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

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26 **ABSTRACT**

27 Cystic ovarian disease (COD) is a major contributor to infertility in cattle, with 30% of cows
28 developing ovarian cysts during a given lactation and becoming anovulatory. The cause of COD has
29 remained elusive and is thought to be multifactorial, with angiogenic and genetic contributions proposed.
30 There is an increasing body of work suggesting that microRNAs (miRNAs) may be involved in a number
31 of ovarian-based reproductive disorders. Changes in miRNA expression can affect a multitude of genes
32 and may be important regulators of the dynamic processes involved in each ovarian cycle. We
33 hypothesized that miRNAs are integral to the regulation of critical ovarian processes such as
34 angiogenesis and follicular development and that altered miRNA expression contributes to the onset and
35 progression of ovarian dysfunction and reproductive disorders. Eight miRNAs (miR-15a, -18a, -20a, -
36 21, -29a, -126, -132, Let7a) known to target vascular endothelial growth factor (VEGF),
37 thrombospondin-1 (TSP-1), and play a role in both angiogenesis and folliculogenesis, were selected for
38 analysis. miRNA, mRNA, and protein expression were analyzed in large and cystic bovine follicles using
39 qPCR, western blot, and immunohistochemistry. miR-29a was found to be upregulated in cystic follicles,
40 whereas miR-132 expression was downregulated. VEGF expression increased in cystic follicles at both
41 the transcript and protein level, while no significant differences in TSP-1 expression was observed. Our
42 findings suggest that miR-29a and miR-132 may play an important role in maintaining the balance
43 between VEGF and TSP-1 expression in the ovary, and if disrupted, could potentiate ovarian-based
44 reproductive disorders such as PCOS and cystic ovarian disorder.

45

46

47

48 **INTRODUCTION**

49 Reproductive insufficiency results in a significant economic burden to the cattle industry. Cystic
50 ovarian disease (COD), is characterized by the development of large, anovulatory follicles, and is a major

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

60

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

73

74 Ovarian physiology is dependent on the establishment and continual remodelling of vasculature
75 in order to provide the follicle and corpus luteum with an adequate supply of nutrients, oxygen, and

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

88
89 There are two key factors involved in the regulation of ovarian angiogenesis; vascular endothelial
90 growth factor (VEGF), a pro-angiogenic factor, and thrombospondin-1 (TSP-1), an anti-angiogenic
91 factor [11,12]. VEGF mRNA has been detected in both the granulosa and thecal cell layers of secondary
92 follicles in cattle [13], with expression increasing throughout follicle development, whereas translated
93 VEGF protein is predominately localized in the granulosa cells [11]. Originally, it was thought that
94 VEGF functions were restricted to promoting blood vessel development, however, Greenaway et al [14]
95 identified the expression of the VEGF receptor, VEGFR2, in granulosa cells and determined that VEGF
96 signaling was cytoprotective for these cells and integral to the regulation of follicular recruitment,
97 progression, and function. Additionally, the inhibition of VEGF signalling results in the disrupted
98 ovulation, inhibition of vascularization of the corpus luteum, and subsequent inhibition of a post-
99 ovulatory rise in progesterone [15,16]. Anti-angiogenic factors such as angiostatin, TSP-1, and TSP-2,

100 inhibit angiogenesis by stimulating endothelial cell apoptosis and by associating with the extracellular
101 matrix in order to prevent endothelial cell migration [17,18].

102

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

115

116 It has been suggested that the cyclicity of ovarian angiogenesis and folliculogenesis may be
117 mediated via changes in gene expression. miRNAs are single-stranded, short non-coding RNAs, 19-24
118 nucleotides in length that play important gene regulatory roles in plants and animals by binding to target
119 mRNAs of protein-coding genes, resulting in post-transcriptional repression [20]. miRNAs are the most
120 abundant small non-coding RNAs in ovarian tissue and are present in a great diversity due to the
121 heterogeneous nature of the ovary, containing both somatic and germ cells [21]. By better understanding
122 the regulatory abilities of miRNAs in the ovary, this can result in the
123 potential for miRNA diagnostics and therapeutics, which could lead to substantial benefits for
124 reproductive medicine and health.

125

126 Gene expression analyses using cloning or sequencing, have shown that miRNAs are widely
127 expressed in the mammalian ovary, with estimates ranging from 373 to 679 different miRNAs being
128 present in whole ovarian tissues of various species [22]. Hossain et al [23] determined that the most
129 abundant miRNAs in bovine ovarian tissue were let-7a, let-7b, let-7c, miR-21, miR-23b, miR-24, miR-
130 27a, miR-126, miR-143, miR-652. In addition, there appears to be similarities between species with miR-
131 21, let-7a, and miR-126 also being highly abundant in human and murine ovarian tissue [24].

132

133 While limited research has been conducted on the role of miRNAs in cystic ovarian disease in
134 cattle, studies have begun to analyze miRNA expression in women with polycystic ovarian syndrome
135 (PCOS), a common endocrine disorder affecting 5-7% of reproductive age women [25]. Analysis of FF
136 from patients with PCOS compared to control healthy follicles revealed a decrease in miR-132 and miR-
137 320 expression and an increase in miR-9, -18b, -32, -34c, and -135a expression [26,27], suggesting that
138 these miRNAs may be playing a role in the development and progression of the disease.

139

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

144

145 This study began by selecting eight miRNAs of interest, miR-15a, -18a, -20a, -21, -29a, -126, -
146 132, and let7a. based on their predicted ability to target VEGF and TSP-1, and their role in angiogenesis
147 and folliculogenesis. The goal of this study was to characterize the transcript and protein expression
148 profiles of key angiogenic factors in large and cystic bovine follicles as well as determine which miRNAs

149 may be playing a key regulatory role in ovarian angiogenesis and folliculogenesis. The results of this
150 study could have implications on future research and therapies for ovarian-based reproductive disorders.

151

152

153 **MATERIALS AND METHODS**

154

155 **Granulosa Cell, Follicular Fluid, and Follicle Collection**

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

159

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

167

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

173

174 **RNA Extraction**

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

192

193 **cDNA Synthesis**

194 ***MicroRNA***

195 Following RNA extraction, microRNA cDNA synthesis using reverse transcription was
196 performed using qScript microRNA cDNA synthesis kit (Quanta Biosciences, Beverly, MA). A two-step

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

206

207 ***mRNA***

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

213

214 **Reference Gene Selection**

215 Candidate reference genes for miRNA qPCR analysis were selected from previous literature on
216 miRNA reference gene analyses [28,29]. A total of 6 candidates were chosen: miR16-5p, miR106a-5p,
217 Let-7a-5p, miR93-5p, miR191-5p, and U6. In addition, candidate reference genes were selected from
218 previous literature on *Bos Taurus* mRNA reference gene analyses [30]. A total of 8 candidates were
219 chosen: ACTB, GAPDH, UBA52, RPS18, RPL19, HPRT1, H3F3B, and YWHAZ. qPCR was performed

220 as described below and reference gene stability was assessed using three different reference gene
221 software systems: geNorm, Normfinder, and Bestkeeper.

222

223 **Quantitative PCR (qPCR)**

224 *MicroRNA*

225 MicroRNA reverse transcribed cDNA was analyzed using quantitative real-time polymerase
226 chain reaction (qPCR) to assess expression profiles of miRNAs of interest. Each analysis was performed
227 on three biological replicates each consisting of three technical replicates. MicroRNA targets of interest
228 included miR15a-5p, miR-18a-5p, miR20a-5p, miR21-5p, miR-29a-3p, miR126-5p, miR132-3p, and
229 Let-7a-5p, with miR16-5p and miR106a-5p used as reference genes (Quanta Biosciences, Beverly, MA).
230 All primer efficiencies were calculated by performing a standard curve using a 2:1 dilution of a pooled
231 reaction sample from 50ng/μL to 0.78ng/μL. Only primer efficiencies between 90-110% were accepted.
232 MiRNA primer sequences and efficiencies can be found in Supplementary Table 1.

233 For miRNA expression analysis, a master mix was created using 5μL of PerfeCTa SYBR Green
234 Supermix (5x), 0.2μL PerfeCTa Universal PCR Primer (10μM), 0.2μL Quanta Biosciences miRNA
235 specific primer (10μM), and 2.6μL of RNase/DNase free water in each reaction well. The master mix
236 was calculated based on the number of wells required for analysis. 8μL of the master mix was then added
237 to the wells followed by 2μL of diluted cDNA (1.5ng/μL per reaction); resulting in a reaction total of
238 10μL per well. Signal detection was acquired using a two-step protocol: 95°C for 2 minutes, followed
239 by 44 repeated cycles of 94°C for 30 seconds and 60°C for 30 seconds, ending with a melt curve
240 acquisition from 65-95°C. The Bio-Rad CFX96 Real-Time PCR system was used for the qPCR analysis.
241 Relative quantity of miRNA targets was log-transformed in BioRad CFX Manager 3.0 and normalized
242 to the relative quantity of the reference genes miR16-5p and miR106a-5p across all samples.

243

244 ***mRNA***

245 mRNA targets of interest included VEGFA, and TSP-1, with RPS18 and RPL19 used as reference
246 genes (Lab Services, University of Guelph). All mRNA primers were either designed using Primer-
247 BLAST NCBI or sequences were selected from previous literature. Primer efficiencies were calculated
248 by performing a standard curve using a 2:1 dilution of a pooled reaction sample from 50ng/μL to
249 0.78ng/μL. Only primer efficiencies between 90-110% were accepted. mRNA primer sequences for *Bos*
250 *Taurus* can be found in Supplementary Table 2.

251 mRNA expression profiles were performed using the Bio-Rad CFX96 Real-Time PCR system.
252 A master mix was created using 5μL SensiFAST SYBR No-ROX (FroggaBio, Scientific Solutions,
253 Toronto, ON), 1μL forward primer (1.25μM), 1μL reverse primer (1.25μM), and 2μL RNase/DNase free
254 water. The master mix was calculated based on the number of wells required for analysis. 9μL of the
255 master mix was then added to the wells followed by 1μL of diluted cDNA (5ng/μL per reaction);
256 resulting in a reaction total of 10μL per well. Signal detection was acquired using a two-step protocol:
257 95°C for 30 seconds, followed by 39 repeated cycles of 95°C for 5 seconds and 60°C for 5 seconds,
258 ending with a melt curve acquisition from 65-95°C. Relative quantity of gene targets was log-transformed
259 in BioRad CFX Manager 3.0 and normalized to the relative quantity of the reference genes RPS18 and
260 RPL19 across all samples.

261

262 **Protein Extraction**

263 Protein was extracted from granulosa cells previously collected and stored at -80 °C. Cell in
264 1.6mL tubes were lysed over ice in a radioimmunoprecipitation assay (RIPA) buffer supplemented with
265 fresh protease inhibitors for 30 minutes. Buffer samples containing lysate were spun at 14,000rpm for 10
266 minutes at 4°C. The aqueous phase of each sample was aliquoted and stored at -80°C. Protein

267 concentration of the cell lysate was determined using a DC Bio-Rad Protein Quantification Kit (Bio-Rad,
268 Mississauga, ON).

269

270 **Western Blot Analysis**

271 A wet/tank blotting system (Bio-Rad Mini Trans-Blot Cell; Bio-Rad, Mississauga, ON) was used
272 to prepare western blot. Equal volume samples (20µg; n=5/group) were prepared in 3x reducing buffer
273 containing dithiothreitol (DTT; Life Technologies Inc., Burlington, ON) denaturing agent and heated to
274 90°C for 5 minutes. Denatured samples were loaded into separating gels and subjected to sodium dodecyl
275 sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) at 120 volts (V) in room temperature running
276 buffer. Precision Plus Protein Dual Colour Standards (Bio-Rad, Mississauga, ON) were used as
277 molecular weight markers. When the desired separation was achieved, gels were equilibrated in transfer
278 buffer for 15 minutes and transferred onto a nitrocellulose membrane (Amersham Hybond ECL; GE
279 Healthcare Bio-Sciences Corp., Piscataway, NJ) at 100V in ice-cold transfer buffer for 90 to 120 minutes.
280 Following the transfer, membranes were washed in Tris-buffered saline with Tween 20 (TBST) and
281 blocked with skim milk in TBST. After blocking for 1 hour, membranes were incubated in the appropriate
282 primary antibody solution: VEGF (1:500, Santa Cruz, sc512) and β -Actin (1:3000, Cell Signalling,
283 cs4967), overnight at 4°C. The following day, membranes were washed with TBST and incubated in
284 IgG, HRP-linked anti-rabbit secondary antibody (1:2000 for VEGF and 1:5000 for β -Actin) for 1 hour
285 at room temperature. Membranes were washed again in TBST and antibody binding was detected by
286 enhanced chemiluminescence (ECL) (Luminata Forte Western HRP Substrate, Millipore Ltd.,
287 Etobicoke, ON). Signals were detected and imaged using a Bio-Rad ChemiDoc XRS+ system and
288 relative densitometry was determined using Bio-Rad Image Lab software.

289

290 **Immunohistochemistry on Paraffin-Embedded Follicle Sections**

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

309

310 **Image Analysis**

311 Follicle sections stained by immunohistochemistry were imaged by bright-field microscopy using
312 a Nikon Eclipse E600 microscope with a QImaging camera. Follicles were imaged at 200x total
313 magnification and analyzed for cytoplasmic factors VEGFA, VEGFR2, TSP-1, CD36, IGF1, and IGF2.

314 For these factors, the percentage of positively stained tissue was determine using Aperio ImageScope
315 software (Aperio, Vista, CA). The “Positive Pixel Count v9” algorithm was optimized and used for each
316 image setting. Selective annotations were made as to only include the granulosa and thecal cell layers of
317 the follicles for analysis. Percent positivity was calculated as a portion of total positive pixels (the sum
318 of the weak positive, positive, and strong positive pixels).

319

320 **Statistical Analysis**

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

325

326

327 **RESULTS**

328

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

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

335

336 GeNorm provides a ranking of the tested genes based on their stability value (M value), which is
337 considered to be stable if below 0.15. In addition, geNorm determines mean pairwise variation (V value)

338 between a candidate gene and all other tested candidates, allowing for the selection of a combination of
339 reference genes, again considered to be stable if below 0.15. MiR16-5p and miR106a-5p were found to
340 have the most stable M value at 0.309 and 0.331, respectively (Figure 2B), and together were found to
341 have a V value of 0.113 (Figure 2C).

342

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

346

347 Bestkeeper calculates the stability measure for each candidate gene and then ranks their stability
348 based on standard error (SE), coefficient of correlation (R) and p-value. Reference genes with the lowest
349 SE, R value closest to 1.0, and lowest p-value are considered to be the most stable. MiR93-5p, miR16-
350 5p, and miR-106a-5p were shown to be the most stable via Bestkeeper analyses (Figure 2F).

351 Based on the data collected from all three reference gene software programs, miR16-5p and
352 miR106a-5p were chosen to be the most stable reference genes as they demonstrated consistent stability
353 throughout all analyses and demonstrated stability when averaged together in pairwise variation. MiR16-
354 5p and miR106a-5p were subsequently used as normalizing genes in all miRNA qPCR expression
355 analyses conducted.

356

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

358 In order to identify the most stable reference genes for mRNA qPCR analyses, candidate
359 reference genes were selected from previous literature [30]. A total of eight candidate reference genes
360 (YWHAZ, H3F3B, ACTB, GAPDH, UBA52, RPL19, RPS18, and HRPT1) were tested across twelve
361 bovine granulosa cell samples from both large and cystic follicles. To determine the most steadily
362 expressed mRNAs, three different software programs were used: geNorm, Normfinder, and Bestkeeper.

363

364 GeNorm analyses found RPS18 and RPL19 to have the most stable M values of 0.167 and 0.159,
365 respectively (Figure 3B). In addition, the pairwise variation (V value) for RPS18 and RPL19 was 0.071
366 (Figure 3C), well below the minimum value of 0.15. Normfinder determined UBA52 to be the most
367 stable with a stability value of 0.061 (Figure 3D). Finally, Bestkeeper analysis indicated UBA52 to be
368 the most stable as it had the lowest standard error, highest R value and lowest p-value (Figure 3F).
369 Ultimately, RPS18 and RPL19 were chosen as the most stable reference genes in bovine granulosa cells
370 as they demonstrated stability across all three software program analyses, a high degree of correlation
371 between M value and stability value (Figure 3E), and a stable pairwise variation (Figure 3C).

372

373 **MicroRNA Expression in Granulosa Cells of Large and Cystic Bovine Follicles**

374 Large and cystic bovine follicles were aspirated to collect follicular fluid and granulosa cells for
375 expression analyses. Expression of eight miRNAs, previously shown to be involved in angiogenesis and
376 folliculogenesis as well as have the ability to target VEGF and TSP-1, were analyzed using qPCR.
377 miRNA expression was normalized to miR16-5p and miR106a-5p. Expression of miR29a-3p was found
378 to be increased in cystic follicles as compared to large ($p<0.05$; Figure 4E), while expression of miR132-
379 3p was significantly reduced in cystic follicles as compared to large ($p<0.05$; Figure 4G). No other
380 significant changes in miRNA expression was found between large and cystic follicles.

381

382 **mRNA Expression in Granulosa Cells of Large and Cystic Bovine Follicles**

383 Granulosa cells aspirated from large and cystic ovarian follicles were collected and total RNA
384 was extracted for qPCR analysis. VEGFA and TSP-1 expression was normalized to RPS18 and RPL19,
385 as determined through reference gene software analyses. VEGFA was found to be increased in cystic

386 follicles as compared to large follicles ($p<0.05$; Figure 5C), however no significant difference in TSP-1
387 expression was observed (Figure 5D).

388

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

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

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

404

405

406 **DISCUSSION**

407

408 This study has characterized the expression of key angiogenic factors in bovine folliculogenesis
409 as well as explored which miRNAs play a regulatory role in ovarian folliculogenesis and angiogenesis.
410 Our study began by selecting eight miRNAs, let-7a, miR-15a, -18a, -20a, -21, -29a, -126, and -132, that

411 have been shown in the literature to have a regulatory role in ovarian follicle development and
412 angiogenesis as well as target either VEGF or TSP-1 [24]. Our goal was to determine which miRNAs
413 play an important compensatory role in maintaining the balance between VEGF and TSP-1 throughout
414 the ovarian cycle, as well as to characterize the expression profile of large, antral follicles compared to
415 cystic, diseased follicles, in order to gain a better understanding of how miRNAs, angiogenic factors, and
416 mitogenic factors potentially mediate cystic ovarian disease (COD).

417

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

432

433

434 MicroRNA target prediction tools were used to determine potential mRNA transcript targets for
435 each miRNA. MiR-29a and miR-132 have over 600 and 350 predicted targets, respectively, thereby

436 having the ability to regulate a wide variety of genes. Within the context of this study, miR-29a has been
437 shown to target VEGF, TSP-2, and IGF1 [32]. Previous studies have shown miR-29a to be highly
438 expressed in the ovarian granulosa cells and during the early phase of granulosa cell luteinization, but
439 interestingly not expressed in mature corpus luteum (CL) [23]. In addition, miR-29a has been shown to
440 induce apoptosis, play an essential role in extracellular matrix remodeling during the follicular phase,
441 and is significantly decreased in serum of patients with PCOS [33–35]. Researchers have also suggested
442 that miR-29a plays a regulatory role in growth and differentiation of cumulus cells, endocrine regulation
443 of theca cells, and early luteinization within the ovary [36].

444

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

460 shows that miR-29a is upregulated in cystic follicles, therefore may be playing a role in enhancing the
461 diseased state or working to downregulate proliferative and pro-angiogenic factors.

462

463 Previous studies of miRNA expression in follicular fluid has shown a decrease in miR-132
464 expression in cystic follicles of PCOS patients compared to healthy follicles [26,27]. Our results also
465 show a significant decrease in miR-132 in cystic bovine follicles as compared to healthy follicles, which
466 suggests that a lack of miR-132 could play a role in the development or progression of cystic follicles in
467 mammalian species. miR-132 has also been shown to increase proliferation, regulate steroidogenesis,
468 increase estradiol production, and inhibit downstream components of the VEGF signalling cascade [38–
469 41]. Therefore, the down regulation of miR-132 found in cystic follicles may result in decreased GC
470 proliferation, decreased estradiol production, and increased VEGF signalling. miR-132 has also been
471 shown to promote angiogenesis, where the use of miR-132 inhibitors results in anti-angiogenic effects
472 [42]. The regulatory effect that miR-132 has within the ovary and specifically in cystic follicles is of
473 great interest. Based on this study and the current literature, miR-132 appears to be a great candidate for
474 future functional studies as it is evident that it plays an important role in follicle and blood vessel
475 development.

476 Previous work in our lab has uncovered an inverse relationship between the expression of VEGF
477 and TSP-1 throughout folliculogenesis. Using bovine follicles ranging in size small (<5mm), medium
478 (5-10mm), and large (10-15mm), protein expression levels of VEGF were shown to increase as the
479 follicle matured, while TSP-1 expression opposed this trend, decreasing in expression as the follicle
480 matured [11]. This inverse relationship coincides with the development of vasculature within the follicle
481 with the vascular density increases as the follicle matures, suggesting a greater amount of pro-angiogenic
482 stimulation. Within the context of COD, this study aimed to characterize the expression of VEGF and
483 TSP-1 in cystic follicles in order to determine if this inverse trend continues in pathological conditions.
484 In order to do this, RNA was extracted from granulosa cells of large and cystic bovine follicles and qPCR

485 was conducted. As hypothesized, VEGF mRNA expression was significantly upregulated in cystic
486 follicles as compared to large, while TSP-1 expression did not differ, although it was expressed at very
487 low levels. In addition to mRNA expression, protein was also collected from granulosa cells of large and
488 cystic follicles and western blots for protein quantification were performed. Similar to mRNA expression,
489 VEGF protein expression was significantly upregulated in cystic follicles. Previous data has shown that
490 VEGF is highly expressed in cystic bovine follicles [4] and correlates with previous studies
491 demonstrating over expression in cystic follicles of women with PCOS [43]. VEGF expression results in
492 enhanced vascular permeability, thereby allowing for serum to leave the vasculature in the thecal cell
493 layer and flow into the follicular antrum, which results in excess fluid buildup [4]. Additionally,
494 Greenaway et al [14] demonstrated that VEGF plays a cytoprotective role in granulosa cells where cell
495 viability of serum starved GCs *in vitro* was enhanced with recombinant VEGF treatment. In this same
496 study, when the receptor for VEGF, VEGFR2, was inhibited *in vitro*, this led to decreased GC viability
497 [14]. Taken together, this data suggests that the pro-angiogenic factor VEGF is playing a pivotal role in
498 promoting blood vessel growth in the follicle, and continuous high expression may be potentiating the
499 cystic follicle state.

500

501 Members of the IGF family are key intraovarian regulators shown to be important for granulosa
502 and theca cell proliferation and steroid development throughout the ovarian cycle. Disruption of IGFs
503 can lead to abnormal follicular development and function, as well as compromise fertility through
504 development of follicular cysts [44,45]. The bioavailability of both IGF1 and IGF2 in the follicle is
505 greatly influenced by the proportion of IGF-binding proteins (IGFBPs). If IGFBPs are degraded via
506 proteolytic enzymes, this results in increased levels of free IGFs, which leads to stimulation of
507 steroidogenesis and mitogenesis [46]. Previous studies have demonstrated that COD is associated with a
508 decreased concentration of IGF1 in follicular fluid and granulosa cells, thereby suggesting that the role
509 that IGF1 plays in the regulation of folliculogenesis may be integral in the pathogenesis of COD [44].

510

511 In order to assess IGF1 and IGF2 expression, as well as angiogenic factors VEGF, VEGFR2,
512 TSP-1, and CD36, immunohistochemistry (IHC) was conducted on the theca and granulosa cell layers
513 of large and cystic follicles. As mentioned previously, VEGF increases with follicle maturity, as does its
514 receptor VEGFR2 [14], therefore it was hypothesized that these factors would be further increased in
515 cystic follicles. While no significant difference in VEGF expression in granulosa and theca cells was
516 detected, a significant increase in VEGFR2 was observed. These results correlate with previous studies,
517 again suggesting that VEGF and the VEGF signalling pathway plays an essential role in the development
518 of cystic follicles due to enhanced blood vessel development within the thecal cell layer [11,12,14].

519

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

530

531 Lastly, while previous studies have indicated a decreased expression of IGF1 in cystic follicles
532 [44], there was no significant difference observed in IGF1 or IGF2 expression between large and cystic
533 follicles, however there is a decreasing trend in both IGF1 and IGF2 expression, which may be further
534 elucidated with a greater sample size.

535

536 Due to a lack of consensus on how best to perform and interpret quantitative real-time PCR
537 (qPCR) experiments, the Minimum Information for Publication of Quantitative Real-Time PCR
538 Experiments (MIQE) guidelines were created. These guidelines included information on various aspects
539 of qPCR analysis including nomenclature, reagents, research applications, sample preparation, primer
540 efficiency, housekeeping genes, and many more [48]. We have, to the best of our abilities, aimed to
541 follow all of the guidelines set for qPCR, with reference gene selection being a main area of investigation
542 and investment of time.

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

550 For miRNA and mRNA expression analyses using qPCR, six and eight candidate housekeeping
551 genes were selected to analyze, respectively, on large and cystic bovine granulosa cells. Reference gene
552 stability was assessed using various software systems, geNorm, Normfinder, and Bestkeeper. These
553 software systems use various algorithms to determine which reference gene has the most stable
554 expression across all samples. In addition, some software will assess combinations of reference genes in
555 order to determine which sets of genes, when averaged together, would be optimal for qPCR
556 normalization, thereby adhering to the MIQE guidelines. This analysis determined that miR-16-5p and
557 miR-106a-5p together are the most stable reference genes to use for miRNA expression analysis of our
558 sample set. Interestingly, the most commonly used reference gene for miRNA expression analysis, U6,
559 was shown to be the least stable reference gene, with great variability across this sample set. Additionally,

560 RPS18 and RPL19 were shown to be the most stable mRNA transcripts and therefore chosen as reference
561 genes for mRNA expression analyses. Overall, these results demonstrate the importance of analyzing
562 reference gene stability before proceeding with qPCR analysis, as improper reference gene selection can
563 result in dramatically altered results.

564

565 **CONCLUSION**

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

578

579 **DECLARATIONS**

580 **Ethics Approval and Consent to Participate**

581 Not applicable.

582

583 **Consent for Publication**

584 Not applicable.

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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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760 Figure Legends

761

762 **Figure 1: Schematic of angiogenesis and angiogenic factors throughout folliculogenesis and luteal**
763 **development.** Blood vessels appear within follicles at the late secondary stage, remaining in the thecal
764 cell layer due to the presence of the basement membrane. VEGF protein expression increases throughout
765 follicular development in cattle, while TSP-1 expression decreases [11]. At ovulation, the basement
766 membrane breaks down, angiogenesis intensifies as blood vessels begin to invade the developing corpus
767 luteum. VEGF mRNA expression in cattle is high throughout luteal development and maturation and
768 then decreases during luteal regression [16]. In contrast, TSP-1 mRNA expression in cattle increases as
769 the CL develops and remains high during CL regression [18]. Follicle images adapted from Fraser et al
770 [16].

771

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

786

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

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802 **Figure 4: miRNA expression in granulosa cells of large and cystic bovine ovarian follicles.**
803 Granulosa cells were collected from follicular fluid aspirates of large and cystic bovine follicles. Total

804 RNA was extracted, and qPCR analysis of miRNA expression was performed. **A)** miR15a-5p
805 expression. **B)** miR18a-5p expression. **C)** miR20a-5p expression. **D)** miR21-5p expression. **E)** miR29a-
806 3p expression. **F)** miR126-5p expression. **G)** miR132-3p expression. **H)** Let7a-5p expression.
807 Expression normalized to reference genes miR16-5p and miR106a-5p. Significance was evaluated
808 using an unpaired t-test with a Welch's correction. Asterisks denote statistical significance (* $p < 0.05$).
809 Error bars represent standard error (n=14/group).
810

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

818
819 **Figure 6: VEGF, VEGFR2, TSP-1, CD36, IGF1, IGF2 protein expression in granulosa and theca**
820 **cell layers in large and cystic bovine follicles.** Follicles were immunostained and selectively annotated
821 to determine protein expression in the granulosa and theca cell layers. Areas with the highest proportion
822 of positive staining were imaged at 200x magnification. Three images were captured per bovine follicle
823 section (VEGF, TSP-1, CD36: n=15 images analyzed/group; VEGFR2, IGF1, IGF2: n=9 images
824 analyzed/group). Significance was evaluated using an unpaired t-test with a Welch's correction.
825 Asterisks denote statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Error bars represent standard
826 error.
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829 Supplemental Data Figure Legends

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

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845 Figure 1.

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Figure 2

Figure 3

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869 Supplemental Figure 1

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873 **Supplementary Table 1: MicroRNA primers for qPCR**

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	CGCCATAGCAGCACGTAAAT	100.4%
miR106a	HSMIR-0106A	hsa-miR-106a-5p	MIMAT0000103	CGCCAAAAGTGCTTACAGTGC	102.3%
miR18a	HSMIR-0018A	hsa-miR-18a-5p	MIMAT0000072	AAGGTGCATCTAGTGCAGATAGAAA	91.1%
miR20a	HSMIR-0020A	hsa-miR-20a-5p	MIMAT0000075	CGCTAAAGTGCTTATAGTGCAGGT	100.3%
miR21	HSMIR-0021-5P	hsa-miR-21-5p	MIMAT0000076	GCTAGCTTATCAGACTGATGTTGAAA	100.7%
Let7a	HSLET-0007A-5P	hsa-let-7a-5p	MIMAT0000062	CCGAGCTGAGGTAGTAGGTTGTATA	95.4%
miR126	HSMIR-0126-5P	hsa-miR-126-5p	MIMAT0000444	CGCATTATTACTTTTGGTACGC	102.0%
miR29a	HSMIR-0029A-3P	hsa-miR-29a-3p	MIMAT0000086	GCACCATCTGAAATCGGTTAAA	103.7%
miR15a	HSMIR-0015A-5P	hsa-miR-15a-5p	MIMAT0000068	TAGCAGCACATAATGGTTTGTGA	99.1%
miR132	HSMIR-0132-3P	hsa-miR-132-3p	MIMAT0000426	CAGTCTACAGCCATGGTCGAAA	93.5%
miR93	HSMIR-0093	hsa-miR-93-5p	MIMAT0000093	TGCTGTTCGTGCAGGTAGAAAA	N/A
miR191	HSMIR-0191-5P	hsa-miR-191-5p	MIMAT0000440	GAATCCCAAAAGCAGCTGAAAA	N/A
U6	RNU6	RNU6	NR_002752.1	GCAAATTCGTGAAGCGTTCC	N/A

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879 **Supplementary Table 2: *Bos Taurus* mRNA primers for qPCR**

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	CACCGCAAATGCTTCTAGGC TGTCACCTTCACCGTTCCAG	186	102.9%
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_001034034.2	Glycolytic enzyme	CCTGCCCGTTCGACAGATAG ATGGCGACGATGTCCACTTT	153	106.6%
H3F3B	H3 Histone	NM_001242571.1	Involved in structure of chromatin and nucleosomes	ATGTGGTGGGGAGTGTGTGTC TCCCCCTCCCCATTAGTGTT	182	N/A
HPRT1	Hypoxanthine Phosphoribosyltransferase-1	NM_001034035.2	Catalyses purine recycling in all cells	ACCACACCACTATTGACTGGAA CTGTCTGTTGGTACGAGAGTG	191	N/A
RPS9	Ribosomal Protein S9	NM_001101152.2	Encodes a ribosomal protein	CGAGTATGGGCTCCGGAACAAACG GGGTCTTTCTCATCCAGCGTCAGC	105	N/A
RPS18	Ribosomal Protein S18	NM_001033614.2	Encodes a ribosomal protein	CCCTGAGAAGTTCCAGCACAT CACACGTTCACCTCATCCT	187	109.0%
RPL19	Ribosomal Protein L19	NM_031103.1	Encodes a ribosomal protein	TGGAGCACATCCACAAACTGA AGACACGAGGGACGCTTCAT	102	89.6%
TSP-1	Thrombospondin-1	NM_174196.1	Anti-angiogenic factor	CCACAATGGAGAGCACAGGT TGCACCTGGCGTTCTTGTTG	188	101.9%
UBA52	Ubiquitin A-52	NM_001076363.2	Protein degradation	AGAAAGAGTCCACCCTGCACTTGG ACACTTGCGGCAGATCATCTTGTCG	113	91.2%
VEGFA	Vascular Endothelial Growth Factor A	NM_174216.2	Pro-angiogenic factor	TTCCAGGAGTACCCAGATGAGA CTGGCTTTGGTGAGGTTTGAT	162	100.1%
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan	NM_174814.2	Mediates signal transduction	AGGAGCCCGTAGGTCATCTT CCTCAGCCAAGTAGCGGTAG	239	N/A

881 **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

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