutea followed by its demise a few days later occur within relatively short cycles and under tight hormonal regulation throughout a female’s reproductive life (Fig. 1). Cyclic ovarian activity is key to reproductive success and the profound changes in tissue composition and function involved require exquisite spatiotemporal co-ordination of proliferation, apoptosis and differentiation of many different cell types within follicles, corpora lutea and ovarian stroma. Two key developments during the last 12 years have dramatically increased knowledge of the molecular control of follicular and luteal development. One is the advent and application of genomic technologies to study ovarian function leading to the identification of hundreds of new genes putatively involved in ovarian processes in different species (Espey & Richards 2002, Evans et al. 2004, Agca et al. 2006, Hamel et al. 2008, Bogan et al. 2009). The second major development has been the discovery and characterisation of animal microRNAs (miRNAs) as key global post-transcriptional regulators of tissue growth and differentiation both in health and disease (Ambros 2004, Croce 2009, Sayed & Abdellatif 2011). Although clear advances have been made in understanding transcriptional gene regulation during follicular and luteal development (Wu & Wiltbank 2002, Liu et al. 2009, Patel et al. 2009, Pisarska et al. 2011), little is known about the post-transcriptional mechanisms involved. Given the dynamic nature of the mammalian ovary, miRNAs are predicted to play important roles in the regulation of ovarian function, and studies over the past 5 years have already begun to identify and characterise such roles. This review will summarise current knowledge on the involvement of miRNAs in normal follicular and luteal development within the adult mammalian ovary. Endogenous siRNAs rather than miRNAs may be functionally important in the oocyte (Suh et al. 2010); the involvement of miRNAs in oocyte biology has been reviewed elsewhere and will not be covered here (Hawkins et al. 2011).
miRNA biology

miRNAs are short, non-coding RNAs, 19–25 nucleotides in length, that act by targeting partially complementary sequences within mRNAs, most commonly in their 3'-UTR, leading to functional repression of target transcripts (Huntzinger & Izaurralde 2011). The latest release of the miRNA database, MiRBase, contains just above 2000 different human miRNAs (release 19.0, http://www.mirbase.org). A single miRNA may target hundreds of genes and each of these genes may in turn be regulated by several different miRNAs. In this way, miRNAs are thought to regulate the activity of most protein-coding genes in mammals (Friedman et al. 2009). In line with their role as master regulators of cell differentiation processes, many miRNAs are highly conserved and their expression is tightly regulated during development (Berezikov 2011). miRNA biogenesis in animal cells is schematically shown in Fig. 2. miRNAs are most commonly transcribed by RNA polymerase II as independent genes (intergenic) or from introns of protein-coding genes, and miRNA-coding sequences may be clustered within specific genomic regions (Berezikov 2011). Primary miRNA transcripts are processed into stem-loop precursors (pre-miRNAs) by the microprocessor complex, which contains the RNAse III endonuclease, DROSHA and DiGeorge syndrome critical region gene 8 (DGCR8), an RNA-binding protein that facilitates cleavage by DROSHA. A type of intronic miRNAs called mirtrons do not follow this general pattern but are generated by conventional intron splicing, thus avoiding the requirement for DROSHA (Ruby et al. 2007).

Figure 1 Schematic representation of ovarian events (lower panel) and associated changes in circulating hormone levels (upper panel) during an ovulatory cycle as exemplified by the bovine. Waves involving several antral follicles periodically develop in the ovaries during each cycle. Depending on the species, a variable number of follicular waves can occur during each ovulatory cycle, e.g. usually one in human, two to three in cattle and ≥5 in porcine, each of which is preceded by a stimulatory surge of circulating FSH concentrations. Only a limited number of follicles within each wave (dominant follicles) usually reach the pre-ovulatory stage and the remaining undergo atresia before they can reach an equivalent stage of development (atretic follicles are shown in grey). Only one dominant follicle typically develops during each wave in monovular species such as humans and cattle. Dominant follicles produce high levels of oestradiol, which, in the absence of a corpus luteum (CL), will trigger a surge in circulating LH followed by ovulation. After ovulation, a transitory blood-filled structure (corpus haemorrhagicum, CH) forms before the development of a fully functional CL whose main role is to produce high levels of progesterone. At about 2 weeks after ovulation and in the absence of pregnancy, luteolysis occurs and the CL involutes into a non-functional corpus albicans (CA), followed shortly after by another ovulation. The two follicular cell compartments, theca and granulosa, are schematically shown (lower panel), which encase the cumulus–oocyte complex within the fluid-filled antral cavity. The top box in this figure schematically summarises steroid synthesis in follicular/luteal tissues showing the main end products and enzymes involved, reference to which is made in this review.
Precursor miRNAs are transferred by Exportin 5 (XPO5) to the cell cytoplasm where a second endonuclease, DICER, in association with TAR RNA-binding protein (TRBP), cleaves the pre-miRNA into a mature double-stranded form. One of the mature miRNA strands is then preferentially loaded onto ribonucleoprotein complexes (RISC) where proteins of the AGO and GW182 families co-operatively mediate the repression of target mRNAs. Although there is still controversy about the precise mechanisms by which miRNAs exert their effects on gene expression, recent evidence suggests that, in general, mRNA destabilisation, followed or preceded by translational repression, accounts for most of the observed effects of miRNAs on the levels of target genes (reviewed in Huntzinger & Izaurralde (2011)).

Identification and profiling of miRNAs in ovarian tissues

Small RNA populations have been identified by cloning-based or next-generation sequencing of normal ovarian tissues from human (Landgraf et al. 2007), mice (Ro et al. 2007, Mishima et al. 2008, Ahn et al. 2010), pigs (Li et al. 2011), cattle (Hossain et al. 2009, Tripuran et al. 2010, Huang et al. 2011, Miles et al. 2012) and sheep (McBride et al. 2012). Most of those studies involved analyses on whole ovaries rather than on specific ovarian tissue components, an approach that, although very useful for comprehensive identification of miRNA sequences, provides very limited insight into their functional relevance. miRNAs constitute the most abundant class of small RNAs in the ovary (Ro et al. 2007, Mishima et al. 2008, Ahn et al. 2010, Huang et al. 2011). In a study using porcine tissues, as many as 673 different miRNA sequences were identified by deep sequencing of whole ovaries (Li et al. 2011), although not all may be bona fide miRNAs (Kozomara & Griffiths-Jones 2011). Ovarian-expressed miRNAs can be mapped throughout the genome; however, chromosomes X and 2 reportedly account for a relatively high number of ovarian miRNAs in several species (Ahn et al. 2010, Huang et al. 2011, Li et al. 2011). Although ovarian tissues used for miRNA sequencing so far include a range of developmental stages (fetal, pre-pubertal or adult) in different species, in all studies as few as ten miRNAs accounted for more than half of all ovarian miRNA sequences (Table 1). Some of the highly abundant miRNAs were common across studies, including miR–21, miR–143, let–7 family, miR–26a and miR–125b, suggesting important roles in mammalian ovarian function.

Lists of miRNAs expressed in specific ovarian compartments including follicular granulosa cells of mice (Fiedler et al. 2008, Yao et al. 2010a) and horses (da Silveira et al. 2012), cumulus–oocyte complexes (COCs) from cows
miRNAs and follicular/luteal development

Table 1 Ten most abundant miRNAs in whole ovaries\(^1\) or follicular/luteal tissues\(^2\) as reported in different studies using cloning-based\(^a\) or next-generation\(^b\) sequencing. The bovine miR-143 sequence (Hossain et al. 2009, Huang et al. 2011) corresponds to miR-143-3p in human, pig and mouse.

<table>
<thead>
<tr>
<th>Human (adult)</th>
<th>Cow (neonatal/adult) (Huang et al. 2011)(^{1b})</th>
<th>Cow (fetal) (Tripuran et al. 2010)(^{1a})</th>
<th>Cow (adult) (Hossain et al. 2010)(^{1a})</th>
<th>Pig (adult) (Li et al. 2011)(^{1b})</th>
<th>Mouse (adult) (Mishima et al. 2008)(^{1a})</th>
<th>Mouse (neonatal) (Ahn et al. 2010)(^{1a})</th>
<th>Sheep (adult) (McBride et al. 2012)(^{1a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-143-3p</td>
<td>miR-143</td>
<td>miR-99a</td>
<td>let-7b</td>
<td>miR-21</td>
<td>miR-125b</td>
<td>miR-320</td>
<td>miR-21</td>
</tr>
<tr>
<td>miR-125b</td>
<td>let-7f</td>
<td>miR-125b</td>
<td>let-7c</td>
<td>let-7b</td>
<td>miR-121</td>
<td>let-7f</td>
<td>miR-125b</td>
</tr>
<tr>
<td>let-7b</td>
<td>let-7a</td>
<td>let-7c</td>
<td>miR-125</td>
<td>let-7i</td>
<td>miR-149</td>
<td>let-7b</td>
<td>let-7b</td>
</tr>
<tr>
<td>let-7a</td>
<td>let-7c</td>
<td>miR-125a</td>
<td>miR-143</td>
<td>let-7i</td>
<td>miR-199a</td>
<td>miR-298</td>
<td>let-7a</td>
</tr>
<tr>
<td>miR-10b</td>
<td>miR-26a</td>
<td>miR-7a</td>
<td>let-7a</td>
<td>miR-143-3p</td>
<td>miR-199a-3p</td>
<td>miR-298</td>
<td>miR-16a</td>
</tr>
<tr>
<td>miR-99a</td>
<td>miR-26a</td>
<td>miR-26a</td>
<td>miR-24</td>
<td>let-7i</td>
<td>miR-145</td>
<td>miR-16b</td>
<td>miR-142-3p</td>
</tr>
<tr>
<td>miR-26a</td>
<td>miR-148a</td>
<td>miR-27a</td>
<td>let-7c</td>
<td>miR-148a-3p</td>
<td>miR-351</td>
<td>miR-30a</td>
<td>let-7c</td>
</tr>
<tr>
<td>miR-29a</td>
<td>miR-21</td>
<td>miR-652</td>
<td>miR-24-3p</td>
<td>miR-21</td>
<td>miR-140</td>
<td>miR-322</td>
<td>miR-202</td>
</tr>
<tr>
<td>let-7c</td>
<td>miR-140</td>
<td>miR-126*</td>
<td>miR-21</td>
<td>miR-29a-3p</td>
<td>miR-34c</td>
<td>miR-152</td>
<td>let-7f</td>
</tr>
</tbody>
</table>

Challenges associated with studying miRNA function during follicular and luteal development

Genetically engineered mice with a conditional deletion of Dicer1 in reproductive tissues or that carry a hypomorphic Dicer1 gene have been used to study the effects of deficient miRNA biogenesis in the ovary (Hong et al. 2008, Nagaraja et al. 2008, Otsuka et al. 2008, Gonzalez & Behringer 2009, Lei et al. 2010). In general, the effects of Dicer1 deficiency on miRNA populations of mouse reproductive tissues were relatively modest as illustrated by the fact that only 28 miRNAs were downregulated in Dicer1 knockout oviducts (Nagaraja et al. 2008). Therefore, although it is useful to study Dicer1 function, these genetically engineered models do not comprehensively test for the effects of miRNA deficiency. In that regard, deletion of specific miRNA genes (Park et al. 2010) or in vivo delivery of miRNA inhibitors (Otsuka et al. 2008, Carletti et al. 2010) are likely to be more useful approaches. Many studies have used gain-of-function or loss-of-function approaches to study the effects of specific miRNAs on cultured ovarian cells (Fiedler et al. 2008, Otsuka et al. 2008, Carletti et al. 2010, Yang et al. 2010a, Xu et al. 2011, Yin et al. 2012), although the physiological relevance of the information obtained may sometimes be limited (see discussion below).

Elucidating the physiological targets mediating the effects of miRNAs in animal tissues remains a challenging task. Different algorithms can be used to identify predicted targets of miRNAs (reviewed in Thomas et al. (2010)). Such computational algorithms use specific criteria to estimate the likelihood of miRNA–mRNA pairing and, depending on the stringency of each algorithm, up to several thousand miRNA targets can be predicted for a given miRNA; however, many targets identified by computational models are not bona fide targets and, in addition, many biologically relevant targets fail to be predicted, which makes this approach alone very unreliable for detecting physiologically relevant targets. A minority of predicted miRNA targets have
been experimentally validated, usually in human or rodent cells; this provides added confidence that they may be actual targets in other cells or species of interest, provided miRNA recognition elements (MREs) in the target 3'-UTR are conserved.

When testing the effects of overexpressing or inhibiting miRNAs on the levels of a given target or reporter gene, consideration needs to be given to the fact that miRNA targets can be cell context dependent (Pasquinelli 2012) and that the effects on a given target may depend on the simultaneous expression of other miRNAs targeting the same gene. In addition, consistent with their fundamental role as micro-managers of gene expression in cells, the effects of individual miRNAs on their targets are often modest and may be difficult to detect experimentally (Berezikov 2011). For example, miR–21 knockdown in granulosa cells had no effect on the levels of four different genes that are known to be targeted by this miRNA in other cell types (Carletti et al. 2010). Target identification is more likely to be successful if the above methods are used in combination with unbiased large-scale approaches, for example, transcriptome and/or proteome analyses or immunoprecipitation of RISC complexes followed by sequencing or microarray screening of pulled-down miRNAs (Chi et al. 2009, Guo et al. 2010); however, these techniques have yet to be reported in relation to the study of ovarian miRNAs.

Roles of miRNAs during follicular development

Results with conditional Dicer1 knockouts are consistent with an involvement of this enzyme in follicle development. Mouse lines were created with a conditional deletion of Dicer1 in tissues that express anti-Müllerian hormone receptor 2 post-natally, that is, oviductal and uterine mesenchyme as well as granulosa cells from pre-antral and small antral follicles (Hong et al. 2008, Nagaraja et al. 2008, Gonzalez & Behringer 2009, Lei et al. 2010). These animals displayed obvious reproductive tract abnormalities including paratubal cysts and hypotrophy of oviduct and uterus, in addition to an ovarian phenotype suggestive of defects in follicle development, oocyte maturation and/or ovulation.
Such defects included accelerated early follicle recruitment, increased follicular atresia, a reduction in the number of pre-ovulatory follicles and the number of natural or induced ovulations, compromised oocyte integrity and the presence of luteinised follicles that contained a trapped oocyte. Further, both in vivo and in cultured cells, knockout of Dicer1 was associated with altered expression of key genes involved in granulosa cell proliferation and steroidogenesis as well as genes involved in oocyte development (Lei et al. 2010). Despite irregularities in the oestrous cycle, circulating levels of FSH were normal in Dicer1-deficient animals (Nagaraja et al. 2008).

The involvement of specific miRNAs in different aspects of follicle development has been precisely examined in several other studies (Table 2) and their results are discussed below.

### Involvement of miRNAs during follicular growth and steroidogenesis

Dynamic changes in the levels of different miRNAs have been reported during follicle development in mice (Yao et al. 2009, Lei et al. 2010, Lin et al. 2012, Yin et al. 2012), pigs (Xu et al. 2011) and sheep (McBride et al. 2012) and also in response to stimulation of murine follicular cells with gonadotropins (Fiedler et al. 2008, Lei et al. 2010, Yin et al. 2012) or growth factors (Yao et al. 2010a). Treatment of granulosa cells from mouse pre-antral follicles with TGFβ1 in culture resulted in the upregulation of three miRNAs and downregulation of 13 miRNAs (Yao et al. 2010a). One of the upregulated miRNAs, miR–224, was shown to mediate the stimulatory effects of TGFβ1 on granulosa cell proliferation, aromatase (Cyp19a1) expression and oestradiol production. In addition,

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**Table 2** Summary of reported roles of miRNAs during ovarian follicular/luteal development

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Site of expression</th>
<th>Reported function(s)</th>
<th>Target(s)*</th>
<th>Reported changes during development</th>
<th>Species (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-224</td>
<td>Granulosa</td>
<td>Stimulates cell proliferation, Cyp19a1, oestradiol levels</td>
<td>Smad4</td>
<td>TGFβ1-induced increase in pre-antral follicles</td>
<td>Mouse (Yao et al. 2010a)</td>
</tr>
<tr>
<td>miR-383</td>
<td>Granulosa, oocyte</td>
<td>Stimulates Cyp19a1, oestradiol levels</td>
<td>Rbms1</td>
<td>TGFβ1-induced decrease in pre-antral follicles, gonadotropin-induced increase in antral follicles, decrease before ovulation</td>
<td>Mouse (Yin et al. 2012)</td>
</tr>
<tr>
<td>miR-378</td>
<td>Granulosa, CL</td>
<td>Inhibits CYP19A1, oestradiol levels</td>
<td>CYP19A1</td>
<td>Decrease during antral follicle growth Increase during luteal development, decrease during luteal regression</td>
<td>Pig (Xu et al. 2011)</td>
</tr>
<tr>
<td>miR-23a</td>
<td>ND</td>
<td>Pro-apoptotic in granulosa cells</td>
<td>XIAP</td>
<td>ND</td>
<td>Human (Yang et al. 2012)</td>
</tr>
<tr>
<td>miR-26b</td>
<td>Follicle</td>
<td>Pro-apoptotic in granulosa cells</td>
<td>ATM</td>
<td>Increase during follicular atresia</td>
<td>Pig (Lin et al. 2012)</td>
</tr>
<tr>
<td>miR-132/miR-212</td>
<td>Granulosa</td>
<td>ND</td>
<td>Ctbp1</td>
<td>Increase during hCG-induced ovulation Increase during hCG-induced ovulation</td>
<td>Mouse (Fiedler et al. 2008)</td>
</tr>
<tr>
<td>miR-21</td>
<td>Granulosa</td>
<td>Anti-apoptotic (shown in vivo)</td>
<td>ND</td>
<td>Decrease during luteinisation</td>
<td>Sheep (McBride et al. 2012)</td>
</tr>
<tr>
<td>miR-125b</td>
<td>Granulosa, theca, CL</td>
<td>ND</td>
<td>(LIF)</td>
<td>Decrease during luteinisation</td>
<td>Sheep (McBride et al. 2012)</td>
</tr>
<tr>
<td>miR-145</td>
<td>Theca, CL</td>
<td>ND</td>
<td>(CDKNA1)</td>
<td>Decrease during luteinisation</td>
<td>Sheep (McBride et al. 2012)</td>
</tr>
<tr>
<td>miR-199a-3p</td>
<td>ND</td>
<td>Inhibits granulosa cell proliferation</td>
<td>Acrv1b, Ccnd2 (PTGS2)</td>
<td>Decrease during luteinisation</td>
<td>Mouse (Yan et al. 2012)</td>
</tr>
<tr>
<td>miR-503</td>
<td>Granulosa, oocyte</td>
<td>ND</td>
<td>Acrv2, Bcl2, Ccnd2, Lhbn, Cyp19a1, Lhcgr, Esr2 and Cdkn1b (Temp1)</td>
<td>Decrease during luteinisation Gonadotropin-induced decrease during follicle development</td>
<td>Mouse (Lei et al. 2010)</td>
</tr>
<tr>
<td>let-7b/miR-17-5p</td>
<td>Luteal</td>
<td>Pro-angiogenic (shown in vivo)</td>
<td>ND</td>
<td>ND</td>
<td>Mouse (Otsuka et al. 2008)</td>
</tr>
</tbody>
</table>

*Targets were identified using luciferase reporter constructs combined with miRNA overexpression/inhibition in follicular cells or, in the studies by Fiedler et al. (2008), Lei et al. (2010) and Yan et al. (2012), only by measuring the effects of miRNA overexpression/inhibition on target levels. For genes in parentheses, only a negative correlation between their levels and the levels of putative targeting miRNAs in follicular cells has been shown.
Smad4 was identified as a target of miR–224 (Yao et al. 2010a); however, it is not clear whether this is indeed the actual target mediating the observed effects of miR–224 on granulosa cells (Wang et al. 2011). A subsequent study (Yin et al. 2012) showed that miR–383, a miRNA downregulated by TGFβ1, positively regulated aromatase expression and oestradiol production by mouse granulosa cells in culture and that these effects resulted, at least partially, from miR–383 targeting of RBMS1, a DNA binding protein that activates MYC. Further, it was shown that miR–383 was transcriptionally activated by steroidogenic factor 1 (NR5A1), a known gonadotropin-induced regulator of steroidogenic genes including STAR, CYP11A1 and aromatase (Parker & Schimmer 1997). These observations, together with the finding that miR–383 levels increased in vitro during equine chorionic gonadotropin (eCG)-induced follicle growth and decreased following administration of hCG, provide strong support for a role for miR–383 in physiologically regulating changes in oestradiol production during follicle development. Whereas miR–224 and miR–383 had indirect stimulatory effects on aromatase and oestradiol production, a different miRNA, miR–378, was shown to directly target aromatase in porcine granulosa cells (Xu et al. 2011). The authors showed that miR–378 levels increased in granulosa cells during antral follicle growth and they subsequently identified two different MREs in the 3′-UTR of the porcine aromatase gene that were targeted by miR–378, resulting in reduced gene expression and oestradiol production in vitro (Xu et al. 2011). Overall, these studies illustrate how a key follicular transcription factor, oestradiol production, can be distinctly regulated, directly or indirectly, by different miRNAs (Fig. 4). Whether, physiologically, miR–224, miR–383 and miR–378 are each involved in regulation of aromatase during different stages of follicle development has not been determined. In addition, large-scale functional testing in human granulosa cells revealed effects of multiple miRNAs on the production of progesterone, testosterone and oestradiol, as well as in the expression of the markers of proliferation and apoptosis, PCNA and BAX, although no attempt was made to investigate the mechanisms involved in these responses (Sirotnik et al. 2009, 2010).

Three recent in vitro studies have also proposed roles of miRNAs in regulating granulosa cell proliferation and apoptosis. Yan et al. (2012) reported attenuation of activin-induced proliferation of mouse granulosa cells by miR-145 targeting of both activin receptor 1B and cyclin D2. In another study, Yang et al. (2012) showed that miR–23a was pro-apoptotic in cultured human luteinised granulosa cells presumably by decreasing the levels of X-linked inhibitor of apoptosis protein (XIAP) and increasing caspase-3 cleavage, although it was not clarified whether these were actually direct targets of miR–23a in granulosa cells. Finally, using a microarray approach in porcine ovarian follicles, Lin et al. (2012) found that miR–26b expression increased during follicular atresia and further showed that this miRNA could induce granulosa cell death by directly targeting ataxia...
of luteinisation was considered in the Fiedler study, and there is evidence that the expression of some miRNAs dynamically changes throughout luteal development (Ma et al. 2011, McBride et al. 2012). Further studies will be essential to more precisely establish the temporal patterns of miRNA expression during the follicular–luteal transition and to determine whether species differences exist.

miRNAs whose expression differentially changes during the follicular–luteal transition (Fig. 3) have been experimentally shown to target a multitude of genes in a variety of (non-ovarian) cell types (lists of experimentally validated targets can be found in [link]). Such targets include genes involved in regulation of the cell cycle, apoptosis and differentiation, many of which are known to be involved in follicular differentiation and/or oocyte maturation (McBride et al. 2012). More specifically, gene expression analyses during in vitro luteinisation of bovine follicular cells identified LIF, CDKN1A and PTGS2 as putative targets of miR-125b, miR-145 and miR-199a-3p respectively in granulosa cells but not in theca cells (McBride et al. 2012); although further testing will be needed to confirm that these are actual follicular miRNA targets, this result suggests that individual miRNAs may have different actions in granulosa and theca cells.

More definitive evidence of specific genes and cell functions targeted by miRNAs during the peri-ovulatory period has been sought by experimentally inhibiting miRNAs using locked nucleic acids (LNAs). Using this approach, Fiedler et al. (2008) provided evidence that miR-132 and miR-212 upregulate protein levels of CTBP1, a transcriptional co-repressor of nuclear receptor target genes, during differentiation of granulosa cells in vitro. This conclusion is in contrast with the view that miRNAs act primarily as translational repressors rather than activators.

CTBP1 can regulate CYP17A1 expression by interacting with NR5A1 in adrenal cells (Dammer & Sewer 2008). However, LNA-mediated inhibition of miR-132 and miR-212 had no effects on oestradiol or progesterone production by granulosa cells and therefore Fiedler’s results did not clarify whether miR-132 and miR-212 are involved in regulating steroidogenesis (or any other function) in granulosa cells. In a subsequent study, Carletti et al. (2010) showed that inhibition of miR-21 induced apoptosis of granulosa cells from mouse pre-ovulatory follicles both in vivo and in vitro. miR-21 inhibition also reduced ovulatory rates by >2-fold; surprisingly, it had little effect on the viability of corpora lutea, a finding likely related to suboptimal efficiency associated with the procedure for in vivo ovarian transfection with miR-21 inhibitor (Otsuka et al. 2008, Carletti et al. 2010). In that regard, alternative gene manipulation strategies (e.g. conditional miRNA knock-out) (Park et al. 2010) or the use of larger animal models (e.g. ruminants) that allow easier experimental access to specific ovarian compartments and developmental stages (Campbell et al. 2003) may provide added benefit for functional studies of ovarian miRNAs in vivo. Nonetheless, the results of Carletti et al. (2010) provided novel evidence that an increase in miR–21 contributes to follicular cell survival associated with the follicle–luteal transition, a conclusion that is well in line with the described anti-apoptotic role of miR–21 in other normal tissues including cardiac and hematopoietic (Thum et al. 2008, Roy et al. 2009, Sheedy et al. 2010). However, the precise targets mediating such effects of miR–21 in the ovary are yet to be identified.

Similar to miR-21, miR-503 has been found to be differentially expressed during the peri-ovulatory period in both mice (Lei et al. 2010) and sheep (McBride et al. 2012). Of interest, in these two species and in humans, the ovary is the main site of miR-503 expression in the body (Landgraf et al. 2007, Ahn et al. 2010, McBride et al. 2012). During the sheep oestrous cycle, miR-503 levels transiently decreased in pre-ovulatory follicles followed by a rebound in corpora lutea (Fig. 3). Consistent with this, ovarian miR-503 levels decreased in response to treatment with eCG in mice, especially when this was followed by administration of an ovulatory dose of hCG (Lei et al. 2010). Interestingly, over-expression of miR-503 in cultured granulosa cells from mice resulted in downregulation of different transcripts associated with cell proliferation and differentiation including cyclin D2, cdkn1b, bcl2, activin receptor, aromatase and LH receptor (Lei et al. 2010), a finding that nonetheless needs to be interpreted with caution as, in general, changes in transcript levels after over-expression of miRNAs in cells may represent both specific and non-specific effects of these miRNAs (Thomas et al. 2010). Taken together, the above findings are consistent with the notion that a gonadotropin-induced decrease in miR-503 levels is involved in regulating follicle maturation and differentiation in the mammalian ovary. Future studies will need to precisely characterise the effects of miR-503 on follicular cells in vitro. Another issue that will need to be resolved is the precise site(s) of expression of miR-503 in the ovary. In mice, miR-503 was reportedly expressed predominantly in granulosa cells and oocytes but not in the CL (Lei et al. 2010), whereas in sheep miR-503 expression occurred predominantly in follicular theca cells and in luteal cells (McBride et al. 2012); these discrepancies will need to be resolved, although they may reflect genuine species differences and/or may be a result of different techniques used to determine miR-503 expression (in situ hybridisation vs qPCR), which may have led to detection of different forms of the miRNA.

In summary, numerous miRNAs have been identified in mice and ruminants that are differentially expressed in association with the follicular–luteal transition; however, the precise spatial and temporal expression patterns of these miRNAs and whether those patterns vary among different species have yet to be determined. In addition, although there is evidence that these miRNAs may be important in regulating cell survival and differentiation during the follicular–luteal transition, their precise roles and target genes have largely not been determined.
Modelling follicular miRNA physiology in vitro

All the studies described earlier used primary granulosa cell cultures to investigate roles of ovarian miRNAs. It has long been known that granulosa cells tend to spontaneously differentiate in culture (reviewed in Murphy (2000)) and that the use of serum-free media conditions can reduce these effects (Gutierrez et al., 1997, Donadeu et al., 2011). During attempts to model in vitro some of the changes in miRNAs associated with follicular differentiation, both Christenson’s (Carletti et al., 2010) and our group demonstrated that culture-induced factors can have a large effect on miRNA expression in follicular cells. Treatment of mouse granulosa cell cultures with cAMP increased the expression of miR-132 and miR-212, mimicking the effects of an ovariolytic dose of hCG on follicular miRNA levels (Fiedler et al., 2008). However, cAMP did not increase the levels of miR-21 in cultured granulosa cells but miR-21 spontaneously increased within 12 h after plating, a response that was not affected by the presence of serum in culture media (Carletti et al., 2010). Similarly, another study reported that treatment of mouse granulosa cells with FSH increased miR-132 levels but not miR-21 (Yao et al., 2010b). Consistent with these findings, we found that treatment of cultured bovine granulosa cells with forskolin (an adenylate cyclase agonist) to promote luteinisation was followed by a decrease in the levels of miR-125b and miR-145, as observed during the follicular–luteal transition in vivo (McBride et al., 2012); in contrast, levels of miR-21 and miR-34a spontaneously increased in the cultured cells regardless of treatment with forskolin. Changes in the levels of these four miRNAs in bovine theca cells during culture were also consistent with changes during the follicular–luteal transition in vivo but they occurred independently of forskolin treatment (McBride et al., 2012). Overall, these results show that, when attempting to model follicular miRNA physiology in vitro, consideration needs to be given not only to the actual cell phenotype that is being studied (granulosa vs theca, undifferentiated vs differentiating cells) but also to the fact that the expression of some miRNAs may be regulated differently in vivo and in vitro (Carletti et al., 2010). This calls for caution when interpreting published in vitro data on ovarian miRNAs.

Roles of miRNAs during luteal development

The highly dynamic nature of the CL is illustrated by the fact that following ovulation, and within the relatively short lifespan of a normal luteal phase, the CL quickly develops into the most vascularised and steroidogenically active tissue of the female body to, within a few days, suddenly and quickly regress into non-functional avascular fibrous tissue (corpus albicans; Fig. 1). Little research has been done to date on the biological involvement of luteal miRNAs. A role of miRNAs in luteal angiogenesis is suggested by the finding that several of the miRNAs that were found to be differentially expressed between follicular and luteal tissues in ruminants (Fig. 3), including miR-125b, miR-145, mir-31, miR-503 and miR-21 (Cordes et al., 2009, Caporali et al., 2011, Liu et al., 2011, Wu et al., 2011, Muramatsu et al., 2012), are known to regulate blood vessel formation. To precisely study the role of miRNAs in the CL, Otsuka et al. (2008) used mice homozygous for a hypomorphic Dicer1 gene, which was associated with impaired luteal development and function leading to an inability to sustain pregnancy. Using both cell culture and in vivo ovarian miRNA transfection, they showed that expression of miR-17-5p and let-7b, two miRNAs that targeted Timp1, was critical for luteal angiogenesis and function during early pregnancy. To this date, this remains the strongest evidence of a physiological requirement of miRNAs during luteal development.

Changes in miRNA expression have been reported during maturation and regression of the CL (Ma et al., 2011, McBride et al., 2012). Northern blot analyses of ovarian tissues collected at different stages of the luteal phase in sheep revealed higher levels of different miRNAs in corpus albicans than CL, particularly miR-199a-3p and miR-145, whereas the expression of miR-503 was much lower in corpus albicans (Fig. 3). These miRNAs have been reported to have a role in tissue fibrosis responses as well as to mediate immune and inflammatory responses in different tissues (Witwer et al., 2010, Cheng et al., 2012), and this may explain their dynamic expression during luteal regression. A different study using microarray identified miR-378 as the most differentially expressed miRNA during bovine luteal development (Ma et al., 2011); specifically, miR-378 expression increased over fivefold during luteal maturation and then decreased during luteal regression. Further, the authors reported changes in protein levels of the IFN receptor 1 that were consistent with this being a target of miR-378 and concluded that miR-378 may be involved in interferon-mediated cell death during bovine luteal regression, an assertion that will need confirmation in future studies. These findings, together with the demonstrated involvement of miR-378 in regulation of aromatase expression in the follicle, suggest a pleiotropic nature for some miRNAs during follicular/luteal development.

Conclusions and future directions

Although the full extent to which miRNAs are involved in mammalian ovarian function remains unknown, important steps are being made to understand how miRNAs regulate follicular and luteal development. Studies have already identified genome-wide miRNA populations putatively involved in follicular atresia, ovulation and the follicular–luteal transition. Further, despite generic challenges in identifying physiologically relevant roles of miRNAs in animal tissues, miRNA regulation of aromatase expression has already been described with some detail, and progress is
also being made in other aspects of follicle/luteal development. Current understanding of the roles of specific miRNAs is summarised in Table 2. The large amount of data generated through genome-wide analyses of ovarian tissues is providing plenty of fertile ground for the identification and functional characterisation of biologically relevant miRNAs, and this will foreseeably lead to a much greater understanding of the molecular regulation of follicular and luteal function in the ovary. Recent progress in understanding small RNA biology and physiology has brought new and exciting perspectives about the regulation of reproductive function by miRNAs. In addition, the clinical relevance of miRNAs has steadily grown about the regulation of reproductive function by miRNAs. In and physiology has brought new and exciting perspectives characterisation of biologically relevant miRNAs, and this plenty of fertile ground for the identification and functional

Declaration of interest

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