Characterization of microRNA, mRNA, and Protein Expression in Large and Cystic Bovine Follicles

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ABSTRACT

Cystic ovarian disease (COD) is a major contributor to infertility in cattle, with 30% of cows developing ovarian cysts during a given lactation and becoming anovulatory. The cause of COD has remained elusive and is thought to be multifactorial, with angiogenic and genetic contributions proposed. There is an increasing body of work suggesting that microRNAs (miRNAs) may be involved in a number of ovarian-based reproductive disorders. Changes in miRNA expression can affect a multitude of genes and may be important regulators of the dynamic processes involved in each ovarian cycle. We hypothesized that miRNAs are integral to the regulation of critical ovarian processes such as angiogenesis and follicular development and that altered miRNA expression contributes to the onset and progression of ovarian dysfunction and reproductive disorders. Eight miRNAs (miR-15a, -18a, -20a, -21, -29a, -126, -132, Let7a) known to target vascular endothelial growth factor (VEGF), thrombospondin-1 (TSP-1), and play a role in both angiogenesis and folliculogenesis, were selected for analysis. miRNA, mRNA, and protein expression were analyzed in large and cystic bovine follicles using qPCR, western blot, and immunohistochemistry. miR-29a was found to be upregulated in cystic follicles, whereas miR-132 expression was downregulated. VEGF expression increased in cystic follicles at both the transcript and protein level, while no significant differences in TSP-1 expression was observed. Our findings suggest that miR-29a and miR-132 may play an important role in maintaining the balance between VEGF and TSP-1 expression in the ovary, and if disrupted, could potentiate ovarian-based reproductive disorders such as PCOS and cystic ovarian disorder.

INTRODUCTION

Reproductive insufficiency results in a significant economic burden to the cattle industry. Cystic ovarian disease (COD), is characterized by the development of large, anovulatory follicles, and is a major
contributor to infertility in cattle [1]. Due to the failure to ovulate and disruption of the ovarian cycle, ovarian cysts result in reproductive insufficiency. Cysts are defined as follicle-like structures on the surface of the ovary that have persisted for more than 10 days, have a diameter greater than 2 cm, and occur in the absence of luteal tissue [1]. As cows do not develop cysts with every lactation, or during every ovarian cycle, it is thought that a change in gene expression may be an important mediator and contributor to this disease. MicroRNAs (miRNAs), small non-coding RNAs that regulate gene expression, have been shown to be central regulators of various cellular processes throughout the ovarian cycle and increasing evidence suggests that miRNAs may be involved in a number of ovarian-based reproductive disorders [2,3].

The accumulation of excess follicular fluid (FF) in a cystic follicle is also accompanied by a highly developed vascular network within the theca interna [4]. This increased density and complexity of vasculature within cystic follicles is believed to play a critical role in the accumulation of FF, thus potentiating COD [5]. While the exact cause of COD is still unknown, it is thought to have multiple contributing factors including environment, genetics, endocrine regulation, and angiogenesis [1]. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, involves a variety of cellular processes and molecular changes. Angiogenesis is essential for organogenesis, cellular proliferation, and cellular differentiation during embryonic development [6]. In adult tissues, angiogenesis can be classified as both pathological and physiological. Pathological angiogenesis involves uncontrolled proliferation of endothelial cells, whereas physiological angiogenesis is a highly regulated process that is observed in wound healing and throughout the female reproductive system including the ovary and endometrium [7].

Ovarian physiology is dependent on the establishment and continual remodelling of vasculature in order to provide the follicle and corpus luteum with an adequate supply of nutrients, oxygen, and
hormonal support, as well as facilitate the systemic distribution of steroid hormones produced within the ovary. If angiogenesis throughout the ovarian cycle is disrupted in any way, this can lead to attenuation of follicle growth, interference of ovulation, and affect the development and function of the follicle and corpus luteum [8]. Primordial and primary follicles receive sufficient nutrients and oxygen via passive diffusion from the ovarian stromal vasculature, however for a follicle to grow beyond the primary stage, formation of an individual capillary network is required. This capillary network is initially very thin, roughly structured, and confined to a single layer in the thecal cells, with the granulosa cell layers remaining avascular throughout folliculogenesis [8,9]. There is a large increase in not only total vasculature but also vascular density during pre-antral follicle growth, where approximately 40% of all proliferating cells in the theca are of endothelial origin [10]. During early follicle growth, there exists a positive correlation between the degree of granulosa cell proliferation and vascular area, suggesting that pre-antral follicle selection may be based on vascular supply [10].

There are two key factors involved in the regulation of ovarian angiogenesis; vascular endothelial growth factor (VEGF), a pro-angiogenic factor, and thrombospondin-1 (TSP-1), an anti-angiogenic factor [11,12]. VEGF mRNA has been detected in both the granulosa and thecal cell layers of secondary follicles in cattle [13], with expression increasing throughout follicle development, whereas translated VEGF protein is predominately localized in the granulosa cells [11]. Originally, it was thought that VEGF functions were restricted to promoting blood vessel development, however, Greenaway et al [14] identified the expression of the VEGF receptor, VEGFR2, in granulosa cells and determined that VEGF signaling was cytoprotective for these cells and integral to the regulation of follicular recruitment, progression, and function. Additionally, the inhibition of VEGF signalling results in the disrupted ovulation, inhibition of vascularization of the corpus luteum, and subsequent inhibition of a post-ovulatory rise in progesterone [15,16]. Anti-angiogenic factors such as angiotatin, TSP-1, and TSP-2,
inhibit angiogenesis by stimulating endothelial cell apoptosis and by associating with the extracellular matrix in order to prevent endothelial cell migration [17,18].

Previous studies have also uncovered an inverse and reciprocal relationship between VEGF and TSP-1, where VEGF protein expression in granulosa cells increases throughout follicle development while TSP-1 decreases (Figure 1) [11]. Protein expression of TSP-1 and its receptor, CD36, have been shown to be present in the early antral phase of folliculogenesis and are localized primarily to the granulosa cell layer [19]. TSP-1 has also been found to have a direct inhibitory effect on the expression of VEGF during follicle development. TSP-1 binds VEGF and internalizes it via LRP-1, a low-density lipoprotein receptor-related protein, resulting in the degradation of VEGF [12]. In vivo murine studies have also shown that inhibition of TSP-1 leads to subfertility, with ovarian hypervasculization and altered ovarian morphology, suggesting that TSP-1 is an essential regulator of VEGF, angiogenesis, and follicle development in the ovary [12]. All of this taken together, it is evident that angiogenic factors such as VEGF and TSP-1 are playing an important regulatory role throughout the ovarian cycle and when dysregulated may result in abnormal ovarian angiogenesis and subsequent ovarian disorders.

It has been suggested that the cyclicity of ovarian angiogenesis and folliculogenesis may be mediated via changes in gene expression. miRNAs are single-stranded, short non-coding RNAs, 19-24 nucleotides in length that play important gene regulatory roles in plants and animals by binding to target mRNAs of protein-coding genes, resulting in post-transcriptional repression [20]. miRNAs are the most abundant small non-coding RNAs in ovarian tissue and are present in a great diversity due to the heterogeneous nature of the ovary, containing both somatic and germ cells [21]. By better understanding the regulatory abilities of miRNAs in the ovary, this can result in the potential for miRNA diagnostics and therapeutics, which could lead to substantial benefits for reproductive medicine and health.
Gene expression analyses using cloning or sequencing, have shown that miRNAs are widely expressed in the mammalian ovary, with estimates ranging from 373 to 679 different miRNAs being present in whole ovarian tissues of various species [22]. Hossain et al [23] determined that the most abundant miRNAs in bovine ovarian tissue were let-7a, let-7b, let-7c, miR-21, miR-23b, miR-24, miR-27a, miR-126, miR-143, miR-652. In addition, there appears to be similarities between species with miR-21, let-7a, and miR-126 also being highly abundant in human and murine ovarian tissue [24].

While limited research has been conducted on the role of miRNAs in cystic ovarian disease in cattle, studies have begun to analyze miRNA expression in women with polycystic ovarian syndrome (PCOS), a common endocrine disorder affecting 5-7% of reproductive age women [25]. Analysis of FF from patients with PCOS compared to control healthy follicles revealed a decrease in miR-132 and miR-320 expression and an increase in miR-9, -18b, -32, -34c, and -135a expression [26,27], suggesting that these miRNAs may be playing a role in the development and progression of the disease.

It has been well established that miRNAs play an important role in gene regulation within the ovary and that VEGF and TSP-1 are essential factor for ovarian physiology. What has yet to be uncovered is how miRNAs play a functional role in governing folliculogenesis and angiogenesis in the ovary and how their dysregulation could potentiate ovarian dysfunction such as COD.

This study began by selecting eight miRNAs of interest, miR-15a, -18a, -20a, -21, -29a, -126, -132, and let7a, based on their predicted ability to target VEGF and TSP-1, and their role in angiogenesis and folliculogenesis. The goal of this study was to characterize the transcript and protein expression profiles of key angiogenic factors in large and cystic bovine follicles as well as determine which miRNAs
may be playing a key regulatory role in ovarian angiogenesis and folliculogenesis. The results of this study could have implications on future research and therapies for ovarian-based reproductive disorders.

MATERIALS AND METHODS

Granulosa Cell, Follicular Fluid, and Folicle Collection

Bovine ovaries were collected from a local slaughter house (Cargill, Guelph, ON). Follicles were selected based on size with large follicles measuring between 10-15mm in diameter and cystic follicles greater than 20mm in diameter (Supplementary Figure 1).

For follicular fluid and granulosa cell collection, follicles were aspirated using an 18G vacutainer needle into collection tubes. The follicular fluid and cells were then transferred to a 15mL tube and centrifuged at 500rpm for 5 minutes in order to pellet the cells. The follicular fluid supernatant was then removed, and flash frozen in 1.6mL tubes and stored at -80°C until future use. The pelleted granulosa cells were then resuspended in 1x PBS, transferred into a 1.6mL tube and centrifuged at 500rpm for 5 minutes. The PBS supernatant was then removed, and the granulosa cell pellet was flash frozen in liquid nitrogen and stored in -80°C until future use.

For immunohistochemistry analyses, large and cystic follicles were categorized based on size and then carefully dissected out from the ovarian tissue. The intact follicle was placed in 10% buffered formalin for 96 hours followed by immersion in 70% ethanol until further tissue processing. Tissues were then de-hydrated following standard procedures, embedded in paraffin wax, and sectioned with a rotary microtome.
RNA Extraction

RNA extraction was conducted on the large and cystic granulosa cells using the Norgen Biotek Corporation RNA/DNA/Protein Plus Micro Extraction Kit (Norgen Biotec, Thorold, ON). 300µL of lysis buffer SKP was added to each granulosa cell sample and vortexed for 30 seconds. In order to homogenize the cells further, the lysate was passed 5-10 times through a 25-gauge needle attached syringe until fully homogenized. The lysate was then centrifuged at 8000rpm for 2 minutes in order to pellet cellular debris. Up to 600µL of the lysate supernatant was then transferred into a gDNA Purification Micro Column with collection tube and centrifuged at 8000rpm for 1 minute. Flowthrough was retained and placed on ice for RNA purification. For every 100µL of RNA flowthrough from gDNA extraction, 180µL of 96-100% ethanol was added and mixed by vortexing. Up to 600µL of RNA flowthrough and ethanol was transferred into an RNA/Protein Purification Micro Column with collection tube and centrifuged at 6000rpm for 1 minute. Wash of RNA/Protein purification column was conducted three times by adding 400µL of Wash Solution A to the column and centrifuging at 6000rpm for 1 minute. Following the final wash, the column was spun at 14,000rpm for 2 minutes in order to thoroughly dry the resin. RNA/Protein purification column was then transferred to a 1.7mL elution tube. 50µL of Elution Solution A was added and centrifuged at 2000rpm for 2 minutes followed by 14,000rpm for 1 minute. RNA/Protein column was retained for protein purification procedure. RNA concentration was the quantified using Nanodrop 2000 and stored at -80°C for future use.

cDNA Synthesis

MicroRNA

Following RNA extraction, microRNA cDNA synthesis using reverse transcription was performed using qScript microRNA cDNA synthesis kit (Quanta Biosciences, Beverly, MA). A two-step
reaction was performed. In the first step, 2µL of 5x Poly(A) Tailing Buffer, 1µL of Poly(A) Polymerase, and a variable amount of RNA and nuclease free water (up to 7µL and 1µg total RNA) were combined per reaction and incubated in a Bio-Rad T100 Thermal Cycler at 37°C for 60 minutes followed by 70°C for 5 minutes. In the second step, 9µL of microRNA cDNA Reaction Mix and 1µL qScript Reverse Transcriptase was added to the reaction product from step 1 and incubated at 42°C for 20 minutes followed by 85°C for 5 minutes. In addition, a No-Reverse-Transcriptase (NRT) control was conducted, where 1µL of qScript Reverse Transcriptase in the second step was replaced with 1µL of nuclease-free water in order to control for potential genomic DNA contamination. The resulting cDNA product was then stored in -20°C until future use.

mRNA

Following RNA extraction, mRNA cDNA synthesis using reverse transcription was performed using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA). 4µL of 5x qScript cDNA SuperMix was combined with a variable amount of RNA and RNase/DNase-free water (up to 16µL and 1µg of RNA) per reaction and incubated in a Bio-Rad T100 Thermal Cycler at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. The resulting cDNA product was then stored in -20°C until future use.

Reference Gene Selection

Candidate reference genes for miRNA qPCR analysis were selected from previous literature on miRNA reference gene analyses [28,29]. A total of 6 candidates were chosen: miR16-5p, miR106a-5p, Let-7a-5p, miR93-5p, miR191-5p, and U6. In addition, candidate reference genes were selected from previous literature on Bos Taurus mRNA reference gene analyses [30]. A total of 8 candidates were chosen: ACTB, GAPDH, UBA52, RPS18, RPL19, HPRT1, H3F3B, and YWHAZ. qPCR was performed
as described below and reference gene stability was assessed using three different reference gene software systems: geNorm, Normfinder, and Bestkeeper.

Quantitative PCR (qPCR)

MicroRNA

MicroRNA reverse transcribed cDNA was analyzed using quantitative real-time polymerase chain reaction (qPCR) to assess expression profiles of miRNAs of interest. Each analysis was performed on three biological replicates each consisting of three technical replicates. MicroRNA targets of interest included miR15a-5p, miR-18a-5p, miR20a-5p, miR21-5p, miR-29a-3p, miR126-5p, miR132-3p, and Let-7a-5p, with miR16-5p and miR106a-5p used as reference genes (Quanta Biosciences, Beverly, MA).

All primer efficiencies were calculated by performing a standard curve using a 2:1 dilution of a pooled reaction sample from 50ng/µL to 0.78ng/µL. Only primer efficiencies between 90-110% were accepted.

MiRNA primer sequences and efficiencies can be found in Supplementary Table 1.

For miRNA expression analysis, a master mix was created using 5µL of PerfeCTa SYBR Green Supermix (5x), 0.2µL PerfeCTa Universal PCR Primer (10µM), 0.2µL Quanta Biosciences miRNA specific primer (10µM), and 2.6µL of RNase/DNase free water in each reaction well. The master mix was calculated based on the number of wells required for analysis. 8µL of the master mix was then added to the wells followed by 2µL of diluted cDNA (1.5ng/µL per reaction); resulting in a reaction total of 10µL per well. Signal detection was acquired using a two-step protocol: 95°C for 2 minutes, followed by 44 repeated cycles of 94°C for 30 seconds and 60°C for 30 seconds, ending with a melt curve acquisition from 65-95°C. The Bio-Rad CFX96 Real-Time PCR system was used for the qPCR analysis.

Relative quantity of miRNA targets was log-transformed in BioRad CFX Manager 3.0 and normalized to the relative quantity of the reference genes miR16-5p and miR106a-5p across all samples.
mRNA targets of interest included VEGFA, and TSP-1, with RPS18 and RPL19 used as reference genes (Lab Services, University of Guelph). All mRNA primers were either designed using Primer-BLAST NCBI or sequences were selected from previous literature. Primer efficiencies were calculated by performing a standard curve using a 2:1 dilution of a pooled reaction sample from 50ng/µL to 0.78ng/µL. Only primer efficiencies between 90-110% were accepted. mRNA primer sequences for Bos Taurus can be found in Supplementary Table 2.

mRNA expression profiles were performed using the Bio-Rad CFX96 Real-Time PCR system. A master mix was created using 5µL SensiFAST SYBR No-ROX (FroggaBio, Scientific Solutions, Toronto, ON), 1µL forward primer (1.25µM), 1µL reverse primer (1.25µM), and 2µL RNase/DNase free water. The master mix was calculated based on the number of wells required for analysis. 9µL of the master mix was then added to the wells followed by 1µL of diluted cDNA (5ng/µL per reaction); resulting in a reaction total of 10µL per well. Signal detection was acquired using a two-step protocol: 95°C for 30 seconds, followed by 39 repeated cycles of 95°C for 5 seconds and 60°C for 5 seconds, ending with a melt curve acquisition from 65-95°C. Relative quantity of gene targets was log-transformed in BioRad CFX Manager 3.0 and normalized to the relative quantity of the reference genes RPS18 and RPL19 across all samples.

Protein Extraction

Protein was extracted from granulosa cells previously collected and stored at -80 °C. Cell in 1.6mL tubes were lysed over ice in a radioimmunoprecipitation assay (RIPA) buffer supplemented with fresh protease inhibitors for 30 minutes. Buffer samples containing lysate were spun at 14,000rpm for 10 minutes at 4°C. The aqueous phase of each sample was aliquoted and stored at -80°C. Protein
The concentration of the cell lysate was determined using a DC Bio-Rad Protein Quantification Kit (Bio-Rad, Mississauga, ON).

**Western Blot Analysis**

A wet/tank blotting system (Bio-Rad Mini Trans-Blot Cell; Bio-Rad, Mississauga, ON) was used to prepare western blot. Equal volume samples (20µg; n=5/group) were prepared in 3x reducing buffer containing dithiothreitol (DTT; Life Technologies Inc., Burlington, ON) denaturing agent and heated to 90°C for 5 minutes. Denatured samples were loaded into separating gels and subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) at 120 volts (V) in room temperature running buffer. Precision Plus Protein Dual Colour Standards (Bio-Rad, Mississauga, ON) were used as molecular weight markers. When the desired separation was achieved, gels were equilibrated in transfer buffer for 15 minutes and transferred onto a nitrocellulose membrane (Ammersham Hybond ECL; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) at 100V in ice-cold transfer buffer for 90 to 120 minutes.

Following the transfer, membranes were washed in Tris-buffered saline with Tween 20 (TBST) and blocked with skim milk in TBST. After blocking for 1 hour, membranes were incubated in the appropriate primary antibody solution: VEGF (1:500, Santa Cruz, sc512) and β-Actin (1:3000, Cell Signalling, cs4967), overnight at 4°C. The following day, membranes were washed with TBST and incubated in IgG, HRP-linked anti-rabbit secondary antibody (1:2000 for VEGF and 1:5000 for β-Actin) for 1 hour at room temperature. Membranes were washed again in TBST and antibody binding was detected by enhanced chemiluminescence (ECL) (Luminata Forte Western HRP Substrate, Millipore Ltd., Etobicoke, ON). Signals were detected and imaged using a Bio-Rad ChemiDoc XRS+ system and relative densitometry was determined using Bio-Rad Image Lab software.
Immunohistochemistry on Paraffin-Embedded Follicle Sections

Immunohistochemistry was performed by citrate buffer antigen retrieval. Slides with paraffin-embedded tissue sections (5µM) were deparaffinized in xylene (Fisher Scientific, Whitby, ON) and rehydrated by immersion in decreasing concentrations of ethanol. Following rehydration, the slides were washed in PBS) and endogenous peroxidase activity was blocked in 1% hydrogen peroxide (H₂O₂) in PBS. Citrate antigen retrieval was performed on the tissue sections, followed by blocking 5% bovine serum albumin (BSA)/0.1% sodium azide. After blocking, slides were incubated in a solution of antibody diluting fluid with one of the following primary antibody solutions: VEGF (1:500, Abcam, ab46154), TSP-1 (1:50, Santa Cruz, sc59887), IGF1 (1:50, Santa Cruz, sc9013), IGF2 (1:50, Santa Cruz, sc5622), VEGFR2 (1:600, Cell Signalling, cs2479), and CD36 (1:1000, NovusBio, NB400-144), overnight at 4°C in a humidity chamber. Tissues were then washed in PBS and incubated in anti-rabbit biotinylated secondary antibody (1:100 dilution) or anti-mouse (1:100 dilution), for 2 hours at room temperature. Tissues were then incubated for 1 hour at room temperature with a 1:50 dilution of avidin and biotinylated horseradish peroxidase (ExtrAvidin-Peroxidase; Sigma-Aldrich Canada Ltd., Oakville, ON) and antibody immunolocalization was detected by incubation with fresh 3,3’-diaminobenzidine (DAB tablets, 10mg; Sigma-Aldrich Canada Ltd., Oakville, ON) dissolved in 5mL of reverse osmosis water (RO H₂O). The DAB colour reaction time varied depending on the antibody used (Supplementary Table 3); however, incubation time was kept constant for both large and cystic follicles. Slides were then counterstained with Carazzi’s Hematoxylin, and mounted on glass coverslips.

Image Analysis

Follicle sections stained by immunohistochemistry were imaged by bright-field microscopy using a Nikon Eclipse E600 microscope with a QImaging camera. Follicles were imaged at 200x total magnification and analyzed for cytoplasmic factors VEGFA, VEGFR2, TSP-1, CD36, IGF1, and IGF2.
For these factors, the percentage of positively stained tissue was determined using Aperio ImageScope software (Aperio, Vista, CA). The “Positive Pixel Count v9” algorithm was optimized and used for each image setting. Selective annotations were made as to only include the granulosa and thecal cell layers of the follicles for analysis. Percent positivity was calculated as a portion of total positive pixels (the sum of the weak positive, positive, and strong positive pixels).

**Statistical Analysis**

GraphPad Prism 6 software was used for statistical analysis and graph preparation. Fourteen biological replicates were used in each experimental group for miRNA and mRNA expression analyses and five biological replicates for immunohistochemistry analysis. Data was analyzed using an unpaired t-test with Welch’s correction. Differences among groups were considered significant if $p<0.05$.

**RESULTS**

**Optimal microRNA Reference Gene Selection for Quantitative-PCR (qPCR) Analyses**

In order to identify the most stable reference genes for miRNA qPCR analyses, candidate reference genes were selected from previous literature [28,29]. A total of six candidate reference genes (U6, let7a-5p, miR16-5p, miR106-5p, miR93-5p, and miR191-5p) were tested across twelve bovine granulosa cell samples. To determine the most steadily expressed miRNAs, three different software programs were used: geNorm, Normfinder, and Bestkeeper.

GeNorm provides a ranking of the tested genes based on their stability value (M value), which is considered to be stable if below 0.15. In addition, geNorm determines mean pairwise variation (V value)
between a candidate gene and all other tested candidates, allowing for the selection of a combination of reference genes, again considered to be stable if below 0.15. MiR16-5p and miR106a-5p were found to have the most stable M value at 0.309 and 0.331, respectively (Figure 2B), and together were found to have a V value of 0.113 (Figure 2C).

Normfinder provides a measure of the stability of genes’ expression (stability value) as well as estimates any bias in gene expression between groups using a two-way ANOVA. MiR93-5p was shown to have the lowest stability value at 0.129 (Figure 2D).

Bestkeeper calculates the stability measure for each candidate gene and then ranks their stability based on standard error (SE), coefficient of correlation (R) and p-value. Reference genes with the lowest SE, R value closest to 1.0, and lowest p-value are considered to be the most stable. MiR93-5p, miR16-5p, and miR-106a-5p were shown to be the most stable via Bestkeeper analyses (Figure 2F).

Based on the data collected from all three reference gene software programs, miR16-5p and miR106a-5p were chosen to be the most stable reference genes as they demonstrated consistent stability throughout all analyses and demonstrated stability when averaged together in pairwise variation. MiR16-5p and miR106a-5p were subsequently used as normalizing genes in all miRNA qPCR expression analyses conducted.

**Optimal Bos Taurus mRNA Reference Gene Selection for Quantitative-PCR (qPCR) Analyses**

In order to identify the most stable reference genes for mRNA qPCR analyses, candidate reference genes were selected from previous literature [30]. A total of eight candidate reference genes (YWHAZ, H3F3B, ACTB, GAPDH, UBA52, RPL19, RPS18, and HRPT1) were tested across twelve bovine granulosa cell samples from both large and cystic follicles. To determine the most steadily expressed mRNAs, three different software programs were used: geNorm, Normfinder, and Bestkeeper.
GeNorm analyses found RPS18 and RPL19 to have the most stable M values of 0.167 and 0.159, respectively (Figure 3B). In addition, the pairwise variation (V value) for RPS18 and RPL19 was 0.071 (Figure 3C), well below the minimum value of 0.15. Normfinder determined UBA52 to be the most stable with a stability value of 0.061 (Figure 3D). Finally, Bestkeeper analysis indicated UBA52 to be the most stable as it had the lowest standard error, highest R value and lowest p-value (Figure 3F). Ultimately, RPS18 and RPL19 were chosen as the most stable reference genes in bovine granulosa cells as they demonstrated stability across all three software program analyses, a high degree of correlation between M value and stability value (Figure 3E), and a stable pairwise variation (Figure 3C).

MicroRNA Expression in Granulosa Cells of Large and Cystic Bovine Follicles

Large and cystic bovine follicles were aspirated to collect follicular fluid and granulosa cells for expression analyses. Expression of eight miRNAs, previously shown to be involved in angiogenesis and folliculogenesis as well as have the ability to target VEGF and TSP-1, were analyzed using qPCR. miRNA expression was normalized to miR16-5p and miR106a-5p. Expression of miR29a-3p was found to be increased in cystic follicles as compared to large (p<0.05; Figure 4E), while expression of miR132-3p was significantly reduced in cystic follicles as compared to large (p<0.05; Figure 4G). No other significant changes in miRNA expression was found between large and cystic follicles.

mRNA Expression in Granulosa Cells of Large and Cystic Bovine Follicles

Granulosa cells aspirated from large and cystic ovarian follicles were collected and total RNA was extracted for qPCR analysis. VEGFA and TSP-1 expression was normalized to RPS18 and RPL19, as determined through reference gene software analyses. VEGFA was found to be increased in cystic
follies as compared to large follicles (p<0.05; Figure 5C), however no significant difference in TSP-1 expression was observed (Figure 5D).

Protein Expression in Granulosa and Theca Cells of Large and Cystic Bovine Follicles

Western blot analysis was used to evaluate expression of VEGF in granulosa cells collected from follicular fluid aspirates of large and cystic bovine ovarian follicles. Levels of VEGF were found to be significantly increased in cystic follicles as compared to large (p<0.05; Figure 5A, B).

Protein expression was also analyzed using immunohistochemistry in order to evaluate expression of additional vascular factors VEGF, VEGFR2, TSP-1, and CD36, as well as mitogenic factors IGF1 and IGF2 in the granulosa and theca cell layers of large and cystic bovine ovarian follicles. The pro-angiogenic factor, VEGF, was expressed at high levels in both the large and cystic follicles, with no statistically significant differences observed (Figure 6). In contrast, the receptor of VEGF, VEGFR2, was found to be enhanced in cystic follicles as compared to large (p<0.05; Figure 6). Interestingly, the anti-angiogenic factor TSP-1 (p<0.01; Figure 6), along with its receptor, CD36 (p<0.001; Figure 6), were both found to be upregulated in cystic follicles as compared to large follicles. Finally, mitogenic factors IGF1 and IGF2, both involved in cellular proliferation and inhibition of apoptosis, had high levels of expression in both large and cystic follicles, however no significant differences were observed between groups (Figure 6).

DISCUSSION

This study has characterized the expression of key angiogenic factors in bovine folliculogenesis as well as explored which miRNAs play a regulatory role in ovarian folliculogenesis and angiogenesis. Our study began by selecting eight miRNAs, let-7a, miR-15a, -18a, -20a, -21, -29a, -126, and -132, that
have been shown in the literature to have a regulatory role in ovarian follicle development and angiogenesis as well as target either VEGF or TSP-1 [24]. Our goal was to determine which miRNAs play an important compensatory role in maintaining the balance between VEGF and TSP-1 throughout the ovarian cycle, as well as to characterize the expression profile of large, antral follicles compared to cystic, diseased follicles, in order to gain a better understanding of how miRNAs, angiogenic factors, and mitogenic factors potentially mediate cystic ovarian disease (COD).

Ovarian cysts are persistent, anovulatory follicular structures that occur in several mammalian species including bovine and humans. Cystic ovarian disease (COD) is a common ovarian-based reproductive disorder that causes infertility and significant economic burden to dairy and beef industries. The cause of COD is still unknown, however experts have suggested that it may comprise a variety of factors including environment, genetics, endocrine and angiogenic factors [1]. As cows do not develop cysts with every lactation, or during every ovarian cycle, a change in gene expression has been suggested as a possible mediator and contributor to the disease [3]. As miRNAs are key regulators of gene expression, they may be integral to the gene regulatory mechanisms involved in COD and thereby regulate key angiogenic factors such as VEGF and TSP-1 that are essential for follicle development, blood vessel growth, and ovarian function [11,12]. In addition, this study also aimed to characterize the mRNA and protein expression patterns of several vascular and mitogenic growth factors along with their receptors in cystic follicles [1,31]. Characterizing the expression patterns in cystic follicles can lead to a better understanding of the role that miRNAs, angiogenic factors, and mitogenic factors potentially play in COD.

MicroRNA target prediction tools were used to determine potential mRNA transcript targets for each miRNA. MiR-29a and miR-132 have over 600 and 350 predicted targets, respectively, thereby
having the ability to regulate a wide variety of genes. Within the context of this study, miR-29a has been shown to target VEGF, TSP-2, and IGF1 [32]. Previous studies have shown miR-29a to be highly expressed in the ovarian granulosa cells and during the early phase of granulosa cell luteinization, but interestingly not expressed in mature corpus luteum (CL) [23]. In addition, miR-29a has been shown to induce apoptosis, play an essential role in extracellular matrix remodeling during the follicular phase, and is significantly decreased in serum of patients with PCOS [33–35]. Researchers have also suggested that miR-29a plays a regulatory role in growth and differentiation of cumulus cells, endocrine regulation of theca cells, and early luteinization within the ovary [36].

In cystic follicles, our study, as well as others, have demonstrated an increase in VEGF expression, therefore one would hypothesize that miRNAs known to target VEGF would potentially be downregulated, thereby reducing the inhibitory effect on VEGF. Interestingly, this study observed a significant increase in miR-29a along with a significant increase in VEGF expression. This upregulation of miR-29a may be present due to the enhanced VEGF expression in the cystic follicles, thereby acting as a compensatory mechanism in order to try to decrease the proliferation of endothelial cells. In addition, miR-29a may be targeting other factors involved in regulating blood vessel formation or folliculogenesis, such as TSP-2 or IGF1. During follicle growth, the IGF family works in synergy with gonadotropins FSH and LH, in order to regulate proliferation and differentiation of granulosa and theca cells [23]. IGF1 has also been shown to initiate follicle growth, suppress granulosa cell apoptosis, and has been suggested as a potential mediator of accelerated preantral follicle growth in PCOS patients [31,37]. This suggests that the enhanced expression of miR-29a could be acting to downregulate IGF1 in order to reduce proliferation and induce apoptosis of granulosa cells in these cystic follicles, although this study does not present mRNA transcript or protein expression levels of IGF1 to further confirm this theory. Overall, the regulatory role that miR-29a has in the context of follicle growth has not been fully elucidated. This study
shows that miR-29a is upregulated in cystic follicles, therefore may be playing a role in enhancing the
diseased state or working to downregulate proliferative and pro-angiogenic factors.

Previous studies of miRNA expression in follicular fluid has shown a decrease in miR-132
expression in cystic follicles of PCOS patients compared to healthy follicles [26,27]. Our results also
show a significant decrease in miR-132 in cystic bovine follicles as compared to healthy follicles, which
suggests that a lack of miR-132 could play a role in the development or progression of cystic follicles in
mammalian species. miR-132 has also been shown to increase proliferation, regulate steroidogenesis,
increase estradiol production, and inhibit downstream components of the VEGF signalling cascade [38–
41]. Therefore, the down regulation of miR-132 found in cystic follicles may result in decreased GC
proliferation, decreased estradiol production, and increased VEGF signalling. miR-132 has also been
shown to promote angiogenesis, where the use of miR-132 inhibitors results in anti-angiogenic effects
[42]. The regulatory effect that miR-132 has within the ovary and specifically in cystic follicles is of
great interest. Based on this study and the current literature, miR-132 appears to be a great candidate for
future functional studies as it is evident that it plays an important role in follicle and blood vessel
development.

Previous work in our lab has uncovered an inverse relationship between the expression of VEGF
and TSP-1 throughout folliculogenesis. Using bovine follicles ranging in size small (<5mm), medium
(5-10mm), and large (10-15mm), protein expression levels of VEGF were shown to increase as the
follicle matured, while TSP-1 expression opposed this trend, decreasing in expression as the follicle
matured [11]. This inverse relationship coincides with the development of vasculature within the follicle
with the vascular density increases as the follicle matures, suggesting a greater amount of pro-angiogenic
stimulation. Within the context of COD, this study aimed to characterize the expression of VEGF and
TSP-1 in cystic follicles in order to determine if this inverse trend continues in pathological conditions.
In order to do this, RNA was extracted from granulosa cells of large and cystic bovine follicles and qPCR

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was conducted. As hypothesized, VEGF mRNA expression was significantly upregulated in cystic follicles as compared to large, while TSP-1 expression did not differ, although it was expressed at very low levels. In addition to mRNA expression, protein was also collected from granulosa cells of large and cystic follicles and western blots for protein quantification were performed. Similar to mRNA expression, VEGF protein expression was significantly upregulated in cystic follicles. Previous data has shown that VEGF is highly expressed in cystic bovine follicles [4] and correlates with previous studies demonstrating over expression in cystic follicles of women with PCOS [43]. VEGF expression results in enhanced vascular permeability, thereby allowing for serum to leave the vasculature in the thecal cell layer and flow into the follicular antrum, which results in excess fluid buildup [4]. Additionally, Greenaway et al [14] demonstrated that VEGF plays a cytoprotective role in granulosa cells where cell viability of serum starved GCs in vitro was enhanced with recombinant VEGF treatment. In this same study, when the receptor for VEGF, VEGFR2, was inhibited in vitro, this led to decreased GC viability [14]. Taken together, this data suggests that the pro-angiogenic factor VEGF is playing a pivotal role in promoting blood vessel growth in the follicle, and continuous high expression may be potentiating the cystic follicle state.

Members of the IGF family are key intraovarian regulators shown to be important for granulosa and theca cell proliferation and steroid development throughout the ovarian cycle. Disruption of IGFs can lead to abnormal follicular development and function, as well as compromise fertility through development of follicular cysts [44,45]. The bioavailability of both IGF1 and IGF2 in the follicle is greatly influenced by the proportion of IGF-binding proteins (IGFBPs). If IGFBPs are degraded via proteolytic enzymes, this results in increased levels of free IGFs, which leads to stimulation of steroidogenesis and mitogenesis [46]. Previous studies have demonstrated that COD is associated with a decreased concentration of IGF1 in follicular fluid and granulosa cells, thereby suggesting that the role that IGF1 plays in the regulation of folliculogenesis may be integral in the pathogenesis of COD [44].
In order to assess IGF1 and IGF2 expression, as well as angiogenic factors VEGF, VEGFR2, TSP-1, and CD36, immunohistochemistry (IHC) was conducted on the theca and granulosa cell layers of large and cystic follicles. As mentioned previously, VEGF increases with follicle maturity, as does its receptor VEGFR2 [14], therefore it was hypothesized that these factors would be further increased in cystic follicles. While no significant difference in VEGF expression in granulosa and theca cells was detected, a significant increase in VEGFR2 was observed. These results correlate with previous studies, again suggesting that VEGF and the VEGF signalling pathway plays an essential role in the development of cystic follicles due to enhanced blood vessel development within the thecal cell layer [11,12,14].

Interestingly, significant increases in both TSP-1 and CD36 in cystic follicles as compared to large were observed, which contrasts the mRNA expression data showing no change in TSP-1 expression. One important difference between the qPCR analyses compared to the IHC analyses is that the former analysed only granulosa cell expression, while the latter analysed both granulosa and theca cells. The thecal cell layer is highly vascularized in antral follicles as well as cystic follicles, while the granulosa cell layer remains avascular [8,9]. As such, the IHC analysis includes the protein expression from the blood vessels within the thecal layer. Previous studies have demonstrated that TSP-1 and CD36 are expressed in granulosa, theca, and endothelial cells [19,47], therefore the increased expression of TSP-1 and CD36 may be attributed to the increased vascularization of the thecal cell layer in cystic follicles as compared to large.

Lastly, while previous studies have indicated a decreased expression of IGF1 in cystic follicles [44], there was no significant difference observed in IGF1 or IGF2 expression between large and cystic follicles, however there is a decreasing trend in both IGF1 and IGF2 expression, which may be further elucidated with a greater sample size.
Due to a lack of consensus on how best to perform and interpret quantitative real-time PCR (qPCR) experiments, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were created. These guidelines included information on various aspects of qPCR analysis including nomenclature, reagents, research applications, sample preparation, primer efficiency, housekeeping genes, and many more [48]. We have, to the best of our abilities, aimed to follow all of the guidelines set for qPCR, with reference gene selection being a main area of investigation and investment of time.

Normalization using housekeeping genes is an essential component of a reliable qPCR assay as this process controls for variations in extraction yield, reverse-transcription yield, and efficiency of amplification; ultimately allowing for the comparison of mRNA or miRNA concentrations across different samples. Normalization against a single reference gene is considered not acceptable unless clear evidence is provided showing the housekeeping genes invariant expression under the experimental conditions. As such, two or more reference genes that have been experimentally determined is considered optimal for qPCR analyses [48].

For miRNA and mRNA expression analyses using qPCR, six and eight candidate housekeeping genes were selected to analyze, respectively, on large and cystic bovine granulosa cells. Reference gene stability was assessed using various software systems, geNorm, Normfinder, and Bestkeeper. These software systems use various algorithms to determine which reference gene has the most stable expression across all samples. In addition, some software will assess combinations of reference genes in order to determine which sets of genes, when averaged together, would be optimal for qPCR normalization, thereby adhering to the MIQE guidelines. This analysis determined that miR-16-5p and miR-106a-5p together are the most stable reference genes to use for miRNA expression analysis of our sample set. Interestingly, the most commonly used reference gene for miRNA expression analysis, U6, was shown to be the least stable reference gene, with great variability across this sample set. Additionally,
RPS18 and RPL19 were shown to be the most stable mRNA transcripts and therefore chosen as reference genes for mRNA expression analyses. Overall, these results demonstrate the importance of analyzing reference gene stability before proceeding with qPCR analysis, as improper reference gene selection can result in dramatically altered results.

CONCLUSION

In conclusion, this study aimed to characterize the mRNA and protein expression profiles of key angiogenic and mitogenic factors in cystic follicles as well as analyze the expression patterns of select miRNAs thought to be heavily involved in the regulation of ovarian angiogenesis and folliculogenesis. This study demonstrated an increase in miR-29a and decrease in miR-132, along with a significant increase in the mRNA and protein levels of VEGF in granulosa cells, and a significant increase in protein expression levels of VEGFR2, TSP-1, and CD36 in the granulosa and theca cell layers of cystic follicles. These results demonstrate the increased vascular density and pro-angiogenic stimulation of cystic follicles and suggest that miR-29a and miR-132 may be potential mediators of cystic ovarian disease. Together, these results suggest that an increase in pro-angiogenic signalling within the granulosa cells of cystic follicles may not be counteracted with anti-angiogenic signalling or appropriate miRNA regulation, thereby leading to proliferation of vasculature and development of cystic, anovulatory follicles.

DECLARATIONS

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.
Availability of Data and Material

The data generated during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors confirm there are no competing interests.

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Author’s Contributions

Allyssa Hooper, Stacey del Castillo, Joshua Antunes, Kathy Matuszewska, and Madison Pereira conducted experiments and assisted in composition of the manuscript. Jim Petrik, Jim Greenaway and Jonathan LaMarre were involved in conceptualizing the project and contributed to writing the manuscript. Jim Petrik was involved in the initiation of the project and oversaw the experimental approach and production of the manuscript.

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Figure 1: Schematic of angiogenesis and angiogenic factors throughout folliculogenesis and luteal development. Blood vessels appear within follicles at the late secondary stage, remaining in the thecal cell layer due to the presence of the basement membrane. VEGF protein expression increases throughout follicular development in cattle, while TSP-1 expression decreases [11]. At ovulation, the basement membrane breaks down, angiogenesis intensifies as blood vessels begin to invade the developing corpus luteum. VEGF mRNA expression in cattle is high throughout luteal development and maturation and then decreases during luteal regression [16]. In contrast, TSP-1 mRNA expression in cattle increases as the CL develops and remains high during CL regression [18]. Follicle images adapted from Fraser et al [16].

Figure 2: Optimal microRNA reference gene selection using geNorm, Normfinder, and Bestkeeper. Stability of candidate non-coding RNA reference genes was assessed using three software programs; geNorm, Normfinder, and Bestkeeper. A) Raw CT values of each candidate reference gene. B) geNorm stability score (M value). MiR16-5p considered to be the most stable across sample set, with U6 being the least stable. Red line indicates minimum M value for a reference gene to be considered stable. C) geNorm pairwise variation score (V value), where candidate reference genes are paired together to determine most stable combination. miR16-5p and miR106a-5p were the most stable combination. Red line indicates minimum V value for a pairwise variation to be considered stable. D) Normfinder stability value, where a lower stability value is considered to be the most stable reference gene. MiR93-5p considered to be the most stable. E) Correlation of the geNorm M value and the Normfinder stability value. miR93-5p considered to be the most stable. F) Bestkeeper analysis of standard error (SE), coefficient of correlation (R value), and p-value. Genes that ranked comparatively better are highlighted with a more intense cell colour. MiR93-5p, miR16-5p, and miR106a-5p were considered to be the most stable.

Figure 3: Optimal Bos Taurus mRNA reference gene selection using geNorm, Normfinder, and Bestkeeper. Stability of Bos Taurus candidate mRNA reference genes was assessed using three software programs; geNorm, Normfinder, and Bestkeeper. A) Raw CT values of each candidate reference gene. B) geNorm stability score (M value). RPL19 considered to be the most stable across sample set, with YWHAZ being the least stable. Red line indicates minimum M value for a reference gene to be considered stable. C) geNorm pairwise variation score (V value), where candidate reference genes are paired together to determine most stable combination. RPS18 and RPL19 were chosen as the most stable combination. Red line indicates minimum V value for a pairwise variation to be considered stable. D) Normfinder stability value, where a lower stability value is considered to be the most stable reference gene. UBA52 considered to be the most stable. E) Correlation of the geNorm M value and the Normfinder stability value. HRPT1, UBA52, ACTB, RPL19, and RPS18 considered to be the stable. F) Bestkeeper analysis of standard error (SE), coefficient of correlation (R value), and p-value. Genes that ranked comparatively better are highlighted with a more intense cell colour. UBA52 considered to be the most stable.

Figure 4: miRNA expression in granulosa cells of large and cystic bovine ovarian follicles. Granulosa cells were collected from follicular fluid aspirates of large and cystic bovine follicles. Total
RNA was extracted, and qPCR analysis of miRNA expression was performed. A) miR15a-5p expression. B) miR18a-5p expression. C) miR20a-5p expression. D) miR21-5p expression. E) miR29a-3p expression. F) miR126-5p expression. G) miR132-3p expression. H) Let7a-5p expression. 

Expression normalized to reference genes miR16-5p and miR106a-5p. Significance was evaluated using an unpaired t-test with a Welch’s correction. Asterisks denote statistical significance (*p<0.05). Error bars represent standard error (n=14/group).

**Figure 5:** Protein quantification of VEGF and mRNA expression of VEGFA and TSP-1 in granulosa cells of large and cystic bovine follicles. Granulosa cells were collected from follicular fluid aspirates of large and cystic bovine follicles. A, B) Immunoblot and densitometric analysis of VEGF from granulosa cells. C) VEGFA mRNA expression. D) TSP-1 mRNA expression. Expression normalized to reference genes RPS18 and RPL19. Significance was evaluated using an unpaired t-test with a Welch’s correction. Asterisks denote statistical significance (*p<0.05). Error bars represent standard error (n=3/group for protein n=14/group for mRNA).

**Figure 6:** VEGF, VEGFR2, TSP-1, CD36, IGF1, IGF2 protein expression in granulosa and theca cell layers in large and cystic bovine follicles. Follicles were immunostained and selectively annotated to determine protein expression in the granulosa and theca cell layers. Areas with the highest proportion of positive staining were imaged at 200x magnification. Three images were captured per bovine follicle section (VEGF, TSP-1, CD36: n=15 images analyzed/group; VEGFR2, IGF1, IGF2: n=9 images analyzed/group). Significance was evaluated using an unpaired t-test with a Welch’s correction. Asterisks denote statistical significance (*p<0.05; **p<0.01; ***p<0.001). Error bars represent standard error.

**Supplemental Data Figure Legends**

**Supplementary Figure 1:** Representative size selection of large and cystic bovine ovarian follicles. Follicles selected for analysis based on diameter. A) Large follicle. Classified as large if the follicle diameter was between 10-15mm. B) Cystic follicle. Classified as cystic if the follicle diameter was greater than 20mm.
Figure 1.
Figure 2

|               | Let7a-5p | miR106a-5p | miR16-5p | miR191-5p | miR93-5p | U6 |
|---------------|----------|------------|----------|-----------|----------|----|
| Standard Error (SE) [± x-fold] | 0.107    | 0.063      | 0.066    | 0.100     | 0.091    | 0.216 |
| Coefficient of Correlation (R value) | 0.919    | 0.972      | 0.972    | 0.910     | 0.978    | 0.250 |
| p-value       | 0.001    | 0.001      | 0.001    | 0.001     | 0.001    | 0.431 |
Figure 3
Figure 4
Figure 5
Figure 6
Supplemental Figure 1
**Supplementary Table 1:** MicroRNA primers for qPCR

| MicroRNA | Quanta Product Number | Primer ID     | Mature Accession Number | Primer Sequence (5’-3’) | Primer Efficiency (%) |
|----------|-----------------------|---------------|-------------------------|-------------------------|-----------------------|
| miR16    | HSMIR-0016            | hsa-miR-16-5p | MIMAT0000069            | GCCATAGCAGACGTAAAT      | 100.4%                |
| miR106a  | HSMIR-0106A           | hsa-miR-106a-5p | MIMAT0000103          | GCGGAGGTTCCTACAGTGC      | 102.3%                |
| miR18a   | HSMIR-0018A           | hsa-miR-18a-5p | MIMAT0000072           | AAGGTGACATCTAGTCAGAAGA   | 91.1%                 |
| miR20a   | HSMIR-0020A           | hsa-miR-20a-5p | MIMAT0000075           | CGCTAAAGTGCTATAGTCAGGT   | 100.3%                |
| miR21    | HSMIR-0021-5P         | hsa-miR-21-5p | MIMAT0000076           | GCTAGCCTATCAGACTGTGGTAA  | 100.7%                |
| Let7a    | HSLET-0007A-5P        | hsa-let-7a-5p | MIMAT000062             | CCGAGCTGAGGTAGGTCTGATA   | 95.4%                 |
| miR126   | HSMIR-0126-5P         | hsa-miR-126-5p | MIMAT0000444           | CGCATTATTACTCTTGGTACGC   | 102.0%                |
| miR29a   | HSMIR-0029A-3P        | hsa-miR-29a-3p | MIMAT0000086           | GCACAGTCGAAATCGGTTAAA    | 103.7%                |
| miR15a   | HSMIR-0015A-5P        | hsa-miR-15a-5p | MIMAT0000068           | TAGCAGCACATAATGGTTGTGA   | 99.1%                 |
| miR132   | HSMIR-0132-3P         | hsa-miR-132-3p | MIMAT0000426           | CAGTCTACAGCCATGGTCGAAA   | 93.5%                 |
| miR93    | HSMIR-0093            | hsa-miR-93-5p | MIMAT0000093           | TGCTGTTCGTCAGGTAGAAAA   | N/A                   |
| miR191   | HSMIR-0191-5P         | hsa-miR-191-5p | MIMAT0000440           | GAATCCAAAAAAGCAGCTGAAAA  | N/A                   |
| U6       | RNU6                  | RNU6          | NR_002752.1            | GCAAAATCGTGAAGCAGTTCC    | N/A                   |
**Supplementary Table 2:** *Bos Taurus* mRNA primers for qPCR

| Gene | Full Gene Name | Accession Number | Function | Forward Primer Sequences (5’-3’) | Reverse Primer Sequences (5’-3’) | Amplicon Size (bp) | Primer Efficiency (%) |
|------|----------------|------------------|----------|----------------------------------|----------------------------------|-------------------|-----------------------|
| ACTB | Beta-actin     | NM_173979.3      | Cytoskeletal structure protein | CACCGAATAAGCTTCTAGGCTGTCACCTTCACCCTGTCAG | CCTGCCGATTGGACAGATAGATGGGAGCATGTCCACTTT | 186 | 102.9% |
| GAPDH| Glyceraldehyde-3-Phosphate Dehydrogenase | NM_0010340 34.2 | Glycolytic enzyme | CACCAACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 153 | 106.6% |
| H3F3B| H3 Histone     | NM_0012425 71.1 | Involved in structure of chromatin and nucleosomes | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 182 | N/A |
| HPRT1| Hypoxanthine Phosphoribosyltransferase-1 | NM_0010340 35.2 | Catalyses purine recycling in all cells | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 191 | N/A |
| RPS9 | Ribosomal Protein S9 | NM_0011011 52.2 | Encodes a ribosomal protein | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 105 | N/A |
| RPS18| Ribosomal Protein S18 | NM_0010336 14.2 | Encodes a ribosomal protein | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 187 | 109.0% |
| RPL19| Ribosomal Protein L19 | NM_031103.1 | Encodes a ribosomal protein | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 102 | 89.6% |
| TSP-1| Thrombospondin-1 | NM_174196.1 | Anti-angiogenic factor | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 188 | 101.9% |
| UBA52| Ubiquitin A-52 | NM_0010763 63.2 | Protein degradation | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 113 | 91.2% |
| VEGFA| Vascular Endothelial Growth Factor A | NM_174216.2 | Pro-angiogenic factor | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 162 | 100.1% |
| YWHAZ| Tyrosine 3-Monooxygenase/Tryptophan | NM_174814.2 | Mediates signal transduction | ACCACACCACTTTGACTGGAACCGGTGAGGTAGTCGGAGATG | ATGTGTGGGGAGTTGTGTCGCCCCCTCCCCATATGTT | 239 | N/A |
### Supplementary Table 3: Immunohistochemistry method details

| Antigen Retrieval | 1° Antibody | Source | 1° Solution | 2° Solution | DAB time (seconds) |
|-------------------|-------------|--------|-------------|-------------|-------------------|
| Citrate           | VEGF        | ab46154| 1:500       | 1:100 (R)   | 300               |
|                   | TSP-1       | sc59887| 1:50        | 1:100 (M)   | 240               |
|                   | IGF1        | sc9013 | 1:50        | 1:100 (R)   | 240               |
|                   | IGF2        | sc5622 | 1:50        | 1:100 (R)   | 240               |
|                   | VEGFR2      | cs2479 | 1:600       | 1:100 (R)   | 240               |
|                   | CD36        | NB400-144| 1:1000   | 1:100 (R)   | 60                |

Antigen retrieval was performed using Citrate Buffer. Antibody solutions consisted of antibody diluting fluid with the appropriate antibody dilution. Biotinylated anti-mouse (M), or anti-rabbit (R) IgG secondary antibodies (Sigma-Aldrich Canada Ltd., Oakville, ON) were used for secondary solutions.