Identification of miRNAs associated with the follicular–luteal transition in the ruminant ovary

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Abstract

Little is known about the involvement of microRNAs (miRNAs) in the follicular–luteal transition. The aim of this study was to identify genome-wide changes in miRNAs associated with follicular differentiation in sheep. miRNA libraries were produced from samples collected at defined stages of the ovine oestrous cycle and representing healthy growing follicles, (diameter, 4.0–5.5 mm), pre-ovulatory follicles (6.0–7.0 mm), early corpora lutea (day 3 post-oestrus) and late corpora lutea (day 9). A total of 189 miRNAs reported in sheep or other species and an additional 23 novel miRNAs were identified by sequencing these libraries. miR-21, miR-125b, let-7a and let-7b were the most abundant miRNAs overall, accounting for 40% of all miRNAs sequenced. Examination of changes in cloning frequencies across development identified nine different miRNAs whose expression decreased in association with the follicular–luteal transition and eight miRNAs whose expression increased during this transition. Expression profiles were confirmed by northern analyses, and experimentally validated targets were identified using miRTarBase. A majority of the 29 targets identified represented genes known to be actively involved in regulating follicular differentiation in vivo. Finally, luteinisation of follicular cells in vitro resulted in changes in miRNA levels that were consistent with those identified in vivo, and these changes were temporally associated with changes in the levels of putative miRNA targets in granulosa cells. In conclusion, this is the first study to characterise genome-wide miRNA profiles during different stages of follicle and luteal development. Our data identify a subset of miRNAs that are potentially important regulators of the follicular–luteal transition.

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Introduction

Cyclic ovarian activity is essential to reproductive success in mammals. Growth and regression of follicles, ovulation, differentiation of mature follicle cells into luteal cells and growth and subsequent demise of corpora lutea occur in the ovary within the short time frame of a reproductive cycle and occur repeatedly throughout a female’s reproductive life. Avoiding ovarian failure and infertility requires sequential changes in cell function and cell fate within follicles and corpora lutea that must be finely synchronised at the molecular level. Although the transcriptional regulation of follicular and luteal cell function has been described in considerable detail, particularly in rodents (Richards 2001, Richards & Pangas 2010), little is known on the post-transcriptional mechanisms involved.

MicroRNAs (miRNAs) are short, non-coding RNAs of around 21 nucleotides in length that act by targeting partially complementary sequences within mRNAs and most commonly lead to translational repression or degradation of the target mRNAs (Krol et al. 2010). There are around 1500 different miRNAs expressed in human tissues, and it is thought that each miRNA may target hundreds of genes. In this way, miRNAs have been predicted to regulate the activity of ~50% of all known protein-coding genes in mammals. miRNAs are most commonly transcribed as independent genes or from introns of protein coding-genes, and miRNA genes may be clustered within specific genomic regions (Krol et al. 2010). miRNA transcripts are processed into stem–loop miRNA precursors by the RNAse III endonuclease, DROSHA, and then exported to the cytoplasm where a second endonuclease, DICER, cleaves the pre-miRNA into the mature form. Mature miRNAs are then assembled into ribonucleoprotein complexes where they interact with specific mRNA targets. A number of miRNAs have been described as master regulators of cell differentiation processes and, in line with this, miRNAs are often transcribed in a tissue- and developmental stage-specific manner (Ivey & Srivastava 2010).

Insight into the roles of miRNAs in the ovary has been provided by deleting Dicer expression specifically in the reproductive tract of mice, leading to reduced follicle growth and ovulatory rates as well as defective oocyte maturation and oviductal and uterine defects.
et al (2007, Tang et al. 2007, Hong et al. 2008, Nagaraja et al. 2008, Lei et al. 2010). In addition, Dicer1 hypomorphism was associated with impaired corpus luteum (CL) function resulting from defects in angiogenesis (Otsuka et al. 2008). Genome-wide miRNA expression has also been examined in whole ovariates from mice (Ro et al. 2007, Mishima et al. 2008), cattle (Hossain et al. 2009, Huang et al. 2009, Tripurani et al. 2010) and pig (Li et al. 2011). While these studies identified novel and ovarian-specific miRNA species, comparisons across different developmental stages were not performed. Separate studies identified hormone-responsive miRNAs in the ovary and proposed different roles for some of those miRNAs including mediation of responsive miRNAs in the ovary and proposed different not performed. Separate studies identified hormone-comparisons across different developmental stages were identified novel and ovarian-specific miRNA species, variations across different developmental stages were not performed. Separate studies identified hormone-responsive miRNAs in the ovary and proposed different roles for some of those miRNAs including mediation of granulosa cell responses to transforming growth factor β1 (TGFβ1) in pre-antral follicles (Yao et al. 2010a), oestradiol (E2) production (Xu et al. 2011), prevention of early granulosa cell differentiation (Lei et al. 2010), promotion of granulosa cell survival during ovulation (Carletti et al. 2010), inhibition of anti-angiogenic factor expression during luteogenesis (Otsuka et al. 2008) and ovarian aging (da Silveira et al. 2012). It has not yet been established whether these proposed functions are conserved in large animal species and humans.

To investigate the physiological involvement of miRNAs in ovarian function, we used the sheep as a model species as this allows detailed analyses of precisely defined follicle and luteal developmental stages. This study was performed with the basic aims of identifying the miRNA populations expressed in late antral follicles and corpora lutea at different stages of development and to characterise changes in miRNA expression associated with the follicular–luteal transition. In a complementary analysis, we also investigated the changes in miRNAs and their putative targets induced upon in vitro differentiation of follicular cells.

Results

Characterisation of miRNAs in follicles and corpora lutea

miRNA profiling was performed on material from pools of medium-sized follicles, pre-ovulatory follicles, early corpora lutea and late corpora lutea collected from cycling mature ewes. Total RNA was extracted from individual tissue pools and used to prepare the miRNA fraction for each stage of development. miRNA libraries were generated for each of the four developmental stages and 300 clones from each library were sequenced, representing a total of 6181 individual small RNA fragments. These corresponded, for the combined libraries, to a total of 702 different sequences of between 18 and 25 nucleotides in length. From these 702 sequences, a total of 212 miRNAs and 35 tRNAs or other small RNAs were identified. Of these 212 miRNAs, 14 had previously been reported in sheep, 146 represented homologues of miRNAs reported in other species including bovine and 29 represented miRNAs reported in species other than sheep and bovine (Supplementary Table 1, see section on supplementary data given at the end of this article). The remaining 23 sequences had not previously been reported as miRNAs in any other species but matched the ovine genome in regions capable of folding into hairpin structures and thus represent entirely novel miRNAs (Supplementary Table 2, see section on supplementary data given at the end of this article).

Several of the miRNA sequences identified corresponded to miRNA pairs derived from different arms of the same hairpin (-5p/-3p) in other species (Supplementary Table 1, see section on supplementary data given at the end of this article). For many of these pairs, the two sequences are designated as major and minor forms (miRNA/miRNA*) in MirBase; however, this designation does not always correspond to the relative abundance found in sheep ovaries. For example, the sequence corresponding to bta-miR-151* was cloned at a higher frequency than the sequence corresponding to bta-miR-151. For some of the miRNAs, sequence variations were identified that had 3’ or 5’ modifications and represent potential isomiRs (Ryan et al. 2010). These variants largely involved the 3’ end and consisted of single-base additions, deletions or replacements (Supplementary Table 1, see section on supplementary data given at the end of this article).

The most commonly expressed miRNA was miR-21, representing an average of 18% of the total miRNA population across all four stages of follicular and luteal development. In combination, the 19 most highly expressed miRNAs represented 70% of the total miRNAs expressed in sheep ovarian tissues, and most of these miRNAs varied in expression level depending on the stage of tissue development (Fig. 1).

Characterisation of miRNAs associated with the follicular–luteal transition

To specifically identify miRNAs whose expression levels change during the transition between follicle and CL, miRNA cloning frequencies were individually examined across developmental stages and changes in frequency involving a greater than or equal to twofold increase or decrease between follicular and luteal tissues were identified; to minimise the number of false positives, only miRNAs that had been cloned overall ≥20 times across developmental stages were included in these analyses. Based on these criteria, 17 different miRNAs were identified, which represent species potentially involved in post-transcriptional regulation during luteinisation (Fig. 2). To ensure that the recorded frequency of individual sequences accurately reflected tissue expression levels, the expression of seven of these miRNAs was examined on an extended range of
Developmental stages by northern analysis. These included miRNAs that were expressed at higher levels (miR-199a-3p, miR-125b, miR-145 and miR-31) and at lower levels (miR-503, miR-21 and miR-142-3p) in follicular than luteal tissues. Developmental stages analysed included small follicles (1.5–3.5 mm), medium follicles (4.0–5.5 mm), pre-ovulatory follicles, early CL, late CL and corpus albicans (Fig. 3). Consistent with the expression profiles obtained from ovarian libraries, northern analysis revealed that miR-199a-3p, miR-125b, miR-145 and miR-31 were robustly expressed at all stages of follicular development and showed a marked reduction in the expression levels on differentiation into luteal tissue, which was then maintained for the lifetime of the CL followed by an increase in levels of the corpus albicans. In contrast, miR-503, miR-21 and miR-142-3p were generally expressed at lower levels during follicular stages and showed a clear increase in the expression levels in luteal tissues.

To establish whether the expression of these miRNAs was confined to a particular somatic cell compartment in the developing follicle, northern analyses were performed on RNA extracted from separate granulosa and theca cell populations isolated from ovine follicles. These analyses showed that while all miRNAs were expressed in the theca compartment, in general, their expression in granulosa cells was lower (Fig. 4). To determine whether the selected miRNAs were expressed in an ovary-specific or tissue-enriched pattern, northern analyses were performed on RNA isolated from a variety of ovine tissues (Fig. 5). This analysis showed that while most miRNAs were expressed in multiple tissues, miR-503 showed a more restricted pattern of expression with significant levels only seen in ovarian tissues, whereas the levels of other miRNAs such as miR-142-3p had relative low abundance in the ovary.

In order to gain insight on the functional relevance of miRNAs whose expression distinctly increased or decreased in association with the follicular transition (Fig. 2), lists of experimentally validated targets were obtained from each group of miRNAs using miRTarBase. In each case, a list with all genes targeted by two or more miRNAs was produced (Fig. 6). Almost all targets identified correspond to genes involved in cell cycle regulation, cell survival and regulation of cell differentiation and, many of them have been shown to be actively involved in regulating differentiation of somatic cells (MYC, AKT1, BAK1, CDK11A, TP53, KLF4, KLF13, CDK6 and VEGF) or the oocyte (AKT1, LIF CDC25A and CDK6) during and after ovulation (SCCPiR Ovarian Kaleidoscope Database; http://ovary.stanford.edu/).

Changes in the levels of miRNAs and putative mRNA targets during differentiation in vitro

To investigate whether changes in the levels of miRNAs observed during the follicular–luteal transition could be recapitulated during in vitro differentiation of follicular cells, we analysed expression of selected miRNAs using a well-established and characterised in vitro model of induced luteinisation (Meidan et al. 1990, Mamlok et al. 1999, Wu & Wiltbank 2002). By doing this, we studied the relative involvement of follicular granulosa and theca cells in the changes in miRNA expression identified in vivo and also determined concomitant changes in the expression of putative targets of these miRNAs. Granulosa and theca cells were isolated from bovine ovaries and separately cultured. One day later (day 0), half of the cultures were treated with standard luteinisation-promoting media containing forskolin. qPCR was used to determine the levels of selected transcripts representing miRNAs decreasing (miR-125b and miR-145) or...
increasing (miR-21 and miR-34a) as a result of the follicular–luteal transition. As expected (Meidan et al. 1990), incubation with luteinisation media resulted in a differential increase in progesterone production by both granulosa cells and theca cells (Fig. 7), confirming that these cells were actively undergoing differentiation. qPCR analyses revealed that the levels of each miRNA were significantly higher ($P<0.04$) in bovine theca cells than in granulosa cells before culture (8.0 $\pm$ 1.1-, 63.4 $\pm$ 23.4-, 1.9 $\pm$ 0.3- and 3.1 $\pm$ 0.1-fold for miR-125b, miR-145, miR-21 and miR-34a, respectively, n=3 replicates), thus confirming results from northern analysis of ovine tissues. Furthermore, levels of both miR-125b and miR-145 dynamically changed after day 0 in the two types of cultured cells (Fig. 7). In granulosa, these changes largely involved a decrease in miRNA levels in forskolin-treated cells; this resulted, for each of miR-125b and miR-145, in significantly lower levels ($P<0.05$) on day 3 than on both day 0 and the day earlier in the cells treated with luteinisation media but not in untreated cells. In theca cells, however, a decrease in miR-125b and miR-145 occurred immediately after day 0 regardless of whether cells had or not had been treated to induce differentiation (Fig. 7). In general, expression of both miR-21 and miR-34a increased during the culture of granulosa cells and theca cells, although these effects were independent of cell treatment (Fig. 7).

Finally, we determined changes in the expression of putative miRNA targets during cell differentiation in vitro (Fig. 8). As changes in miR-125b and miR-145 could be readily induced in response to treatment with luteinisation media (Fig. 7), we focused on putative targets of these miRNAs (identified in miRTarBaSe; Fig. 6). miRTarBaSe only covers humans, mice and rats; hence, we looked for the presence of potential target sequences in the 3'-UTR of corresponding bovine orthologues using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid); this yielded LIF and CDKN1A as containing putative 3'-UTR target sequences of bovine miR-125b and miR-145 respectively (Fig. 8). In addition, we examined PTGS2, a putative target of miR-199a-3p (miRTarBase) with a well-known role during the physiological follicular–luteal transition. We found that bovine PTGS2 contained a putative 3'-UTR target sequence for bovine miR-199a-3p (Fig. 8). In agreement with the above results of in silico analyses, levels of each of LIF and CDKN1A distinctly increased ($P<0.05$) in granulosa cells in response to treatment with luteinisation media, as did PTGS2 (Fig. 8). In contrast, levels of these transcripts in theca cells decreased ($P<0.05$) or did not change during luteinisation (Fig. 8).

### Discussion

This is the first study characterising genome-wide changes in miRNA profiles across follicular/luteal development in any species. Using a large animal model, ovaries were collected during different days of the oestrous cycle, and follicular and luteal structures were isolated for miRNA expression analyses. Through this approach, we characterised miRNA populations specifically associated with follicles and corpora lutea at defined developmental stages and identified a subset of putative miRNA regulators of the follicular–luteal transition.

Compared with other domestic species, the ovine genome is poorly annotated and only a limited number of miRNA sequences were previously reported, thus only 14 of the 212 miRNAs sequences reported in this study had already been registered as ovine sequences in MirBase. Most of the remaining sequences represent novel sheep miRNAs, which have already been identified in other species, with 70% of these previously registered in bovine. Our analyses also identified...
23 miRNA sequences that appeared to be novel in any species. These were expressed at relatively low levels (0.02–0.8% of clones), and this may explain in part why these miRNAs have previously escaped detection.

Other studies in this area only reported small-RNA sequencing of whole ovaries, providing little or no information on the potential functional relevance of the identified miRNAs (Ro et al. 2007, Mishima et al. 2008, Hossain et al. 2009, Huang et al. 2011, Li et al. 2011). This study compared expression profiles in defined tissue samples encompassing all stages of the physiological follicular–luteal transition. Five miRNAs (miR-21, miR-125b, let-7b, let-7a and miR-16b) accounted for over 45% of the total miRNA population, and although, based on cloning frequencies, their expression changed throughout development, they remained the five most abundant miRNAs at each developmental stage. These five miRNA sequences, together with miR-143 and miR-199a-3p, were also reported to be among the most prevalent in the whole ovaries of cattle, pigs and mice (Mishima et al. 2008, Tesfaye et al. 2009, Huang et al. 2011, Li et al. 2011).

Based on the changes in cloning frequencies of individual miRNAs across developmental stages, a total of 17 sequences were identified whose expression levels clearly varied between follicular and luteal stages (Fig. 2). Northern blotting analyses confirmed that these represented actual differences in tissue miRNA expression and therefore they are likely to play a role in the regulation of follicular differentiation. Interestingly, four of the nine miRNAs identified as being highly expressed in follicles were previously reported to be upregulated by FSH in rat granulosa cells whereas miR-21, which was expressed at low levels in sheep follicles, was downregulated by FSH (Yao et al. 2010b).

Wang et al. 2011), including roles in modulating inflammation, innate immunity and angiogenesis (Chen et al. 2004, Otsuka et al. 2008, Yamakuchi et al. 2008, Liu et al. 2009, Guo et al. 2010, Jennewein et al. 2010, Kuhn et al. 2010, Sheedy et al. 2010, Caporali et al. 2011, Shatseva et al. 2011), all of which are intrinsically associated with ovulation and early luteogenesis (Espey & Richards 2002). Among these miRNAs, the let-7 family has been shown to be critical for angiogenesis in the developing CL (Otsuka et al. 2008).

To identify transcripts potentially targeted by miRNAs during the follicular–luteal transition, a list of genes was produced that have been experimentally verified as being targeted in other systems by at least two of the differentially expressed miRNAs identified in this study. In total, 27 genes were identified as targets of the miRNAs associated with the follicular–luteal transition (Fig. 6). As expected, these targets predominantly represent regulators of cell proliferation and survival (over half of validated targets) as well as genes associated with cell differentiation and development in different tissues. In addition, a majority of identified targets (21 of 27) are known to be expressed in follicular and luteal tissues and many of them have been experimentally shown to be involved in follicular differentiation and/or oocyte maturation (SCCPiR Ovarian Kaleidoscope Database; http://ovary.stanford.edu/). Further, for many of the genes for which changes in expression have been reported during terminal follicular differentiation, the nature of these changes is consistent with their designation as miRNA targets, for example levels of MYC, CDKN1A and LIF increase in response to human chorionic gonadotropin (hCG) during ovulation (Arici et al. 1997, Robker & Richards 1998, Chaffin et al. 2003) and during in vitro granulosa cell differentiation (Fig. 8), which is consistent with these being putative targets of miRNAs whose expression decreases in corpora lutea.

Among the miRNAs whose expression levels dynamically changed between follicles and corpora lutea in sheep, miR-21 and miR-503 were previously studied...
in relation to follicular development (Carletti et al. 2010, Lei et al. 2010). Microarray profiling of mouse pre-ovulatory follicles revealed an increase in miR-21 expression in granulosa cells following ovulation induction with hCG up to at least 12 h post-hCG (Fiedler et al. 2008). The authors further showed that miR-21 was important in mediating the anti-apoptotic effects of the ovulatory LH surge on granulosa cells, although the precise targets mediating this effect were not identified (Carletti et al. 2010). Another study reported downregulation of miR-21 by E2 in mammary cells (Wickramasinghe et al. 2009). The present results of a differential increase in miR-21 levels between ovine follicles and corpora lutea are consistent with those studies and further extend their findings by showing that miR-21 levels continue to increase throughout luteal development to reach the highest levels in late CL and, surprisingly, in corpus albicans. Multiple roles have been recently described for miR-21 in addition to its best-known function as an anti-apoptotic factor (Kumarswamy et al. 2011). The demonstration in different tissues that miR-21 can mediate immune and fibrotic responses, both of which play a central role in luteal demise, may well explain the observed increase in miR-21 in late CL and corpus albicans of sheep. A relatively large increase in the expression levels in corpus albicans was also found for each of miR-142-3p and miR-145, and this may be related to the reported roles of these miRNAs in mediating immune and inflammatory responses (Witwer et al. 2010, Sun et al. 2011). These novel findings suggest an involvement of miRNAs in multiple aspects of the follicular/luteal developmental cycle. They also indicate the need for studying the involvement of miRNAs specifically in relation to luteal regression.
This study shows miR-503 to be enriched in the ovary relative to the majority of other ovine tissues, in agreement with previous studies on rodents and pig (Landgraf et al. 2007, Ahn et al. 2010, Li et al. 2011). Furthermore, miR-503 levels visibly decreased in sheep pre-ovulatory follicles, corresponding to the earliest phase of luteinisation, and this was followed by a recovery in miR-503 levels during subsequent CL development. Consistent with these results, a previous study on mice reported a decrease in the follicular expression of miR-503 in response to an ovulatory gonadotropin surge (Lei et al. 2010). The authors further showed that miR-503 overexpression in granulosa cells led to suppression in the levels of different transcripts involved in cell proliferation and differentiation. In other tissues, miR-503 has been shown to repress the cell cycle and to act as an angiogenesis inhibitor (Sarkar et al. 2010, Caporali et al. 2011). These findings are consistent with a role of miR-503 in timely coordination of key cellular events during follicular differentiation.

An interesting and novel observation in this study was that many of the miRNAs examined in follicles of sheep and cattle were predominantly expressed in the theca compartment, with only a small fraction expressed in granulosa cells. This difference may be partly attributed to the heterogeneous nature of the theca compartment including vascular tissue, which, as indicated earlier, is a well-described functional target of a multitude of miRNAs (Suárez & Sessa 2009). Additionally, miRNAs may be produced in the theca compartment and act in a paracrine manner to regulate gene expression in neighbouring granulosa cells, a mechanism that is well
described in other tissues (Camussi et al. 2010) and that warrants further investigation in follicles.

Our studies with cultured follicular cells revealed that, in general, changes in miRNA levels induced upon in vitro differentiation of bovine granulosa and theca cells, specifically a decrease in levels of miR-125b and miR-145 and an increase in miR-21 and miR-34a, were consistent with expression changes observed ex vivo in sheep tissues and further support the involvement of these miRNAs in the follicular–luteal transition. Granulosa cell levels of miR-125b and miR-145 robustly decreased (>80%) in response to chemically induced differentiation, whereas no net change in expression levels of these two miRNAs occurred during culture of non-treated cells. Consistent with this result, changes in cell morphology (data not shown) and progesterone synthesis indicative of differentiation (Meidan et al. 1990) were clearly apparent in both cultured granulosa and theca cells by day 3. However, levels of miR-125b and miR-145 in theca cells and of miR-21 and miR-34a in both granulosa and theca cells significantly changed during culture regardless of cell treatment. In the study by Carletti et al. (2010), miR-21 levels increased on plating of murine granulosa cells and could not be further stimulated by treatment with cAMP or hCG. The authors attributed this to spontaneous luteinisation presumably undergone by granulosa cells once released from the follicular environment and placed in culture (Murphy 2000, Carletti et al. 2010). The present results in bovine cells are consistent with that observation and, overall, they show that the relative effects that in vitro culture and chemical-induced luteinisation can have separately on changes in miRNA levels in follicular cells will depend on the specific miRNA analysed and on the cell type. This fact will need to be carefully considered when using in vitro models to study miRNA function in future studies.

To gain insight on genes targeted by miRNAs in follicular cells, we sought to investigate changes in levels of putative targets (Fig. 6) associated with the decrease in the expression of miR-125b and miR-145 during chemically induced luteinisation of follicular cells (Fig. 7). Using RNAhybrid, we found that the bovine 3′-UTRs of LIF and CDKN1A indeed contained potential target sequences for bovine miR-125b and miR-145. It has become increasingly clear that, in general, mRNA degradation accounts for most of the effects of miRNAs on target levels in animal cells (reviewed in Huntzinger & Izaurralde (2011)). In agreement with this principle, mRNA levels of each of LIF and CDKN1A increased during luteinisation of granulosa cells. These changes were consistent with the results of previous studies (Arici et al. 1997, Robker & Richards 1998a) and with the downregulation of these genes by miR-125b and miR-145 in non-luteinised granulosa cells. A similar conclusion resulted from examining granulosa cell changes in PTGS2 expression, a putative target of miR-199a-3p in bovine. In contrast, levels of LIF, CDKN1A and PTGS2 decreased during in vitro luteinisation of theca cells, simultaneously with a decrease in levels of miR-125b, miR-145 and, presumably, miR-199a-3p, an observation that was not consistent with those three genes being miRNA targets in theca cells. These findings suggest that miRNA targeting within ovarian follicles may occur in a cell-specific manner, a possibility that warrants further investigation.

In summary, genome-wide sequencing of sheep ovarian tissues identified 212 different miRNAs expressed in follicles and corpora lutea. A subset of 17 miRNAs was differentially expressed in association with the follicular–luteal transition. Putative targets for these miRNAs include genes that are involved in the regulation of cell proliferation, survival and differentiation during ovulation and luteogenesis. Furthermore, changes in miRNA levels identified ex vivo in ovarian tissues were recapitulated during luteinisation of follicular cells in vitro and were negatively correlated with changes in levels of putative mRNA targets in granulosa cells. In conclusion, we report for the first time global miRNA profiles associated with different stages of follicle and luteal development and identify a specific subset of miRNAs that are potentially important regulators of the follicular–luteal transition.

Materials and Methods
Experimental animals and tissue collection
Ovaries were collected from 22 normally cycling, mature ewes of a Blue Faced Leicester and Scottish Blackface cross during the natural breeding season (September–October). Animal procedures were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Before collection of tissues, ewes were checked daily for signs of oestrus with a teaser ram. Ewes were then randomly allocated to one of three experimental groups and killed on the first day of oestrus, 3 days after oestrus or 9 days after oestrus (n=7–8 animals/group). Ovaries were removed immediately after killing, stored on ice and were processed within 15 min of collection. Ovaries were dissected and all follicles >1 mm, corpora lutea and corpora albicantia were collected. Follicular fluid was gently aspirated from follicles ≥4.0 mm and stored at −20°C for hormone analyses, and corpora lutea and corpora albicantia were weighed. All tissues were then individually snap frozen in liquid nitrogen until RNA extraction. In some instances, follicles were cut in half and granulosa and theca cell fractions collected separately. A variety of additional tissues were also collected and stored frozen for later miRNA expression analysis.

E2 levels in fluid from follicles ≥4 mm were measured by RIA as described (Doyle et al. 2010) without previous extraction of samples. Follicular fluid concentrations of progesterone were measured by a commercial RIA kit (Siemens Healthcare Diagnostics, Surrey, UK) validated in our laboratory by showing parallelism between serial dilutions of ovine
follicular fluid and the provided assay standard curves. The oestrogen to progesterone (O/P) ratio was determined as an indicator of follicle health and ovulatory LH surge-induced terminal follicle differentiation (Murdoch & Dunn 1982). Follicles were grouped according to size into small follicles (1.5–3.5 mm), medium follicles (4.0–5.5 mm) and large (pre-ovulatory) follicles (6.0–7.0 mm). A total of 20 small follicles were used for analyses. Only follicles with an O/P ratio > 1, i.e. healthy, growing follicles (a total of nine of 12 follicles collected), were included in the medium follicle group. A total of seven pre-ovulatory follicles were collected on the day of oestrus. Of these, five had an O/P ratio > 2 mm and deemed healthy based on specific visual criteria (Yang & Rajamahendran 2000), i.e. a well-vascularised follicular wall and transparent, amber-coloured follicular fluid lacking debris. Granulosa cells were harvested by dissecting follicles into hemispheres and gently scraping follicle walls with blunt-ended forceps. Theca cells were harvested by enzymatic digestion of follicular walls as described (Campbell et al. 1998). The two cell types were separately washed in culture media and then cultured in plates pre-coated with fibronectin in a humidified atmosphere with 5% CO2 at 37°C under serum-free conditions (McCoy's 5a with 0.02 M HEPES, 3 mM L-glutamine, 0.1% (w/v) BSA, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) with 10 ng/ml bovine insulin, 2.5 μg/ml transferrin and 4 ng/ml sodium selenite, as described (Donadeu & Ascoli 2005, Doyle et al. 2010).

One day after plating (day 0), cells were either treated with forskolin (10 μM), bovine insulin (1 μg/ml) and FCS (1%, v/v) to induce luteinisation or left untreated. Media were replaced 1 day later. On each of days 0, 1 and 3, media were collected and stored for later analyses of progesterone levels, and cells were lysed and processed for qPCR as described below.

**RNA preparation, miRNA cloning and sequencing**

Total RNA was extracted from pools of ovarian tissue at different stages of development using RNA Bee reagent (AMS Biotechnology (Europe) Ltd., Abingdon, UK), according to manufacturers' instructions. Low-molecular weight RNAs (<40 nucleotides long) were isolated from total RNA using a FlashPAGE fractionator (Ambion, Life Technologies Ltd., Paisley, UK). miRNA libraries were constructed using a modification of the method by Lau et al. (2001) and Lee & Ambros (2001), in which 3' and 5' linkers are sequentially added to RNA fragments followed by RT-PCR amplification, concatamerisation of resulting cDNA fragments and bacterial transformation. Libraries were plated on 384-well plates containing ampicillin (50 μg/ml) and grown overnight at 37°C. Individual colonies were picked onto 384-well plates containing 2×LB broth with ampicillin (50 μg/ml) and grown overnight at 37°C. Plates were replicated by direct inoculation into fresh plates and stored frozen at −80°C. Plasmid DNA was prepared using a Qiagen Bio-Robot, and quality and quantity of DNA were checked by agarose gel electrophoresis.

DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, DD1 5EH, Scotland; http://www.dnaseq.co.uk/) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

**miRNA northern analysis**

Total RNA (1–5 μg) was separated on a 15% (w/v) TBE/urea polyacrylamide gel run for 3 h at 80 V. The RNA was then transferred to Hybond-N+ membranes using a semi-dry transblotter (Bio-Rad Laboratories Ltd.) and cross-linked using a u.v. Stratalinker (Stratagene, La Jolla, CA, USA). Locked nucleic acid oligonucleotides, complementary to the target miRNA, were labelled with 32P-R-ATP (Exiqon, Vedbaek, Denmark). Hybridisation was performed overnight in ULTRAhyb oligo (Ambion) at 42°C. Membranes were washed at low stringency in 2×SSC/0.1% (w/v) SDS at 42°C or high stringency in 0.1×SSC/0.1% SDS at 42°C. Membranes were exposed to autoradiographic film for between 2 and 48 h (Kodak Biomax MS; Sigma).

**Follicular cell cultures**

Bovine ovaries were obtained from a local abattoir and transported to the laboratory and kept at 38°C in PBS containing kanamycin (50 μg/ml). Ovaries were then quickly trimmed free of extraneous tissues and rinsed in PBS before submerison in pre-warmed McCoy's 5a media containing penicillin/streptomycin. Follicles were dissected from ovarian stroma and the cross-sectional diameter was determined. All follicles were >2 mm and deemed healthy based on specific visual criteria (Yang & Rajamahendran 2000), i.e. a well-vascularised follicular wall and transparent, amber-coloured follicular fluid lacking debris. Granulosa cells were harvested by dissecting follicles into hemispheres and gently scraping follicle walls with blunt-ended forceps. Theca cells were harvested by enzymatic digestion of follicular walls as described (Campbell et al. 1998). The two cell types were separately washed in culture media and then cultured in plates pre-coated with fibronectin in a humidified atmosphere with 5% CO2 at 37°C under serum-free conditions (McCoy's 5a with 0.02 M HEPES, 3 mM L-glutamine, 0.1% (w/v) BSA, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) with 10 ng/ml bovine insulin, 2.5 μg/ml transferrin and 4 ng/ml sodium selenite, as described (Donadeu & Ascoli 2005, Doyle et al. 2010).

One day after plating (day 0), cells were either treated with forskolin (10 μM), bovine insulin (1 μg/ml) and FCS (1%, v/v) to induce luteinisation or left untreated. Media were replaced 1 day later. On each of days 0, 1 and 3, media were collected and stored for later analyses of progesterone levels, and cells were lysed and processed for qPCR as described below.

**qPCR analyses**

Total RNA was collected using MiRNeasy kit (Qiagen Ltd.), and RT-qPCR was performed using the miScript PCR System (Qiagen) following manufacturer's instructions and using commercially designed primers that were fully complementary to bovine miRNA and U6B sequences. Primers were also designed for quantification of putative bovine miRNA targets, LIF (forward, 5'-CTTGGGGCGCAGGAGTTGTCG-3' and reverse, 5'-GCTCCCACCCTGGCGCGTAA-3'), CDKN1A (forward, 5'-GGGCGCGACACCAGATGACAGA-3' and reverse, 5'-GTGGGTTGTTGGAAGGTTG-3'), PTGS2 (forward, 5'-TCCTGAAAAACCTCCCA-3' and reverse, 5'-TGGGCAATCGACAGC-3') and 18S (forward, 5'-GGGATCATGCGGTTCG-3' and reverse, 5'-GTCGCCACCAGACTTG-3'). Quantitative PCR was performed using a Mx3000P real-time PCR system (Stratagene). The relative abundance of each RNA was calculated with Mx3000P real-time PCR system analysis software using the cycle threshold for each sample relative to a standard curve constructed from a pool of follicles of different sizes. The resulting values were normalised by dividing the corresponding value for U6B or 18S for miRNAs and mRNAs respectively.
Bioinformatics and statistical analyses

For bioinformatics analysis, programmes written in Perl (http://www.perl.org) were run on a UNIX platform. Adaptor sequences were identified and discarded. The remaining short-RNA sequences were catalogued and the frequency of occurrence of individual sequences was calculated. Sequences were compared with those present within miRBase (release 17, http://www.mirbase.org) to identify previously reported miRNAs and matched against an available ovine genome assembly (http://www.sheephapmap.org) to identify the genomic locations of the identified miRNAs. The sequence around genomic matches was extracted and assessed for the potential to form a hairpin structure by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Changes in miRNAs associated with the follicular–luteal transition were identified by selecting miRNAs that had been cloned overall ≥20 times and by individually examining their cloning frequencies across developmental stages. miRNAs that displayed a greater than or equal to twofold difference in cloning frequencies between the medium follicle stage and at least each of the pre-ovulatory follicle and early luteal stages, and/or a greater than or equal to twofold difference between the pre-ovulatory follicle stage and each of early luteal and late luteal stages, were selected for further study. Lists of gene targets for these miRNAs that have been experimentally validated were identified using miRTarBase (release 2.5, http://mirTarBase.mbc.nctu.edu.tw/index.html).

For experiments monitoring day-to-day levels of miRNAs in cultured cells, between-experiment variation was minimised by expressing levels for each day relative to the average value on day 0 within each experiment. The Kolmogorov–Smirnov normality test was applied to each data set (P<0.01) and data log-transformed if necessary followed by statistical analysis using the General Linear Models procedure to determine main effects of day and treatment, using each as a block. If a main effect or the interaction was significant (P<0.05), Tukey’s test was performed for pair-wise comparison of multiple means. In all cases, mean differences were considered at P<0.05.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-12-0025.

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References

Donadeu FX & Ascoli M 2005 The differential effects of the gonadotropin receptors on aromatase expression in primary cultures of immature rat granulosa cells are highly dependent on the density of receptors expressed and the activation of the inositol phosphate cascade. Endocrinology 146 3907–3916. (doi:10.1210/endo.2005-04043)


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