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1 # Embryo culture in presence of oviductal fluid induces DNA
2 methylation changes in bovine blastocysts

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25 **Short title:** Epigenetic effect of oviductal fluid on embryo

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ABSTRACT

During the transit through the oviduct, the early embryo initiates an extensive DNA methylation reprogramming of its genome. Given that these epigenetic modifications are susceptible to environmental factors, components present in the oviductal milieu could affect the DNA methylation marks of the developing embryo. The aim of this study was to examine if culture of bovine embryos with oviductal fluid (OF) can induce DNA methylation changes at specific genomic regions in the resulting blastocysts. *In vitro* produced zygotes were cultured in medium with 3 mg/mL bovine serum albumin (BSA) or 1.25% OF added at the one- to 16-cell stage (OF1-16), one- to 8-cell stage (OF1-8) or 8- to 16-cell stage (OF8-16), and then were cultured until Day 8 in medium with 3 mg/mL BSA. Genomic regions in four developmentally important genes (*MTERF2*, *ABCA7*, *OLFM1*, *GMDS*) and within LINE-1 retrotransposons were selected for methylation analysis by bisulfite sequencing on Day 7-8 blastocysts. Blastocysts derived from OF1-16 group showed lower CpG methylation levels in *MTERF2* and *ABCA7* compared with the BSA group. However, CpG sites within *MTERF2*, *ABCA7* and *OLFM1* showed higher methylation levels in groups OF1-8 and OF8-16 than in OF1-16. For LINE-1 elements, higher CpG methylation levels were observed in blastocysts from the OF1-16 group than in the other experimental groups. In correlation with the methylation changes observed, mRNA expression level of *MTERF2* was increased, while LINE-1 showed a decreased expression in blastocysts from OF1-16 group. Our results suggest that embryos show transient sensitivity to OF at early stages, which is reflected by specific methylation changes at the blastocyst stage.

Keywords: bovine, blastocyst, DNA methylation, oviduct, oviductal fluid

51 **INTRODUCTION**

52 The embryo environment during the first stages of development can influence
53 the developmental potential and physiology of the future conceptus, and this may have
54 long-term consequences for the health of the offspring (Fazeli & Holt 2016). In
55 mammalian species, including mice and cattle, the early cleavage stages in which the
56 embryo transits through the oviduct represent a very dynamic developmental window,
57 involving molecular events related to morphological and metabolic changes, embryonic
58 genome activation (EGA) and epigenetic reprogramming of parental and embryonic
59 genomes (Bell *et al.* 2008, Duranthon *et al.* 2008). Among these events, reprogramming
60 of epigenetic marks is one of the most critical processes affecting the embryo potential.
61 As an epigenetic mark, DNA methylation is essential for normal embryo development
62 through mechanisms such as regulation of gene expression, differentiation, cell cycle
63 control and maintenance of genome stability (Okano *et al.* 1999, Golding *et al.* 2011,
64 Messerschmidt *et al.* 2014).

65 Studies in mouse zygotes and developing embryos have revealed that shortly
66 after fertilization, parental genomes undergo an active global demethylation that
67 continues up to blastocyst stage, after which cells of the inner cell mass and
68 trophectoderm reacquire methylation marks (Smith *et al.* 2012, Gkountela & Clark
69 2014, Guo *et al.* 2014). In cattle, immunofluorescence analysis using specific 5-
70 methylcytosine antibodies demonstrated that global DNA methylation decreases
71 between the 2- to 8-cell stages, followed by a subsequent *de novo* DNA methylation that
72 increases progressively from 8-cell to blastocyst stage (Dean *et al.* 2001, Dobbs *et al.*
73 2013). These findings suggest that, similar to mouse embryos, the DNA methylation
74 pattern in bovine embryos changes dynamically during the first stages of
75 preimplantation development.

76 Recent studies in bovine blastocysts developed *in vivo* from embryos that were
77 previously cultured *in vitro* until zygote, 4-cell or 16-cell stage, have indicated that
78 during early embryo development the *in vitro* culture conditions can also modify the
79 methylation levels of the embryonic epigenome in a developmental stage-dependent
80 manner (Salilew-Wondim *et al.* 2015). Given that the embryo environment in *in vitro*
81 culture conditions lacks several maternally derived molecules, a fact that could
82 contribute to the epigenetic modifications observed previously, an interesting question
83 that arises is whether embryonic DNA methylation marks are influenced by maternal-
84 embryo signals.

85 In the *in vivo* setting, luminal fluid of the oviduct is the first maternal
86 microenvironment that makes contact with the embryo. The composition of OF is
87 complex and changing both in the ampullary and isthmic region depending on the
88 phases of the estrous cycle (Killian 2004, Seytanoglu *et al.* 2008). It contains growth
89 factors, cytokines and other candidate macromolecules that act as pivotal mediators of
90 maternal-embryo communication (Lee & Yeung 2006, Aviles *et al.* 2010). Moreover,
91 gene expression of some of these factors in the oviduct seems to be induced by the
92 presence of the embryo via specialized cross-talk (Maillo *et al.* 2015). Signaling
93 pathways triggered by oviductal molecules not only help mediate the maternal effect on
94 embryo survival, development, plasticity and quality (Buhi *et al.* 2000, Coy &
95 Yanagimachi 2015), but may also exert a long-term impact through epigenetic
96 modifications of the embryonic genome. Interestingly, the presence of OF in *in vitro*
97 culture induces changes in mRNA levels of *DNMT1* and *DNMT3A* genes both in bovine
98 early embryos and blastocysts, suggesting that oviductal factors could affect DNA
99 methyltransferase (DNMT) expression during preimplantation development (Barrera *et*
100 *al.* 2013, Lopera-Vasquez *et al.* 2015). However, to date there is no experimental

101 evidence of the impact of the oviductal milieu on DNA methylation marks in the
102 preimplantation embryo.
103 Since epigenetic marks are susceptible to environmental influence, we
104 hypothesized that OF can affect DNA methylation pattern at specific genomic regions in
105 the developing embryo. To evaluate this hypothesis, bovine embryos produced *in vitro*
106 were cultured in the presence or absence of OF at different time points during the first
107 four days of embryo development (period when *in vivo* the embryo is still in the
108 oviduct). The methylation state in specific genomic targets was determined by bisulfite
109 sequencing at the blastocyst stage. The present study particularly focused on regions
110 within CpG islands in developmentally significant genes (*MTERF2*, *ABCA7*, *OLFM1*
111 and *GMDS*) and in CpG sites within LINE-1 repetitive elements. Moreover, to evaluate
112 the correlation between the DNA methylation pattern and the transcriptional levels,
113 relative mRNA abundance of the selected genes and LINE-1 expression were
114 determined in blastocysts derived from embryos cultured in the presence or absence of
115 OF during the examined time points of the early embryogenesis.
116
117 **MATERIALS AND METHODS**
118
119 ***Chemicals***
120 Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich
121 Quimica (Madrid, Spain).
122
123 ***Experimental design***
124 The experimental design was established with two purposes: I) to evaluate if the
125 exposure of the embryos to the OF during early stages of development can induce

126 changes and have subsequent effect on the DNA methylation pattern at blastocyst stage;
127 and II) to evaluate if there are specific embryonic stages more sensitive to the influence
128 of OF before (from the 1-cell to 8-cell stage) or during the main phase of the EGA
129 (from the 8-cell to 16-cell stage) in bovine embryos (Gad *et al.* 2012, Graf *et al.* 2014).
130 In total, 2047 presumptive zygotes were produced by *in vitro* maturation and
131 fertilization. These presumptive zygotes were randomly assigned to different
132 experimental groups: SOF supplemented with 3 mg/mL BSA (group BSA, n=586) or
133 SOF supplemented with 1.25% (v/v) OF for three different periods of time (Fig. 1): i)
134 from the 1-cell to 16-cell stage (group OF1-16, n=483), ii) from the 1-cell to 8-cell stage
135 (group OF1-8, n=488), and iii) from the 8-cell to 16-cell stage (group OF8-16, n=490).
136 Thus, in these three experimental groups, OF was present respectively: i) from the start
137 of culture (18 hpi) to 98 hpi (Day 4), ii) from 18 hpi to 52 hpi (Day 2) and iii) from 52
138 hpi (Day 2) to 98 hpi (Day 4). In all four groups, embryo culture was continued until
139 Day 8 post-insemination (pi). In the three OF groups, the culture medium outside the
140 treatment windows was SOF supplemented with 3 mg/mL BSA. The concentration of
141 OF used was chosen according our previous observations indicating that this
142 concentration added to embryo culture medium supports *in vitro* development and
143 positively affects the quality of the produced blastocysts in bovine (Lopera-Vasquez *et*
144 *al.* 2015).

145 For this design, normally developing embryos that reached the ≥8-cell stage at
146 52 hpi and ≥16-cells at 98 hpi were selected and separately cultured from slowly
147 developing embryos (referred to as “late 52 hpi” and “late 98 hpi”, respectively).
148 Cleavage rates were assessed at 52 hpi and the blastocyst rate was determined on Day 7-
149 8 pi as the percentage of blastocysts obtained from embryos cultured under each
150 experimental condition in both the normal and late development group. The experiment

151 was carried out eight times under the same assay conditions. Expanding blastocysts
152 obtained from embryos developing in a timely manner (Day 7-8) were frozen in liquid
153 nitrogen (LN2) and stored at -80°C. A total of four pools of 20 expanding blastocysts
154 per experimental group derived from five experimental replicates were used for
155 methylation analysis. As result of bisulfite sequencing, 480 positive clones were
156 sequenced, including a total of 20 individual clones from each target loci from each
157 treatment group. On the other hand, three pools of 10 blastocysts obtained from the
158 additional three experimental replicates were used to evaluate the relative mRNA
159 expression.

160

161 ***In vitro oocyte maturation***

162 Bovine cumulus-oocyte complexes (COCs) were recovered and *in vitro* matured
163 as previously described by Lopera-Vasquez *et al.* (2015). Briefly, immature COCs were
164 obtained by aspirating follicles (2-8 mm) from the ovaries of heifers collected at the
165 slaughterhouse. Class 1 and 2 COCs were matured for 22 h in 500 µL maturation
166 medium [TCM 199 (M4530) supplemented with 10% (v/v) fetal calf serum (FCS) and
167 10 ng/mL epidermal growth factor (E4127)] in groups of approximately 50 COCs per
168 well in four-well dishes (NUNC, Roskilde, Denmark). The culture conditions were
169 38.5°C, 5% CO_2 in air and maximum humidity.

170

171 ***In vitro fertilization***

172 Frozen semen from a single Asturian Valley bull, previously tested for IVF
173 (ASEAVA, Asturias, Spain), was thawed at 37°C in a water bath for 1 min and sperm
174 was selected on a Bovipure® gradient (Nidacon Laboratories AB, Gothenburg, Sweden)
175 as previously described by Lopera-Vasquez *et al.* (2015). Sperm concentration was

176 determined and adjusted to a final concentration of 1×10^6 sperm cells/mL for IVF.
177 Gametes were co-incubated for 18 h in 500 µL of fertilization medium (Tyrode's
178 medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/mL
179 fatty acid-free BSA) supplemented with 10 µg/mL heparin sodium salt (Calbiochem,
180 San Diego, CA, USA) in groups of 50 COCs per well in four-well dishes at 38.5ºC in an
181 atmosphere of 5% CO_2 in air at maximum humidity.
182
183 ***In vitro embryo culture***
184 After the fertilization period, presumptive zygotes were denuded of cumulus
185 cells by vortexing for 3 min, randomly divided into groups of 25 and cultured in 25 µL
186 droplets of synthetic oviductal fluid (SOF) supplemented with 4.2 mM sodium lactate
187 (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL/mL BME amino acids (B6766), 10
188 µL/mL MEM non-essential amino acids (M7145) and 1 µg/mL phenol red (P0290).
189 Droplets were placed under mineral oil at 38.5°C in an atmosphere of 5% CO_2, 5% O_2
190 and 90% N_2. Depending on the experimental group (see experimental design section),
191 SOF was supplemented with either 3 mg/mL bovine serum albumin (BSA; A9647) or
192 with 1.25% (v/v) OF.
193
194 ***Bovine oviductal fluid***
195 The bovine OF added to the *in vitro* embryo culture medium was obtained from
196 Embryocloud, Murcia, Spain (www.embryocloud.com). According to information
197 provided by the company, the OF (NatuArts BOF-EL) was collected from oviducts
198 ipsilateral to the ovary containing a corpus hemorrhagicum obtained from heifers
199 slaughtered during the early luteal phase of the estrous cycle (Day 1-4). Briefly,
200 oviducts were transported to the laboratory on ice, washed twice with cold phosphate-

201 buffered saline (PBS) and transferred to a stainless steel tray on a bed of ice before
202 dissection from surrounding connective tissues. Following the protocol described by
203 Carrasco *et al.* (2008), each oviduct was squeezed gently from the utero-tubal junction
204 towards the ampulla and the OF was collected by aspiration using a 200-μL automatic
205 pipette. A volume between 10 and 30 μL per oviduct was collected and a pool of OF
206 from five oviducts was centrifuged at 7,000 x g for 10 min at 4°C to remove cellular
207 debris. The supernatant was aliquoted and stored at -80°C until use.

208

209 ***Genomic DNA bisulfite conversion***

210 Genomic DNA from four pools of 20 blastocysts obtained from each
211 experimental group and derived from five IVF replicates was subjected to bisulfite
212 treatment using the MethylEdge Bisulfite Conversion System (Promega, Madison, WI,
213 USA) following the manufacturer's instructions. Samples were digested with proteinase
214 K for 1 h at 55°C in a 20 μL reaction volume containing 1 μg/μL proteinase K and 1 x
215 STES buffer (20 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS).
216 Samples were then incubated at 95°C for 5 min to inactivate proteinase K. Next, 130 μL
217 of bisulfite conversion reagent was added directly to the samples which were incubated
218 at 98°C for 8 min and then at 54°C for 1 h. For bisulfite DNA clean-up, bisulfite treated
219 samples were transferred to a spin column preloaded with 600 μL of binding buffer and
220 centrifuged at 10,000 x g. After washing, 200 μL of desulfonation buffer were added to
221 each spin column and the DNA bound to the column was desulfonated for 15 min at
222 room temperature. Bisulfite-converted DNA was then eluted from the column with 36
223 μL of elution buffer and immediately used for PCR amplification.

224

225

226 ***Primer design and bisulfite PCR***

227 To amplify the bisulfite-converted DNA sequences, primers were designed using
228 MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Table 1).
229 The four genes chosen for this study contain regions that showed significant DNA
230 methylation changes among porcine blastocysts collected *in vivo* and derived from
231 embryos produced *in vitro* in the presence or absence of reproductive fluids during the
232 entire procedure (Canovas *et al.* 2017). The candidate regions examined from bovine
233 genome belong to CpG islands localized at proximal promoters or gene bodies within
234 the genes mitochondrial transcription termination factor 2 (*MTERF2*), olfactomedin 1
235 (*OLFM1*), ATP binding cassette subfamily A member 7 (*ABCA7*) and GDP-mannose
236 4.6-dehydratase (*GMDS*). Given the fact that DNA methylation is a key epigenetic
237 modification controlling the transcriptional activity of mammalian retrotransposable
238 elements, the other regions selected for methylation analysis corresponded to LINE-1
239 (long interspersed nuclear element class-1 or L1) repetitive elements. LINE-1 sequences
240 are the most abundant interspersed sequences throughout the bovine genome and
241 probably the most active type of LINE elements (Adelson *et al.* 2009). It should be
242 mentioned that the CpG sites analyzed allowed assessment of changes in methylation
243 patterns in three different contexts: a) transcription start sites (e.g. *MTERF2*), b)
244 intragenic regions like introns (e.g. *GMDS*), last exons (e.g. *OLFM1*) and exon-intron
245 junctions (e.g. *ABCA7*) and c) regions located across the genome associated with
246 transposable elements (e.g. LINE-1).

247 Bisulfite-modified DNA (3 μL) was used to amplify each sequence by nested
248 PCR. The first PCR reaction with outer pairs of primers consisted of one cycle at 95ºC
249 for 2 min, 5 cycles at 95ºC for 20 s, 50ºC for 1 min, and 72ºC for 1 min 15 s, 30 cycles
250 at 95ºC for 20 s, 50ºC for 1 min, and 72ºC for 1 min 30 s, and a final step at 72ºC for 4

251 min. For nested PCR, 2 μL of the primary PCR product were used in a first step at 95ºC
252 for 3 min followed by 35 cycles at 95ºC for 20 s, 54ºC for 30 s, 72ºC for 45 s, and a
253 final extension at 72ºC for 20 min. PCR reactions were run in a 25 μL volume
254 consisting of 10 μM of each primer, 0.1 mM of dNTPs mix (Biotools, Madrid, Spain), 2
255 mM of $MgCl_2$, 5 x GoTaq Flexi Buffer and 1U GoTaq DNA Polymerase (Promega,
256 Madison, WI, USA). Presence of each PCR product was confirmed by loading 5 μL of
257 the product on 2% (w/v) agarose gels stained with SYBR Safe (Life Technologies,
258 Carlsbad, CA, USA) and electrophoresis was carried out at 120 V for 15-20 min.
259
260 ***Cloning of PCR products and sequencing analysis***
261 PCR products derived from bisulfite-treated DNA were purified from the PCR
262 reaction mixture using the FavorPrep™ PCR Clean-UP Kit (Favorgen Biotech Corp.,
263 Vienna, Austria) following the manufacturer's instructions. Each purified PCR fragment
264 was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and
265 *Escherichia coli* competent cells were transformed. The bacterial cells were plated onto
266 selective LB agar plates containing ampicillin/IPTG/X-gal and incubated overnight at
267 37°C. After incubation, independent white colonies were selected to verify presence of
268 the insert using PCR. Sequence data analysis of purified recombinant plasmids was
269 performed using CpGviewer software (http://dna.leeds.ac.uk/cpgviewer/). The bisulfite
270 conversion rate of cytosines located at non-CpG sites was checked and sequences
271 included in the analysis showed conversion efficiency >99%. The sequences examined
272 were localized in the genome using the *Bos taurus* genome assembly UMD 3.1.1
273 available at the National Center for Biotechnology Information (NCBI) as reference
274 sequence.
275

276 ***RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)***

277 Isolation of embryonic RNA was performed using three pools of 10 expanding
278 blastocysts (Day 7-8) obtained from independent experiments per treatment group.
279 Poly(A) RNA was extracted using the Dynabeads® mRNA DIRECT™ Micro Kit
280 (Ambion, Thermo Fisher Scientific Inc., Oslo, Norway) following the manufacturer's
281 instructions and with minor modifications described by Bermejo-Alvarez *et al.* (2008).
282 After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the
283 Dynabeads was extracted with a magnet and washed twice with washing buffer A and
284 washing buffer B. RNA was eluted with 30 μL of 10 mM Tris-HCl and immediately
285 used for cDNA synthesis. Reverse transcription (RT) was carried out following the
286 manufacturer's instructions (Bioline, Ecogen, Madrid, Spain). Briefly, oligo-dT (0.2
287 μM) and random hexamer primers (0.5 μM) were added to the RNA and the mixture
288 was heated at 70ºC for 5 min for denaturation of the secondary RNA structure. Then,
289 RNA was reverse-transcribed in a final volume of 40 μL containing 0.375 mM dNTPs
290 (Biotools, Madrid, Spain), 6.25U RNasin RNAse inhibitor (Promega), 10X MMLV-RT
291 buffer with 8 mM dithiothreitol, and 5U MMLV (Moloney Murine Leukemia Virus)
292 high performance reverse transcriptase (Epicentre, Madison, WI, USA), followed by
293 incubation at 70°C for 10 min to inactivate the RT enzyme. cDNA was stored at -20°C
294 until further use.

295 The expression levels of the selected genes and LINE-1 retrotransposons were
296 determined by quantitative real-time PCR (qRT-PCR) using specific primers, which are
297 listed in Table 2. Each pair of primers was verified to achieve efficiencies close to 1. All
298 qRT-PCR reactions were performed in a final volume of 20 μL, containing 2 μL of each
299 cDNA sample (60 ng/μL), 0.25 mM of forward and reverse primers and 10 μL of
300 GoTaq® qPCR Master Mix (Promega) using a Rotorgene 6000 Real Time Cycler™

301 (Corbett Research, Sydney, Australia) and SYBR Green as double-stranded DNA-
302 specific fluorescent dye. Three cDNA samples per experimental group were used in two
303 repetitions for all genes of interest. PCR amplification conditions were as follows: an
304 initial denaturalization step at 94°C for 2 min, followed by 35 denaturalization cycles at
305 94°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 30 s and 10 s of
306 fluorescence acquisition at a temperature which was specific for each product (76-86°C)
307 and higher than the melting temperature of primer dimers.

308 The comparative cycle threshold (CT) method was used to quantify expression
309 levels of the genes analyzed. Housekeeping gene H2A histone family, member Z
310 (*H2AFZ*), previously used for relative quantification of mRNA transcripts in bovine
311 blastocysts (Bermejo-Alvarez *et al.* 2008) was used for normalization. In our
312 determinations, *H2AFZ* produced uniform expression levels varying less than 0.5 CT
313 between control and treated cDNA samples. Fluorescence was acquired in each cycle to
314 determine the threshold cycle during the log-linear phase of the reaction during which
315 fluorescence increased above the background for each sample. According to the
316 comparative CT method, the ΔCT value was determined by subtracting the
317 housekeeping gene mean CT value for each sample from each gene CT value of the
318 sample. Calculation of ΔΔCT involved the use of the highest sample ΔCT value (i.e. the
319 sample with the lowest target expression) as an arbitrary constant to subtract from all
320 other ΔCT sample values. Fold changes in the relative gene expression of the target
321 were determined using the formula $2^{-\Delta\Delta CT}$. The entire study was carried out following
322 the Minimum Information for Publication of Quantitative Real-Time PCR Experiments
323 guidelines (Bustin *et al.* 2009).
324
325

326 ***Statistical analysis***

327 Data were analyzed using SigmaStat 3.5 and SigmaPlot 10.0 (Systat Software,
328 Richmond, CA, USA). Embryo developmental variables and relative mRNA expression
329 among the experimental groups were compared using one-way analysis of variance
330 (ANOVA) followed by multiple pair-wise comparisons using the Tukey method.
331 Significance was set at $P<0.05$.

332 Methylation percentages obtained from the sequenced clones were compared
333 among the different groups using the statistical z-test.

334

335 **RESULTS**

336 ***Developmental rates of embryos cultured in the presence of oviductal fluid at***
337 ***different time points during in vitro development***

338 No significant differences were observed in cleavage rates between control
339 group embryos and embryos cultured in the presence of OF (Fig. 2A). Proportions of
340 embryos reaching the ≥8-cell stage at 52 hpi were similar in the four experimental
341 groups with no significant differences between them. Overall, 51.2-59.2% of the
342 embryos reached the 8-cell stage or more at 52 hpi, and a significantly lower proportion
343 of embryos was observed in a delayed developmental stage, irrespective the addition of
344 OF to the embryo culture medium (Fig. 2B). Embryos that developed in a timely
345 manner reaching the stage of ≥8-cells at 52 hpi were then separately cultured. A
346 percentage between 52.8-61.3% of these embryos reached the 16-cell stage or more at
347 98 hpi, with no differences observed between the control and OF groups (Fig. 2C).

348 Cumulative blastocyst yields on Day 7 and Day 8 post-insemination were similar
349 for embryos cultured with or without OF (Fig. 2D). In all experimental groups,
350 normally developing embryos gave rise to significantly higher blastocyst rates than

351 embryos that showed slower developmental kinetics at 52 hpi (late 52 hpi) or 98 hpi
352 (late 98 hpi).
353
354 ***Effects of oviductal fluid on the DNA methylation state of gene-specific regions***
355 To determine whether addition of OF to the embryo culture medium during
356 different time intervals would affect DNA methylation marks at the blastocyst stage,
357 bisulfite sequencing was performed to assess the methylation status of specific genomic
358 regions within the CpG islands of four developmentally important genes.
359 As shown in Fig. 3A, the region analyzed for the *MTERF2* gene spanned a 240
360 bp segment with 21 CpG sites, covering the entire first exon and part of its neighboring
361 regions. Blastocysts from groups OF1-16, OF1-8 and OF8-16 showed a reduced level of
362 methylation at the *MTERF2* region compared to BSA control (20.0%, 26.2% and 32.9%
363 vs 56.2% respectively, $P<0.001$). Additionally, embryos in the OF8-16 group showed a
364 significantly higher methylation level of these CpG sites compared to those in OF1-16
365 ($P<0.05$). In contrast, comparable methylation levels of the *MTERF2* region were
366 observed in blastocysts in OF1-16 versus OF1-8 ($P>0.05$).
367 The fragment of 261 bp examined within *ABCA7* gene spanned 19 CpG sites
368 covering part of exon 14 and exon 15 and the entire intron between them (Fig. 3B). This
369 CpG sequence showed a lower methylation level in blastocysts from OF1-16 (31.1%)
370 than blastocysts from OF1-8 (56.8%), OF8-16 (57.9%) or the control group (65.8%)
371 ($P<0.001$). However, methylation values in embryos from OF1-8, OF8-16 or the control
372 group were not significantly different (Fig. 3B, $P>0.05$).
373 For the *OLFM1* gene, a fragment of 249 bp was amplified from bisulfite-treated
374 DNA and this amplicon included a total of 17 CpG sites within the last exon of the gene
375 (Fig. 3C). The first 9 CpGs cover the last coding region of the exon and the other 8

CpGs encompass the first part of the non-coding region. The methylation pattern of this CpG sequence did not differ between blastocysts derived from the OF1-16 (19.4%) and control groups (24.1%) (Fig. 3C, $P>0.05$). However, in embryos from OF1-8 this region was more methylated (47.1%) than in those from OF1-16 (19.4%), OF8-16 (29.4%) and the control group (24.1%) (Fig. 3C, $P<0.001$). Although OF8-16 blastocysts showed a lower methylation level in the *OLFM1* region than OF1-8 blastocysts, this methylation level was significantly higher than that observed for embryos in OF1-16 (Fig. 3C, $P<0.05$).

In contrast with these results, no significant differences among experimental groups were detected in the methylation patterns of CpG sites in the *GMDS* region, which comprises a 137 bp genome segment containing five CpG sites within intron 6 of the gene (Fig. 3D, $P>0.05$).

Effects of oviductal fluid on the DNA methylation state of LINE-1 repetitive elements

Since repetitive elements present in the genome are also important target sequences for DNA methylation, the methylation status of two regions (A and B) within the bovine LINE-1 elements was also examined.

Percentages of methylated cytosines observed in the CpG sequence of region A in LINE-1 were significantly higher in OF1-16 blastocysts than those in the OF8-16 ($P<0.001$) and BSA group ($P<0.05$) (Fig. 4A). However, no significant differences in the methylation level were observed between embryos in OF1-16 and OF1-8, though OF1-8 blastocysts showed a significantly higher methylation rate than OF8-16 blastocysts (Fig. 4A, $P<0.05$).

The CpG sequence within region B in LINE-1 showed low methylation levels in blastocysts from all the experimental groups (Fig. 4B).

401 ***Analysis of mRNA expression in blastocysts derived from embryos cultured with or***
402 ***without oviductal fluid during the first stages of development***

403 In addition to the DNA methylation analysis, we investigated whether the
404 changes in methylation marks, as a consequence of exposure to OF during different
405 periods of the early development, are correlated with relative mRNA abundance
406 changes. Therefore, relative transcriptional levels of *MTERF2*, *ABCA7*, *OLFM1* and
407 *GMDS* were determined in blastocysts derived from control and OF-treated groups
408 according to the experimental design. Likewise, LINE-1 expression was also examined.

409 As shown in Fig. 5, relative mRNA expression for *MTERF2* was found
410 increased in blastocysts obtained from OF1-16, OF1-8 and OF8-16 groups compared to
411 transcript level in BSA group (Fig. 5, $P<0.05$). However, the relative mRNA expression
412 levels for *ABCA7, OLFM1* and *GDMS* did not differ significantly among blastocysts
413 from the different treatment groups (Fig. 5). The transcription level for LINE-1 was
414 found significantly lower in blastocysts derived from OF1-16 group than blastocysts
415 from BSA group and the other OF-treated groups (Fig. 5, $P<0.05$).
416

417 **DISCUSSION**

418 Epigenetic reprogramming during preimplantation is critical for the development
419 of the mammalian embryo (Beaujean 2014). Part of this reprogramming occurs when
420 the embryo passes through the oviduct. Several studies have evidenced that mammalian
421 embryos developed in *in vitro* culture conditions, outside the maternal environment, are
422 susceptible to errors in the epigenetic reprogramming, leading to alterations in their
423 DNA methylation pattern (Fernandez-Gonzalez *et al.* 2004, Niemann *et al.* 2010,
424 Salilew-Wondim *et al.* 2015). In this sense, there is a growing body of evidence
425 suggesting that the ovarian follicle microenvironment and the reproductive tract fluids

426 can impact on the DNA methylation marks of the oocyte and the embryo, respectively
427 (O'Doherty *et al.* 2014, Canovas *et al.* 2017). However, the influence of OF on early
428 embryo epigenetic marks is still poorly understood. Thus, the aim of the present study
429 was to determine if the methylation pattern in particular genomic regions of bovine
430 blastocysts changes in response to addition of OF to the culture medium during the first
431 stages of development. Particularly, the attention was focused on genomic regions
432 contained within CpG islands of four developmentally important genes including
433 *MTERF2* (Gustafsson *et al.* 2016), *OLFM1* (Kodithuwakku *et al.* 2011), *ABCA7*
434 (Morales *et al.* 2008) and *GMDS* (Haliburton *et al.* 2016), and also within LINE-1
435 retrotransposons.

436 Addition of OF to the embryo culture medium at different time points during
437 embryonic development had no effect on the kinetics of development and blastocyst
438 yield. Similar findings were observed in a previous study using the same OF
439 concentration (Lopera-Vasquez *et al.* 2015). As expected, a greater proportion of
440 embryos reached the blastocyst stage in the subgroup of embryos that developed in a
441 timely manner regardless addition of OF compared to the subgroup of embryos showing
442 slower developmental kinetics. Despite this difference, similar blastocyst yields were
443 observed when embryos were cultured in the presence or absence of OF. This suggests
444 that there is no impact of OF on embryo developmental kinetics during *in vitro* culture.

445 In contrast, methylation analysis revealed that bovine embryos exposed to OF
446 for the first four days of culture (Group OF1-16), showed significantly reduced
447 methylation levels in CpG sites of *MTERF2* and *ABCA7* genes when compared with
448 levels in the BSA group. This four-day period of *in vitro* culture in medium
449 supplemented with OF mimics the window of development in which the embryo makes
450 contact with oviductal components in an *in vivo* situation (Hackett *et al.* 1993).

451 One plausible explanation for these results is that the higher levels of methylation
452 detected in the BSA group for the genomic regions analyzed (compared with OF1-16)
453 could be triggered by the absence of oviductal factors in the culture medium. This
454 finding reinforces the idea that oviductal factors, that are naturally present in the OF,
455 may be important for maintaining a more reduced methylation level at least in these
456 genomic regions. As highlighted in a recent study by Canovas *et al.* (2017), porcine
457 blastocysts produced *in vitro* in the presence of reproductive fluids during all steps of *in*
458 *vitro* production, show in general DNA methylation patterns diminished compared to
459 blastocysts cultured without biological fluid supplementation. However, in this genomic
460 study it is not possible to differentiate a precise effect of OF on specific DNA
461 methylation marks in the resulting blastocysts as porcine embryos were cultured *in vitro*
462 first with OF and then with uterine fluid. It has been well established that embryos of
463 different mammalian species produced *in vitro* show higher DNA methylation levels
464 than embryos developed *in vivo* (Corcoran *et al.* 2007, Deshmukh *et al.* 2011, Wright *et*
465 *al.* 2011). An interesting fact observed by Salilew-Wondim *et al.* (2015) is that bovine
466 embryos exposed to *in vitro* culture conditions up to the 16-cell stage produced higher
467 genome methylation in the resulting blastocysts than those derived from embryos that
468 were transferred at earlier stages to *in vivo* conditions. This finding, together with our
469 results, emphasizes the idea that suboptimal culture conditions and particularly the
470 absence of maternal signals from the 1-cell to 16-cell stage may affect DNA
471 methylation reprogramming with a subsequent effect on later stages of development.

472 In the current study, the effect of addition of OF in a stage-specific manner,
473 particularly at embryonic stages before or during the main phase of EGA in bovine
474 embryos was also investigated. In cattle, the main phase of EGA occurs at the 8- to 16-
475 cell stage (Graf *et al.* 2014). Even though the precise mechanisms involved in EGA

476 have not yet been determined in the bovine specie; it is thought that DNA methylation
477 remodeling at specific gene-regulatory regions could play some role in EGA (Bogliotti
478 & Ross 2015). This seems to be reflected by changes in the embryo gene expression
479 profile at the blastocyst stage (Rizos *et al.* 2002, Rizos *et al.* 2003, Gad *et al.* 2012). The
480 present study showed that methylation levels in the analyzed CpG sites of *MTERF2*
481 *ABCA7* and *OLFM1* were higher in blastocysts exposed to the OF at embryo stages
482 before the 8-cell stage (group OF1-8) or during major embryonic genome activation
483 (group OF8-16) than after addition of OF during the entire 1- to 16-cell stage (group
484 OF1-16). One feasible interpretation of this finding is that *in vitro* culture without OF,
485 before or during the major phase of EGA, may induce greater deregulation in DNA
486 methylation, suggesting that the embryonic stages in both phases of development are
487 susceptible to the oviductal factors missing *in vitro*. Our observations are somewhat
488 consistent with recent work indicating that the methylation profile of CpG islands and
489 repetitive elements within the bovine genome in blastocysts is affected when embryos
490 are subjected to *in vitro* culture during early stages of development (up to zygote, 4-cell
491 or 16-cell stage) and then transferred to *in vivo* conditions (Salilew-Wondim *et al.*
492 2015). Thus, it seems that the stages of development before and during EGA are
493 sensitive to embryonic DNA methylation changes induced by the embryo environment.

494 Interestingly, contrary to the effect of OF on specific gene regions in blastocysts
495 developed from OF1-16 embryos (e.g. *MTERF2* and *ABCA7*), methylation level
496 significantly increased within the CpG sites of LINE-1 retrotransposons. Bovine LINE-
497 1 repeats are among the evolutionarily younger active repetitive elements (Adelson *et*
498 *al.* 2009). Other authors have proposed that LINE-1 repeats can serve as a surrogate
499 marker for global genomic DNA methylation for the human or bovine genome (Yang *et*
500 *al.* 2004, Li *et al.* 2017). In a physiological situation, the activity of interspersed

501 repetitive elements is regulated by DNA methylation (Liang *et al.* 2002). Indeed, loss of
502 methylation has been associated with reactivation of retrotransposons and consequently
503 with modified chromosome integrity and/or modified gene expression by insertion
504 events (Thurston *et al.* 2007, Jones 2012). Hence, it could be expected that OF help
505 keep these sequences methylated to prevent their activation and ensure the genomic
506 stability of the embryos. The expression analysis showed lower transcriptional level of
507 LINE-1 in blastocysts from OF1-16 group than blastocysts from groups OF1-8, OF8-16
508 and BSA. This result suggests that the observed differences in DNA methylation status
509 of LINE-1 in blastocysts derived from OF1-16 have a positive correlation with their
510 expression level. Therefore, the addition of OF during the first four days of embryo
511 culture may help the suppression of LINE-1 transposable elements.

512 It should be mentioned that the genome regions examined in the present study
513 are situated at different loci either within genes or in intergenic regions. The biological
514 significance of methylation changes at these loci in response to OF is not known.
515 However, the role of DNA methylation in gene expression depends on the genomic
516 context in which they occur. While methylation at promoters within CpG islands or the
517 first exon is frequently associated with gene repression (Brenet *et al.* 2011, Smith *et al.*
518 2012), gene body specific methylation is often linked to active transcription (Hellman &
519 Chess 2007) and it also seems to be related to silencing of intragenic retrotransposons
520 and modulation of alternative splicing promoting exon recognition (Maunakea *et al.*
521 2013). In order to further investigate the influence of changes in methylation marks
522 within the genomic regions analyzed, the relative mRNA abundance for the selected
523 genes was determined. The increased relative expression of *MTERF2* in blastocysts
524 derived from groups OF1-16, OF1-8 and OF8-16 suggests a correlation with the lower
525 methylation levels observed in this genomic region compared to their BSA group

526 counterparts. Considering that DNA methylation changes occurred very close to the
527 promoter region of the gene, the *MTERF2* transcriptional level could be epigenetically
528 regulated via methylation changes as a consequence of the influence of the OF during
529 early embryogenesis. In contrast to this finding, a similar relative mRNA abundance of
530 *ABCA7* and *OLFM1* in blastocysts derived from OF-treated and BSA groups suggests
531 that the changes in methylation of the genomic regions analyzed (exon-intron junction
532 and last exon, respectively) would not be involved in the control of the transcript level
533 of these genes.

534 In conclusion, our findings indicate that bovine embryos are transiently sensitive
535 to the presence of OF at early embryonic stages *in vitro*. This sensitivity was reflected
536 by the OF effect on methylation marks in CpG sites within certain genomic regions and
537 repetitive elements at the blastocyst stage. The changes in embryo DNA methylation
538 induced by the presence of OF in the culture environment could partly affect the mRNA
539 expression level for specific genes (e.g. *MTERF2*) and LINE-1 elements. Although the
540 present study is only focused on methylation analysis of specific genomic CpG
541 sequences, thus giving only a partial view on the impact of OF on embryo epigenetics, it
542 provides evidence to suggest that embryo could be able to respond to oviductal
543 signaling modifying the methylation pattern of genome-specific loci. Our findings
544 provide new evidence that may help reveal the role of maternal factors in the
545 communication between the oviduct and the embryo in the early postconception period.

546

547 **DECLARATION OF INTEREST**

548 The authors declare that there is no conflict of interest that could be perceived as
549 prejudicing the impartiality of the research reported.

550

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555

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560

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706
707

1 **FIGURE LEGENDS**

2 **Figure 1. Diagram of the experimental design**. Bovine embryos were subjected to *in*
3 *vitro* culture under four experimental groups: 1) Group BSA = synthetic oviductal fluid
4 (SOF) + 3 mg/mL bovine serum albumin (BSA); 2) Group OF1-16 = SOF + 1.25%
5 (v/v) oviductal fluid (OF) added to culture from the 1-cell stage to 16-cell stage; 3)
6 Group OF1-8 = SOF + 1.25% (v/v) OF added to culture from the 1-cell stage to 8-cell
7 stage; and 4) Group OF8-16 = SOF + 1.25% (v/v) OF added to culture from the 8-cell
8 stage to 16-cell stage. Expanding blastocysts derived from embryos developing in a
9 timely manner were collected at Day 7-8 post-insemination (pi) for DNA methylation
10 analysis.

11

12 **Figure 2. Development rates of bovine embryos cultured in the presence or absence**
13 **of bovine oviductal fluid (OF) at different time periods during *in vitro***
14 **development**. A) Cleavage rate at 52 h post-insemination (hpi). B) Percentage of
15 cleaved embryos reaching the 8-cell stage or beyond ("normal development") at 52 hpi.
16 C) Percentage of embryos reaching the 16-cell stage or beyond at 98 hpi derived from
17 embryos showing "normal development" at 52 hpi. D) Blastocyst rate on Day 7-8 (*in*
18 *vitro* fertilization = Day 0) derived from the "normally developed" (normal dev.), "late
19 98 hpi" and "late 52 hpi" groups. Results are expressed as mean ± standard error of the
20 mean. Different superscripts in each column indicate significant differences based on
21 ANOVA ($P<0.05$). BSA: embryos cultured in SOF + BSA; OF1-16: embryos cultured
22 in SOF + OF from the 1-cell stage to 16-cell stage; OF1-8: embryos cultured in SOF +
23 OF from the 1-cell stage to 8-cell stage; OF8-16: embryos cultured in SOF + OF from
24 the 8-cell stage to 16-cell stage.

25

26 **Figure 3. DNA methylation changes produced in genomic regions in blastocysts**
27 **derived from embryos cultured in the presence or absence of bovine oviductal fluid**
28 **as determined by bisulfite sequencing**. A) DNA methylation pattern around the first
29 exon of *MTERF2*. B) DNA methylation changes around intron 14 and its adjacent exons
30 in *ABCA7*. C) DNA methylation changes in the last exon of *OLFM1*. D) DNA
31 methylation profile in a region within intron 6 in *GMDS*. Exons are represented by a
32 vertical rectangle, and the location of the region analyzed is indicated by a shaded
33 horizontal rectangle. The black arrow indicates the transcription start site (TSS). Each
34 row represents the average methylation level of the clones sequenced for each
35 experimental group. Changes in methylation of each CpG site are indicated by shaded
36 circles and shading indicates average percentage of DNA methylation. Black color
37 denotes the presence of methylation, whereas white color indicates lack of methylation.
38 Average methylation percentage for each experimental group is given on the right.
39 Numbers across the top indicate specific positions of CpG dinucleotides in the genomic
40 region. Different superscripts indicate significant differences between treatments based
41 on the statistical z-test ($P<0.05$).
42
43 **Figure 4. DNA methylation profiles in regions within LINE-1 repetitive elements in**
44 **blastocysts derived from embryos cultured with or without bovine oviductal fluid**
45 **as determined by bisulfite sequencing**. A) DNA methylation changes in the A region
46 of LINE-1 repeated sequences. B) DNA methylation pattern in the B region of LINE-1
47 repeated sequences. Each row represents the average methylation level of the clones
48 analyzed for each experimental group. Changes in methylation of each CpG site are
49 indicated by shaded circles and shading indicates the average percentage of DNA
50 methylation. Black color denotes the presence of methylation, while white indicates

51 lack of methylation. Average methylation percentage for each experimental group is
52 given on the right. Significant differences based on the statistical z-test ($P<0.05$) are
53 indicated with different letters.
54
55 **Figure 5. Relative mRNA abundance of selected genes and LINE-1 in blastocysts**
56 **developed from embryos cultured with or without bovine oviductal fluid at**
57 **different time points of the early embryogenesis.** Bars represent the relative
58 abundance of *MTERF2*, *ABCA7*, *OLFM1*, *GMDS* and LINE-1 transcripts in blastocyst
59 stage embryos normalized to the *H2AFZ* housekeeping gene. The BSA group is
60 represented by white columns and oviductal fluid (OF)-treated embryos are represented
61 with grey (group OF1-16), black (group OF1-8) and hatched (OF8-16) columns. Results
62 are expressed as mean ± standard error of the mean. Different superscripts indicate
63 significant differences ($P<0.05$) between treatments. Data are obtained from three
64 replicates of independent groups of 10 expanding blastocysts (Day 7-8).
65

Table 1. Details of primers used for the amplification of bisulfite treated DNA

DNA region	Outer and inner* primer sequences (5´- 3´)		Amplicon length (bp)	Amplicon location in bovine genome and number of CpG analyzed
MTERF2	Forward	GTTAGGYGGAGTTGAGGTAGTT	437	Chromosome 5 From: 70,601,882 To: 70,602,121 (240 bp – 21 CpG)
	Reverse	CTTCTCACGAACTATAACATTCC		
	Forward*	GAGTATTTATAGGTGTGTAGG	240	
	Reverse*	CRATCRAAATAAACTCCRCCACCCTA		
OLFM1	Forward	TGGAGAAAYGTGGGGGTAAYGTT	476	Chromosome 11 From: 106,675,747 To: 106,675,995 (249 bp – 17 CpG)
	Reverse	TACAAACCTAAAACACCAACTACC		
	Forward*	GAGTTTAYGTTGTTGATGAGTTTG	249	
	Reverse*	ACATCTCCATACTAAACTACAACC		
ABCA7	Forward	TGTTTTAYGTGTTGTGYGTGGTT	305	Chromosome 7 From: 45,170,162 To: 45,170,422 (261 bp – 19 CpG)
	Reverse	ACAACAAAAAACCAAAAACCTAAAC		
	Forward*	GTTTGGAGGGATTAGTTGTTAATG	261	
	Reverse*	CCAAACTAAAAACATCTACTATAA		
GMDS	Forward	AAGTTTGYGGGATTTTATATGGTG	337	Chromosome 23 From: 51,395,536 To: 51,395,672 (137 bp – 5 CpG)
	Reverse	AAAATCTCCTCTACCCCATACTACC		
	Forward*	GTTATTTGTTAGGGTGGGTTTGTT	137	
	Reverse*	CACAAAACCACTTTCTAATCTACTC		
LINE-1 Region A	Forward	TAAAATTTATAGATTATGAGTTTTAT	357	Region located across the genome (317 bp-11 CpG)
	Reverse	TAAATAATATCTCTCTAAAACTA		
	Forward*	ATGAGTTTTATGGTAATTTTTATAG	317	
	Reverse*	ATTACCCCCTTCCAACTCTAACTAC		
LINE-1 Region B	Forward	ATAGGTTGTATTGTTTAGAGTAAGGAT	244	Region located across the genome (201 bp-7 CpG)
	Reverse	AATACATTTCTAAAACTACGATTAA		
	Forward*	GAGTAAGGATAGGGTTTGAATGTTT	201	
	Reverse*	CCCTTCCAACTCTAACTACCTATCAC		

bp: base pairs. Y= C/T, R= A/G.

Table 2. Primers used for qRT-PCR analysis

Gene/Repetitive element		Primer sequences (5´- 3´)	Amplicon length (bp)	GenBank accession number
MTERF2	Forward Reverse	AGTTTATTGCAGGGAAGTGACA GCTGGGATCTCATCAGCAACT	198	NM_001191174
ABCA7	Forward Reverse	ATGGGCGGTTGAAGGGTCTGAG CCCACAAAGGCAATGGCCACTG	153	NM_001205705
OLFM1	Forward Reverse	AACCAGATGAAAGGGCTGGAG TGGACTGCTTCTTCAGTCTGC	128	NM_001101879
GMDS	Forward Reverse	AAGTTTGYGGGATTTTATATGGTG AAAATCTCCTCTACCCCATACTACC	216	NM_001080331
H2AFZ	Forward Reverse	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	208	NM_174809
LINE-1	Forward Reverse	CCCAGGTCCAGACGGCTT GGGTGATGGTGGCCTCATAGA	137	-

bp: base pairs.

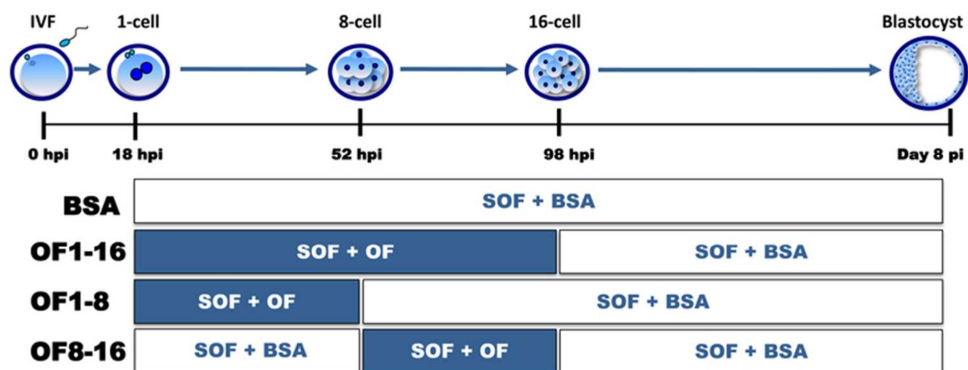


Figure 1. Diagram of the experimental design.

68x31mm (300 x 300 DPI)

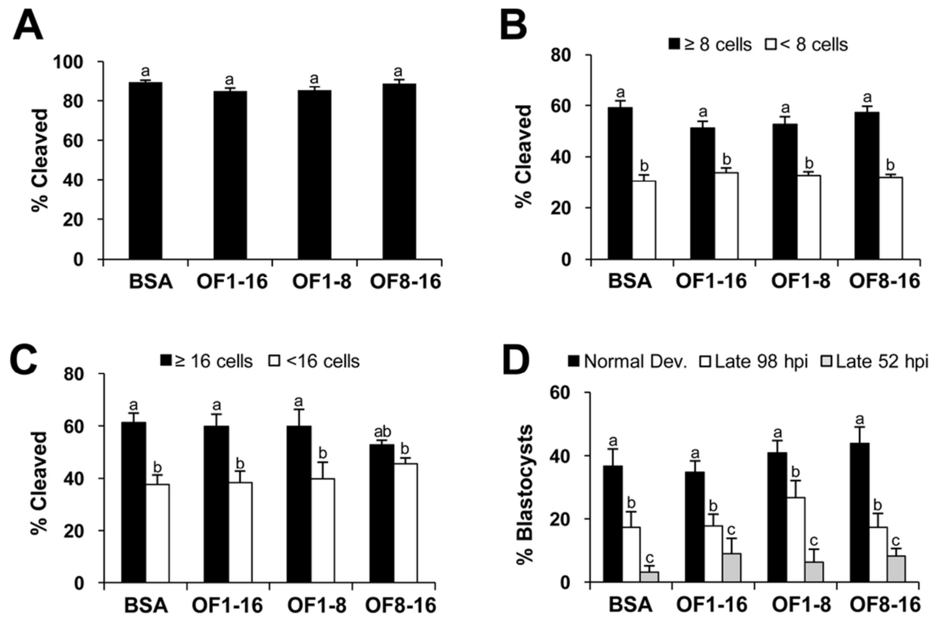


Figure 2. Development rates of bovine embryos cultured in the presence or absence of bovine oviductal fluid (OF) at different time periods during *in vitro* development.

99x66mm (300 x 300 DPI)

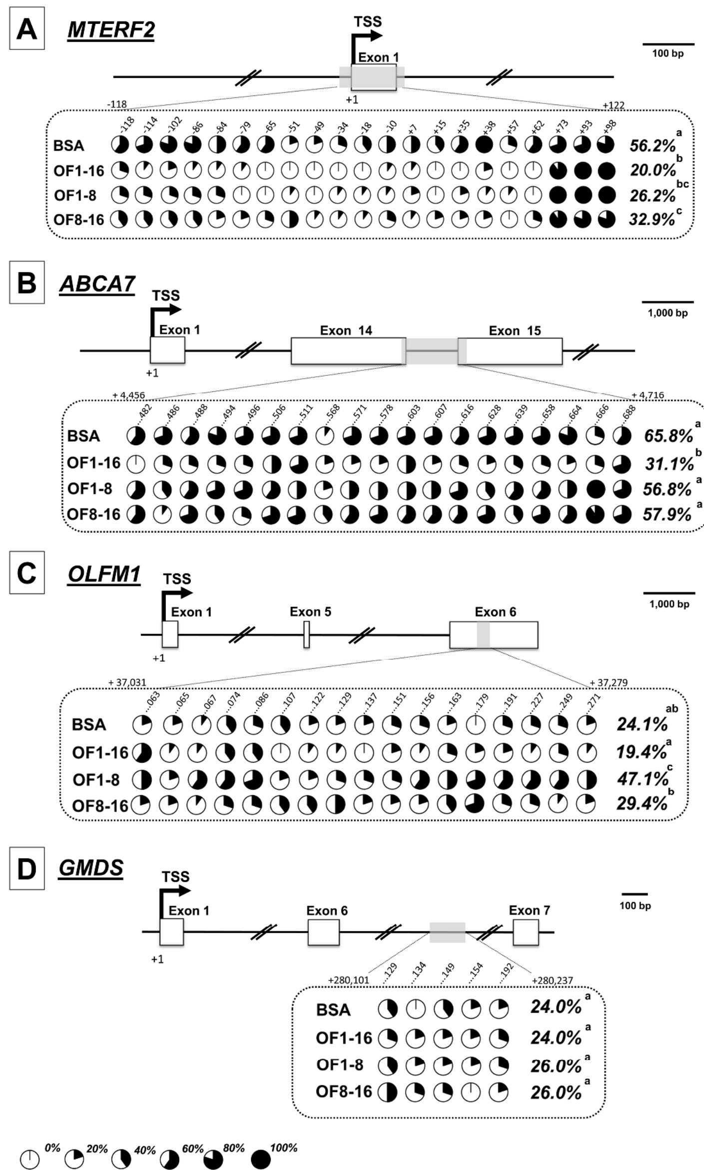
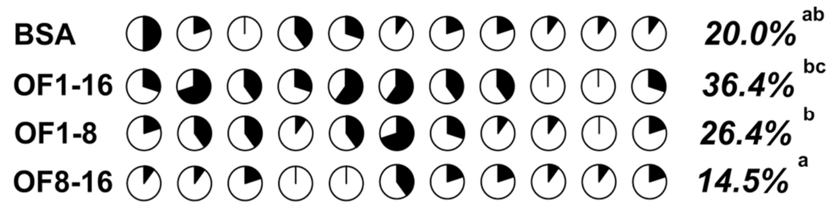


Figure 3. DNA methylation changes produced in specific genomic regions in blastocysts derived from embryos cultured in the presence or absence of bovine oviductal fluid as determined by bisulfite sequencing.

231x356mm (300 x 300 DPI)



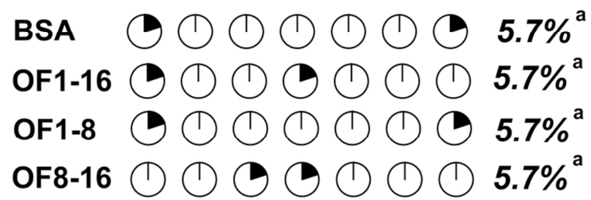


Figure 4. DNA methylation profiles in regions within LINE-1 repetitive elements in blastocysts derived from embryos cultured with or without bovine oviductal fluid as determined by bisulfite sequencing.

110x81mm (300 x 300 DPI)

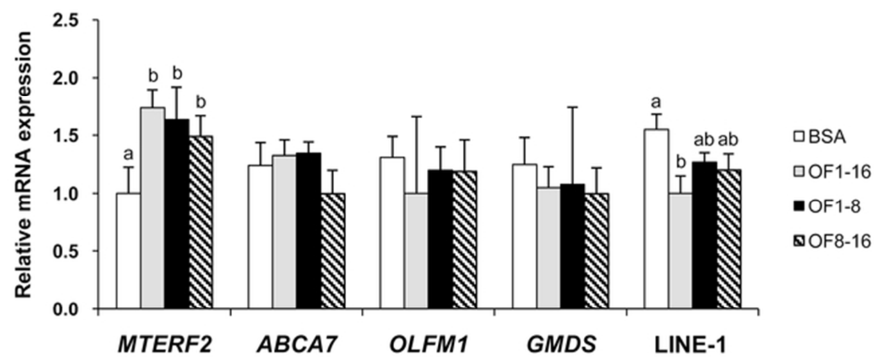


Figure 5. Relative mRNA abundance of selected genes and LINE-1 in blastocysts developed from embryos cultured with or without bovine oviductal fluid at different time points of the early embryogenesis.

63x26mm (300 x 300 DPI)