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MicroRNA expression profile in bovine cumulus–oocyte complexes: Possible role of let-7 and miR-106a in the development of bovine oocytes☆

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ABSTRACT

The objectives of this study included: (1) identify the expression of miRNAs specific to bovine cumulus–oocyte complexes (COCs) during late oogenesis, (2) characterize the expression of candidate miRNAs as well as some miRNA processing genes, and (3) computationally identify and characterize the expression of target mRNAs for candidate miRNAs. Small RNAs in the 16–27 bp range were isolated from pooled COCs aspirated from 1- to 10-mm follicles of beef cattle ovaries and used to construct a cDNA library. A total 1798 putative miRNA sequences from the cDNA library of small RNA were compared to known miRNAs. Sixty-four miRNA clusters matched previously reported sequences in the miRBase database and 5 miRNA clusters had not been reported. TaqMan miRNA assays were used to confirm the expression of let-7b, let-7i, and miR-106a from independent collections of COCs. Real-time PCR assays were used to characterize expression of miRNA processing genes and target mRNAs (*MYC* and *WEE1A*) for the candidate miRNAs from independent collections of COCs. Expression data were analyzed using general linear model procedures for analysis of variance. The expression of let-7b and let-7i were not different between the cellular populations from various sized follicles. However, miR-106a expression was greater ($P < 0.01$) in oocytes compared with COCs and granulosa cells. Furthermore, all the miRNA processing genes have greater expression ($P < 0.001$) in oocytes compared with COCs and granulosa cells. The expression of potential target mRNAs for let-7 and let-7i (i.e., *MYC*), and miR-106a (i.e., *WEE1A*) were decreased ($P < 0.05$) in oocytes compared with COCs and granulosa cells. These results demonstrate specific miRNAs within bovine COCs during late oogenesis and provide some evidence that miRNAs may play a role regulating maternal mRNAs in bovine oocytes.

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1. Introduction

Reproductive efficiency is a major factor determining the success of cow-calf and dairy production systems. A primary reason cows are culled from a production herd is that they fail to produce a calf (Lucy, 2007), resulting in significant economic losses for the industry. A recent report has indicated that annual financial losses due to reproductive failure in the U.S. beef cattle industry exceed $1.2 billon (Geary, 2005). Therefore, by limiting reproductive failure in

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cattle there is a significant potential to increase the profitability of the cattle industry.

To limit reproductive failure, we must understand the mechanisms that regulate fertility in cattle. Abnormal preimplantation embryonic development is a key mechanism that decreases fertility in cattle (Geary, 2005; Lucy, 2007). Although many factors impact early embryonic development, there is a general consensus that the quality and competence of the oocyte plays a key role in determining the success of an embryo developing (Krisher, 2004). During oogenesis, many processes occur within the oocyte, which dictate its quality and competence. One of these processes is the accumulation of maternal mRNAs that are necessary to guide the early stages of embryonic development prior to the activation of embryonic transcription (Bettegowda and Smith, 2007). Many of these maternal mRNAs are stored for an extended time, and thus, require post-transcriptional regulatory mechanisms to coordinate mRNA stability prior to activation. Furthermore, there is a rapid turnover of maternal mRNAs during the maternal-to-embryonic transition, indicating selective degradation of maternal mRNAs during this transition. As a result, many post-transcriptional regulatory mechanisms are known to occur within the oocyte and these include mechanisms that repress translation as well as subsequent activation of translation and also mechanisms that degrade maternal mRNAs (Bettegowda and Smith, 2007).

MicroRNAs (miRNAs) are a class of recently identified regulatory RNAs, which are short non-coding RNAs of approximately 22 nucleotides in length in their mature form (Bartel, 2004). MiRNAs function through sequence complementation of target genes and can regulate gene expression and translation through translational repression or mRNA degradation (Bushati and Cohen, 2007). Recently, miRNAs have been identified and thoroughly characterized in mice oocytes and early embryos by several independent groups (Murchison et al., 2007; Tang et al., 2007; Watanabe et al., 2006). Interestingly, specific deletion of dicer1, the key ribonuclease that cleaves pre-miRNAs to mature length miRNAs, resulted in depletion of many of the miRNAs identified in the mouse oocyte (Murchison et al., 2007). These mutant oocytes also failed to complete maturation resulting in a block at first cleavage following fertilization (Tang et al., 2007). Furthermore, dicer1 knockout oocytes showed dysregulation of many genes, indicating that a large portion of maternal mRNAs are regulated by miRNAs in the mouse oocyte (Murchison et al., 2007). Two recent studies have identified the expression of several miRNAs within the fetal (Tripurani et al., 2010) and adult (Hossain et al., 2009) ovary of the cow. Characterization of some of these identified miRNAs within the various ovarian, embryonic, and somatic cell types has illustrated a potential role for miRNAs in ovarian function and early embryonic development within the cow (Hossain et al., 2009; Tripurani et al., 2010). Taken together, these studies demonstrate that miRNAs likely play a critical role in the development of mammalian oocytes and early embryos.

The miRNA processing pathway is linear and conserved for all mammalian miRNAs (Winter et al., 2009). First, primary miRNAs, or pri-miRNAs, are transcribed by either RNA polymerase II or III from within typically polycistronic genes often found within introns (Kim and Kim, 2007). These pri-miRNAs fold back onto complementary sequence to form the characteristic hair-pin structure common to all pre-miRNAs. The pri-miRNAs are then cleaved to pre-miRNAs within the nucleus by the complex of ribonuclease type III, nuclear (RNASEN) and diGeorge syndrome critical region 8 (DGCR8). The pre-miRNA is exported to the cytoplasm by the complex of exportin 5 (XPO5) and RAN, member RAS oncogene family (RAN). Within the cytoplasm, dicer1, ribonuclease type III (DICER1) and TAR (HIV-1) RNA binding protein 2 (TARBP2) cleave the pre-miRNA to its mature length. The single stranded mature miRNA is then loaded together with eukaryotic translation initiation factor 2C, 2 (EIF2C2) to form the RNA-induced silencing complex (RISC). The targeting of specific mRNAs by RISC results in mRNA cleavage, translational repression or mRNA deadenylation (Winter et al., 2009).

This study identified the specific expression profile of miRNAs in bovine cumulus–oocyte complexes (COCs) during late oogenesis by sequencing a small RNA cDNA library. Secondly, this study validated the expression of candidate miRNAs in bovine oocytes, COCs, and granulosa cells from various sized follicles. And finally, this study characterized the expression of potential miRNA target genes and miRNA processing genes in bovine oocytes, COCs, and granulosa cells from various sized follicles.

2. Materials and methods

All experimental procedures were reviewed and approved by the U.S. Meat Animal Research Center Animal Care and Use Committee.

2.1. Collection of ovaries and isolation of RNA for sequencing

Ovaries from beef cattle of mixed population were obtained at a local abattoir in five replicate runs over a 4-week period. For each replicate run, ~100 ovaries were collected and processed. Ovaries were stored in 0.9% NaCl with 0.75 μg/mL penicillin for ~4 h during collection and transport to the laboratory. Cumulus–oocyte complexes were aspirated from 1- to 10-mm follicles and washed three times in PBS supplemented with 0.1% fetal bovine serum. Groups of ~100 COCs, consisting of COCs from class 1 to 6 according to the classification system for bovine COCs (Blondin and Sirard, 1995), were snap frozen in liquid nitrogen and stored at −80 °C. Total RNA from the pooled COCs (n = 2241) was extracted using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The total RNA concentration and quality was determined using the Agilent 2100 Bioanalyzer RNA Nano chip assay (Agilent Technologies, Santa Clara, CA).

2.2. Construction, sequencing, and bioinformatic analysis of the small RNA cDNA library

Single insert cDNA libraries were constructed as described previously (Lu et al., 2005; McDaneld et al., 2009). Briefly, small RNA in the 16–27 bp size was

isolated using Flash polyacrylamide gel electrophoresis (Ambion, Austin, TX). The 5′ and 3′ RNA adaptors (5′-GGUCUUACUCGCAUCCUGUAGAUGGAUC-3′ and 5′-AUGCACACUGAUGCUGACACCUGC-idT-3′, respectively) were ligated to the purified small RNA using T4 RNA ligase (Ambion). Purified ligation products were reverse transcribed using a reverse transcription (RT) primer (5′-GCAGGTGTCAGCATCAGTGT-3′) and SUPERSCRIPT II reverse transcriptase (Invitrogen). PCR amplification of the small RNA was performed using the primers (5′-GGTCTTACTCGCATCCTGTA-3′ and 5′-GCAGGTGTCAGCATCAGTGT-3′) specific to sequence within the 5′ and 3′ RNA adaptors. Following PCR, amplified products were digested with EcoRI and cloned by ligating products into pBLUEscript, electroporating the vector into EC100 electrocompetent cells, and growing individual colonies in 384-well plates containing ampicillin selective LB media. Purified plasmids from individual colonies were sequenced using an Applied Biosystems 3730 sequencer (Foster City, CA).

Nucleotide sequence quality was assessed using PHRED (Ewing et al., 1998) and sequence was aligned based on the identification of flanking vector and linker sequences. Putative miRNA were clustered based on a 14-bp consensus sequence in which each member matched 14 consecutive bases to the most common member of the cluster. This approach was taken due to the high frequency of similar miRNAs having different length and sequence at their 3′ ends (Coutinho et al., 2007). The resulting putative miRNAs were screened against known miRNAs listed in miRBase Release 17, provided by the Sanger Institute (http://www.mirbase.org/). A positive match to a known miRNA was determined if the putative miRNA contained an exact sequence match to positions 4–17 within a known miRNA. The remaining putative miRNAs were screened using BLAST against tRNA, rRNA, snoRNA and mitochondrial sequences. If the putative miRNA had no known miRNA match within the miRBase or was not a result of tRNA, rRNA, snoRNA and mitochondrial contamination, the putative miRNA was identified as novel. Prediction of potential target mRNAs for selected candidate miRNAs was performed using Microcosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). This software identifies target mRNAs using the miRanda scores and targets were sorted by most significant *P*-value.

2.3. Collection of ovaries and isolation of RNA for validation and characterization

For validation and characterization of candidate miRNAs and associated mRNAs, beef cattle ovaries were obtained from the same abattoir used for generating the small RNA cDNA library; however, these ovaries were collected in three independent replicate runs ~8 months after the initial collections. For each replicate collection, ~50 ovaries were collected and processed in a similar manner as described above. COCs were aspirated and pooled according to follicular size (1–5-mm or 6–10-mm) and consisted of only class 1 or 2 COCs (Blondin and Sirard, 1995). Within the pools of COCs from the 1- to 5-mm follicles, a random subsample of COCs were denuded by vortexing for 2 min in 50 μL of PBS supplemented with 0.1% FBS. In addition, granulosa cells were collected from the aspirate of the 1- to 5-mm follicles. All cellular components from the 1- to 5-mn follicles and the COCs from the 6- to 10-mm follicles were snap frozen in which denuded oocytes and COCs were collected in pools ~20 oocytes or COCs from each replicate collection. Large and small RNA fractions were isolated from samples using the miRNeasy Mini kit (Qiagen, Valencia, CA). Total RNA from both large and small RNA fractions was quantified using RiboGreen (Turner Biosystems, Sunnyvale, CA).

Table 1
Bovine-specific primers for real-time RT-PCR analysis.

Gene ID[a]	Accession number[b]	Primer sequences[c]
GAPDH	TC302944	F 5′-GGGTCATCATCTCTGCACCT-3′ R 5′-GGTCATAAGTCCCTCCACGA-3′
RPLP2	TC354572	F 5′-GCCGCAGCAGAGGAGAAGAAGGA-3′ R 5′-TTTGCAGGGGAGCGGGACTCTAGT-3′
18S	TC378273	F 5′-ATGGCCGTTCTTAGTTGGTG-3′ R 5′-CGCTGAGCCAGTCAGTGTAG-3′
RNASEN	TC360353	F 5′-GGAACAAGTAGGCTCCGTGA-3′ R 5′-AGAGCAGGTGCTGTCCTCAT-3′
DGCR8	TC381697	F 5′-GCAGGAGTGAGGACAGGAAG-3′ R 5′-TCGAGCACTGCATACTCCAC-3′
XPO5	TC363295	F 5′-CCTCTGGTGCTCTTCTGTCC-3′ R 5′-TCTCTTGCGACTCTGGGTTT-3′
DICER1	TC301019	F 5′-GTGGCTCTCATTTGCTGTGA-3′ R 5′-CGTTTTGTGGAACCTGGTCT-3′
TARBP2	TC306919	F 5′-GAGGAGTTGAGCCTGAGTGG-3′ R 5′-AGCAGGGAGCAGAGGATGTA-3′
EIF2C2	TC312997	F 5′-AAGTCGGACAGGAGCAGAAA-3′ R 5′-TGGCACTTCTCATCAGCTTG-3′
MYC	TC303381	F 5′-ATACGGAACTCTTGCGCCTA-3′ R 5′-GCCAAGGTTGTGAGGTTGTT-3′
WEE1A	TC332019	F 5′-GACTGGGCATCCAACAAAGT-3′ R 5′-TTCGCAAGGGCAAAAATATC-3′

[a] Abbreviations according to the human Gene ID (National Center for Biotechnology Information): glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein, large P2 (*RPLP2*), 18S ribosomal rRNA (*18S*), ribonuclease type III, nuclear (*RNASEN*), exportin 5 (*XPO5*), and dicer 1, ribonuclease type III (*DICER1*), eukaryotic translation initiation factor 2C2 (*EIF2C2*).
[b] Accession numbers are from the Dana-Farber Cancer Institute bovine gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=cattle; release 12, 06/18/06).
[c] F = forward primer; R = reverse primer.

2.4. TaqMan MiRNA assays

In selecting candidate miRNAs to be validated, the miRNAs were required to have a relatively high abundance and a high homology with the human miRNA sequence because validation assays were based on human sequence. The miRNAs that fit these characteristics included let-7b, let-7i, and miR-106a. The percentages of abundance of total miRNA identified from sequencing for let-7b, let-7i, and miR-106a were 25.6%, 7.6%, and 3.1%, respectively. Bovine let-7b and let-7i have 100% homology with these human miRNAs and

Table 2
Abundantly expressed putative miRNAs identified in bovine cumulus–oocyte complexes.

miR[a]	Sequence	Number	Frequency[b]	Putative location[c]
bta-let-7b	UGAGGUAGUAGGUUGUGUGGUUU	464	25.55	5q 123.3
hsa-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU	325	17.90	NH
bta-let-7i	UGAGGUAGUAGUUUGUGCUGUUU	138	7.60	5q 55.1
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	79	4.35	19q 21.2
bta-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	73	4.02	25q 38.5
bta-miR-31	AGGCAAGAUGCUGGCAUAGCUGU	62	3.41	NH
bta-miR-106a	CAAAGUGCUUACAGUGCAGGUAG	57	3.14	12q 64.7
bta-miR-210	CUGUGCGUGUGACAGCGGCUGA	47	2.59	18q 1.2
bta-miR-15b	UAGCAGCACAUCAUGGUUUACA	42	2.31	1q 108.9
bta-let-7f	UGAGGUAGUAGAUUGUAUAGUU	41	2.26	Xq 58.7
bta-miR-16a	UAGCAGCACGUAAAUAUUGGUG	37	2.04	1q 108.9
bta-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	37	2.04	12q 64.7
bta-miR-92	UAUUGCACUUGUCCCGGCCUGU	27	1.49	12q 64.7
bta-miR-151-5p	UCGAGGAGCUCACAGUCUAGU	26	1.43	14q 2.3
BN1	UCUGACAUCGGUUGCUCUGCUU	23	1.27	17q 63.7
bta-let-7e	UGAGGUAGGAGGUUGUAUAGUU	21	1.16	18q 57.6
bta-miR-195	UAGCAGCACAGAAAUAUUGGCA	18	0.99	19q 27.2
bta-miR-497	CAGCAGCACACUGUGGUUUGUA	17	0.94	19q 27.2
hsa-miR-202	AGAGGUGUAGGGCAUGGGAA	15	0.83	26q 51.6
hsa-miR-1308	GCAUGGGUGGUUCAGUGGUAGA	14	0.77	NH
bta-miR-206	UGGAAUGUAAGGAAGUGUGUGA	14	0.77	23q 25.1
PN5	CGAACCGAACUCCUCACUAAA	13	0.72	NH
BN2	AACCGAAUUUCUCACUAAA	12	0.66	NH
bta-miR-27a	UUCACAGUGGCUAAGUUCUGC	11	0.61	8q 86.0
BN3	UCCUAUAGGGUGAGCAUGUAGUG	10	0.55	9q 2.8
PN3	CGAACCGAAUCCCUCACUAAA	10	0.55	20q 47.5

[a] Putative miRNAs were screened against known miRNAs using the miRBase Release 17.0 (http://microrna.sanger.ac.uk/sequences/). miRNAs not corresponding to known sequences were classified as novel and labeled BN1-3. PN5 corresponds to porcine new 5 reported by McDaneld et al. (2009).
[b] Percentage from the total putative miRNAs identified (1798).
[c] Position of the mature putative miRNA based on *Bos taurus* Genome Build 4.0 (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9913). NH = no hit.

miR-106a has one nucleotide miss-match at the 5′ cytosine, which is an adenine in the human sequence. However, this position is not critical for the assay because the specific RT primer and probe lay closer to the 3′ end.

TaqMan MiRNA assays (Applied Biosystems) for let-7b, let-7i, and miR-106a were utilized to validate the expression of these candidate miRNAs in independent sources of bovine oocytes, COCs, and granulosa cells from various sized follicles. Briefly, 1 ng of the small fraction RNA isolated from the ovarian samples was reversed transcribed using the specific miRNA RT primers to generate cDNA corresponding to the specific miRNA. For each candidate miRNA, 66.5 pg equivalents cDNA were subjected to PCR using the Chromo4 real-time PCR detection system (Bio-Rad, Hercules, CA) and followed the TaqMan microRNA protocols provided by the manufacture. Expression levels for each miRNA were based on the threshold cycle (Ct) values determined using the Opticon Monitor 3 software (Bio-Rad). For each miRNA, one assay was performed containing all the experimental samples in triplicate, with intra-assay CV of 9.4%, 8.6%, and 11.4% for let-7b, let-7i, and miR-106a, respectively, after converting the exponential Ct to the linear Ct using the formula 2^{-Ct} (Livak and Schmittgen, 2001).

2.5. Real-time PCR for measuring mRNA expression

For the expression of potential target mRNAs for let-7 and miR-106a, bovine-specific primers (Table 1) were designed using the Primer3 software (Rozen and Skaletsky, 2000) and validated to amplify mRNA specific for myc proto-oncogene protein (*MYC*) and wee1A protein kinase (*WEE1A*). For the expression of transcripts involved in the miRNA processing pathway, bovine-specific primers (Table 1) were designed using the Primer3 software (Rozen and Skaletsky, 2000) and validated to amplify mRNA specific for *RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, and *EIF2C2*. Furthermore, previously designed and validated bovine-specific primers (Table 1) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein, large, P2 (*RPLP2*), and RNA, 18S ribosomal 1 (*RN18S1*) (Miles et al., 2009) were used to normalize TaqMan miRNA and mRNA expression data based on normalization factors calculated using the geNorm algorithm (Vandesompele et al., 2002) from these three stable (M value < 0.6) reference genes.

A two-step, real-time PCR method was used for the analysis of all mRNA expression levels. Briefly, 1 ng of the large fraction RNA isolated from ovarian samples was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Each real-time PCR was assayed in duplicate and consisted of 100 pg equivalents of cDNA, 0.4 μM of the appropriate forward and reverse primer, and 12.5 μL of 1× iTaq SYBR Green Supermix with ROX (Bio-Rad) in a 25 μL reaction. All PCR conditions included denaturation (95 °C for 2 min) followed by amplification (95 °C for 15 s, 60 °C for 15 s, and 70 °C for 45 s). Melting curve analysis and gel electrophoresis was used to confirm the amplification of a single product of the predicted size. The PCR product from a representative sample of PCR for each transcript was verified by

sequence analysis to confirm the amplification of the correct cDNA. For each transcript, one assay was performed containing all the samples, with intra-assay CVs of 14.9%, 11.5%, 18.7%, 13.3%, 15.5%, 14.6%, 5.6%, 9.6%, 5.6%, 10.9%, and 4.9% for *RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, *EIF2C2*, *MYC*, *WEE1A*, *GAPDH*, *RPLP2*, and *18S*, respectively, after converting the exponential Ct to the linear Ct using the formula 2^{-Ct} (Livak and Schmittgen, 2001).

2.6. Statistical analysis

For the validation and characterization experiments, two statistical comparisons were made: (1) expression was compared between denuded oocytes, COCs, and granulosa cell from 1- to 5-mm follicles, and (2) expression was compared between COCs from 1- to 5-mm and 6- to 10-mm follicles. TaqMan miRNA and real-time PCR data were analyzed using general linear model procedures for analysis of variance (SAS, 2003; Steel et al., 1997) and results are reported as least square means ± SEM. When a significant *F*-statistic was determined, means were separated using Tukey–Kramer multiple comparison test (SAS, 2003; Steel et al., 1997). The model for the first comparison included the fixed effects of cellular components (i.e., oocyte, COC, or granulosa cell), replication collection, and their interactions. The model for the second comparison included the fixed effects of follicular size (i.e., 1–5-mm or 6–10-mm), replicate collection, and their interactions. All expression data were log-transformed prior to analysis to normalize the data and then back-transformed for reporting observable values.

3. Results

Following construction of the small RNA cDNA library from bovine COCs, 2304 clones from the library were sequenced, producing 2214 successful small RNA sequences that were clustered based on matching 14 consecutive bases to the most common member of the cluster (Table S1). The consensus sequences for each cluster were screened for mitochondrial RNA, rRNA, tRNA, and snoRNA contamination, resulting in the removal of 416 sequences (19% of the total). The remaining 1798 (81% of total) sequences were compared to known miRNA sequences in the miRBase miRNA database, revealing 64 clusters that matched previously known miRNAs, 2 clusters that matched only to previously reported novel miRNAs in pig muscle libraries (McDaneld et al., 2009), and 3 clusters with no match (labeled BN1-3). The cluster with the largest number of sequences identified in bovine COCs (Table S1) matched sequence of the let-7 miRNA family (31% of the total). The 5 clusters that did not match sequences in miRBase represented putative novel miRNA and were labeled BN1-3 and PN3/PN5 (McDaneld et al., 2009). Table 2 illustrates the most abundantly expressed individual miRNAs in the bovine COC library. The most abundant individual miRNA identified in bovine COCs was let-7b at 26.5% of the total miRNA identified. Several other let-7 members were also abundantly expressed in the bovine COC library and included let-7i, let-7f, and let-7e. The three novel miRNA (BN1, BN2, and BN3) unique to this library had low relative expression (10–23 sequence) accounting for only 3% of the total miRNA.

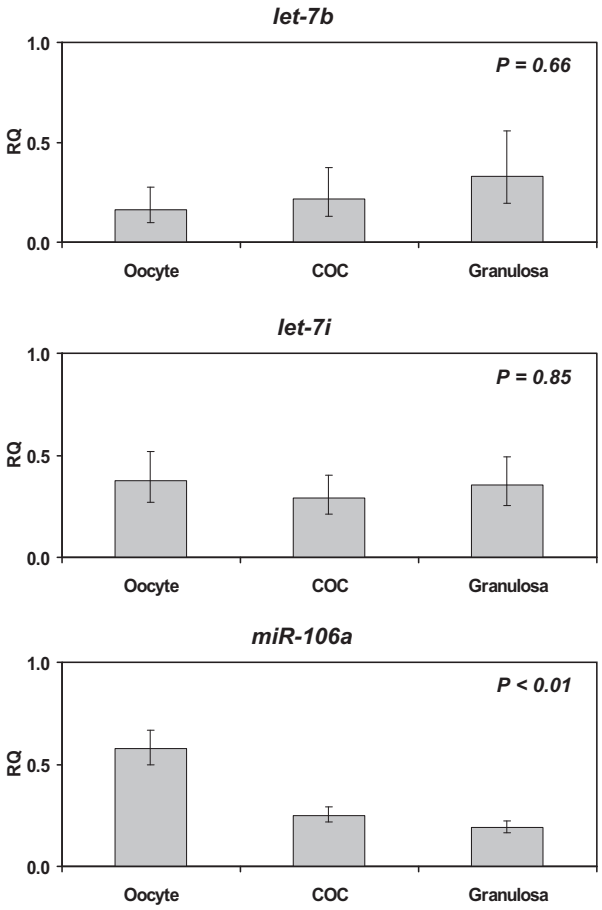

Fig. 1. Expression of *let-7b*, *let-7i*, and *miR-106a* in bovine oocytes, COCs and granulosa cells. TaqMan miRNA assays were performed from three independent replicate collections of oocytes, COCs, and granulosa cells obtained from 1- to 5-mm follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each miRNA.

To validate expression of selected miRNAs, TaqMan miRNA assays were performed in independent sources of oocytes, COCs and granulosa cells from various size follicles. Fig. 1 illustrates the expression of selected miRNAs in oocytes, COCs, and granulosa cells obtained from small (1–5-mm) follicles. There was no difference in the expression of let-7b or let-7i between oocytes, COCs, or granulosa cells. However, miR-106a was significantly increased in oocytes compared with COCs and granulosa cells, demonstrating that this miRNA is up-regulated in the oocyte compared with other somatic cells of the follicle. Fig. 2 illustrates the expression of the selected miRNAs in COCs obtained from small (1–5-mm) and large (6–10-mm) follicles. There was no significant difference in the expression of let-7b, let-7i, or miR-106a in COCs obtained from small and large follicles.

The Microcosm software was used to identify potential target mRNAs for the candidate miRNAs. There were

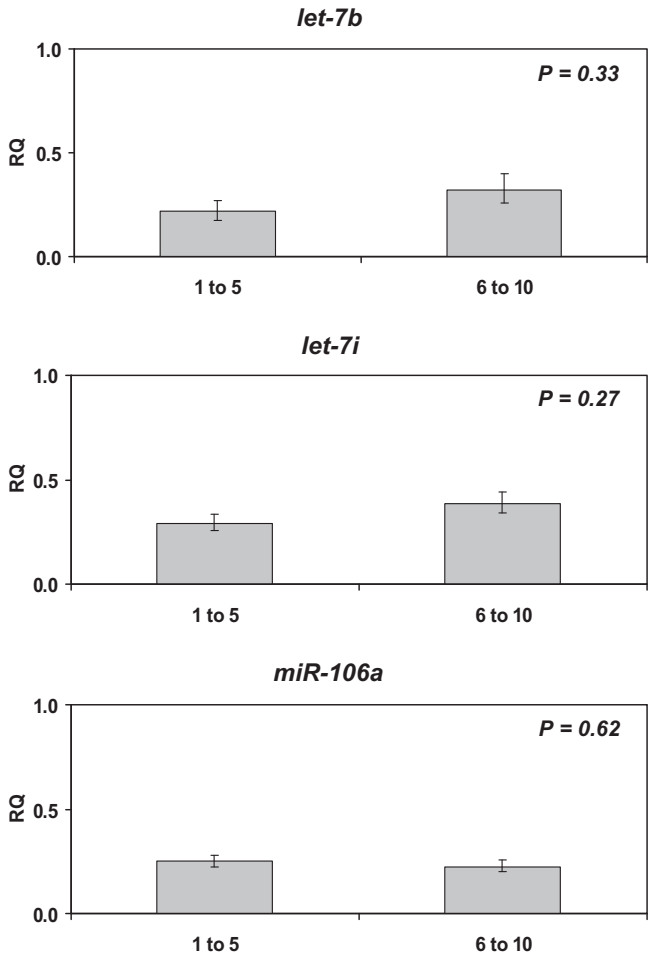

Fig. 2. Expression of *let-7b*, *let-7i*, and *miR-106a* in bovine COCs obtained from small and large follicles. TaqMan miRNA Assays were performed from three, independent replicate collections of COCs obtained from small (1- to 5-mm) and large (6- to 10-mm) follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each miRNA.

920, 923, and 1091 potential mRNA targets identified for let-7b, let-7i, and miR-106a, respectively. Myc proto-oncogene protein was the 8th most significant ($P < 0.0001$) target mRNA identified for let-7b and let-7i. Fig. 3A and B illustrates the alignment of let-7b and let-7i, respectively, within the 3′UTR of the *MYC* gene. Wee1A protein kinase was the 4th most significant ($P < 0.0001$) target mRNA identified for miR-106a. Fig. 3C illustrates the alignment of miR-106a within the 3′UTR of the *WEE1A* gene.

Using real-time PCR, the expression of *MYC* and *WEE1A* mRNA was assessed in independent sources of oocytes, COCs and granulosa cells from various size follicles. Fig. 4 illustrates the expression of *MYC* and *WEE1A* mRNA in oocytes, COCs, and granulosa cell obtained from small (1–5-mm) follicles. The expression of *MYC* mRNA was decreased ($P < 0.05$) in the oocyte compared with the COC and granulosa cells. The expression of *WEE1A* mRNA was decreased ($P < 0.01$) in the oocyte compared with the COC and granulosa cells. Fig. 5 illustrates the expression of *MYC* and *WEE1A* mRNA in COCs obtained from small (1–5-mm) or large (6–10-mm) follicles. The expression of *MYC* mRNA was decreased ($P < 0.01$) in COCs obtained from large follicles compared with small follicles. In contrast, the expression of *WEE1A* mRNA was not different in COCs obtained from small or large follicles.

To further characterize the functionality of miRNAs in bovine COCs, the expression of critical miRNA processing genes was assessed in independent sources of oocytes, COCs and granulosa cells from various size follicles. Fig. 6 illustrates the expression of *RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, and *EIF2C2*, respectively, in oocytes, COCs, and granulosa cells obtained from small (1–5-mm) follicles. The expression of all of these miRNA processing genes were significantly ($P < 0.001$) increased in the oocyte compared with the COCs and granulosa cells, suggesting that miRNA processing and function are likely greater in the oocyte itself. Fig. 7 illustrates the expression of *RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, and *EIF2C2*, respectively, in COCs obtained from small (1–5-mm) or large (6–10-mm) follicles. The expression of *RNASEN*, *DGCR8*, *XPO5*, *TARBP2*, and *EIF2C2* was not different in COCs from the two follicle size categories. However, *DICER1* tended ($P = 0.08$) to

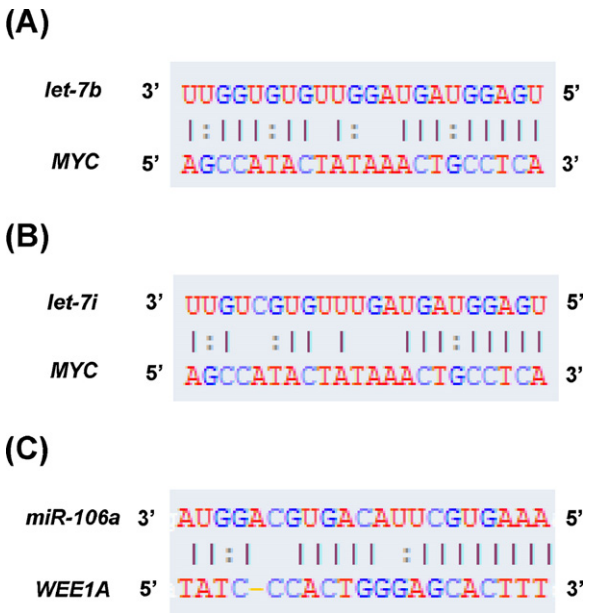

Fig. 3. Alignment of *let-7b* (A), *let-7i* (B), and *miR-106a* (C) within the 3′ UTR of putative mRNA target genes. Potential target mRNAs for selected candidate miRNAs were identified using Microcosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). (A) Alignment of *bta-let-7b* within the 3′-UTR of *MYC*. (B) Alignment of *bta-let-7i* within the 3′-UTE of *MYC*. (C) Alignment of *bta-miR-106a* within the 3′-UTR of *WEE1A*.

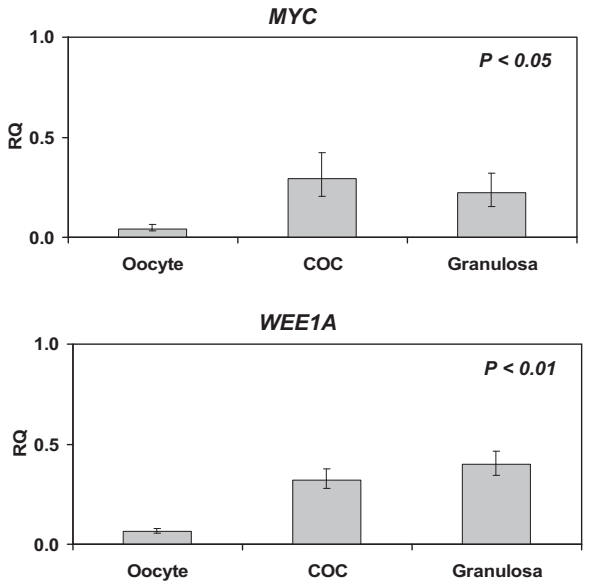

Fig. 4. Expression of *MYC* and *WEE1A* in bovine oocytes, COCs, and granulosa cells. Real-time PCR Assays were performed from three independent replicate collections of oocytes, COCs, and granulosa cells obtained from 1- to 5-mm follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each transcript.

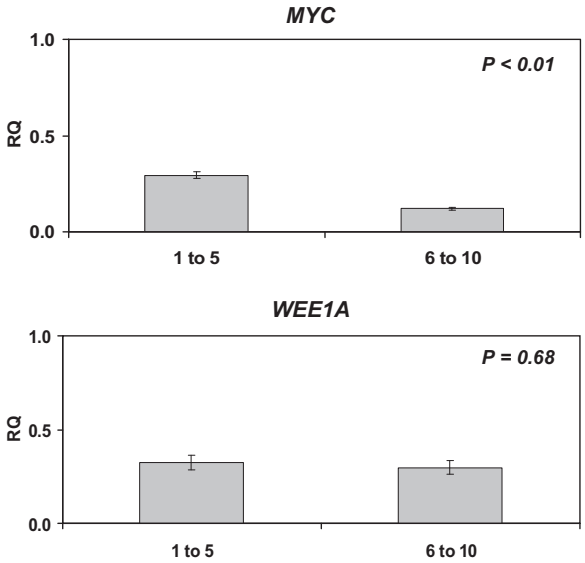

Fig. 5. Expression of *MYC* and *WEE1A* in bovine COCs obtained from small and large follicles. Real-time PCR Assays were performed from three independent replicate collections of COCs obtained from small (1–5-mm) and large (6–10-mm) follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each transcript.

be increased in COCs from larger follicles compared with smaller follicles. Increased levels of *DICER1* and increased numerical levels of let-7b and let-7i may reflect the demonstrated decrease in *MYC* mRNA expression in larger follicles compared with smaller follicles.

4. Discussion

Small RNA cDNA libraries have been previously used in cattle to evaluate miRNA abundance in adipose (Jin et al., 2010) and mammary tissues (Gu et al., 2007), various immune and embryonic tissues (Coutinho et al., 2007), pooled liver, brain, heart, and lung (Long and Chen, 2009), alveolar macrophages (Xu and Huang, 2009), and fetal (Tripurani et al., 2010) and adult (Hossain et al., 2009) ovaries. To our knowledge, the current study is the first to report a miRNA expression profile specific to bovine COCs using the small RNA cDNA library method described herein. Furthermore, this study is the first to characterize the expression of key miRNA processing genes within a variety of ovarian cell types (i.e., granulosa cells, COCs, and oocytes) in the cow. Together, these data provides evidence that let-7 and miR-106a may have an involvement in oocyte development through regulation of potential targets transcriptions, *MYC* and *WEE1A*, respectively. The inhibition of mitotic proliferation and proper activation of factors that regulate the oscillations of meiotic maturation are critical to ensure the proper progression of the oocyte through maturation (Murray and Kirschner, 1989). Potential regulation of let-7 and miR-106a on the expression of *MYC* and *WEE1A*, respectively, may play a role in the proper

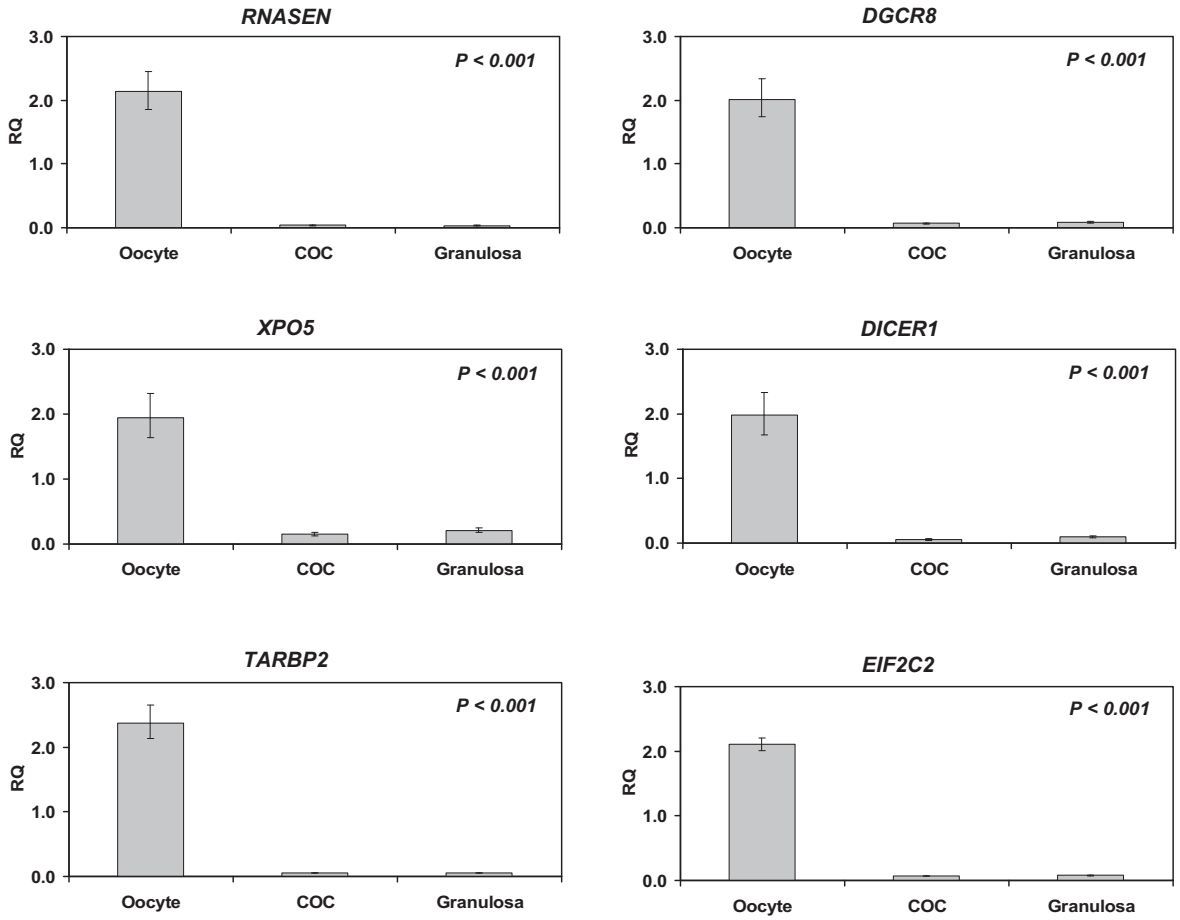

Fig. 6. Expression of miRNA biogenic transcripts (*RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, *EIF2C2*) in bovine oocytes, COCs, and granulosa cells. Real-time PCR Assays were performed from three independent replicate collections of oocytes, COCs, and granulosa cells obtained from 1- to 5-mm follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each transcript.

maturation of the oocyte by regulating mitotic and meiotic activity.

The let-7 miRNA family was the most abundant cluster of miRNAs identified within bovine COCs, with let-7b being the most abundantly expressed individual miRNA in bovine COCs. This supports previously reported expression profiles of miRNAs using cDNA libraries and massively parallel sequencing that identified let-7 miRNA family as an abundantly expressed miRNA family in the bovine (Tripurani et al., 2010) and mouse (Reid et al., 2008; Wyman et al., 2009) ovaries. Many studies focusing on cancer cell development in a variety of tissues have illustrated that let-7 is a tumor suppressive miRNA functioning by inhibiting the expression of the proto-oncogene, MYC (Mendell, 2009; Sampson et al., 2007). Myc proto-oncogene protein is a transcription factor that increases cellular proliferation and drives cell cycle progression (Gearhart et al., 2007). Although the involvement of MYC in oogenesis of mammalian oocytes is unclear, MYC plays a key role in driving the initial cleavages after fertilization in *Xenopus* (Gusse et al., 1989). In *Xenopus* oocytes, *MYC* mRNA is abundantly expressed throughout early oogenesis but decreases during late oogenesis and early cleavage. However, there is a significant lag in the level of MYC protein which remains low during early oogenesis and does not reach its highest level until fertilization. This pattern of mRNA/protein expression illustrates a post-transcriptional regulation for MYC mRNA such that the protein does not accumulate until the time of fertilization (Gusse et al., 1989). It is possible that high amounts of let-7 in bovine COCs may inhibit the activity of MYC in oocytes; thereby, reducing the proliferative activity and maintaining the oocyte in a mitotically inactive state during oogenesis.

In the current study, we provide evidence that *MYC* is a likely target for *let-7b* and *let-7i* in cattle. The expression of *MYC* mRNA was decreased in denuded oocytes compared with COCs and the surrounding granulosa cells. Interestingly, there was no difference in the expression of let-7b or let 7i miRNAs in denuded oocytes, COCs, and granulosa cells, which suggests that let-7b or let-7i miRNAs are not affecting the decreased expression of *MYC* in denuded oocytes. However, all of the miRNA processing genes were highly expressed in the denuded oocyte, which likely enhance the function of available miRNAs within the

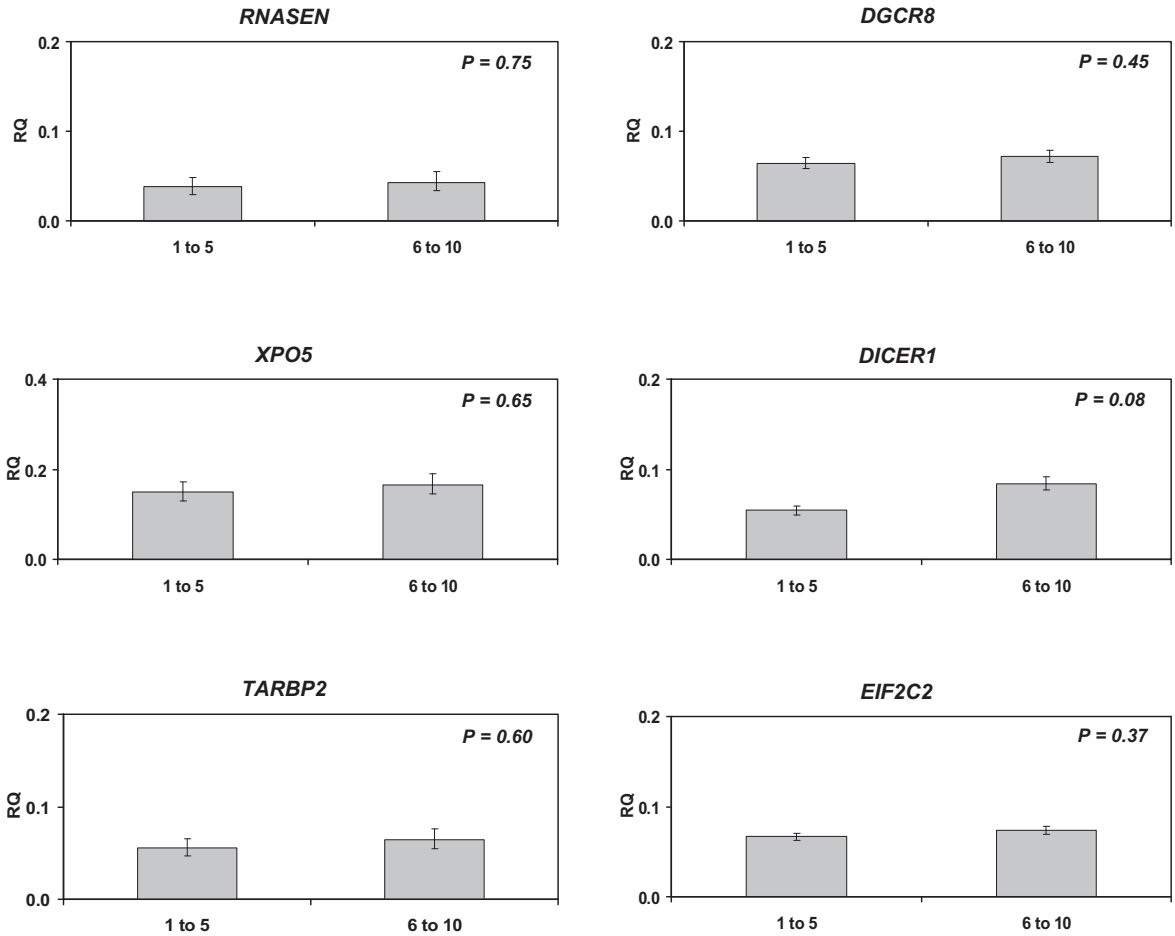

Fig. 7. Expression of miRNA biogenic transcripts (*RNASEN*, *DGCR8*, *XPO5*, *DICER1*, *TARBP2*, *EIF2C2*) in bovine COCs obtained from small and large follicles. Real-time PCR Assays were performed from three independent replicate collections of COCs obtained from small (1–5-mm) and large (6–10-mm) follicles. Data were log transformed before analysis and back-transformed to observed values. Values are reported as least-squares means ± SEM and expressed as relative quality (RQ) after adjusting expression data based on normalization factors calculated using the geNorm algorithm. Statistical significance is reported for each transcript.

oocyte; thereby, resulting in decreased expression of *MYC* in denuded oocytes. Furthermore, other uncharacterized miRNAs could be targeting *MYC* within the oocyte resulting in decreased expression of *MYC*. It is not currently known whether the decreased expression of *MYC* in the oocytes was due to transcriptional repression or mRNA degradation. A similar pattern of decreased *MYC* mRNA expression was observed in COCs from 6- to 10-mm follicles compared with 1- to 5-mm follicles, but there was no significant difference in let-7b and let-7i in COCs from the two size ranges of follicles. It is important to note that there was a numerical increase in let-7b and let-7i and a tendency for increased *DICER* in COCs from 6- to 10-mm follicles, which may reflect differences in *MYC* expression from these COCs.

During prenatal development, oogonia undergo mitosis from primordial germ cells to form diploid oocytes (Wassarman and Albertini, 1994). These diploid oocytes enter into meiosis I, which ultimately generate haploid oocytes that can be fertilized with haploid sperm to form viable zygotes. Shortly before (i.e., cattle) or after birth (i.e., mice), the oocyte undergoes its first meiotic arrest (Wassarman and Albertini, 1994). The oocyte then undergoes a growth phase to develop from immature oocytes into mature oocytes. This growth phase is the time that many maternal mRNAs accumulate within the oocyte (Bettegowda et al., 2008). Mature oocytes have the ability to resume meiosis, at which time they have the ability to complete their first meiotic reductive divisions and progress into meiosis II. The formation of the antrum within the follicle signifies meiotically competent, mature oocytes (Mehlmann, 2005). The COCs evaluated in the current study to identify miRNAs, corresponded to these types of oocytes (i.e., meiotically competent, mature oocytes). Shortly after ovulation, the oocyte enters its second meiotic arrest. The final resumption and completion of meiosis occurs after fertilization or parthenogenetic activation at which time the embryo undergoes subsequent cleavages by means of mitosis (Wassarman and Albertini, 1994). This entire process is referred to as meiotic maturation and is regulated by the maturation promoting factor, which is a complex of CDK1 and cyclinB (Murray and Kirschner, 1989).

The activity of the maturation promoting factor remains elevated in mature oocytes until shortly after ovulation (Murray and Kirschner, 1989). The protein kinase, WEE1A, inhibits the maturation promoting factor by phosphorylating CDK1 at the Thr14 and Try15, which inactivates CDK1 and causes decreased activity and subsequent meiotic arrest (Mehlmann, 2005). A previous study using immature and mature mouse oocytes demonstrated that a reduction of WEE1A protein was necessary for the acquisition of meiotic competence and the ability of the mature oocytes to resume meiosis (Mitra and Schultz, 1996). In the current study, we provide evidence that *WEE1A* is a putative target for miR-106a in cattle. Interestingly, miR-106a has been associated with proliferation of a number of carcinomas and shown to accelerate the cell cycle of these carcinomas (Jiang et al., 2011; Xiao et al., 2009). Therefore, it is possible that miR-106a may inhibit WEE1A in mature oocytes; thereby, ensuring proper acquisition of meiotic competence in these oocytes. From the small RNA cDNA library, miR-106a was abundantly expressed in the bovine COCs. Additionally, Taqman miRNA assays demonstrated that miR-106a was significantly increased in denuded oocytes compared with COCs and granulosa cells, suggesting that miR-106a is specifically up-regulated in the bovine oocyte. In contrast, *WEE1A* mRNA expression was reduced in denuded oocytes compared with COCs and granulosa cells. However, there was no difference in the expression of miR-106a expression in COCs from small and large follicles. Similarly, *WEE1A* mRNA expression did not differ in COCs from small and large follicles. Furthermore, all of the miRNA processing genes were highly overexpressed in the denuded oocyte, which likely enhance the function of available miRNAs within the oocyte. Taken together; these observed expression patterns for miR-106a and *WEE1A* mRNA illustrate a classic pattern of post-transcriptional regulation such that miR-106a may decrease the expression of *WEE1A* within the oocyte.

In summary, this study identified 64 known and 5 novel miRNA clusters in the bovine COC. Let-7b, let-7i, and miR-106a expression was confirmed in denuded oocytes, COCs and granulosa cells from various sized follicles. The expression of miR-106a and the miRNA processing genes were greater in denuded oocytes, suggesting greater function of the miRNA pathway within the oocyte. As a result, there was a decrease in the expression of MYC and WEE1A mRNA in these oocytes, which could have an inhibitory effect of the mitotic cell cycle in the case of MYC or a stimulatory effect of the resumption of meiosis in the case of WEE1A. In conclusion, these results demonstrate specific miRNAs within bovine COCs during late oogenesis and provide evidence that miRNAs may play a role regulating maternal mRNAs in bovine oocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.anireprosci.2011.12.021.

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