Expression of miRNAs in ovine fetal gonads: potential role in gonadal differentiation

Katie J Torley1†, Juliano C da Silveira1†, Peter Smith2, Russell V Anthony1, DN Rao Veeramachaneni1, Quinton A Winger1, Gerrit J Bouma1*

Abstract

Background: Gonadal differentiation in the mammalian fetus involves a complex dose-dependent genetic network. Initiation and progression of fetal ovarian and testicular pathways are accompanied by dynamic expression patterns of thousands of genes. We postulate these expression patterns are regulated by small non-coding RNAs called microRNAs (miRNAs). The aim of this study was to identify the expression of miRNAs in mammalian fetal gonads using sheep as a model.

Methods: We determined the expression of 128 miRNAs by real time PCR in early-gestational (gestational day (GD) 42) and mid-gestational (GD75) sheep ovaries and testes. Expression data were further examined and validated by bioinformatic analysis.

Results: Expression analysis revealed significant differences between ovaries and testes among 24 miRNAs at GD42, and 43 miRNAs at GD75. Bioinformatic analysis revealed that a number of differentially expressed miRNAs are predicted to target genes known to be important in mammalian gonadal development, including ESR1, CYP19A1, and SOX9. In situ hybridization revealed miR-22 localization within fetal testicular cords. As estrogen signaling is important in human and sheep ovarian development, these data indicate that miR-22 is involved in repressing estrogen signaling within fetal testes.

Conclusions: Based on our results we postulate that gene expression networks underlying fetal gonadal development are regulated by miRNAs.

Background
Genetic sex in mammals is determined at the time of fertilization; fertilization of eggs with X or Y-bearing sperm will yield XX (female) or XY (male) embryos, respectively. Normally, distinct genetic pathways will subsequently direct undifferentiated genital ridges in XX and XY fetuses to develop into fetal ovaries or fetal testes, respectively. Mammalian fetal gonadal differentiation is a developmental process involving a dose-dependent balance between promoting and antagonizing factors. That is, the testicular developmental pathway involves genetic networks both promoting testis development and preventing ovarian development and vice versa [1,2]. Critical genes involved in initiation of the testicular and ovarian developmental pathways are the Y-linked gene, Sry (sex determining region of chromosome Y) [3,4], and Rspo1 (R-spondin homolog), Wnt4 (wingless-related MMTV integration site 4) and β-catenin [5,6], respectively. These genes are expressed in the somatic support cells of the fetal gonads directing differentiation of the supporting cell lineages, i.e., Sertoli cells in the testis and granulosa cells in the ovary [5-9]. Genome profiling experiments further have demonstrated that both testicular and ovarian developmental pathways are characterized by dynamic expression patterns of thousands of genes [10-14]. How the expression and function of these genes are regulated is unknown.

Small non-coding RNA molecules called microRNAs (miRNAs) are ~22 nt cytoplasmic RNAs that regulate gene expression and function in many tissues [15-17]. MiRNAs are transcribed by RNA polymerase II generating hairpin loop containing structures called primary-miRNAs which are cleaved by the endonuclease RNase
III DROSHA and its partner DGCR8, yielding a 70-90 nt hairpin stem-loop precursor miRNA (pre-miRNA). Pre-miRNAs are exported into the cytoplasm by Exportin-5 and processed by Dicer1, yielding a ~22 nt mature miRNA. MiRNAs recognize transcript targets through base-pairing to the 3’-untranslated region (UTR), and are able to repress translation or cause degradation, depending upon sequence complementarity. Previous work has demonstrated that miRNA sequences are highly conserved across species and can be expressed in a tissue specific manner [18]. The importance of miRNAs in reproduction was demonstrated using transgenic mouse models [19-24]. Conditional gene targeting approaches demonstrated that Dicer is important for primordial germ cell and spermatogonia proliferation [19,20], Sertoli cell function [21], and development of the oviducts and uteri [22-24].

The above-mentioned studies indicate that miRNAs are important for reproductive development and function, but do not indicate which miRNAs. MiRNA cloning experiments have demonstrated differences in expression profiles between ovaries and testis of adult mice [25], and analysis of a bovine fetal ovarian miRNA library revealed miRNAs predominantly expressed in fetal ovaries compared to somatic tissue pools [26]. Little is known about the expression of miRNAs during fetal gonadal development in mammals. Based on the observation that fetal ovarian and testicular development involves coordinate expression of thousands of genes, we predict that miRNAs are expressed and are involved in regulating gene expression and function during fetal gonadal development.

The aim of this study was to identify the expression of miRNAs in mammalian fetal gonads using the ovine as a model. In addition, expression levels were examined of a number of key genes involved in fetal ovarian (ESR1, ESR2, CYP19, FST, and WNT4) and testicular development (SOX9) as potential target genes of miRNAs. The study of gonadal differentiation and reproductive development in sheep provides both insight into human gonadal development, and a better understanding of reproductive development of economically important livestock species.

**Methods**

**Sheep breeding and tissue collection**

All experimental procedures using animals were approved by the Colorado State University Animal Care and Use Committee. Estrous cycles of twenty-four ewes were synchronized using prostaglandin F2α (5 mg: Lutalyse; Pfizer Animal Health), and ewes and rams were mated and kept together for twelve to twenty-four hours to ensure successful mating (up to 4 ewes with one ram, and the ram replaced with a new ram after ~12 hours). Around gestational day (GD) 40, pregnancy status was confirmed by ultrasonography.

Tissues were collected at either GD42 or 75 (term is ~150 days). GD42 corresponds to the period of testicular cord differentiation and ovigerous cord development in XY and XX gonads respectively, whereas GD75 coincides with primordial follicle formation in the ovary [27,28]. Ewes were taken off feed and water at least twelve hours before necropsy to facilitate unhindered tissue collection. Ewes were euthanized by intrajugular injection of sodium pentobarbital (90 mg/kg). Each fetus was measured crown to rump and lengths were used to estimate and confirm gestational age. Fetal gonads were removed and one was homogenized in 350 μl of RLT Plus lysis buffer (RNeasy Plus Mini Kit, Qiagen) and the other was fixed in 4% paraformaldehyde (PFA; GD42) or Bouin’s fixative (GD75). Gonads used for RNA isolation were stored at -80°C. Gonads for histology were fixed overnight, transferred to 70% ethanol, and stored at 4°C until embedded in paraffin.

**Fetal sheep sex genotyping**

Genetic sex was determined by PCR genotyping using fetal tail tissue lysate, and ovine SRY primers (Forward primer: 5’- CATTGTTGTTCTCGTGAACG-3’; Reverse primer: 5’-GTCTCGGTGTATACTAGTAGTAG-3’) designed based on the ovine SRY sex chromosome (GenBank Accession number Z30265). Polymerase chain reaction was run using the following program: 95°C - 3 minutes; 60°C - 5 minutes; 72°C - 5 minutes (1 cycle); 95°C - 30 seconds, 58°C - 30 seconds, 72°C - 45 seconds (35 cycles); and 72°C - 5 minutes. PCR products were run on a 2% agarose gel, and visualized using ethidium bromide.

**Total RNA isolation**

Total RNA, including miRNAs, was isolated using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer’s instructions. To ensure the small RNA fraction was retained, the first washing step with RW1 buffer was replaced by 100% ethanol (1.5× volume of the tissue lysate) according to manufacturer’s specification. RNA was eluted in 40 μl RNAse-free water, and treated with DNase (4 μl 10× DNase buffer and 1 μl DNase-I (Ambion)) to eliminate genomic DNA contamination. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Samples were then stored at -80°C.

**Reverse Transcription of miRNAs**

Small non-coding RNAs were reverse transcribed using the QuantiMirTM RT kit (Systems Biosciences (SBI), Mountain View, CA) according to the manufacturer’s instructions. Briefly, ~500 ng of total RNA including the small RNA fraction was anchor-tailed with polyA by
incubating RNA, 5× PolyA Buffer, 25 mM MnCl2, 5 mM ATP, and polyA polymerase at 37°C for 30 minutes. Oligo dT adaptors were annealed at 60°C for 5 minutes, and reverse transcription first strand synthesis reaction was carried out by incubating the samples at 42°C for 60 minutes followed by 95°C for 10 minutes.

Real-time PCR expression of conserved miRNAs in fetal gonads

A preliminary experiment was conducted to examine the expression of 211 miRNAs whose mature sequence was identical in human, mouse, bovine and/or goat [29]. Of these 211 miRNAs, 128 were selected that were expressed (arbitrarily set at crossing-point value (Cp) < 37) according to real-time RT-PCR analysis, and showed a single melt peak following dissociation curve analysis. The relative expression of 128 mature miRNAs (see Additional file 1, Table S1) in fetal sheep gonads was assessed using real-time PCR using a custom designed primer plate containing the mature miRNA sequences as a forward primer (SBI).

Each analysis was performed in 6 μl reactions containing 2× SYBR Green I master mix (Roche Applied Sciences), 10 μM Universal reverse primer and miRNA specific forward primer (SBI), and 1 μl cDNA. Real time PCR was conducted using the LightCycler480 PCR system (Roche Applied Sciences) with 384-well plates each containing 3 biological replicates. The PCR cycle conditions were as follows: 95°C for 5 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 30 seconds followed by a melt curve analysis to confirm amplification of single PCR products. This experiment was repeated twice (n = 2-4). Relative expression level of transcripts was determined by calculating the geometric mean of GAPDH and RNU18S expression values, and using this as a normalization factor. Statistical differences were assessed at P < 0.05 using a Students t-test. PCR amplification efficiencies were present as 1.8 - 2.1, and relative expression levels were calculated based on 10 normalizers (miRNAs in the data set that are expressed and not significantly different).

Reverse transcription of mRNAs

Messenger RNA was reverse transcribed into cDNA using the MessageSensorTM RT kit (Ambion Inc.) as described previously [31]. Briefly, 5 μl of RNA (50 ng/μl) was combined with 10× RT buffer, dNTPs, 10 μM random decamers, RNase Inhibitor, and M-MLV reverse transcriptase. RNA was reverse transcribed by incubating the samples at 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 10 minutes. cDNA was used immediately for real-time PCR analysis.

Real-time PCR expression of mRNAs in fetal gonads

The relative expression level of mRNAs involved in fetal gonadal development (ESR1, ESR2, CYP19, SOX9, WNT4, FST) and two housekeeping genes (GAPDH and RNU18S) was examined using real-time PCR. Preliminary experiments in our laboratory revealed the expression level of these two housekeeping genes were consistent and did not change. Gene specific primers were designed using Primer3 [32] using ovine, bovine, and/or porcine sequences (see Additional file 2, Table S2). Amplification efficiencies were determined using a 10 fold serial dilution series of a GD75 XX and XY gonadal cDNA pool for each primer set. In addition, cDNA products were sequenced to validate primer specificity.

Analysis was performed in 10 μl reactions containing 2× SYBR Green Master Mix I (Roche Applied Sciences), 0.5 μM gene specific forward and reverse primer, and cDNA (diluted 1:4) using the LightCycler480 PCR system. The reaction conditions were as follows: 95°C for 5 minutes and then 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by a melt curve analysis, to confirm amplification of single PCR products. This experiment was repeated twice (n = 2-4). Relative expression level of transcripts was determined by calculating the geometric mean of GAPDH and RNU18S expression values, and using this as a normalization factor. Statistical differences were assessed at P < 0.05 using a Students t-test. PCR amplification efficiencies were between 1.8 - 2.1, and relative expression levels were calculated based on 10 normalizers (miRNAs in the data set that are expressed and not significantly different).

Histology

Fixed fetal gonads were dehydrated and paraffin embedded using routine procedures. 5 μm-thick tissue sections were cut and stained with hematoxylin and eosin, and examined using a light microscope equipped with plan aposromatic objectives. Additional 4% paraformaldehyde fixed, unstained 5 μm GD75 and GD90 tissue sections were provided by Dr. Peter Smith (AgResearch Limited, Invermay, New Zealand) and used for in situ hybridization analysis.

In situ hybridization

Cellular localization of miR-22 was examined in GD75 and GD90 ovaries and testes using non-radioactive in situ hybridization. Non-radioactive in situ hybridization was performed using DIG labeled LNA miRNA probes for U6 (positive control), miR-22, and a scramble (negative control) (Cat # 99002-15, 300500-15, and 99004-01 respectively; Exiqon, Vedbaek, Denmark) according to the manufacturer’s instruction and included a Tyramide Signal Amplification (TSA) step (Perkin-Elmer, Walltham, MA). Briefly, following deparaffinization with xylene, tissue sections were rehydrated through a graded series of ethanol washes (100%, 70%, 50%, 25%), and rinsed in phosphate buffered saline (PBS). After endogenous peroxidase activity was blocked by incubating
tissue sections in 0.03% H2O2 (30 minutes) and proteins were digested with 10 μg/ml proteinase K, tissue sections were washed and post-fixed in 4% PFA, and incubated overnight in hybridization buffer (50% deionized formamide, 5× SSC, 0.1% Tween 20, 50 μg/ml yeast tRNA). The next day, tissue sections were incubated overnight with hybridization buffer containing 0.25 μM probe at 50°C in a humidified chamber. Following washes and incubation in blocking buffer (2% sheep serum and 2 mg/ml BSA in PBS plus 0.01% Tween (PBST)), tissue sections were incubated in blocking buffer containing anti-DIG conjugated horseradish peroxidase (HRP) antibody (Abcam, Cambridge, MA; 1:2000 dilution) for 30 minutes at room temperature. TSA signal amplification was conducted by adding 100-300 μl fluorephore tyramide solution per tissue section for 12 minutes at room temperature, followed by washes in PBST, and incubation with anti-fluorescein alkaline phosphatase (AP) conjugated antibody (Rockland Inc., Gilbertsville, PA; 1:75 dilution) for 30 minutes. Antibody staining was visualized using BCIP/NBT in AP buffer (100 mM Tris-HCl, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, 2.4 mg Levamisole (Honeywell, Seelze, Germany)). Color development was monitored and stopped by washing the slides in PBST (5 hours for U6, 48 hours for miR-22 and scramble).

Results
MiRNA expression in fetal gonads
Using a custom designed 128-miRNA profiler PCR plate, the relative expression level of 128 conserved miRNA sequences was assessed in sheep fetal gonads. At GD42, when testicular cords develop in XY gonads (Figure 1), 24 miRNAs exhibited a sexual dimorphic expression pattern with at least 2 fold difference (Table 1). Of these, 12 miRNAs were significantly higher in XX and 12 were expressed significantly higher in XY gonads. At GD75, when the ovary is filled with ovigerous cords (Figure 1) and primordial follicles start to form, 43 miRNAs exhibited a sexually dimorphic expression pattern with at least a 2 fold change; 26 miRNAs were expressed significantly higher in GD75 ovaries, and 17 were expressed significantly higher in GD75 testes (Table 2).

In addition to identifying miRNAs exhibiting sexual dimorphic expression patterns, relative expression level of miRNAs was examined during development within fetal ovaries and fetal testes by comparing miRNA expression in GD42 and GD75 gonads. Comparing miRNA expression in GD42 and GD75 ovaries, 62 miRNAs with at least a 2 fold change were differentially expressed; 31 were expressed significantly higher in GD42 ovaries and 31 were expressed significantly higher in GD75 ovaries (Table 3). Within fetal testes, 30 miRNAs with at least a 2 fold change were expressed differentially when comparing GD42 to GD75; 13 were expressed significantly higher in GD42 testes and 17 were expressed significantly higher in GD75 testes (Table 3).

To gain further insight into the possible function of these miRNAs, TargetScan 5.1, Meta Mir:Target Infer- ence, and miRGator [34-36] were used as tools to identify potential genes known to be involved in mammalian fetal gonadal differentiation targeted by miRNAs. Examining the differentially expressed miRNAs in GD42 gonads revealed a number of genes, including SOX9, ESR1 (estrogen receptor 1), and CYP19A1 (cytochrome P450, family 19, subfamily a, polypeptide 1) as potential targets (Table 4). Furthermore, differently expressed miRNAs in GD75 ovaries and testes are predicted to target a number of genes including ESR1, CYP19A1, FST (follistatin), and WNT4 (Table 4).
Figure 1 Tissue sections of GD42 and GD75 ovaries and testes stained with hematoxylin and eosin. A) GD42 ovary illustrating a thin ovarian cortex containing oogonia and precursor granulosa cells. Arrow in insert points to an oogonium. B) GD75 ovary illustrating a thickened cortex containing ovigerous cords. Arrow in insert depicts oocyte within an ovigerous cord. C) GD42 testis demonstrating presence of testicular cords containing gonocytes and Sertoli cells. Arrow in insert points to a gonocyte; arrowhead points to a Sertoli cell nucleus. D) GD 75 testis revealing prominent testicular cords. Arrow in insert points to a gonocyte; arrowhead points to an interstitial (Leydig) cell. All bars are 20 µm.
Table 1 Significant differently expressed miRNAs exhibiting a fold change > 2 in GD42 sheep ovaries and testes, according to real time RT-PCR analysis

| miRNA     | GPR Score | Fold-Change |
|-----------|-----------|-------------|
| miR-431  | 0.836     | 5.35        |
| let-7c    | 0.789     | 3.98        |
| miR-328  | 0.688     | 3.85        |
| miR-195  | 0.734     | 3.59        |
| miR-486  | 0.695     | 3.40        |
| let-7d   | 0.727     | 3.13        |
| miR-7    | 0.594     | 3.07        |
| miR-484  | 0.672     | 2.99        |
| miR-423  | 0.656     | 2.81        |
| let-7a   | 0.703     | 2.75        |
| miR-320  | 0.531     | 2.67        |
| let-7e   | 0.586     | 2.23        |

**Significantly higher in GD42 ovary**

| miRNA     | GPR Score | Fold-Change |
|-----------|-----------|-------------|
| miR-758  | 0.734     | 5.00        |
| miR-192  | 0.844     | 4.50        |
| miR-223  | 0.797     | 3.74        |
| miR-101  | 0.477     | 2.74        |
| miR-411  | 0.656     | 2.58        |
| miR-369-5p | 0.523   | 2.55        |
| miR-301  | 0.594     | 2.21        |
| miR-142-5p | 0.484   | 2.19        |
| miR-27b  | 0.516     | 2.19        |
| miR-379  | 0.555     | 2.18        |
| miR-142-3p | 0.484   | 2.08        |
| miR-376a | 0.414     | 2.04        |

**Significantly higher in GD42 testis**

Significance (indicated by GPR score) and fold change were determined using Global Pattern Recognition v2.0.

### Cellular localization of miR-22 in fetal sheep gonads

Estrogen signaling plays an important role during sheep ovarian development [27,38,39]. In situ hybridization was conducted to examine the localization of miR-22, predicted to target ESR1 in fetal sheep gonads. MiR-22 is down-regulated during fetal ovarian development (GPR score 0.953, ~1900 fold; Table 3) and up-regulated during testicular development (GPR score 0.500, 2.6 fold; Table 3), according to real time PCR. Using a LNA-modified probe specific to miR-22, in situ hybridization analysis revealed specific localization of miR-22 in Sertoli cells within testicular cords of GD90 testis sections, but not GD75 testis sections (Figure 4). No staining was observed within ovaries, or when a scrambled miRCURY LNA detection probe was used.

### Discussion

In mammals the activation of both fetal ovarian and testicular genetic pathways involve dynamic changes in the

Table 2 Significant differently expressed miRNAs exhibiting a fold change > 2 in GD75 sheep ovaries and testes, according to real time RT-PCR analysis

| miRNA     | GPR Score | Fold-Change |
|-----------|-----------|-------------|
| let-7c    | 0.953     | 100.07      |
| miR-146b | 0.781     | 18.52       |
| miR-103  | 0.844     | 17.23       |
| miR-125b | 0.859     | 15.92       |
| let-7d   | 0.758     | 15.73       |
| miR-484  | 0.859     | 15.55       |
| let-7a   | 0.758     | 14.90       |
| miR-25   | 0.773     | 10.89       |
| miR-125a | 0.758     | 8.91        |
| miR-99a  | 0.805     | 8.78        |
| miR-100  | 0.828     | 8.39        |
| miR-150  | 0.664     | 6.25        |
| miR-130a | 0.734     | 5.76        |
| miR-19b  | 0.664     | 5.33        |
| miR-28   | 0.695     | 4.81        |
| miR-362  | 0.602     | 4.68        |
| miR-16   | 0.484     | 4.35        |
| miR-200c | 0.688     | 4.16        |
| miR-200b | 0.688     | 4.11        |
| miR-210  | 0.688     | 3.85        |
| miR-19a  | 0.555     | 3.59        |
| miR-10a  | 0.648     | 3.12        |
| let-7g   | 0.602     | 2.82        |
| let-7e   | 0.469     | 2.48        |
| miR-92   | 0.430     | 2.42        |
| miR-183  | 0.422     | 2.42        |

**Significantly higher in GD75 ovary**

| miRNA     | GPR Score | Fold-Change |
|-----------|-----------|-------------|
| miR-101  | 0.477     | 2.74        |
| miR-369-5p | 0.523   | 2.55        |
| miR-301  | 0.594     | 2.21        |
| miR-142-5p | 0.484   | 2.19        |
| miR-27b  | 0.516     | 2.19        |
| miR-379  | 0.555     | 2.18        |
| miR-142-3p | 0.484   | 2.08        |
| miR-376a | 0.414     | 2.04        |

**Significantly higher in GD75 testis**

Significance (indicated by GPR score) and fold change were determined using Global Pattern Recognition v2.0.
### Table 3 Significant differently expressed miRNAs exhibiting a fold change ≥ 2 in GD42 and GD75 sheep ovaries and testes, according to real time RT-PCR analysis

| miRNA   | GPR Score | Fold-Change | miRNA   | GPR Score | Fold-Change | miRNA   | GPR Score | Fold-Change |
|---------|-----------|-------------|---------|-----------|-------------|---------|-----------|-------------|
| miR-22  | 0.953     | 1935.53     | let-7c  | 0.883     | 46.01       | miR-302d| 0.891     | 16.36       |
| miR-302d| 0.945     | 119.16      | miR-142-5p | 0.898           | 35.40       | miR-200c| 0.867     | 11.85       |
| miR-206 | 0.875     | 14.76       | miR-19b | 0.906     | 27.28       | miR-222 | 0.734     | 5.32        |
| miR-222 | 0.875     | 8.43        | miR-19a | 0.883     | 16.43       | miR-200b| 0.695     | 4.44        |
| miR-196a| 0.844     | 7.40        | miR-135a| 0.781     | 12.28       | miR-362 | 0.609     | 3.95        |
| miR-328 | 0.672     | 7.34        | miR-125b| 0.883     | 12.18       | miR-99b | 0.539     | 3.95        |
| miR-433 | 0.781     | 7.23        | let-7a  | 0.727     | 10.89       | miR-485-5p | 0.578   | 3.61        |
| miR-486 | 0.813     | 6.81        | miR-130a| 0.852     | 9.93        | miR-382 | 0.586     | 2.22        |
| miR-216 | 0.727     | 6.63        | miR-146b| 0.648     | 9.92        | miR-149 | 0.484     | 3.01        |
| miR-221 | 0.820     | 5.88        | miR-100 | 0.883     | 9.48        | miR-15b | 0.523     | 2.82        |
| miR-196b| 0.758     | 5.69        | miR-99a | 0.859     | 8.91        | miR-92  | 0.430     | 2.72        |
| miR-574 | 0.750     | 4.83        | let-7d  | 0.453     | 8.76        | miR-17-5p| 0.484    | 2.27        |
| miR-485-5p| 0.742   | 4.52        | miR-101 | 0.688     | 8.52        | miR-107 | 0.414     | 2.15        |
| miR-7   | 0.727     | 4.45        | miR-484 | 0.750     | 5.00        |         |           |             |
| miR-423 | 0.617     | 4.39        | miR-150 | 0.750     | 4.66        | miR-142-3p| 0.938   | 15.89       |
| miR-382 | 0.680     | 3.76        | miR-30b | 0.758     | 4.41        | miR-142-5p| 0.938   | 14.89       |
| miR-134 | 0.656     | 3.57        | miR-103 | 0.664     | 4.32        | miR-33  | 0.922     | 11.50       |
| miR-181b| 0.703     | 3.54        | let-7g  | 0.664     | 3.94        | miR-211 | 0.758     | 6.22        |
| miR-200c| 0.625     | 2.99        | miR-497 | 0.773     | 3.82        | miR-193a| 0.633     | 4.21        |
| miR-668 | 0.648     | 2.96        | miR-25  | 0.688     | 3.71        | miR-19b | 0.586     | 3.59        |
| miR-149 | 0.586     | 2.90        | miR-331 | 0.664     | 3.59        | miR-19a | 0.602     | 3.53        |
| miR-379 | 0.672     | 2.90        | miR-143 | 0.734     | 3.36        | miR-199b| 0.539     | 3.52        |
| miR-598 | 0.656     | 2.55        | miR-199a| 0.695     | 3.18        | miR-27a | 0.406     | 3.35        |
| miR-615 | 0.430     | 2.54        | miR-33  | 0.656     | 2.99        | miR-199a| 0.414     | 3.22        |
| miR-214 | 0.500     | 2.50        | miR-335 | 0.680     | 2.89        | miR-143 | 0.453     | 3.18        |
| miR-539 | 0.656     | 2.40        | miR-21  | 0.680     | 2.84        | let-7g  | 0.563     | 2.79        |
| miR-652 | 0.453     | 2.35        | miR-15a | 0.648     | 2.76        | miR-22  | 0.500     | 2.62        |
| miR-17-5p| 0.578    | 2.31        | miR-148a| 0.641     | 2.31        | miR-30e-5p| 0.500  | 2.46        |
| miR-15b | 0.641     | 2.27        | let-7e  | 0.492     | 2.31        | miR-204 | 0.414     | 2.27        |
| miR-296 | 0.602     | 2.21        | miR-204 | 0.563     | 2.11        | miR-30b | 0.492     | 2.25        |
| miR-212 | 0.539     | 2.01        | miR-148b| 0.633     | 2.08        | let-7e  | 0.430     | 2.16        |

Significance (indicated by GPR score) and fold change were determined using Global Pattern Recognition v2.0.

### Table 4 Selected miRNAs and their predicted gene targets according to TargetScan 5.1 [34] and Meta Mir: Target Inference (MAMI) [35]

| Predicted Gene Target | miRNA            |
|-----------------------|------------------|
| CYP19                 | Let7 (a, c, d, e, g) |
| ESR1                  | miR-22           |
| FOG2 (ZPFM2)          | miR-200c, miR-142-5p, miR-302d |
| FOX2L2                | miR-302d         |
| FST                   | miR-410          |
| GATA4                 | miR-200c         |
| SOX9                  | miR-101          |
| WNT4                  | miR-211          |

expression of hundred's of transcripts [10-14, 40, 41]. In this study we used sheep as an animal model to study the regulation of gene expression and function by miRNAs during fetal gonadal differentiation. Currently, only 4 miRNA sequences have been reported for sheep [29], however, miRNA sequences are highly conserved across species. Therefore, in order to examine miRNA expression in sheep fetal gonads, 128 miRNAs were selected that were expressed (Cp < 37; [31]) and demonstrated a single melt peak in a preliminary profiling experiment (see Additional file 1, Table S1).

Real time PCR analysis revealed that XX and XY fetal gonads contain significantly different amounts of several
miRNAs. At GD42, when fetal ovaries and testes differentiate, miR-101 and several members of the Let7 family are preferentially expressed in testes and ovaries, respectively. One of the predicted targets of miR-101 is SOX9, which is expressed at higher relative amounts in sheep fetal testes compared to ovaries, suggesting miR-101 regulates SOX9 expression and/or function. Except for 137 bp (NCBI accession number AF012022), no SOX9 3’-UTR sequence is available for sheep. However, the 8 bp seed sequence of miR-101 predicted to target 3’UTR of SOX9 (~450 bp down stream; 5’-GUACUGU-3’) is conserved (TargetScan v5.1). It is unclear how miR-101 regulates SOX9 expression or function at the transcriptional and/or post-transcriptional level during fetal testicular development, but it is possible that miR-101 acts to fine-tune SOX9 expression [42].

In sheep, estrogen signalling plays a role during fetal ovarian development, and formation of primordial follicles (~ GD75) may be dependent on estrogen and ESR1 signalling pathways [27,39]. CYP19A1 (aromatase) is expressed during fetal gonadal differentiation in ovaries and estrogen receptors (ESRs) are expressed as early as GD30 [37,39]. Similarly, recent studies in cows indicate that estrogen receptors and aromatase are expressed during the early stages of fetal ovarian development [43], further suggesting that estrogen signalling maybe important in fetal ovarian development in non-rodent mammalian species. MiRNA expression analysis revealed that Let7 and miR-22 are preferentially expressed in GD75 ovaries and testes, respectively. Potential targets of Let7 and miR-22 are CYP19A1 and ESR1 (ERα), respectively [34]. CYP19A1 is expressed during fetal ovarian development, whereas Let7 expression level is up-regulated in GD75 compared to GD42 ovaries. This suggests a possible role for Let7 in regulating CYP19A1 expression and/or function during fetal ovarian development. Similarly, miR-22 expression is down-regulated possibly explaining increased ESR1 expression level during fetal ovarian development. Importantly, Pandey and Picard [44] recently demonstrated that miR-22 targets and reduces ESR1 mRNA in breast cancer cells in vitro.

Contrary, in testes CYP19A1 and ESR1 expression levels are significantly lower compared to ovaries at GD42 and GD75, whereas Let7e, Let7g, and miR-22 expression are significantly increased in GD75 compared to GD42 testes. To examine the cellular localization of miR-22, LNA-modified probes and in situ hybridization was performed on GD75 testicular tissue sections. Although real time PCR indicated increased miR-22 expression in GD75 testes, we were unable to detect miR-22 using in situ hybridization at this stage. One possibility is that in situ hybridization was not sensitive enough to detect miR-22 at this stage. Because GD90 tissue sections were available, we explored the possibility that miR-22 can be detected by in situ hybridization at later stages of fetal gonadal development. MiR-22 localization was evident in GD90 testicular sections, and appeared to be confined within the testicular cords, localizing to the cytoplasm in Sertoli cells. Based on these results, we postulate that Sertoli cell development requires suppression of estrogen signalling during sheep testicular development involving miR-22.
Table 5 Functional enrichment analysis (miRGator; [36]) of predicted miRNA targets to uncover selected pathways targeted by miRNAs that are significantly more highly expressed in GD42 (downregulated) and GD75 (upregulated) ovaries and testes

| Pathway                                                   | GD42 | GD75 | GD42 | GD75 |
|-----------------------------------------------------------|------|------|------|------|
| Cell Cycle                                                | 13   | 20   | 15   | 20   |
| Chromatin remodelling by hSWI/SNF ATP-dependent complexes | 3    | 1    | 2    | 5    |
| Cytokine Network                                           | 6    | 6    | 2    | 3    |
| EGF signaling                                              | 4    | 3    | 3    | 2    |
| Electron Transport chain                                   | 4    | 3    | 9    | 2    |
| G-protein signaling                                        | 8    | 16   | 6    | 15   |
| Gap junction proteins connexins                           | 2    | 1    | 3    | 2    |
| Glycolysis and gluconeogenesis                             | 4    | 3    | 1    | 1    |
| Growth hormone signaling                                   | 2    | 2    | 3    | 1    |
| Insulin signaling                                          | 3    | 4    | 4    | 2    |
| MAPKinasen signaling                                       | 10   | 13   | 9    | 12   |
| NGF pathway                                                | 3    | 2    | 3    |      |
| PDGF signaling pathway                                     | 4    | 3    | 3    |      |
| Phospholipids as signaling intermediates                   | 6    | 1    | 4    |      |
| Proteasome degradation                                     | 1    | 7    | 1    | 6    |
| Rac 1 cell motility signaling pathway                     | 1    | 3    | 3    | 2    |
| Regulation ER                                              | 5    | 2    | 1    |      |
| TGFbeta signaling pathway                                  | 2    | 1    | 3    |      |
| TNFR Signaling                                             | 3    | 3    |      |      |
| Transcription factor CREB and its extracellular signals    | 1    | 6    | 3    | 4    |
| WNT signaling                                              | 5    | 5    | 7    | 8    |

Figure 4 Tissue sections of GD75 and GD90 testes. A) GD75 testis section incubated with U6 control miRCURY LNA probe indicating positive ubiquitous staining in germ and somatic cells. B) GD75 testis section incubated with scrambled miRCURY LNA probe indicating absence of any staining. C) GD75 testis section incubated with miR-22 DIG labeled probe indicating no positive staining. D) GD90 testis section incubated with miR-22 DIG labeled probe. Arrows point to unstained gonocytes within testicular cords; arrowheads point to positive staining within testicular cords between gonocytes. All images were captured using a 40x objective, and the bar is 20 μm.
MiRNA binding to 3'UTR target sequences occurs through complementary binding of the miRNA “seed” sequence (miRNA nucleotides 2-7). This partially explains how miRNAs potentially can regulate expression and/or function of more than one mRNA or whole signalling networks and complex biological processes. Using a bioinformatics approach we explored the possibility that the different biological processes or pathways underlying fetal ovarian and testicular development are regulated by miRNAs. This analysis revealed that estrogen signalling (regulation of ESR and modulation ESR activity) is targeted less frequently by miRNAs as fetal ovarian development proceeds. For example, during ovarian development (comparing GD42 to GD75 ovaries) the number of miRNAs targeting ESR function decreases, suggesting that estrogen function is necessary for proper ovarian and follicle development at GD75.

Another example of signalling pathways targeted by miRNAs in fetal sheep gonads is G-protein signalling (includes G-protein signalling, G13 signalling pathway, signalling pathway from G protein families). Both in fetal ovaries and testes, the number of miRNAs targeting G-protein signalling increases during gonadal development, suggesting that miRNAs potentially are involved in regulating G-protein signalling mediated functions during the latter stages of fetal gonadal development. G-protein signalling encompassing many different protein families and different cell signalling pathways is known to regulate reproduction at the hypothalamic, pituitary, and gonadal level [45]. During ovarian development G-protein-coupled receptor (GPR) 30, which has high affinity for estradiol, is involved in primordial follicle formation in hamster ovaries [46]. A predicted binding site for miR-130a (significant more highly expressed in GD75 compared to GD42 ovaries) is present within the 3'UTR of (human) GPR30.

Although this bioinformatics approach suggests the potential regulation of signaling pathways (or other biological processes) by miRNAs, future studies demonstrating direct interaction of selected miRNAs with their target sequences will need to be provided.

Conclusions

In summary, data presented in this study indicate that miRNAs are present during fetal gonadal development and differentiation in sheep. Based on the correlations observed between miRNA expression and their predicted targets, we postulate that miRNAs are important regulators of gene expression and function during fetal gonadal development. Similar to what has been proposed for the cow [47], the sheep is a useful model to study gonadal development and differentiation in mammals. For example, estrogen signalling appears to play a role in human, cow, and sheep fetal ovarian development [38,39,43,48]. Based on our results, we further suggest that Let7 and mir-22 regulate estrogen signalling during fetal sheep gonadal development, and mir-22 may be necessary for suppressing the estrogen-signalling pathway during fetal testicular development. Finally, bioinformatic analysis revealed several pathways that are possibly regulated by miRNAs during fetal ovarian as well as testicular development.

Additional material

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Acknowledgements

The authors are especially indebted to Travis Antes for his help constructing the miRNA profiling plates. We are grateful to Scott Purcell for his help with sheep husbandry. Finally, we thank Brittany Fromme, Vanessa Enrizquez, Samantha Roth, and Carol Moeller for their help in the lab. This project was supported by CSU-CVMBS Department of Biomedical Sciences and College Research Council (GJB).

Author details

1Animal Reproduction and Biotechnology Laboratory, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA.
2AgResearch Limited, Invermay, Research Centre Mosgiel, New Zealand.

Authors’ contributions

KJT and JCS performed the experiments, and DNRV helped with the immunohistochemistry analyses. PS, RVA, DNRV, and QAW provided reagents and materials. GJB designed the experiments, supervised the study, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 6 October 2010 Accepted: 11 January 2011
Published: 11 January 2011
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