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Implications of miRNA expression pattern in bovine oocytes and follicular fluids for development competence

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Credit author statement

R.P. and F.G. designed the study and worked on the manuscript. E.F.M. helped to perform RT-qPCR validation. N.F., V.A. and P.P. helped to perform the experiments and data analysis. T.A.L.B. and J.L.W. helped in interpreting the results and writing the manuscript. All authors discussed the results and commented on the manuscript.

1 **Title:**

2 **Implications of miRNA expression pattern in bovine oocytes and follicular fluids**

3 **for development competence**

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23

Abstract

Developmental competence determines the oocyte capacity to support initial embryo growth, but the molecular mechanisms underlying this phenomenon are still ill-defined. Changes in microRNA (miRNA) expression pattern have been described during follicular growth in several species. Therefore, aim of this study was to investigate whether miRNA expression pattern in cow oocyte and follicular fluid (FF) is associated with the acquisition of developmental competence. Samples were collected from ovaries with more than, or fewer than, 10 mid-antral follicles (H- and L-ovaries) because previous studies demonstrated that this parameter is a reliable predictor of oocyte competence. After miRNA deep sequencing and bioinformatic data analysis, we identified 58 miRNAs in FF and 6 in the oocyte that were differentially expressed between H- and L-ovaries. Overall, our results indicate that miRNA levels both in FF and in the ooplasm must remain within specific thresholds and that changes in either direction compromise oocyte competence. Some of the miRNAs found in FF (miR-769, miR-1343, miR-450a, miR-204, miR-1271 and miR-451) where already known to regulate follicle growth and their expression pattern indicate that they are also involved in the acquisition of developmental competence. Some miRNAs were differentially expressed in both compartments but with opposite patterns, suggesting that miRNAs do not flow freely between FF and oocyte. Gene Ontology analysis showed that the predicted gene targets of most differentially expressed miRNAs are part of a few signalling pathways. Regulation of maternal mRNA storage and mitochondrial activity seem to be the processes more functionally relevant in determining oocyte quality. In conclusion, our data identified a few miRNAs in the follicular fluid and in the ooplasm that modulate the oocyte

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48 developmental competence. This provides new insights that could help with the

49 management of cattle reproductive efficiency.

1. Introduction

51 The growth and development of mammalian follicles are critically dependent on the

52 bidirectional communication between the oocyte and its companion somatic cells [1].

53 In the last few years, there has been increasing interest focused on unraveling the

54 biological processes related to oogenesis and the associated molecular mechanisms

55 related to oocyte growth and maturation. These processes involve complex spatio-

56 temporal expression of genes that contribute to the gradual acquisition of the oocyte

57 competence [2, 3]. The correct regulation of gene expression is essential for oocyte

58 development, and alteration in expression patterns can result in poor oocyte quality

59 [4, 5]. Despite considerable work has been done in several species, including

60 humans [6], mice [7] and cattle [8], the molecular mechanisms that lead to a

61 complete gain of oocyte developmental competence are still unknown. However, for

62 cattle, it is known that developmental potential is gained during the final period of

63 oocyte growth [5] and that maternal mRNAs play an important role in this process

64 [9].

65 Recently, microRNAs (miRNAs), which regulate gene expression at the mRNA level,

66 have been associated with folliculogenesis and oogenesis [10, 11]. MiRNAs, which

67 range in size from 18 to 25 nucleotides (nt), have been found in the different

68 compartments of ovarian follicles, including granulosa cells [12, 13], theca cells [14],

69 follicular fluid and the oocyte itself [15]. Studies on the role of miRNAs during follicle

70 development in humans [16-18], mice [19, 20], cattle [10, 21, 22], pigs [23] and

71 horses [24] suggest that they regulate the cellular differentiation processes which

72 occur during follicular development.

73 Dynamic changes in miRNA expression have been described during mouse oocyte

74 maturation, where a large proportion of maternal genes are directly or indirectly

75 under the control of miRNAs [25]. Similarly, miRNAs may be involved in controlling

76 bovine oocyte growth as follicular fluid miRNA levels have been found to change

77 during folliculogenesis in bovine ovarian follicles [26]. Furthermore, bovine granulosa

78 cells of preovularory dominant and subordinate follicles have been related to the

79 differential expression of miRNAs [27]. Interestingly, bovine miRNAs have been

80 found both free and associated with exosomes in follicular fluid; the latter may

81 facilitate transport of specific miRNAs into follicular cells [21]. However, these studies

82 have only described miRNAs changes during the physiological processes of follicle

83 growth. Aim of this work is to identify which miRNAs are directly involved in the

84 acquisition of oocyte compence. To this purpose we contrasted the miRNA content

85 of high and low competence oocytes identified using ovarian morphology.

86 We have previously demonstrated that the developmental competence of oocytes

87 collected from ovaries with less than 10 follicles of 2-5 mm in diameter (L-ovaries)

88 was six times lower than those collected from ovaries with more than 10 follicles of

89 2-5 mm in diameter (H-ovaries), measured as blastocyst rate (5.5 ± 2.1% vs. 28.9

90 ±3.6% respectively)[28]. Subsequent studies demonstrated that oocytes whose low

91 competence was predicted with the same method showed higher frequency of

92 chromosomal aberrations [67], as well as altered levels of maternal mRNA

93 polyadenilation [2].The application of the same selection criteria to the ovaries of live

94 animals showed that animals whose ovaries had less that 10 antral follicles have

95 several features associated with poor fertility, including poor response to

96 superovulation and poor pregnancy rate [30]. Furthermore, these ovaries are small,

97 have poor vascularization and few healthy follicles [31, 32]. The follicular fluids of L-

98 ovaries have high levels of ovarian hormones, including Growth Hormone (GH) and

99 Progesterone (P4) [29].

100 Here we analysed the miRNA pattern of both the oocytes and the follicular fluids of

101 low and high AFC ovaries. In particular, we analysed the miRNA pattern of follicular

102 fluid because it constitutes the mileu in which oocytes grow and it contains both local

103 and systemic components thereby providing a comprehensive picture of the whole

104 animal physiological situation.

105 The data described here are useful to identify the molecular pathways that are

106 important for the bovine oocytes to gain its optimal developmental competence, and

107 to better understand fertility-related problems in cattle.

108 **2. Materials and methods**

109 All chemicals were obtained from Sigma-Aldrich Company (Italy, Europe) unless

110 otherwise stated.

111 *2.1. Follicular fluid and germinal vesicle oocyte collection*

112 Ovaries were collected at a commercial abattoir and were transported to the

113 laboratory in warmed (27-30°C) Dulbecco Phosphate Buffered Saline (PBS). Ovaries

114 were classified into low and high antral follicle count categories according to the

115 methods used in previous works [28, 29]. Briefly, the ovaries were assigned to high

116 antral follicle count ovaries (H ovaries) when more than 10 mid-antral follicles (2-5

117 mm in diameter) and a dominant follicle (> 8 mm) were observed, while ovaries with

118 fewer than 10 follicles of the same size and with no dominat follicle were classified

119 as low antral follicle count ovaries (L-ovaries).

120 Only 2-5 mm follicles were aspirated from both ovary types using a syringe and 19-

121 gauge needle and both follicular fluids and cumulus-oocyte complexes (COCs) were

122 pooled, according to the classification, into 50 mL Falcon tubes, kept at 38.8°C.

123   Granulosa cells (GC) were separated from the follicular fluids by centrifugation at

124   1500xg per 10 min and supernatants was were frozen and stored at -80° C until

125   analysis. The remaining granulosa cell pellets were washed 2 times with PBS, snap

126   frozen in liquid nitrogen and stored at -80° C for real time quantitative PCR validation

127   as specified below.

128   Cumulus-oocyte complexes (COCs) were identified using a stereomicroscope and

129   COCs that were medium brown in colour with five or more complete layers of

130   cumulus cells were collected [33]. After collection, germinal vesicle oocytes (oocytes)

131   were washed 2 times with PBS (supplemented with 36 μg/L pyruvate, 50 μg/mL

132   gentamycin, 0.5 mg/mL Bovine Serum Albumine (BSA, Sigma-Aldrich, Italy, Europe).

133   Oocytes were denuded by incubating the COCs in PBS and 100 UI/mL

134   hyaluronidase (Sigma-Aldrich, Italy, Europe) for 3 minutes and then by pipetting.

135   Resulting oocytes were snap frozen in liquid nitrogen before storage at -80° C.

136   **2.2.   RNA extraction from follicular fluids and germinal vesicle oocytes**

137   Small RNAs were extracted from three independent pools of follicular fluid from H

138   and L-ovaries using Nucleospin MiRNA plasma (MACHEREY-NAGEL GmbH & Co.

139   KG, Germany, Europe) following the manufacturer's instructions. The H ovary

140   follicular fluid samples are denoted in the rest of the manuscript as FFH1, FFH2 and

141   FFH3 and the L ovary follicular fluid samples as FFL1, FFL2 and FFL3.

142   Total oocyte RNA was obtained from 3 pools of 25 oocytes for each class, using the

143   Trizol Reagent procedure (Life Technologies, CA, USA), which yielded sufficient

144   RNA to construct the libraries for deep sequencing. The oocytes were lysed in 1 mL

145   Trizol reagent following supplier's instructions. Elution of RNA samples was carried

146   out using 8 μL RNAse free-water and samples were stored at -80°C. The samples

147   are denoted in the manuscript as OVOH1, OVOH2 and OVOH3 for the H-ovaries

148   and OVOL1, OVOL2 and OVOL3 for the L-ovaries.

149

150   **_2.3._**   **_miRNA-seq and bioinformatic data analysis_**

151   Preparation of libraries and bioinformatic analysis were performed as previous

152   described [34]. Briefly, libraries were constructed from 5 µL of total RNA from each

153   sample using the TruSeq Small RNA kit (Illumina, CA, USA) with some

154   modifications, as follows: to minimize primer–dimer formation, total RNA was mixed

155   with half the TruSeq Small RNA sample reagents, followed by 15 cycles of

156   polymerase chain reaction (PCR) to amplify the library. A 5 µL aliquot of each unique

157   indexed library was pooled in 6-plex and resolved on a Pippin gel cassette 3%

158   Agarose Dye free (BluPippin; Sage Science, MA, USA). Library RNA fragments in

159   the 140- to 160-nucleotide (nt) size range (the length of miRNA inserts plus the 30

160   and 50 adaptors) were recovered in 40 µL Pippin elution buffer and then purified

161   using the MinElute PCR purification kit (Qiagen, Germany, Europe). The indexed

162   libraries were quantified in triplicate on an ABI9700 Real time PCR instrument

163   (Applied Biosystems, CA, USA) using the KAPA Library Quantification Kit (Sigma

164   Aldrich, Italy, Europe) according to the manufacturer's instructions. Then, 10 µL of

165   the pooled libraries, at a final concentration of 2 nM, were used for sequencing on an

166   Illumina HiSeq 2000 using a 50-bp single-read sequencing protocol. Finally, 6

167   libraries from follicular fluids (FFH1-3 and FFL1-3) and 6 from oocytes samples

168   (OVOH1-3 and OVOL1-3) were sequenced into two different lanes of an Illumina

169   HiSeq2000 flow cell.

170   After sequencing, the quality of Illumina sequences was assessed using FastQC

171   v0.11.2 (Babraham Bioinformatics). The Illumina sequences were trimmed using

172 Trimmomatic software (RWTH Aachen University, Institute for Biology) to remove

173 sequences of adapters. MiRNA sequences passing the quality threshold were

174 annotated using miR-Deep2 software [35]. Bovine miRNAs available at mirBase

175 (http://www.mirbase.org/) were used to identify known miRNA sequences. Human

176 miRNAs that were annotated in miRBase were used to support the annotation of

177 novel bovine miRNAs. The relative abundance of mapped miRNAs was expressed

178 as read counts and the raw counts were analyzed using the EdgeR package of R

179 software [36]. A general linear model was used in the Bioconductor EdgeR package

180 to generate lists of miRNAs with statistically significant different expression levels

181 between sample groups. EdgeR uses negative binomial-based models to assess the

182 quadratic mean–variance relationship observed in sequencing data and to

183 distinguish between biological and technical sources of variation. MiRNAs exhibiting

184 an adjusted P (*i.e.* False Discovery Rate, FDR) < 0.1 and $\log_2$ fold change (logFC)

185 >1 between H- and L-ovaries were considered to be differentially expressed (DE).

186 ### *2.4. MiRNA target prediction and gene enrichment analysis*

187 Gene targets of the DE miRNAs were predicted. These gene targets were further

188 analyzed to identify the most enriched Kyoto Encyclopaedia of Genes and Genomes

189 (KEGG) pathways and Gene Ontology (GO) terms to determine the biological

190 processes that were putatively affected in follicular fluids and oocytes of the L-

191 ovaries.

192 DE follicular fluid miRNAs were analyzed using DIANA miRPath v2.0. This software

193 was run using homologous human miRNA and gene union options. DIANA miRPath

194 predicts miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-

195 CDS algorithm, or experimentally validated miRNA interactions derived from DIANA-

196 TarBase v6.0 [37]. This information is used by DIANA miRPath to identify the KEGG

197 pathways enriched with the gene targets.

198 DE oocyte miRNAs were analyzed using miRWalk 2.0, which is a web-based

199 database capable of predicting miRNA-gene target interactions [38]. In this study,

200 the gene targets were taken into account only when they were predicted by all the

201 following software: miRanda [39], PicTar2 [40], PITA [41], RNA22v2 [42],

202 RNAhybrid2.1 [43] Targetscan6.2 [44]. Then, the lists of gene targets were imported to

203 DAVID Bioinformatics systems in order to run KEGG pathway and GO analyses [45].

2.5. Validation of candidate miRNAs by Real time PCR

205 A selection of DE miRNAs identified by deep sequencing was validated using real

206 time quantitative PCR (RT-qPCR validation), as follows: bta-miR-10a, bta-miR-150

207 and bta-miR-240 for follicular fluids; bta-miR-145, bta-miR-450b for oocytes.

208 Furthermore, bta-miR-10a and bta-miR-150 were analysed in granulosa cells to

209 investigate whether the expression of these DE follicular fluid miRNAs was

210 correlated with that of granulosa cells. We used the locked nucleic acids (LNA)

211 miRNA PCR system (Qiagen, USA), which is based on the use of miRNA-specific,

212 LNA-based primers designed for sensitive and accurate detection of miRNA by RT-

213 qPCR using sybr green. The list of selected miRNAs including indication of the

214 miRCURY LNA miRNA PCR assays used for RT-qPCR is reported (see

215 supplementary table S1). Briefly, total RNA was extracted from pools of 10 oocytes

216 using the miRCURY RNA Isolation Kit (Exiqon, Denmark, Europe) following the

217 manufacturer's protocol. Total RNA of granulosa cell samples was extracted using

218 Trizol Reagent (Invitrogen, Life Technologies, CA, USA) as described above, and

219 small RNA samples from follicular fluid were obtained using the NucleoSpin miRNA

220 Plasma Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany, Europe).

221   Complementary DNA (cDNA) was obtained using a miRCURY LNA, Universal cDNA

222   Synthesis Kit II (Exiqon, Denmark, Europe). Each reaction was run in 10 µL: 2 µL

223   RNA sample (5ng/µL), 2 µL 5x buffer, 4.5 µL RNAse-free water, 0.5 µL UniSp6 RNA

224   spike-in (Exiqon, Denmark, Europe) and 1 µL enzyme mix. The reaction was

225   incubated at 42°C for 1 h and 95 °C for 5 min before cooling at 4°C. The cDNA

226   samples were stored at −20 °C until qPCR analysis, which was performed on a Bio-

227   Rad CFX96™ Real time machine (Bio-Rad, Hercules, CA). Each PCR reaction was

228   performed in triplicate using 5 µL 2X ExiLENT SYBR Green Master Mix (Exiqon,

229   Denmark, Europe), 1 µL primers (Exiqon, Denmark, Europe) and 4 µL cDNA sample.

230   The qPCR program was: 95 °C for 10 min for first cycle followed by 40 cycles of

231   amplification at 95 °C for 10 s and 60 °C for 1 min. Melting curve analysis was run

232   for each experiment to assess the specificity of the primer amplification. Relative

233   gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [46]. In this study we

234   observed that the expression of bta-miR-10b and bta-miR-27b was consistent and

235   not different between each sample of follicular fluids and oocytes of both categories

236   after deep sequencing and therefore we used them as housekeeping miRNA in all

237   our analysis. The Ct values of these miRNAs were indeed not different between

238   follicular fluid, oocyte and granulosa cell samples of both categories, confirming that

239   bta-miR-10b and bta-miR-27b were valid candidate for normalization of RT-qPCR

240   data (supplementary Figure S1). Results were reported as mean ± S.E.M. unless

241   specified. Differences were considered significant for $P < 0.05$ after student's t-test

242   analysis (SPSS 19.1; IBM).

243   ***2.6.   Determination of mitochondrial activity in the oocytes***

244   Mitochondrial activity was analyzed using MitoTracker FM Green (MTG) (Invitrogen,

245   Life Technologies, CA, USA), which allows the analysis of relative mitochondrial

246   activity by staining all the mitochondria without taking into account the membrane

247   potential ($\Delta\psi$, MMP), and MitoTracker Orange CMTMRos (MTO), which stains only

248   active mitochondria with a high MMP value.

249   Briefly, oocytes were incubated in 280 nM MTG and 200 nM MTO in PBS with 0.4%

250   BSA (Sigma Aldrich, Italy, Europe) for 30 minutes at 38.8°C in an atmosphere of 5%

251   CO_2 in air and maximum humidity. After the staining, oocytes were washed three

252   times in PBS and 0.1% polyvinylpyrrolidone (PVP), mounted on slides using PBS

253   and 300 nM DAPI (Sigma Aldrich, Italy, Europe) and immediately observed using an

254   epifluorescence microscope (Axio scope A1; ZEISS, Germany, Europe) at the

255   specific wavelengths for MTG (488 nm), MTO (546 nm) and DAPI (380 nm) dyes

256   (Figure 4A).

257   Quantification of fluorescence was carried out using NIH ImageJ free software [47].

258   Results are expressed as the average of MTO/MTG Ratio per oocyte in arbitrary

259   units. All experiments were repeated 5 times on oocytes from ovaries collected on

260   different slaughter days. In total, 26 oocytes of the L-ovaries and 29 of the H-ovaries

261   were analyzed. Differences were considered significant for $P < 0.05$ after student's t-

262   test analysis.

263   **3. Results**

264   ***3.1. miRNA deep sequencing data***

265   A mean of 13.7 x 10^6 (95.6 %) and 16.5 x 10^6 (95.7 %) reads of the L and H follicular

266   fluids, respectively, were selected after filtering (Table S2A, supplementary material)

267   together with a mean of 75.2 x 10^6 (94.8 %) and 51.3 x 10^6 (91.8 %) reads of the L

268   and H oocytes, respectively (Tables S2B, supplementary material). After annotation

269   of known miRNA sequences, a mean of 285,375 and 290,714 sequences were

270   mapped to known bovine miRNAs for L and H follicular fluids, respectively,

11

271 comprising 3.3% and 1.9% of the total quality reads obtained. A mean of 329,575

272 (0.5%) and 328,237 (0.7%) of L and H oocyte reads, respectively, matched known

273 bovine miRNA sequences. The most abundant length of all miRNA sequences in all

274 the samples was 23 nucleotides (nt) for both follicular fluid and oocyte miRNAs of L-

275 and H-ovaries, which corresponds to mature miRNA size (Figures S2A and S2B,

276 supplementary material). Raw data counts of miRNA deep sequencing data from

277 both follicular fluid and oocyte samples are available in supplementary materials

278 (Table S3 and S4)

279 **_3.2. MiRNAs expressed in follicular fluids and oocytes_**

280 A total of 1236 unique bovine and homologous human miRNA sequences were

281 identified after annotation and discovery from follicular fluid and oocyte samples.

282 The mean number of expressed follicular fluid miRNAs was similar between L- and

283 H-ovaries: 497 ± 85 in the L- ovaries and 520 ± 22 in the H-ovaries ($P > 0.05$, Table

284 1), of which 203 were in common. In contrast the mean number of expressed

285 miRNAs in oocytes was higher in the L-ovaries compared with the H-ovaries: 373 ±

286 12 vs 285 ± 36 ($P < 0.05$), respectively, of these 301 were in common between both

287 L- and H-ovaries. Furthermore, follicular fluids and oocytes of H-ovaries had a

288 different mean number of expressed miRNAs ($P<0.0001$), whereas follicular fluids

289 and oocytes of L-ovaries had a similar number of miRNAs ($P > 0.05$, Table1; Figure

290 1).

291 Bta-miR-10b, bta-miR-27b, bta-miR-143 and bta-miR-22 were the most abundant

292 miRNAs expressed in follicular fluids of both L- and H-ovaries. Bta-miR-10b had the

293 highest level of expression as measured by the 3.0×10^6 reads in L- and the $1.5 \times$

294 10^6 reads in H-ovaries, accounting for 50.1% and 39.2% of the sequence reads

295 aligned to known miRNAs, respectively (Table 1; Table S5, supplementary material).

296   Bta-miR-10b, bta-miR-92a and the human homologous hsa-miR-6509-3p had the

297   highest level of expression in the oocytes of L- and H-ovaries (Table1; Table S6,

298   supplementary material). Bta-miR-10b was the most abundant miRNA with a read

299   count of 1.7×10^6 in L and 758,461 in H-ovaries, which represented the 32.3 % and

300   66.4 % of the mapped reads to miRNA sequences, respectively (Table 1; able S6,

301   supplementary material). Moreover, the miRNA homologous to hsa-miR-6509-3p

302   and hsa-miR-513c-5p were abundantly expressed in the oocytes of both H- and L-

303   ovaries (Table 1; Table S6, supplementary material). In particular, the reads of hsa-

304   miR-6509-3p accounted the 8.5 % and 9.7 % in L and H-ovaries, respectively, while

305   those of hsa-miR-513c-5p were represented by 1.0 % in L and 1.2 % in H-ovaries

306   (Table 1; Table S6, supplementary material).

307   Among the top 10 abundantly expressed miRNAs, four (bta-miR-10b, bta-miR-423,

308   bta-miR-22 and bta-miR-148) were highly expressed in both follicular fluids and

309   oocytes of L and H-ovaries (Table 1; Table S5 and S6, supplementary material).

310   ### 3.3. Differential expression of follicular fluid miRNAs

311   In total, 58 miRNAs were found to be differentially expressed in the follicular fluids; of

312   these, 45 were reduced and 13 were increased in the L compared with the H-ovaries

313   (logFC > +/- 1; $P \leq 0.05$, FDR$\leq$ 0.1; see Table S7, supplementary materials). Some

314   of these miRNAs (miR-450a and miR-450b, miR-99a and let-7c, miR-24-3p and miR-

315   195 and miR-30c, miR-18a and miR-92a) were clustered on the same chromosomal

316   region. The Log$_2$ fold change values between L and H-ovaries ranged from -4.11

317   (bta-miR-150) up to 2.71 (bta-miR-885).

318   ### 3.4. Differential expression of oocyte miRNAs

319   In total, 6 miRNAs were differentially expressed between oocytes of L and H-ovaries

320   (logFC > +/-1; $P \leq 0.05$ and FDR $\leq$ 0.1; see Table S8, supplementary materials): 5

321   (miR-145, miR-150, miR-342, miR-450b and miR-380) were more abundant in the L

322   vs H-ovaries while the opposite was observed for bta-miR-10a. Three of the

323   differentially expressed miRNAs in the oocytes (miR-10a, miR-150 and miR-450b)

324   were also differentially expressed in the follicular fluids, but with an inverse pattern

325   within the same category meaning that a miRNA that was more abundant in the

326   oocyte was less abundant in the follicular fluid and viceversa. The Log_2 fold change

327   values between L- and H-ovaries ranged from -1.62 (bta-miR-10a) up to 9.51 (bta-

328   miR-145).

329

330   ***3.5. KEGG pathways enriched by differentially expressed miRNAs in follicular***

331   ***fluids***

332   GO and KEGG pathway analyses of the gene targets of the first 25 differentially

333   expressed miRNAs of follicular fluid samples identified a total of 53 KEGG pathways

334   ($P < 0.005$) that were enriched by these miRNAs (see Table S9, supplementary

335   material). Among these KEGG pathways, there were several canonical pathways,

336   which were associated with cellular processes, including: cell adhesion (Focal

337   adhesion signaling pathway), cell proliferation (Cell cycle, MAPK, RNA transport,

338   Wnt, mTOR, PI3K-Akt and ErbB signaling pathways), cell survival and growth

339   (Neurotrophin signaling pathway, Oocyte meiosis, TGF-beta signaling pathway) and

340   cell metabolism (Insulin signaling pathway).

341   The gene targets in the 10 most enriched KEGG pathways were further examined by

342   GO analysis. There were 148 genes in these signaling pathways that are

343   predominantly involved in biological processes which included cell cycle regulation,

344   cell proliferation, apoptosis, post-translational modifications, macromolecule

345 biosynthesis and cell migration (Table S10, supplementary material). Among these

346 148 genes, 126 (85.1%) are known to be involved in ovarian follicle development.

347 ***3.6. Gene ontology and pathways enriched by differentially expressed miRNAs***

348 ***in oocytes***

349 KEGG pathway analysis identified 36 signaling pathways for which expression is

350 likely to be significantly reduced because of the higher expression of miR-145, miR-

351 150, miR-342, miR-450b and miR-380 in the oocytes of L-ovaries (Figure 2). In

352 contrast, 18 signaling pathways were found to be associated with a lower expression

353 of miR-10a in the oocytes of L-ovaries.

354 The enriched GO terms of the biological processes that were highly repressed were:

355 cell proliferation, RNA transport and localization, catabolic modification of

356 macromolecules and response to hormone stimulus (Table S11, supplementary

357 material). In contrast, GO terms that were repressed by bta-miR-10a in the oocytes

358 of the L-ovaries, were mainly related to mechanisms involving RNA transcription

359 (Table S12, supplementary material).

360 **3.7. Quality validation of experimental data**

361 *3.7.1. Differential oocyte quality*

362 As detailed above the number of 2-5 mm follicle on a single ovary is a robust

363 parameter for predicting bovine oocyte developmental competence. However, to

364 confirm that the two groups of oocytes and follicles used in our experiments had

365 indeed a different competence we measured two further specific markers.

366 One was mitochondrial activity whose reduced level has been correlated with lower

367 oocyte competence not only in cows [68, 69, 70] but also in many species including

368 pigs [61, 62] and women [64]. We assessed whether mitochondrial function was

369 correlated with the different miRNA expression between oocytes of L- and H-ovaries.

15

370   We found that oocytes of L-ovaries had a lower MTO/MTG ratio than those of H-

371   ovaries: 1.0 ± 0.1 versus 1.4 ± 0.1, respectively ($P < 0.05$, figure 3 A-B).

372   Furthermore, we measured the follicular fluid concentration of P4 that it is known to

373   be higher in follicles of L-ovaries [29]. Our measurement showed that indeed P4

374   concentration was higher in follicular fluid of L than H-ovaries: (82.9 ± 7.0 versus

375   53.8 ± 7.6, respectively ($P < 0.05$) figure 3C).

376   These findings confirm the notion that follicle count on individual ovaries is a reliable

377   marker of oocyte quality.

378

379   *3.7.2. Deep sequencing data quality*

380   To confirm the quality of deep sequencing data we randomly selected three DE

381   miRNAs in follicular fluid and 2 in oocytes.

382   In the follicular fluid, RT-qPCR validated the differential expression of bta-miR-10a

383   and bta-miR-150, but not of bta-miR-240. However, we did not find a correlation

384   between follicular fluid and granulosa cell expression levels of these miRNAs.

385   In the oocytes, the expression of bta-miR-145 and bta-miR-450b was consistent with

386   that of deep sequencing data. (Figure 4 A-C). Overall, these results confirmed the

387   differential expression of the selected miRNAs by RT-qPCR in both follicular fluid

388   and oocyte samples, demonstrating the reliability of data obtained by deep

389   sequencing.

390   4. **Discussion**

391   Several differences of miRNAs expression levels have been described along follicle

392   development both in cattle [10, 22, 23, 26, 27, 48, 49] and humans [16-18, 50], but

393   no attempts to correlate miRNA expression with oocyte developmental competence

394   has been performed. On the basis of previous studies demonstrating that the

16

395 number of 2-5 mm follicles is a reliable parameter for predicting oocyte

396 developmental potential and quality [2, 29, 31], we were able to determine that

397 follicular fluid and oocyte miRNA expression pattern is correlated with oocyte

398 developmental competence. This result broadens our knowledge on the molecular

399 pathways that make a bovine oocyte capable to support full embryonic development.

400 Previous studies indicated that bovine miR-769, miR-1343, miR-450a, miR-204,

401 miR-1271 and miR-451 have an important functional role in follicle development

402 because their level increases during the transition from the subordinate to the

403 preovulatory stage [27]. This observation is consistent with our finding that these

404 miRNAs are found at higher levels in the follicular fluid of H-ovaries and suggests

405 that their expression is beneficial for the acquisition of oocyte developmental

406 compentence. Furthermore, we observed that, while miR-92a level was lower in L-

407 ovaries, that of miR-100 was higher. This observation confirms that not only lower

408 but also higher levels of specific miRNAs are correlated with low ovarian reserve as

409 previously described in human patients and, interestingly, the same two miRNAs

410 were involved [65].

411 The analysis of miRNA levels in the oocytes indicated that also in this compartment

412 too much of some miRNA has a negative impact. In fact, miR-150 was present at

413 higher levels in low competence oocytes consistently with the previous observation

414 that miR-150 level decreases during bovine oocytes maturation [51]. We observed

415 that also miR-145 is more abundant in low quality oocytes in agreement with a

416 previous report indicating that the increased expression of this miRNA has a

417 negative impact on the growth of mouse primordial follicles [52]. Taken together,

418 these results suggest that miRNAs are required at specific thresholds of expression

419 and levels that do not match such thresholds, in either way, can affect oocyte

17

420   developmental competence.

421   The possible effects on oocyte competence of the most differentially expressed

422   miRNAs present in follicular fluid were inferred analysing their gene targets and the

423   molecular pathways that these genes regulate. This analysis revealed that miRNA

424   pattern affects several genes in few signalling pathways.

425   In particular, we found that miR-204, miR-197, miR-146b, miR-30d and miR-383

426   whose levels in the follicular fluid were lower in L- than in H-ovaries, are predicted to

427   down-regulate several genes belonging to the PI3K-Akt signalling pathway that

428   control growth, differentiation and survival of the ovarian follicle [53, 54]. Their

429   targets include: BCL2 apoptosis regulator (*BCL2*), forkhead box O3 (*FOXO3*), tumor

430   protein p53 (*TP53*), vascular endothelial growth factor A (*VEGFA*) and KIT proto-

431   oncogene receptor tyrosine kinase (*KIT*). Therefore, a lower level of these miRNAs is

432   expected to determine an up-regulation of all these genes which would be consistent

433   with the reduced competence of these oocytes. However, since early atresia is

434   associated with high oocyte developmental competence in cattle [71, 72, 73] this

435   suggests that the degree of apopoptosis and disrupted vascularisation in L-ovaries

436   must have exceded the levels typical of ealy atretic follicles.

437   On the contrary, levels of miR-24, miR-10a and miR-320a were higher in the

438   follicular fluid of L- than of H-ovaries. These miRNAs are predicted to down-regulate

439   the neurotrophin signalling pathway that supports follicle formation and development

440   in different species including rodents, sheep, cows, nonhuman primates and humans

441   [55]. They also downregulate the TGF-beta (TGF-β) signalling pathways that controls

442   the production of ovarian peptide hormones, including Antimüllerian hormone [56].

443   TGF-β also modulates transforming growth factor beta 1 and bone morphogenetic

444   protein expression during follicle development from the primordial to the late

18

445   secondary stage in human [57]. Therefore, our results identified a number of

446   miRNAs present in the follicular fluid that have a possible functional role in follicle

447   development and acquisition of oocyte competence.

448   In the oocytes, we found that the differential expression was confined to 6 miRNAs.

449   Levels of miR-145, miR-150, miR-342, miR-450b, miR-380 were higher in low

450   competence oocytes while miR-10a levels were lower. Whereas miR-342 and miR-

451   380 are not differentially expressed in the follicular fluid the others were differentially

452   expressed in both compartements. It is interesting to note, however, that their levels

453   had opposite direction: i.e. when the level was higher in follicular fluid it was lower in

454   the oocyte and viceversa. A lack of correlation was also observed between the

455   miRNA levels of follicular fluid and granulosa cells. This suggests that, in these

456   districts, miRNAs are vehiculated by exosomes and therefore cannot simply flow

457   among different follicle compartments [11, 66].

458   The miRNAs that regulate RNA synthesis, RNA translation and RNA transport are

459   up-regulated in L-ovaries oocytes and, therefore, the associated processes are

460   perturbed. These processes are associated with the accumulation of maternal RNAs

461   that occurs during oocyte growth and is linked to the acquisition of oocyte

462   competence [5]. We previously demonstrated that polyadenylation levels of maternal

463   mRNAs are critical for a correct maternal to embryonic transition and that

464   polyadenylation is reduced in oocytes from L-ovaries [2]. Furthermore, we also

465   observed that the misregulation of maternal mRNA polyadenilation correlates with

466   the defective embryo development to the blastocyst stage [58]. Therefore, these

467   observations suppot the hypothesis that the differential expression of miRNAs

468   involved in RNA metabolism is functionally relevant.

469   Interestingly, we showed that oocytes obtained from L-ovaries had a lower

470 mitochondrial activity, a parameter that is considered a reliable indicator of poor

471 oocyte quality [59, 60, 63, 64]. This is a retrospective confirmation that antral follicle

472 count is indeed a dependable parameter for predicting oocyte quality. Consistent

473 with these observations, the expression of *BCL2,* which is one of the predicted target

474 genes of differentially expressed miRNAs in the oocyte, has been correlated with low

475 mitochondrial activity in poor quality porcine oocytes [61, 62]. Furthermore, the

476 alteration of mitochondrial function has been associated with aberrations of

477 chromosomal alignment in mouse and humans aged oocytes [63].

478 In conclusion, our data identified a few miRNAs in the follicular fluid and in the

479 ooplasm that modulate oocyte developmental competence. The converging

480 evidences coming from different aspects of follicular and oocyte physiology together

481 with the RT-PCR validation of 4 out of 5 miRNAs support the functional meaning of

482 our findings. However, a more extensive validation would further strengthen our

483 data.

484 The results confirm and extend previous finding that demonstrated the role of

485 miRNAs during follicle development and oocyte growth [27, 51, 52] and could help to

486 better understand the mechanisms leading to oocyte competence in cattle.

487 Finally, since bovine oogenesis is a good model for human reproduction, these

488 findings can be relevant also for our own species since low antral follicle count is a

489 common cause of age-related infertility.

Conflicts of Interest

The authors declare no conflicts of interest.

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719   **Table 1.** Summary of miRNA deep sequencing data.

720

miRNA expression	H-ovaries*	L-ovaries*
Follicular fluids (Mean ±SEM)	520 ± 22	497 ± 85
Oocytes (Mean ±SEM)	285 ± 36	373 ± 12
Abundant miRNAs in the follicular fluids	bta-miR-10b, bta-miR-27b, bta-miR-143, bta-miR-22	
Abundant miRNAs in the oocytes	bta-miR-10b, bta-miR-92a, hsa-miR-6509-3p, hsa-miR-513-5p, bta-miR-27b	
Abundant miRNAs in the follicular fluids and oocytes	bta-miR-10b, bta- miR-423, bta-miR-22, bta- miR-148	

721

722   * = number of miRNA molecules expressed for each category.

31

Figure legends

Figure 1. Venn diagram. Comparison of miRNAs that were expressed in the follicular fluid and oocyte samples of high (H) and low (L) antral follicle count ovaries after annotation and discovery of miRNA sequences using bovine and human miRNome.

Figure 2. Venn diagram. Comparison of the signaling pathways that were differentially regulated by the highly (up regulated) and lowly (down regulated) expressed miRNAs of bovine oocytes of high (H) and low (L) antral follicle count ovaries.

Figure 3. A) Images of oocytes obtained from high (H) and low (L) antral follicle count ovaries after the simultaneous staining using mitoTracker Orange CMTMRos (MTO = red), MitoTracker FM Green (MTG = green) and DNA (DAPI = blue), scale bar = 50 μm; B) Red/Green (i.e. MTO/MTG) ratio was calculated to assess differences of mitochondrial membrane potential (MMP) between oocytes of high (H) and how (L) antral follicle count ovaries. Results are represented as mean value ± S.E.M.

Figure 4. Validation of selected miRNAs of follicular fluid and oocyte samples that were differentially expressed after deep sequencing of high (H) and how (L) antral follicle count ovaries. Validation was run using relative gene expression (qPCR) analysis of: (A) bta-miR-145 and bta-miR-450b of the oocyte; B) bta-miR-10a, bta-bta-miR-150 and bta-miR-204 of the follicular fluids; C) bta-miR-10a and bta-miR-150 of the granulosa cells. Results are reported as mean ± S.E.M. and the different superscripts indicate statistical difference (P < 0.05).
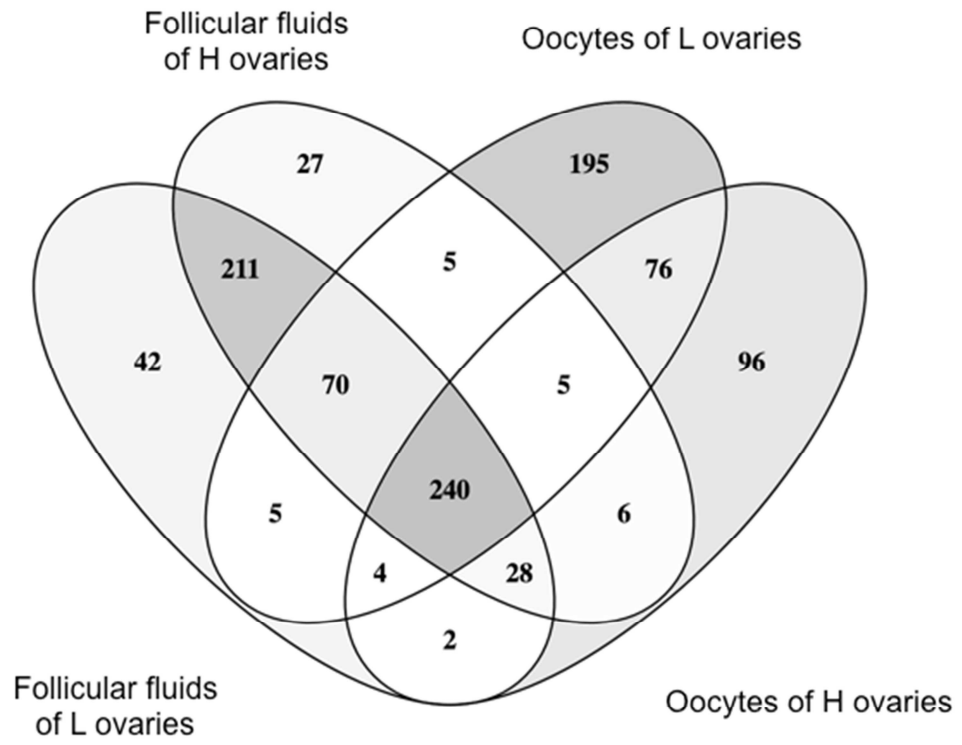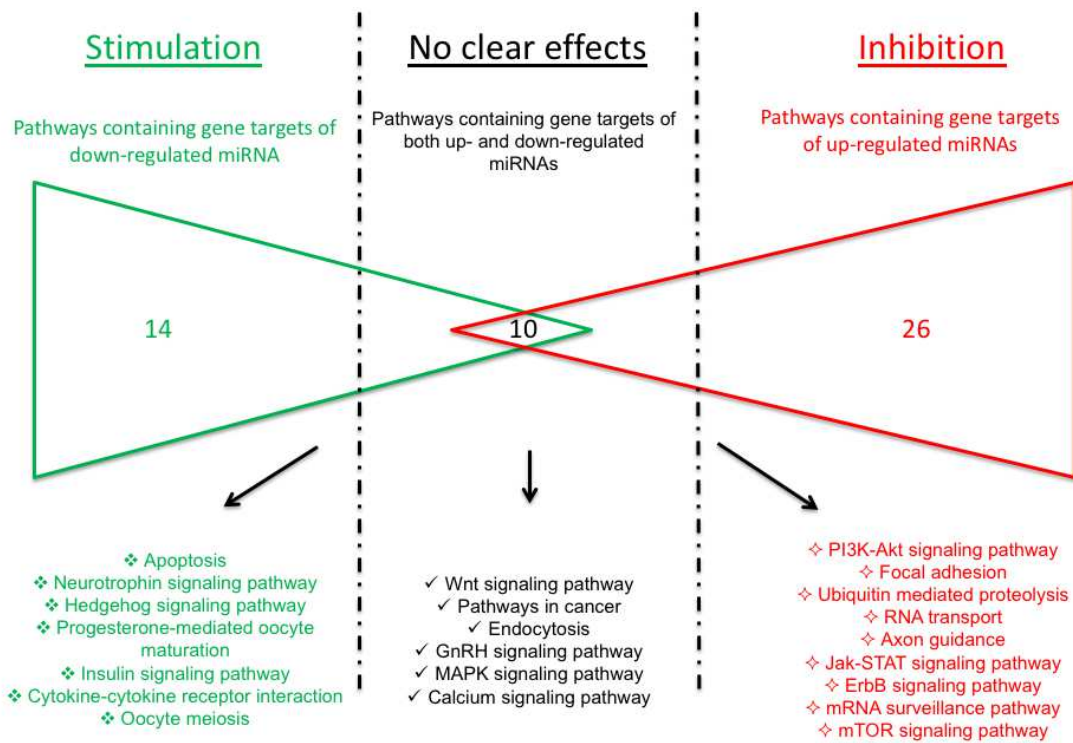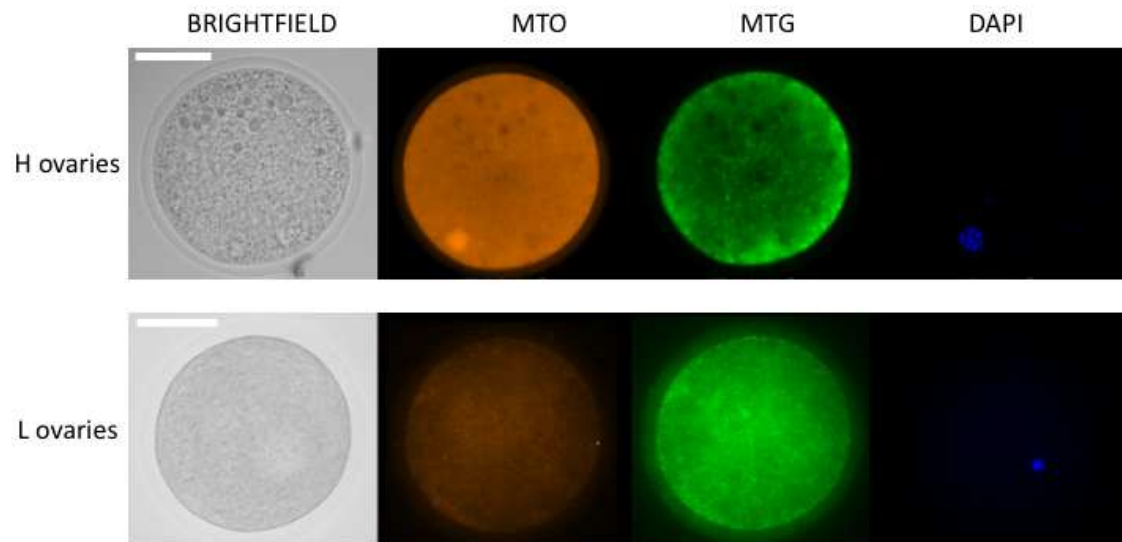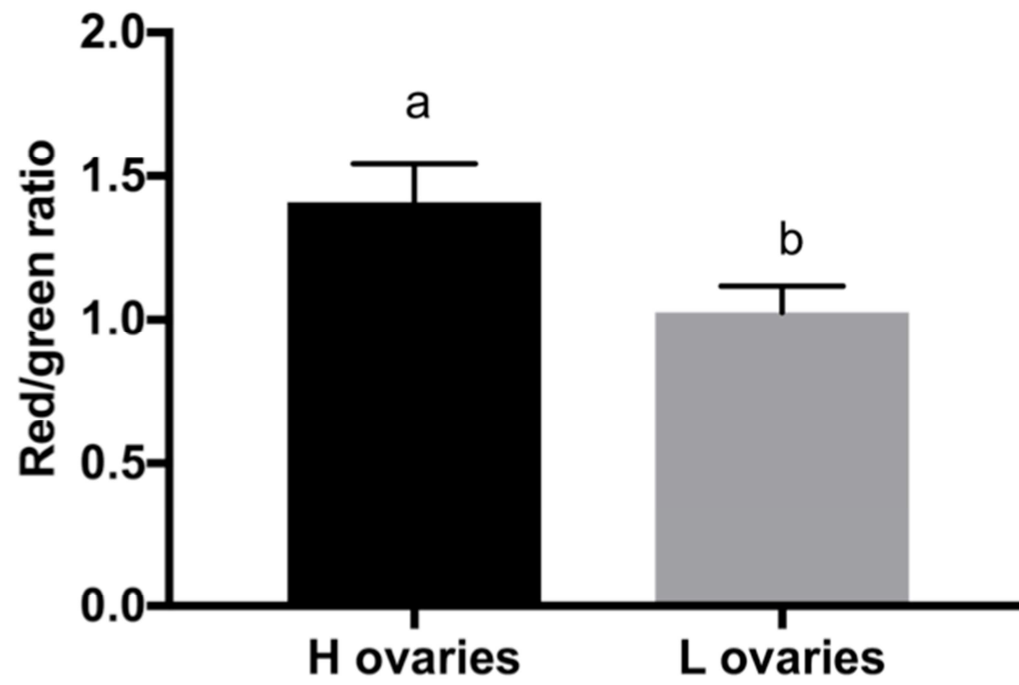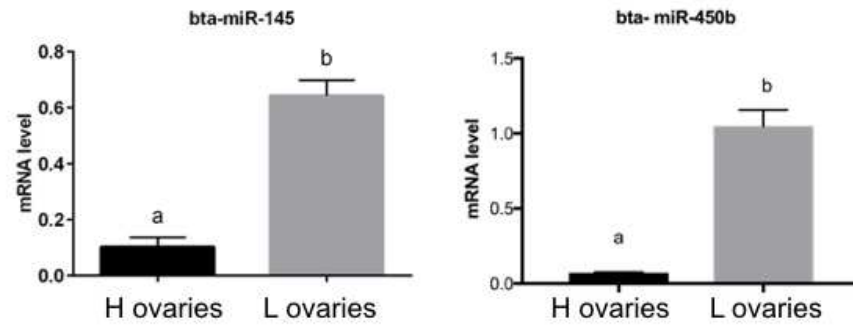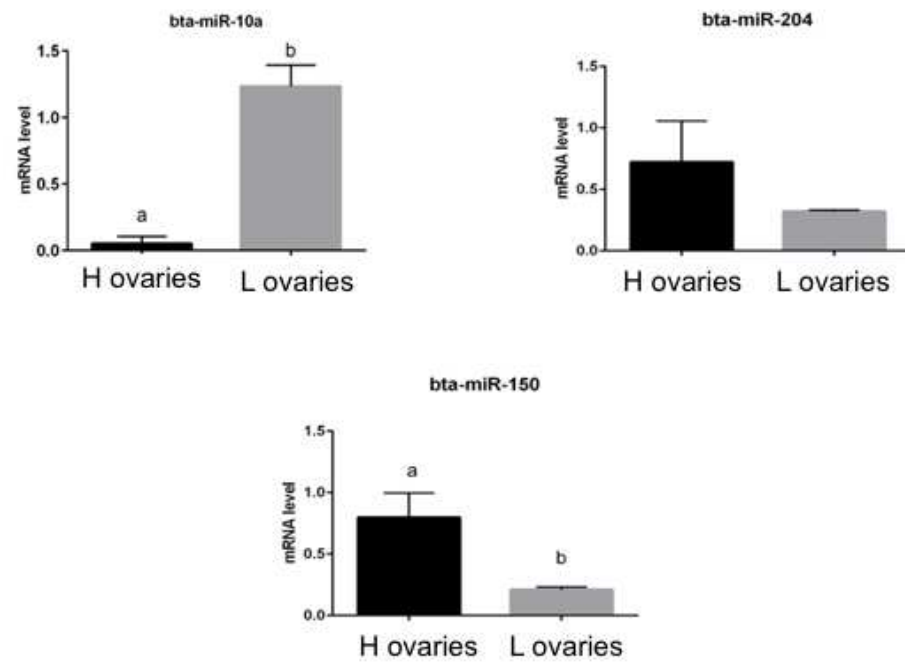
Fig. 1.

Fig. 2.

A

Fig. 3.

B
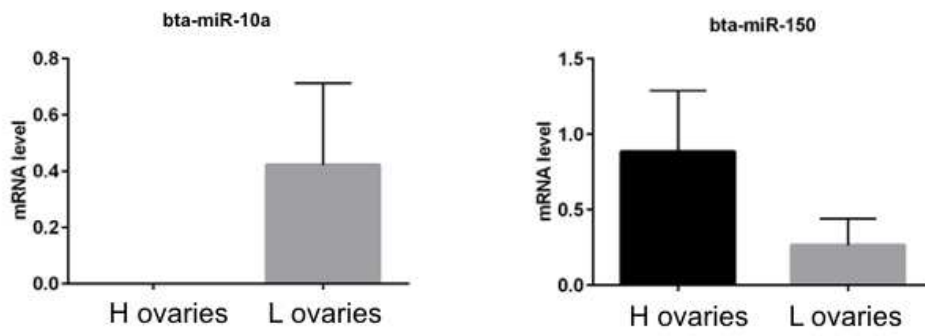

Fig. 3.

A

Fig. 4.

37

B

Fig. 4.

C

Fig. 4.

2. Materials and methods

2.7. Quantification of Progesterone in the follicular fluid

Quantitative analysis of progesterone (P4; sensitivity limit 0.5 ng/mL) was carried out as previous work [74] using a competitive enzyme immunoassay using an in-house produced anti-P4 monoclonal antibody as capture antibody and progesterone-11-HS-HRP (Fitzgerald Industries Inter- national, Concord, MA) as labeled hormone. A total of 5 biological replicates of follicular fluids from the H and L groups that were collected on different days of ovary collection were quantified.

References

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[74] Borromeo V, Berrini A, De Grandi F, Cremonesi F, Fiandanese N, Pocar P, Secchi C. A novel monoclonal antibody-based enzyme-linked immunosorbent assay to determine luteinizing hormone in bovine plasma. Domest Anim Endocrinol. 2014 Jul;48:145-57. doi: 10.1016/j.domaniend.2014.03.004. Epub 2014 Apr 5.

Figure legends

Figure 3. A) Images of oocytes obtained from high (H) and low (L) antral follicle count ovaries after the simultaneous staining using mitoTracker Orange CMTMRos (MTO = red), MitoTracker FM Green (MTG = green) and DNA (DAPI = blue), scale bar = 50 μm; B) Red/Green (i.e. MTO/MTG) ratio was calculated to assess differences of mitochondrial membrane potential (MMP) between oocytes of high (H) and low (L) antral follicle count ovaries. C) Progesterone (P4) quantification of follicular fluids from high (H) and low (L) antral follicle count ovaries. Results are represented as mean value ± S.E.M.
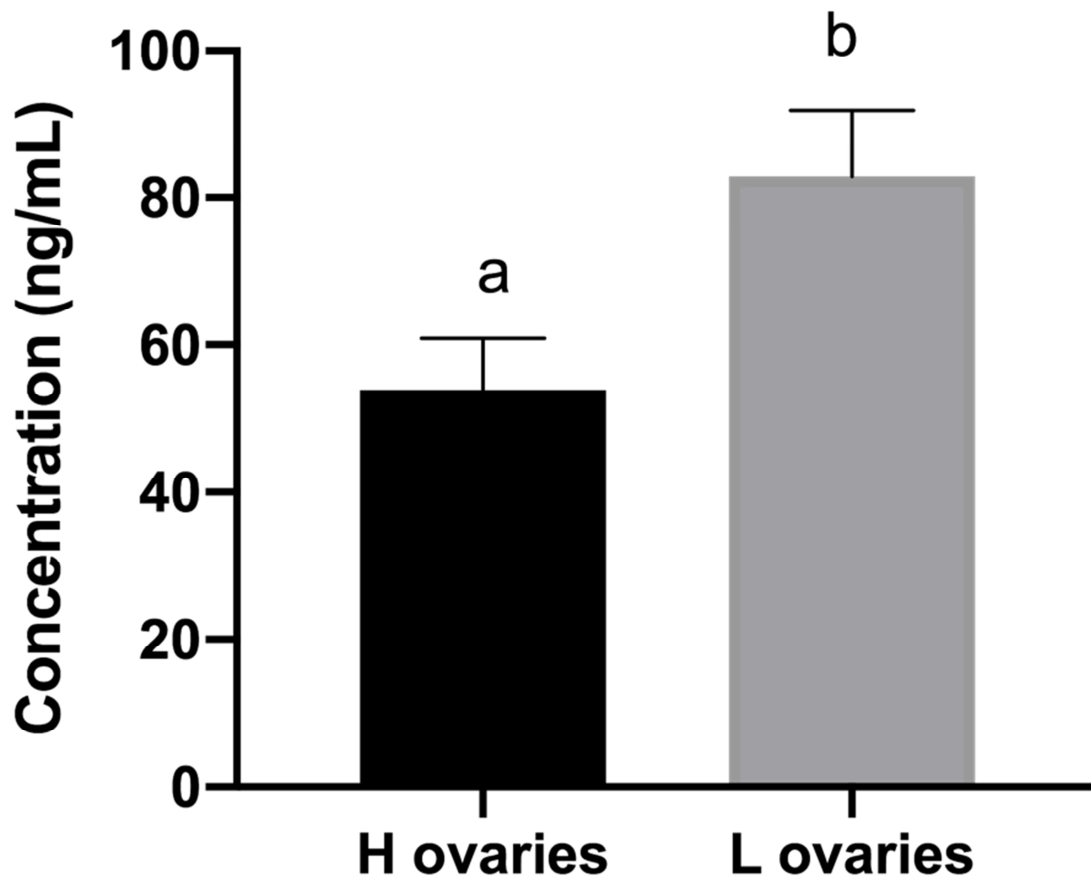
C

Fig. 3

Manuscript highlights

- Follicular fluid and oocyte microRNA expression patterns change between high and low antral follicle count ovaries and could impact developmental competence

- In low antral follicle count ovaries, changes in microRNA expression are correlated with higher progesterone concentration of follicular fluids and lower mitochondrial function of oocytes

- Maternal mRNA storage in the ooplasm could be regulated by specific microRNAs involved in controlling RNA synthesis, RNA translation, RNA transport and mRNA surveillance