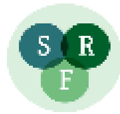

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MicroRNA-146b negatively affects bovine embryo development and quality

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1 # MicroRNA-146b negatively affects bovine embryo development and
2 quality

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32 **Abstract**

33

34 MicroRNAs (miRNAs), which can be carried inside extracellular vesicles (EVs), play a crucial role in
35 regulating embryo development up to the blastocyst stage. Yet, the molecular mechanisms underlying
36 blastocyst development and quality are largely unknown. Recently, our group identified 69 differentially
37 expressed miRNAs in extracellular vesicles (EVs) isolated from culture medium conditioned by bovine
38 embryos that either developed to the blastocyst stage or arrested (non-blastocysts). We found miR-
39 146b to be more abundant in the EVs derived from media conditioned by arrested embryos. Using RT-
40 qPCR, we here confirmed the upregulation of miR-146b in arrested embryos compared to blastocysts
41 ($p<0.005$), which coincides with the upregulation of miR-146b in EVs derived from the medium of these
42 non-blastocysts. To evaluate a functional effect, bovine embryo culture media were supplemented with
43 miR-146b mimics, resulting in significantly decreased embryo quality, with lower blastocyst rates at day
44 7 and lower total cell numbers, while the opposite was found after supplementation with miR-146b
45 inhibitors, which resulted in reduced apoptosis rates ($P < 0.01$). Transcriptomic analysis of embryos
46 treated with miR-146b mimics or inhibitors showed differential expression ($P < 0.01$) of genes associated
47 with apoptosis, cell differentiation, and the RNA Pol II transcription complex, including *WDR36, MBNL2,*
48 *ERCC6l2, PYGO1,* and *SNIP1*. Overall, miR-146b is overexpressed in non-blastocyst embryos and in
49 EVs secreted by these embryos, and it regulates genes involved in embryo development and apoptosis,
50 resulting in decreased embryo quality.

51

52 **Keywords:** Extracellular vesicles, miRNAs, embryo development, embryo quality

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63 **Introduction**
64
65 In the last decades, there has been considerable progress in the development of embryo culture media
66 for cattle (Eskew and Jungheim 2017, Gruber and Klein 2011), that allow extended development up to
67 the blastocyst stage (Blake, et al. 2007, Gardner and Balaban 2016). Almost 1.5 million bovine embryos
68 were produced globally in 2021, the majority of which were *in vitro* produced (IVP) (Joao et al., 2022).
69 About 90% of immature bovine oocytes go through nuclear maturation *in vitro*, and about 80% of
70 fertilized zygotes proceed through initial cleavage to reach the 2-cell stage. Only 30–40% of zygotes
71 develop to the blastocyst stage *in vitro* (Lonergan and Fair 2008, Pavani, et al. 2018, Wydooghe, et al.
72 2014), and 50% of the transferred blastocysts give rise to a pregnancy (Poppicht, et al. 2015), whereas
73 this is about 65 % for *in vivo* derived embryos (Hansen 2020). Here, it can be deduced that the quality
74 of IVP bovine blastocysts is intrinsically lower than that of *in vivo* derived embryos. In human embryo
75 culture, also about 30 to 50% of presumptive zygotes reach the blastocyst stage (Glujovsky, et al. 2022).
76 Implantation and ongoing pregnancy rates in human are higher with top grade blastocyst transfers,
77 whereas transfer of lower quality blastocysts is associated with more early pregnancy loss (Irani, et al.
78 2017). Early embryonic loss can therefore be overcome by only choosing those blastocysts with the
79 best potential for ongoing pregnancy after transfer (Perkel, et al. 2015). Underlying mechanisms
80 affecting blastocyst formation and quality remain largely unknown, although specific signaling factors
81 are likely to be involved and may be used as detector molecules for prediction of blastocyst quality.
82 Signaling factors such as growth factors, miRNAs, extracellular vesicles, morphogens, cytokines,
83 hormones, neurotransmitters, and extracellular matrix (ECM) molecules have been shown to modulate
84 embryo development (Wydooghe, et al. 2017). Throughout the early embryonic stages, microRNA
85 (miRNA) levels undergo dynamic changes (Goossens, et al. 2013, Mineno, et al. 2006, Tang, et al.
86 2007, Viswanathan, et al. 2009, Yang, et al. 2008), indicating their potential role in embryonic
87 development. MicroRNAs are endogenous 21-23 nucleotides non-coding RNAs, capable of controlling
88 gene expression through post-transcriptional regulation. MiRNAs are essential to many cellular and
89 biological processes within a cell and can be transferred between cells to serve as a mode of cell-cell
90 communication (Valadi, et al. 2007). Their structure and size allows them to remain within various
91 biological fluids (Larrea, et al. 2016, Turchinovich, et al. 2011). It has been shown before that free
92 circulating miRNAs are selectively secreted by human and bovine pre-implantation embryos into their
93 culture media and may regulate embryo development and quality (Kropp and Khatib 2015a, b, Kropp,

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94 et al. 2014, Rosenbluth, et al. 2014). We have identified earlier miR30c and miRNA10b in embryo
95 conditioned culture medium, as indicators of hampered preimplantation development (Lin, et al. 2019a,
96 Lin, et al. 2019b). To the best of our knowledge, only a few studies have been conducted on free
97 circulating miRNAs isolated from conditioned media (CM) generated by group cultured blastocysts or
98 degenerated embryos, and they suggested a link with embryo quality and development (Del Rio and
99 Madan 2021, Kropp, et al. 2014, Lin, et al. 2019b)
100 Apart from free circulating miRNAs, extracellular vesicles (EVs) also carry regulatory molecules,
101 including miRNAS as well as messenger RNAs (mRNAs), metabolites, lipids, and proteins (da Silveira,
102 et al. 2012, Pavani, et al. 2016, Simons and Raposo 2009). Given that the membrane of EVs shields
103 encapsulated cargo contents (miRNA, mRNAs) from RNases present in the media, these EVs may
104 contain biomarkers for competent embryos with high implantation potential (Pavani et al., 2018;
105 Dissanayake et al., 2021; Pavani et al., 2022). As such, EVs derived miRNAs are more reliable potential
106 biomarkers than free floating miRNAs (Théry, 2015; Vlaeminck-Guillem, 2018). We recently identified
107 21 known and 48 novel secreted differentially expressed (DE) miRNAs present in EVs isolated from
108 medium conditioned by individually cultured bovine embryos that either reached the blastocyst stage or
109 arrested (non-blastocysts). Of these miRNAs, miR-378a-3p had a significant regulatory impact on the
110 embryo's ability to hatch because it was abundant in EVs released by blastocysts and lacking in non-
111 blastocyst EVs. In this same study, we also identified miR-146b, which was more abundant in EVs
112 secreted by non-blastocysts, indicating that overexpression of miR-146b may be associated with a
113 negative influence on preimplantation embryo development in cattle (Pavani, et al. 2022). Previously,
114 miR-146b has been shown to regulate cell proliferation and apoptosis in bovine male germline stem
115 cells (Gao, et al. 2020).
116 Here, we analyzed the functionality of miR-146b by means of biological assays using inhibitors and
117 mimics for miR-146b during bovine embryo culture. As such, we demonstrate that miR-146b negatively
118 affects bovine embryo development and quality and alters the expression of genes involved in apoptosis
119 and embryo development.
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124 **Materials and Methods**

125

126 **Media and Reagents**

127 Tissue culture media (TCM)-199-medium, minimal essential medium (MEM) non-essential amino acids
128 (100×), synthetic basal medium eagle amino acids, gentamycin, and kanamycin were purchased from
129 Life Technologies Europe (Ghent, Belgium). Phosphate-Buffered Saline (PBS) was obtained from
130 Gibco™ (20012019, Thermo Fisher Scientific, ,Waltham, MA, USA). All other chemicals were obtained
131 from Sigma-Aldrich (Diegem, Belgium). All media were filtered before use (0.22 µM Pall Corporation,
132 Ann Arbor, MI, USA).

133

134 **MicroRNA functional analysis**

135 **Supplementing miR-146b mimics and inhibitors**

136 The objective of this study was to determine whether embryos can effectively internalize the miR-146b
137 present in culture media and use it to influence early embryo development. A summary of the research
138 method is provided in Fig. 1. Conventional *in vitro* techniques were used for producing bovine embryos,
139 as previously described by Wydooghe et al. (2014). Ovaries were procured from a slaughterhouse, and
140 follicular fluid was collected with syringe and needle from 4- to 8-mm-diameter follicles in order to obtain
141 immature cumulus oocyte complexes. Thereafter, 60 cumulus oocyte complexes were cultured in
142 groups of 500-µl of modified bicarbonate buffered TCM-199 (supplemented with 50 mg/mL gentamicin
143 and 20 ng/mL epidermal growth factor) in 5% CO_2 in air for 22 h at 38.5 °C. After 22 h, frozen–thawed
144 bovine spermatozoa were separated using a Percoll gradient (GE Healthcare Biosciences, Uppsala,
145 Sweden). The final sperm concentration for fertilization was 1×10^6 spermatozoa/ml IVF–TALP medium
146 enriched with BSA (Sigma A8806; 6 mg/mL) and heparin (20 µg/mL). After 22 h of maturation, bovine
147 oocytes were washed in 500 µL of IVF–TALP and subsequently co-incubated with bull spermatozoa.
148 After 21 h, presumed zygotes were moved to 50-µl drops or individual 20-µl of synthetic oviductal fluid
149 (SOF) supplemented with ITS (5 ng/ml selenium, 5 ng/ml insulin, and 4 mg/ml transferrin) and 4 mg/mL
150 BSA (Sigma A9647), based on the experimental design (as detailed in Fig. 1 A). MicroRNA mimics and
151 inhibitors with a unique, locked nucleic acid (LNA) enhanced, triple-RNA strand designed for mimicking
152 mature endogenous miR-146b, were purchased from Qiagen (Germantown, USA). These miR-146b
153 mimics and inhibitors (miRCURY LNA Power mimics and inhibitors, Product No. 339173, 339131,

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154 respectively) were supplemented to the culture medium of presumed zygotes (n = 1,155, 8 replicates)
155 with a final concentration of 1 μM. Culture occurred in groups of 25, covered with mineral oil at 38.5°C
156 in 5% CO_2, 5% O_2, and 90% N_2. In parallel, a negative control (NC) mimic or inhibitor (cel-mIR-39-3p,
157 labeled miRNA mimic and inhibitor, Product No. 339173, Qiagen) was supplemented to the culture
158 medium. Finally, a control group was included by adding an equal volume of RNA-free water to the
159 culture medium.
160 *In vitro* embryo culture was performed in two treatments (mimic and inhibitor). For the mimic treatment,
161 the presumed zygotes (n = 557, 4 replicates) were allocated to three different groups (control, NC mimic,
162 miR-146b mimic). Similarly, for the inhibitor treatment, the presumed zygotes (n = 598, 4 replicates)
163 were allocated to three different groups (control, NC inhibitor, miR-146b inhibitor). The presumed
164 zygotes were cultured individually in 20-μL droplets, or in groups of 25 in 50-μL droplets (control)
165 covered with paraffin oil (SAGE oil for tissue culture, ART-4008-5P, Cooper Surgical Company), at
166 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. Embryo development was evaluated on 7 days post
167 insemination (dpi) and 8 dpi. All blastocysts (8 dpi) were collected for differential apoptotic staining and
168 transcriptomic analysis. Additionally, on 8 dpi, some control embryos, cultured in group or individually,
169 were split into arrested (non-blastocyst) and blastocyst groups and stored in -80°C for RT-qPCR analysis
170 (as detailed in Fig. 1B).
171

172 **Differential Apoptotic Staining**

173 Differential apoptotic staining was performed using a previously described protocol (Wydooghe, et al.
174 2011). Briefly, for each group (around n = range of 15-19, 3 replicates), blastocysts were fixed in 4%
175 paraformaldehyde for 1 h and put in a 4-well dish in permeabilization solution (0.5% Triton X-100 C
176 0.05% Tween) in phosphate-buffered saline (PBS) at RT for 1 h. After washing the blastocysts 3 times
177 for 2 min in PBS-BSA, they were incubated in 2N HCl at RT for 20 min and then in 100 mM Tris–HCl at
178 RT for 10 min. The blastocysts were washed (3 times for 2 min) and then put into 500 ml of blocking
179 solution at 4°C overnight. On the second day, the blastocysts were washed again (3 times during 2 min)
180 and incubated in primary CDX-2 antibody (Biogenex, San Ramon, United States) at 4°C overnight. On
181 the third day, the blastocysts were washed twice for 15 min and subsequently incubated in a blocking
182 solution containing the rabbit active caspase-3 antibody (Cell Signaling Technology, Leiden,
183 Netherlands) overnight at 4°C. On day four, the blastocysts were incubated in a blocking solution

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184 containing the goat anti-mouse Texas Red antibody at RT for 1 h and were subsequently incubated in
185 a blocking solution containing the goat anti-rabbit FITC antibody at RT for 1 h. The blastocysts were
186 washed twice for 15 min and incubated at RT for 20 min in a dilution of 1: 200 Hoechst in PBS-BSA in
187 the dark. All slides were examined using a 63 × water immersion objective on a Leica TCS-SP8 X
188 confocal microscope. This staining protocol allowed instantaneous evaluation of three crucial
189 parameters of embryo quality, i.e., the total cell number (TCN), trophectoderm (TE), the proportion of
190 inner cell mass (ICM) relative to the TCN (ICM ratio), and the apoptotic cell ratio (ACR), defined as the
191 percentage of apoptotic cells relative to the TCN.
192
193 **Transcriptomics**
194 Five groups of blastocysts were collected on 8 dpi (control, NC mimic, NC inhibitor, miR-146b mimic,
195 miR-146b inhibitor)). For each group, total RNA was isolated from three pools of ten blastocysts using
196 the RNeasy Micro kit (Qiagen, Germantown, USA) according to the manufacturer's protocol. The quality
197 and concentration of the RNA samples were examined using an RNA 6000 Pico Chip (Agilent
198 Technologies, Carlsbad, CA, United States) and a Quant-iT RiboGreen RNA Assay kit (Life
199 Technologies, Carlsbad, CA, United States), respectively. Transcriptome library preparation was done
200 by the Qiaseq UPX 3' transcriptome kit (Qiagen) according to the manufacturer's instructions. The
201 quality of the library preparation was checked with a high sensitivity DNA chip (Agilent Technologies)
202 and library quantification was performed by qPCR according to the Illumina qPCR quantification protocol
203 (NXTGNT sequencing facility, Gent, Belgium), followed by equimolar pooling of libraries based on qPCR
204 (NextSeq500, Read 1: 57 cycles; read 2: 27 cycles and index 6 cycles). First, the raw reads were quality
205 and adapter trimmed with Trim Galore version 0.6.6 (https://github.com/FelixKrueger/TrimGalore). The
206 trimmed reads were mapped against the *Bos taurus* genome (ARS-UCD1.2, release 95) with STAR
207 version 2.7.6a (Dobin, et al. 2013). Unique Molecular Identifiers (UMIs) were used during the sequencing
208 to characterize the expression levels more accurately and were processed with UMI-tools version: 1.1.1
209 (Smith, et al. 2017). The RSEM software (version 1.3.1) was used to generate the count tables (Li and
210 Dewey 2011). Differential expression analysis between the groups of samples (control, NC mimic, NC
211 inhibitor, miR-146b mimic, miR-146b inhibitor) was performed with DESeq2 in a pairwise fashion (Love,
212 et al. 2015). Genes were considered differentially expressed if the Benjamini-Hochberg adjusted p-value
213 was ≤ 0.05 and the absolute value of the $\log_2$ foldchange ≥1. This resulted in a list of enriched pathways

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214 and differentially expressed genes that are part of those pathways. These results were visualized with
215 simplifyEnrichment analysis (Korotkevich, et al. 2019).
216

217 **RT-qPCR analysis**

218 Bovine embryos in individual or group culture that reached the blastocyst stage as well as embryos that
219 failed to reach the blastocyst stage (non-blastocyst or arrested) were used to isolate total RNA (including
220 miRNAs). Total RNA extraction was performed on blastocyst and non-blastocysts in triplicates (as
221 detailed Fig. 1B) using the miRNeasy Mini kit (Qiagen, Germantown, USA). Reverse transcription was
222 performed in 20 µl reaction volume on a PCR system using a miScript II RT kit (Qiagen, Germantown,
223 USA), including 4 µl 5 × miScript Hispec Buffer, 2 µl 10 × miScript Nucleics Mix, 2 µl miScript Reverse
224 Transcriptase Mix and 12 µl RNA. The reverse transcription program consisted of incubation at 37 °C
225 for 60 min, inactivation of miScript Reverse Transcriptase Mix at 95 °C for 5 min. The miRNA levels
226 were subsequently quantified with a miScript SYBR Green PCR Kit (Qiagen, Germantown, USA)
227 containing 10× miScript Universal Primer. The RT-qPCR was performed in 10 µl reaction volume on a
228 BioRad CFX 96 PCR Detection system, including 5 µl 2 × QuantiTect SYBR Green PCR Master Mix, 1
229 µl 10 × miScript primer assay, 1 µl 10 × miScript Universal primer and 1 µl diluted cDNA. The PCR
230 program consisted of an initial activation step at 95 °C for 15 min, followed by 40 cycles of denaturation
231 for 15 s at 94 °C, a combined primer annealing-extension step for 30 s at specific primers
232 (Supplementary Table S1) annealing temperatures and extension at 70 °C for 30 s. U6 (RNA, U6 small
233 nuclear 1) (Abd El Naby, et al. 2013, Mondou, et al. 2012) and bta-mir-92a was quantified to normalize
234 miRNA expression levels. All reactions were performed in triplicate, and the $2^{-\Delta\Delta Ct}$ method was used to
235 analyze the data.
236

237 **Statistical analyses**

238 Manually collected data were exported to Microsoft Excel (Microsoft Corp., Redmond, WA), where data
239 exploration and organization were done using the PivotTables function (Microsoft Excel). The statistical
240 analyses were performed using R-core (version 4.0.2; R Core Team, Vienna, Austria). Generalized
241 mixed-effects models were used to test the effects of miRNA mimics and inhibitor supplementation
242 (negative control vs. positive control vs miRNA-146b) on developmental parameters (cleavage, day 7,
243 day 8, and hatched/hatching blastocyst rates) expressed as a percentage from presumed zygotes.

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244 Similarly, differential staining parameters (TCN, TE, ICM, ICM/TCN, AC, and AC/TCN) were fitted in
245 mixed linear regression models to test the effect of miRNA mimics and inhibitor supplementation. Each
246 differential-apoptotic staining parameter was analyzed by Shapiro-Wilk's test and when the residuals of
247 simple models were not normally distributed ($P < 0.05$), the outcome variable was $\log_{10}$ transformed.
248 For all transformed variables, the residuals were normally distributed (Shapiro-Wilk's $P > 0.05$). For each
249 model, the replicate was set as a random effect. The differences between treatment groups were
250 assessed using Tukey's post hoc test. Results are expressed as least squares means and standard
251 errors. The significance and tendency levels were set at $P < 0.05$ and $P < 0.1$, respectively.

252
253

254 **Results**

255

256 **Higher miR-146b expression is associated with non-blastocyst embryos**
257 In a previous study, we performed miRNA-sequencing of EVs secreted in the medium of individually
258 cultured bovine embryos, which revealed upregulation of miR-146b in the EVs from non-blastocysts,
259 when compared to blastocyst EVs ((Pavani, et al. 2022); Fig. 2A). Here, using RT-qPCR, we confirm
260 that there is a higher expression of miR-146b in non-blastocysts compared to blastocysts, derived from
261 both group and individual culture (Fig. 2A). Overall, these results show an overexpression of miR-146b
262 in non-blastocysts or arrested embryos and in EVs secreted by these embryos.

263

264 **MiR-146b mimics and inhibitors modulate embryo development and quality**
265 To study the effect of miR-146b on embryo development, miR-146b mimics or inhibitors were
266 supplemented to culture medium containing presumed zygotes at 1 day post insemination (dpi), which
267 were then cultured until 8 dpi, allowing miR-146b mimics or inhibitors to affect further embryo
268 development. Although cleavage rates were not affected, addition of miR-146b mimics reduced the
269 blastocyst rate on day 7 (31.8 ± 4.5) in comparison to the control group (42.2 ± 4.8) ($p = 0.045$) (Table
270 1). In parallel, supplementing miR-146b inhibitors resulted in a similar day 7 blastocyst rate (35.3 ± 3.8)
271 compared to the control group (39.2 ± 3.7). Additionally, differential apoptotic staining showed that
272 miR146b mimics repressed blastocyst quality, while miR146b inhibitors had a beneficial effect on
273 embryo quality (Table 2). Specifically, embryos cultured in the presence of miR-146b mimics showed a
274 lower total cell number (TCN) (107 ± 9.5) compared to the control group (123 ± 9.5) ($p<0.05$), whereas

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275 supplementation with miR146b inhibitors resulted in a reduced apoptotic cell (AC) count (3.8 ± 1.2),
276 compared to the control (6.0 ± 1.0) ($p<0.05$). These results indicate that miR-146b affects embryo
277 development and quality.
278
279 **MiR-146b mimics and inhibitors modulate the transcriptome profile of blastocysts**
280 Transcriptome profiling was performed on blastocysts cultured in the presence of miR-146b mimics in
281 three comparisons (Control vs. negative control (NC) mimic; Control vs. miR-146b mimics, NC mimic
282 vs. miR-146b mimics), resulting in a total of 217 DE genes. In order to distinguish the effect of the miR-
283 146b mimics, the 23 common DE genes in control vs. NC mimic were omitted from further comparison
284 between miR-146b mimics and both control groups, as described previously (Pavani, et al. 2022) (Fig.
285 3A). As such, a total of 137 DE genes were identified in the miR-146b mimic supplemented group
286 compared to both controls (Dataset S1). Supplementation of miR-146b mimic resulted in 58 DE genes
287 (24 up and 34 down) compared to the non-supplemented control and of 66 DE genes (28 up and 38
288 down) compared to the NC mimic, while 13 common DE genes were found for the miR-146b mimic
289 group compared to both control groups (Supplementary Table S2).
290 Similarly, transcriptional analysis on miRNA-146b inhibitor treatment groups (Control vs. NC inhibitor;
291 Control vs. miR-146b inhibitor, NC mimic vs. miR-146b inhibitor) resulted in the identification of 182 DE
292 genes. A total of 80 DE genes were identified in the miR-146b inhibitor supplemented group, after
293 excluding commonly expressed genes in both control groups (Fig. 3B, Dataset S2). Supplementation of
294 miR-146b inhibitor resulted in 35 DE genes (14 up and 21 down) compared to the non-supplemented
295 control and of 38 DE genes (17 up and 21 down) compared to the NC inhibitor, while 7 genes were DE
296 for the miR-146 inhibitor group compared to both control groups (Supplementary Table S3).
297 Finally, we made a direct comparison between blastocysts treated with miR-146 mimic vs. miR-146b
298 inhibitor, resulting in 89 DE genes (Dataset S3). These were compared with the above identified DE
299 genes of miR-146b mimic vs. controls (n=137, Dataset S1) and miR-146b inhibitor vs. controls (n=80,
300 Dataset S2) (Fig. 3C), resulting in a total of 31 commonly expressed DE genes among these groups.
301 Hierarchical clustering and heatmap imaging revealed that these 31 significantly altered genes could be
302 categorized into two groups (miR-146b mimic and miR-146b inhibitor) (Fig. 3D). Further, narrowing
303 down from these 31 DE genes, we identified three genes (*WDR36 (WD repeat-containing protein 36),*

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304 *ERCC6L2 (ERCC excision repair 6 like 2),* and *SNIP1 (Smad Nuclear Interacting Protein 1)*), which are
305 downregulated and upregulated by miR-146b mimic and miR-146b inhibitor respectively.
306
307 **MiR-146b targets genes involved in embryo quality and development**
308 To detect the key pathways regulated by miR-146b, GO gene set overrepresentation analysis with
309 simplify Enrichment package was performed on the 31 DE genes that are common between miR-146b
310 mimic vs. miR-146b inhibitor (Fig. 3C) (Fig. 4A-C). The major pathways linked with these 31 DE genes
311 are apoptosis, protein binding, cell differentiation, DNA demethylase, kinase activity, phosphatase
312 activity, positive regulation, signaling receptor, and centriole.
313
314
315 **Discussion**
316 In this study, we demonstrate (1) that miR-146b is significantly upregulated in arrested embryos
317 compared to blastocysts, (2) that presence of miR-146b leads to significantly reduced embryo
318 development and quality, more specifically lower total cell numbers with increased apoptotic cell counts,
319 while (3) it also regulates the expression of genes involved in apoptosis, cell differentiation and embryo
320 development.
321 This study follows up on our previous study, where we found a higher expression of miR-146b in EVs
322 secreted by non-blastocysts (Pavani, et al. 2022). To examine a potential correlation with the miR-146b
323 expression in the embryos themselves, in the current study, we performed RT-qPCR analysis on
324 blastocysts and non-blastocysts (Fig. 2). Higher expression of miR-146b in non-blastocysts compared
325 with blastocysts confirmed the association between the embryo and its EV cargo content. It was shown
326 in another study that high expression of miR-146b has been connected with the stimulation of apoptosis:
327 indeed transfection of miR-146b mimics to porcine granulosa cells led to cell apoptosis (Li, et al. 2019),
328 which supports our observation that non-blastocyst EVs and embryos had higher levels of miR-146b.
329 The supplementation of miR-146b mimics significantly reduces embryo quality, especially TCN and AC
330 (Table 1 and 2). Explicitly, apoptotic cell count was significantly reduced in the presence of miR-146b
331 inhibitors, further indicating a role in apoptotic pathways. This is in conformity with literature, where
332 overexpression of miR146b in human dendritic cells was associated with increased apoptosis (Li, et al.,
333 2019). Supplementing culture medium with miR-146b mimics also increased apoptosis in porcine

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334 intestinal epithelial cell (Tao, et al. 2017). MiR-146b was further shown to regulate the gene expression
335 levels of apoptosis related genes *Bcl-2* (B-cell lymphoma 2) and *Bax* (Bcl-2 Associated X-protein) and
336 apoptotic cells were more prevalent in bovine male germline stem cells after supplementing them with
337 miR146b mimics (Gao, et al. 2020).
338 Transcriptomics of blastocysts exposed to miR-146b mimics and inhibitors confirmed involvement in
339 apoptotic pathways, as well as other embryo development related pathways, including protein binding,
340 cell differentiation and DNA demethylase (Fig. 4). Interestingly, among the common DE genes (Fig. 4C,
341 D) WDR36, ERCC6L2 and SNIP1 were downregulated in the presence of miR-146b mimics, and
342 upregulated by miR-146b inhibitors. These three genes have been shown to play an important role in
343 apoptosis, cell differentiation and in the RNA Pol II transcription complex (Gallenberger, et al. 2010, Kim,
344 et al. 2001, Tummala, et al. 2018). Knockdown of WDR36 can lead to early embryonic death in mice
345 and lack of WDR36 is causing apoptotic death of blastomeres (Gallenberger, et al. 2010). This coincides
346 with our observations in the current study, that supplementation of miR-146b mimics to the embryo
347 culture media leads to the downregulation of WDR36 (Fig. 3D) and subsequently to a lower blastocyst
348 rate and quality and higher apoptotic cell ratio (Table 1 and 2), whereas supplementation of miR-146b
349 inhibitors results in upregulation of WDR36 (Fig. 3D) and a lower apoptotic cell ratio in the blastocysts.
350 Similarly, *SNIP1* and *ERCC6L2* genes, downregulated by supplementation of miR-146b mimic, are also
351 connected to apoptosis, and developmental signaling pathways respectively (Matsui, et al. 2022,
352 Tummala, et al. 2018), as represented in Fig 4.
353
354 In conclusion, we examined the effects of miR-146b derived from non-blastocyst EVs, on embryo
355 development, apoptosis, cell differentiation, and gene expression in bovine embryos. We showed that
356 miR-146b was associated with apoptosis, as apoptotic cell rates were lower in embryos after being
357 supplemented with miR-146b inhibitors. This study uncovered that miR-146b is transcriptionally
358 regulating apoptosis, cell differentiation and RNA Pol II transcription complex by targeting 32 DE genes,
359 including *WDR36*, *ERCC6L2* and *SNIP1*. Our findings about the functionality of miR-146b in embryos
360 are improving our understanding on gene regulation of embryo development and quality and may
361 provide further indirect evidence for the importance of EVs as signaling factors in *in vitro* culture
362 conditions.
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364 **Data Availability**
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366 The transcriptomics datasets generated during the current study were deposited in the National Center
367 for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession
368 number GSE197878.

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385 experiments, analyzed the data, and wrote the manuscript. A.H., A.V.S., B.H., L.P., F.VN., D.D., K.T.,
386 and K.S helped in all experiments and discussed the draft manuscript. T.M. worked on miRNA
387 sequencing and data analysis. J.C. helped with molecular dynamic simulations. G.X assisted in IVF
388 experiments and miRNA RT-qPCR analysis. All authors reviewed and approved the final manuscript.

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568 **Tables**
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570 **Table 1. Blastocyst development and hatching rates of bovine embryos treated with miRNA**
571 **mimics/inhibitors**
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Treatment	No. of presumed zygotes	Cleavage	Blastocyst day 7	Blastocyst day 8	Hatched/Hatching day 8
Control	161	88.6 ± 2.9[a]	42.2 ± 4.8[a]	49.7 ± 3.9[a]	10.7 ± 2.6[a]
NC mimics	225	90.1 ± 2.5[a]	37.4 ± 4.1[a]	46.2 ± 3.3[a]	12.5 ± 2.4[a]
miRNA-146b mimics	158	94.1 ± 2.1[a]	31.8 ± 4.5[b]	43.0 ± 3.9[a]	12.6 ± 2.8[a]
Control	239	88.3 ± 2.0[a]	39.2 ± 3.7[a]	45.5 ± 4.5[a]	10.8 ± 2.0[a]
NC inhibitor	165	89.7 ± 2.3[a]	29.7 ± 3.6[a]	32.2 ± 4.8[a]	6.0 ± 1.8[b]
miRNA-146b inhibitor	167	91.6 ± 2.1[a]	35.3 ± 3.8[a]	45.7 ± 5.2[a]	9.5 ± 2.2[a]

573 Cleavage, 7 and 8 dpi blastocyst rates, and hatched/hatching rates expressed as a percentage of
574 presumed zygotes. For the miRNA mimic experiment, culture media were not supplemented (Control),
575 or were supplemented with control mimics (NC mimics) or with miRNA-146b mimics. For the miRNA
576 inhibitor experiment, culture media were not supplemented (Control), or were supplemented with control
577 inhibitors (NC inhibitor) or with miRNA-146b inhibitor. Different superscripts per column (a and b)
578 represent statistical differences ($P < 0.05$) among treatments. Results are expressed as least-square
579 mean ± SE.

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593 **Table 2. Embryo quality assessment of bovine embryos treated with miRNA mimics/inhibitor**
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Treatment	No. of blastocysts	Cell numbers				ICM/TCN ratio	AC/TCN ratio
		TCN	ICM	TE	AC		
Control	17	123 ± 9.5^{a}	43.6 ± 3.1^{a}	79.8 ± 8.1^{a}	8.1 ± 1.3^{a}	37.5 ± 2.8^{a}	6.4 ± 1.0^{a}
NC mimic	16	106 ± 9.8^{b}	46.9 ± 3.2^{a}	59.1 ± 8.3^{a}	5.4 ± 1.4^{a}	46.3 ± 2.9^{a}	5.0 ± 1.1^{a}
miRNA-146b mimic	17	107 ± 9.5^{b}	41.1 ± 3.1^{a}	65.8 ± 8.1^{a}	9.0 ± 1.3^{a}	42.2 ± 2.8^{a}	8.0 ± 1.0^{a}
Control	22	120 ± 7.9^{a}	52.3 ± 3.2^{a}	68.0 ± 6.1^{a}	6.0 ± 1.0^{a}	45.7 ± 2.2^{a}	4.9 ± 0.9^{a}
NC inhibitor	17	102 ± 8.9^{b}	42.1 ± 3.7^{b}	60.4 ± 7.0^{a}	7.3 ± 1.2^{a}	43.2 ± 2.5^{a}	7.2 ± 1.0^{b}
miRNA-146b inhibitor	17	121 ± 7.9^{a}	54.7 ± 3.7^{a}	66.8 ± 7.0^{a}	3.8 ± 1.2^{b}	45.8 ± 2.5^{a}	3.3 ± 1.0^{a}

595 TCN, AC, ICM/TCN ratio, and AC/TCN ratio of differentially stained day 8 blastocysts. For the miRNA
596 mimic experiments, culture media were not supplemented (Control), or were supplemented with control
597 mimics (NC mimics) or miRNA-146b mimics. For the miRNA inhibitor experiment, culture media were
598 not supplemented (Control), or were supplemented with control inhibitors (NC inhibitor) or miRNA-146b
599 inhibitor. Different superscripts per column (a, b, and c) represent statistical differences ($P < 0.05$)
600 among groups. Results are expressed as least-square mean ± SE.

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601 **Figures**
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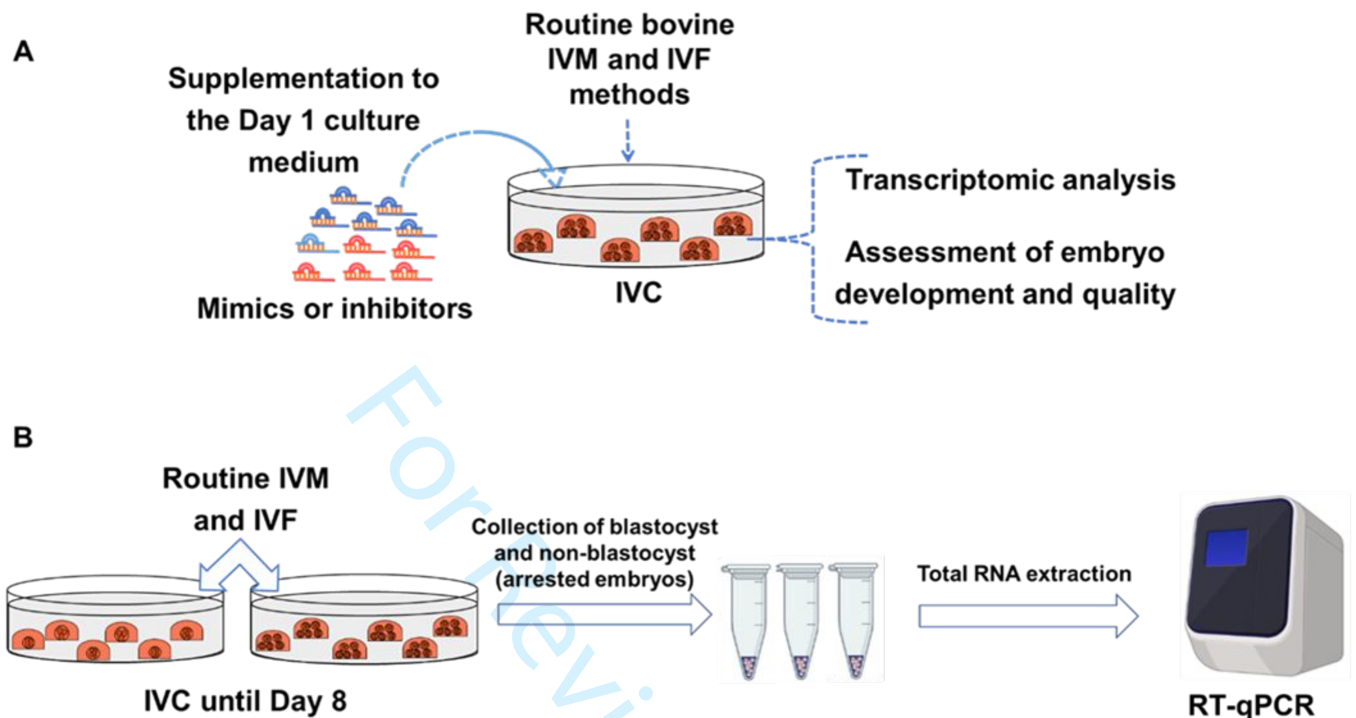

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604 **Fig. 1.** (A) Functionality of miR-146b was tested by supplementing mimics and inhibitors (bta-
605 miR-146b, miRCURY LNA Power) to the culture medium of presumed zygotes with a final
606 concentration of 1 μM. Embryo development was evaluated on 7 and 8 dpi, and all blastocysts
607 were collected on 8 dpi for differentially staining and transcriptomic analysis. (B) To test
608 miRNA-146b expression levels from the embryos directly. Bovine oocytes were subjected to
609 routine, in vitro maturation (IVM) and IVF in groups, and presumed zygotes were cultured
610 individually or in groups until 8 dpi. On 8 dpi, bovine embryos that developed to the blastocyst
611 stage and embryos that were not able to reach the blastocyst stage (non-blastocyst or
612 arrested) were collected separately and 8 pooled embryos per group (three replicates). Further
613 total RNA extraction was performed on these collected embryos, followed by cDNA synthesis
614 and qRT-PCR analysis.

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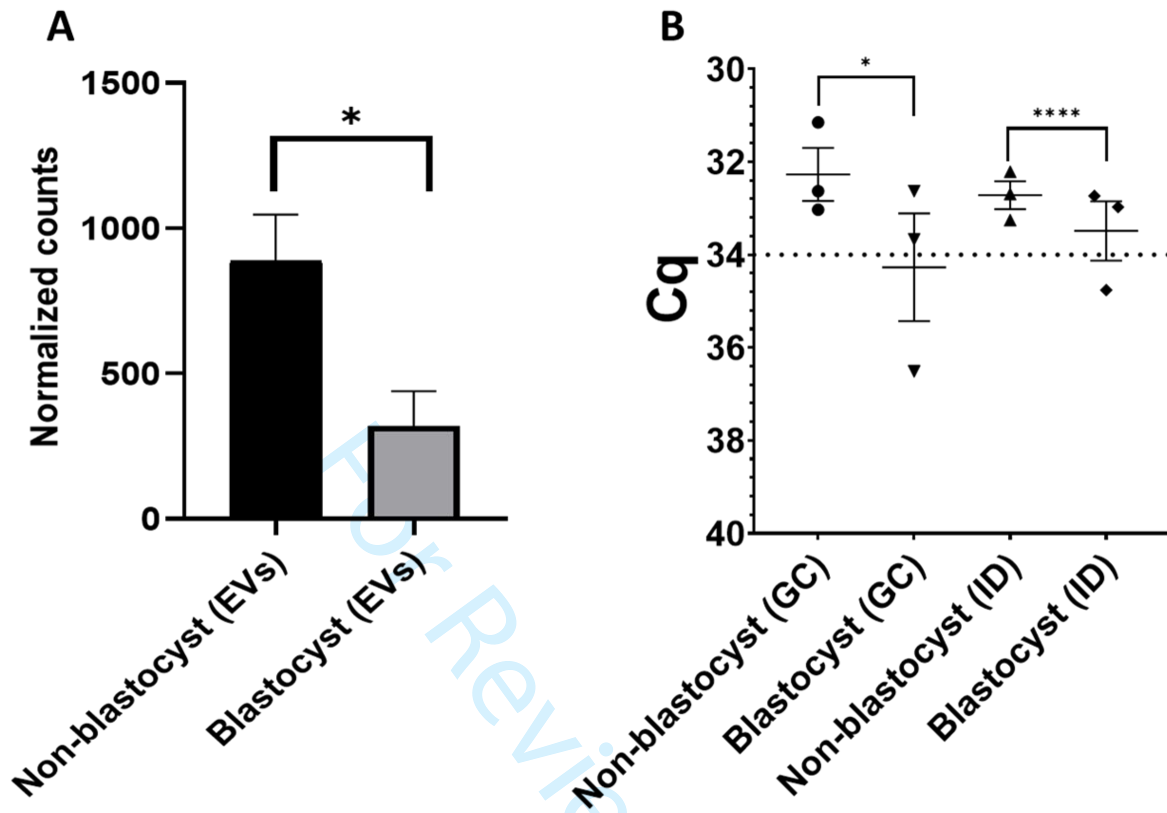

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621 **Fig.2.** (A) Normalized counts of miR-146b obtained in blastocyst and non-blastocyst EV samples. (B)

622 Quantification of miR-146b is indicated by raw Cq values obtained from blastocyst and non-blastocyst

623 embryos (derived from group (GC) and individual culture (IC) conditions). Data from three replicates of

624 all the samples are shown, ** P<0.001, *** P<0.0002, ANOVA with Tukey's HSD post-hoc test.

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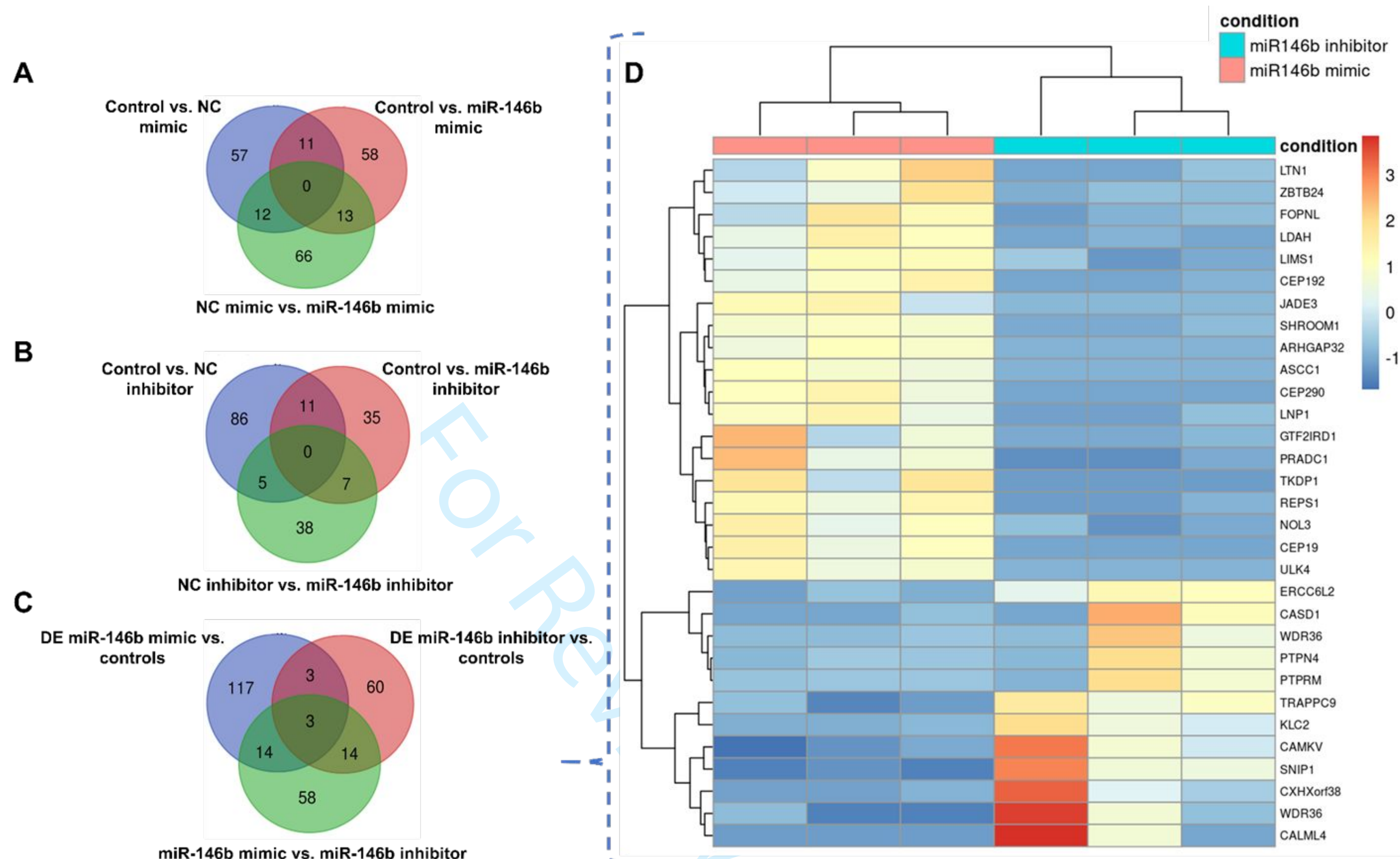

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630 **Fig.3.** Venn diagrams showing the number of DE genes in bovine blastocysts after treatment with miR-
631 146b mimic and miR-146b inhibitor vs. NC and unsupplemented controls. (A) Control vs. miR-146b
632 mimic vs. NC mimic; (B) control vs. miR-146b inhibitor vs. NC inhibitor; (C) miR-146b mimic vs. controls,
633 miR-146b inhibitor vs. controls, and miR-146b mimic vs. miR-146b inhibitor. (D) Hierarchical clustering
634 (Euclidean distance) and heatmap imaging of the 31 DE genes that are common among DE genes of
635 miR-146b mimic vs. controls or miR-146b inhibitor vs. controls, and miR-146b mimic vs. miR-146b
636 inhibitor. Up-regulated expression is shown in red, and down-regulated expression is shown in blue.

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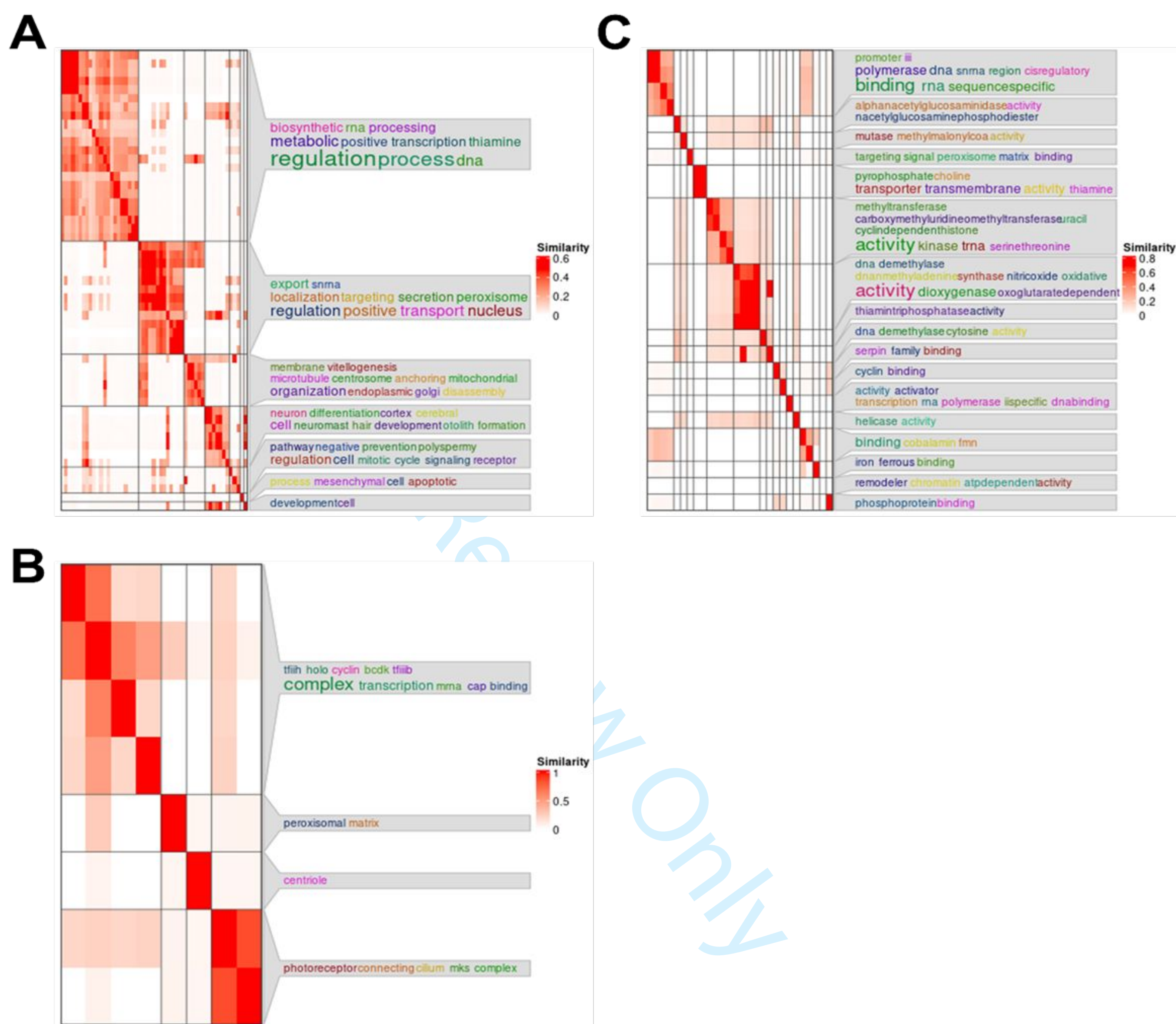

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642 **Fig.4.** Overrepresentation analysis on 31 differentially expressed genes that were common among miR-
643 146b mimic vs. controls, miR-146b inhibitor vs. controls, and miR-146b mimic vs. miR-146b inhibitor
644 enriched in top 10 GO/KEGG pathways. The resulting GO terms were clustered by binary cut enriched
645 (A) Biological Process (BP), (B) Molecular Function (MF), (C) Cellular Component (CC).

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2 **Table S1. RT-qPCR primers**

Target miRNA	Accession	Sequence (5'–3')
bta-miR-127	MIMAT0003787	UCGGAUCCGUCUGAGCUUGGCU
bta-miR-146b	MIPF0000103	UGAGAACUGAAUUCCAUAGGCUGU
bta-miR-93	MIMAT0003837	CAAAGUGCUGUUCGUGCAGGUA

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6 **Table S2. Differentially expressed genes common among the comparison of miR-146b mimic vs. control and miR-146b mimic vs.**
7 **negative control mimic**

Ensembl ID	Gene name	log2(FC)	P-value	FDR	Regulation
ENSBTAG00000002490	CHPT1	-5,491966441	0,000161	0,020117	Down
ENSBTAG00000051466	ULK4	8,405874027	0,000195	0,022553	UP
ENSBTAG00000018313	MBNL2	-7,556954859	0,000169	0,020117	Down
ENSBTAG00000020855	PTPN4	-7,000138229	5,48E-05	0,011609	Down
ENSBTAG00000007658	CTNNBL1	5,611484322	7,95E-06	0,002885	UP
ENSBTAG00000023736	CDC42EP3	-9,743313126	2,86E-07	0,000221	Down
ENSBTAG00000013821	RIOK1	-9,904030867	2,31E-07	0,00022	Down
ENSBTAG00000008584	CLN8	-3,787134592	0,000167	0,020117	Down
ENSBTAG00000018745	CEP290	7,152270082	0,000129	0,017714	UP
ENSBTAG00000013192	CEP19	7,929839039	4,54E-05	0,010479	UP
ENSBTAG00000017502	RIMKLA	8,53644049	0,000114	0,016797	UP
ENSBTAG00000018084	CASTOR1	-7,664806022	3,00916E-06	0,001355666	Down
ENSBTAG00000037996	ARMCX3	8,713624327	3,33631E-05	0,008648647	UP

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19 **Table S3. Differentially expressed genes common among the comparison of miR-146b inhibitor vs. control and miR-146b inhibitor vs**
20 **negative control inhibitor**

Ensembl ID	Gene name	log2(FC)	P-value	FDR	Regulation
ENSBTAG00000010355	VPS37A	8,995722	1,42E-06	0,001079	UP
ENSBTAG00000008765	PRADC1	-6,88735	2,4E-05	0,006548	Down
ENSBTAG00000010381	PRMT9	8,556106	4,45E-05	0,011231	UP
ENSBTAG00000032640	FOPNL	-6,86684	6,13E-07	0,000597	Down
ENSBTAG00000004878	CAMKV	5,152639	0,000195	0,029603	UP
ENSBTAG00000012600	GTF3A	6,69129	6,79E-05	0,014454	UP
ENSBTAG00000025822	CALML4	11,05775	2,52E-07	0,00055	UP

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