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1 **Effect of urokinase type plasminogen activator on *in vitro* bovine oocyte**
2 **maturation**
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4 Roldán-Olarte, Mariela[1,2]; Maillo, Verónica[1]; Sánchez-Calabuig, María Jesús[1];
5 Beltrán-Breña, Paula[1]; Rizos, Dimitrios[1]; Gutiérrez-Adán, Alfonso[1]
6
7 [1]Dept. Reproducción Animal, INIA, Crta. de la Coruña Km 5.9, Madrid 28040,
8 Spain.
9 [2]Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-Universidad
10 Nacional de Tucumán (UNT), and Instituto de Biología 'Dr. Francisco D. Barbieri',
11 Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI –
12 San Miguel de Tucumán, Argentina.
13
14 **Correspondence to:**
15 Mariela Roldán-Olarte, Instituto Superior de Investigaciones Biológicas (INSIBIO),
16 CONICET-UNT, and Instituto de Biología 'Dr. Francisco D. Barbieri', Facultad de
17 Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI – San Miguel de
18 Tucumán, Argentina.
19 Phone: 54-381-4247752 (ext. 7099); Fax: 54-381- 4247752 (ext. 7004).
20 e-mail: emroldanolarte@fbqf.unt.edu.ar
21
22 **Short title:**
23 uPA on *in vitro* bovine oocyte maturation
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25 **Abstract**

26 This study examines the impacts of the urokinase type plasminogen activator
27 (uPA) on the *in vitro* maturation (IVM) of bovine oocytes. Cumulus-oocyte
28 complexes in IVM medium were treated with uPA, amiloride (an uPA inhibitor),
29 DMSO or left untreated (control group). After 24 h of IVM, oocytes were recovered
30 for testing or were *in vitro* fertilized and cultured to the blastocyst stage. The factors
31 examined in all groups were: i) oocyte nuclear maturation (Hoëscht staining), ii)
32 oocyte cytoplasmic maturation (cortical granules, CG, distribution assessed by
33 LCA-FITC), iii) oocyte and cumulus cell (CC) gene expression (RT-qPCR), and iv)
34 embryo development (cleavage rate and blastocyst yield). Oocytes subjected to
35 uPA treatment showed rates of nuclear maturation and CG distribution patterns
36 similar to controls ($p>0.05$), whereas lower rates of oocyte maturation were
37 recorded in the amiloride group ($p<0.05$). Both in oocytes and CC, treatment with
38 uPA did not affect the transcription of genes related to apoptosis, cell junctions, cell
39 cycle, or serpin protease inhibitors. In contrast, amiloride altered the expression of
40 genes associated with cell junctions, cell cycle, oxidative stress and CC serpins.
41 No differences were observed between the control and uPA group in cleavage rate
42 or in blastocyst yield recorded on Days 7, 8 or 9 post-insemination. However,
43 amiloride led to drastically reduced cleavage rate (28.5% vs 83.2%) and Day 9
44 embryo production (6.0% vs 21.0%) over the rates recorded for DMSO. These
45 results indicate that the proteolytic activity of uPA is needed for successful oocyte
46 maturation in bovine.

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48

49

50 **Introduction**

51 The structural integrity of the cumulus cell extracellular matrix (ECM) is essential
52 for oocyte maturation (Zhuo & Kimata 2001, Salustri *et al.* 2004). Several cumulus
53 proteins linked to hyaluronan are present around cumulus cells and have been
54 attributed a role in cumulus expansion and oocyte maturation (Lu *et al.* 2013). The
55 task of ECM remodeling is the responsibility of proteolytic enzymes. As a
56 proteolytic system, the plasminogen activation system has the capacity to act on a
57 wide broad spectrum of substrates (Collen 1980, Saksela 1985, Liu *et al.* 2004,
58 Deryugina & Quigley 2012). Plasminogen is an extracellular proenzyme that is
59 abundant in blood plasma and most extracellular fluids (Plow *et al.* 1995),
60 especially follicular and oviductal fluids (Beers 1975, Mondèjar *et al.* 2012).
61 Plasminogen is activated to plasmin by two types of plasminogen activators:
62 urokinase-type (uPA) and tissue-type (tPA). Both activators are produced by
63 bovine cumulus-oocyte complexes during their *in vitro* maturation (Park *et al.*
64 1999). A role for uPA has been established in the pericellular proteolysis and is
65 required for cell migration and tissue remodeling (Andreasen *et al.* 2000,
66 Deryugina & Quigley 2012). Binding to specific receptors (uPAR) localizes uPA
67 activity at the cell surface (Blasi & Sidenius 2010, Smith & Marshall 2010). The
68 activity of uPA is controlled by the specific inhibitor type 1 (PAI-1), a member of the
69 serpin proteinase inhibitor superfamily (Potempa *et al.* 1994). The uPA substrate,
70 plasminogen, has been detected in the plasma membrane and zona pellucida of
71 hamster oocytes (Jiménez-Díaz *et al.* 2002), immature pig oocytes (Roldán-Olarte
72 *et al.* 2005) and in *in vitro* matured porcine and bovine oocytes (Mondèjar *et al.*
73 2012). Recently, it was observed that the gene that codifies uPA (*PLAU)* is only
74 expressed in the cumulus cells of immature or *in vitro* matured COCs, while
75 *PLAUR* and *PAI-1* are expressed in both cumulus cells and in immature and *in*
76 *vitro* matured oocytes (García *et al.* 2016). These authors propose that the
77 plasminogen activation system could play a critical role in the oocyte maturation
78 process. When tPA activity was determined in cortical granule extracts (Rekkas *et*
79 *al.* 2002), it was related to post-fertilization events such as the cortical reaction and
80 the block of polyspermy at the zona pellucida (Mondèjar *et al.* 2012). Plasmin, the

81 central protease of this system, is required for physiological processes such as
82 ovulation (Liu *et al.* 2004), cumulus cell expansion (Liu *et al.* 2004), oocyte
83 maturation (Dow *et al.* 2002), fertilization (Smokovitis *et al.* 1992, Huarte *et al.*
84 1993), zona reaction (Zhang *et al.* 1992, Cannon & Menino 1998, Rekkas *et al.*
85 2002) and embryo hatching (Menino & Williams 1987, Kaaekuahiwi & Menino
86 1990).

87 Despite numerous works addressing the role of the plasminogen activation
88 system during the initial stages of reproduction (Papanikolaou *et al.* 2008, Coy *et*
89 *al.* 2012, Grullon *et al.* 2013, Krania *et al.* 2015a, Krania *et al.* 2015b), the
90 contributions of each of its components to each stage of *in vitro* embryo production
91 have not been well established. What has been established is that the inhibition of
92 endogenous uPA compromises cumulus expansion during the *in vitro* maturation of
93 COCs in mice and humans (Lu *et al.* 2013). The latter authors reported that the
94 over-expression of *SERPINE2,* or exogenous supplementation with high levels of
95 SERPINE2 impaired cumulus expansion and oocyte maturation. This protein is a
96 member of the serpin family, a group of proteins that inhibit serine proteases such
97 as thrombin, uPA, plasmin and trypsin. Amiloride, a specific inhibitor of uPA,
98 produced a similar effect on cumulus expansion. Amiloride competitively inhibits
99 the catalytic activity of uPA while it has no effects on tPA (Vassalli & Belin 1987).
100 These different effects can be attributed to structural differences between the two
101 activator types (Jankun & Skrzypczak-Jankun 1999, Zhu *et al.* 2007).

102 The present study was designed to address the effects of uPA on *in vitro*
103 bovine oocyte maturation by separately determining the impacts of adding uPA or
104 a specific inhibitor of uPA (amiloride) to the IVM medium.
105
106

107 **Materials and methods**

108 Unless stated otherwise, all chemicals were purchased from Sigma Aldrich
109 Química (Madrid, Spain).

110

111 **Oocyte collection and IVM**

112 Immature cumulus–oocyte complexes (COCs) were obtained by aspirating follicles
113 (2–8 mm diameter) from the ovaries of mature heifers (i.e. at least one corpus
114 luteum or remained scars from previous ovulations in one or both ovaries)
115 collected at slaughter from a local abattoir (Transformación Ganadera de Leganés
116 S.A., Madrid, Spain). Class 1 and class 2 COCs (homogeneous cytoplasm and
117 intact cumulus cells) were matured for 24 h in 500 µL of maturation medium, TCM
118 199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/mL epidermal
119 growth factor in four well dishes, in groups of 50 COCs per well at 38.5ºC under an
120 atmosphere of 5% CO_2 in air, with maximum humidity (Rizos *et al.* 2002). Each
121 experiment consisted of four groups of 50 COCs, in accordance to each treatment
122 given at the start of IVM: I) no treatment (control); II) uPA (10 nM, Sigma, U0633-
123 25UG; solubilized in sterile-filtered water, to obtain a stock solution of 1 µM); III)
124 dimethyl sulfoxide (DMSO, 0.02% as the amiloride vehicle) and IV) amiloride (100
125 µg/mL, Sigma, A0370000; solubilized in DMSO to obtain a stock solution of 50
126 mg/mL).

127 The concentration of uPA was based on the findings of other studies in
128 which the effect of uPA on expression levels of *C-FOS* in oviductal epithelial cells
129 (García *et al.* 2014) and ovarian cancer cells (Dumler *et al.* 1994) was analyzed.
130 The amiloride concentration used was selected according to the findings of Lu *et*
131 *al*. (2013) and Ding *et al.* (2012). The first study evaluated the effect of 0.3 mM
132 amiloride on the expansion of murine and human COCs and the latter study
133 reported that 0.01, 1 and 1 mM amiloride decreases *PLAU* expression levels in
134 human gastric cancer cell lines after 24 of incubation (Ding *et al.* 2012).

135 COCs matured under different conditions were employed to evaluate:
136 developmental competence after *in vitro* fertilization, nuclear maturation, cortical
137 granules (CG) distribution and gene expression in oocytes and cumulus cells (CC).

138 From each group, 10 oocytes were employed to the evaluation of nuclear
139 maturation and CG distribution, 10 COCs to the gene expression analysis and the
140 remaining were destined to *in vitro* fertilization and posterior embryo development.
141 Four replicates of each experiment were set up.
142

143 **Sperm preparation and *in vitro* fertilization (IVF)**

144 Frozen semen straws (0.25 mL) from an Asturian Valley bull previously tested for
145 IVF (ASEAVA, Asturias, Spain) was thawed at 37°C in a water bath for 1 min and
146 centrifuged for 10 min at 280 x *g* through a gradient of 1 mL of 40% and 1 mL of
147 80% Bovipure® (Nidacon Laboratories AB, Göthenborg, Sweden Bovipure)
148 according to the manufacturer's instructions. The sperm pellet was isolated and
149 washed in 3 mL of Boviwash® (Nidacon Laboratories AB, Göthenborg, Sweden
150 Bovipure) by centrifugation at 280 x *g* for 5 min. The pellet was re-suspended in
151 the remaining 300 µL of Boviwash®. Sperm concentration was determined and
152 adjusted to a final concentration of 1 X 10^6 sperm mL $^-$1 for IVF. Gametes were
153 coincubated for 18–22 h at 38.5°C in four-well plates in groups of 50 COCs per
154 well under an atmosphere of 5% CO_2 in air and maximum humidity. Each well
155 contained 500 µL of Tyrode's fertilization medium containing 25 mM bicarbonate,
156 22 mM Na-lactate, 1 mM Na-pyruvate, and 6 mg/mL fatty acid-free bovine serum
157 albumin (BSA) supplemented with 10 mg/mL heparin sodium salt (Calbiochem,
158 San Diego, CA, USA) (Lopera-Vásquez *et al.* 2016).
159

160 ***In vitro* culture of presumptive zygotes**

161 At approximately 20 h post insemination (hpi), presumptive zygotes were denuded
162 of cumulus cells by vortexing for 3 min and then cultured in groups of 25 in 25 µL
163 droplets (control: n=129; uPA: n=205; DMSO: n=137; amiloride: n=209) of
164 synthetic oviductal fluid (SOF) containing 4.2 mM sodium lactate (L4263), 0.73 mM
165 sodium pyruvate (P4562), 30 µL/mL BME amino acids (B6766), 10 µL/mL
166 minimum essential medium (MEM) amino acids (M7145) and 1 µg/mL phenol red
167 (P0290) under mineral oil at 38.5°C under an atmosphere of 5% CO_2, 5% O_2 and
168 90% N_2, as the embryo culture is routinely performed (Lopera-Vásquez *et al.*

169 *2016*). SOF was supplemented with 5% FCS (Gutiérrez-Adán *et al.* 2001, Rizos *et*
170 *al.* 2008).
171
172 **Embryo development and quality**
173 Cleavage rates were recorded on Day 2 (48 hpi) and cumulative blastocyst yields
174 on Days 7, 8, and 9 post insemination under a stereomicroscope. Four different
175 experiments, each of them with 4 experimental conditions were performed.
176
177 **Nuclear and cortical granules distribution patterns**
178 Nuclear maturation and CG distribution, as one parameter of cytoplasmic oocyte
179 maturation, were assessed by confocal microscopy following a method described
180 previously (Coy *et al.* 2005). Briefly, first 10 *in vitro* matured COCs from each
181 treatment were suspended in 100 µL of phosphate-buffered saline (PBS) without
182 calcium or magnesium supplemented with 1% BSA and their cumulus cells were
183 removed by gently pipetting. Next, oocytes were treated with 0.5% w/v pronase in
184 PBS to digest the zona pellucida. Zona-free oocytes were washed in PBS three
185 times and fixed in 3.7% w/v buffered neutral paraformaldehyde solution (pH 7.2–
186 7.4) for 30 min at room temperature and treated with permeabilization solution
187 (0.01% v/v Triton X-100 in PBS) for 10 min. The oocytes were then treated for 30
188 min with blocking solution (7.5% w/v BSA in PBS) and incubated in 100 µg/mL
189 FITC-labeled *Lens culinaris* agglutinin (LCA-FITC Sigma, L9262) for 40 min in a
190 dark chamber. Chromatin was stained with 1 µg/mL Hoëchst 33342 for 5 min. After
191 staining, oocytes were washed, mounted in 2 µL of mounting medium (50% v/v
192 PBS, 50% v/v glycerol (Sigma G-S150), 0.0025 µg/mL Hoëchst) between a
193 coverslip and a glass slide and sealed with nail polish. Slides were examined using
194 a laser-scanning confocal microscope (MRC 175 1024, Bio-Rad, Hercules, CA,
195 USA) equipped with an argon laser excited at 488 nm and whose detection
196 spectrum is 515-530 nm. Nuclear maturation was observed in an epifluorescence
197 microscope (Nikon 141731) equipped with a fluorescent lamp (Nikon HB-10104AF)
198 and UV-1 filter and oocytes were classified as follows: germinal vesicle stage (GV).
199 For the nuclear maturation, all the nucleus and polar bodies were evaluated,

200 oocytes (control: n=55; uPA=41; DMSO: n=59; amiloride: n=53) were classified as
201 GV: germinal vesicle stage (nucleus well defined) MI: metaphase I (first
202 metaphasic plate visible); or MII: metaphase II (nucleus mature, represented by the
203 presence of first polar body, observed before ZP dissolution, or second metaphasic
204 plate). As a parameter of cytoplasmatic maturation, cortical granules were
205 analysed (control: n=39; uPA=28; DMSO: n=39; amiloride: n=35 oocytes) and the
206 distribution of cortical granule was classified as three types (type I, distributed in
207 clusters; type II, dispersed and partly clustered; type III, small CG arranged at the
208 periphery) (Hosoe & Shioya 1997). Four replicates were carried out.
209

210 **Oocytes and cumulus cells for gene expression analysis**

211 After 24 h of IVM, pools of 10 COCs were collected from each treatment group
212 (four replicates) and cumulus cells physically separated from oocytes by gentle
213 pipetting. Oocytes, in pools of 10 per treatment group, were washed in PBS, snap
214 frozen in liquid N_2 and stored at -80°C until mRNA extraction. Their corresponding
215 cumulus cells were also washed in PBS, centrifuged at 10000 *x g* and then snap
216 frozen in liquid N_2 and stored at -80°C until mRNA extraction.
217

218 **Gene expression**

219 For gene expression studies, pools of 10 oocytes and their corresponding cumulus
220 cells from each experimental group were analyzed separately in 4 replicates.

221 Poly(A) RNA was extracted using the Dynabeads® mRNA DIRECT™ Micro
222 Kit (Ambion®, Thermo Fisher Scientific Inc., Oslo, Norway) according to
223 instructions with minor modifications (Bermejo-Alvarez *et al.* 2008). Immediately
224 after extraction, the reverse transcription (RT) reaction was run according to the
225 manufacturer's instructions (Epicentre Technologies Corp., Madison, U.S.A.) using
226 poly(T) primer, random primers and Moloney murine leukemia virus (MMLV)
227 reverse transcriptase. Tubes were heated to 70°C for 5 min to denature the
228 secondary RNA structure and then the RT mix was completed with the addition of
229 50 units of reverse transcriptase. The tubes were incubated at 25ºC for 10 min to
230 induce the annealing of random primers, followed by 37ºC for 60 min to allow the

231 RT of RNA, and finally 85ºC for 5 min to denature the enzyme. All qPCR reactions
232 were run in duplicate in the Rotorgene 6000 Real Time Cycler TM (Corbett
233 Research, Sydney, Australia). In each run, 2 µL aliquots of each sample were
234 added to the PCR mix (GoTaq® qPCR Master Mix, Promega Corporation,
235 Madison, USA) containing specific primers to amplify the genes contained in Table
236 1. The selection of genes to be evaluated in oocytes and CC was carried out
237 considering that they are representatives of key processes, i.e. apoptosis (*BAX*,
238 *BCL2*, *TP53*, *SHC1*), cell junctions (*GJA1*, *TJP1*), oxidative stress (*SOD2*, *GPX1*),
239 cell cycle (*CCNB1*), oocyte quality (*GDF9*, *BMP15*) and serpin protease inhibitors
240 (*SERPINE1*, *SERPINA5*) as previously described by several authors (Feuerstein *et*
241 *al.* 2007, Assidi *et al.* 2008, Assou *et al.* 2010, Bermejo-Alvarez *et al.* 2010,
242 Dovolou *et al.* 2014, Blaha *et al.* 2015). Primer sequences and sizes of the
243 amplified fragments of all transcripts are provided in Table 1. Cycling conditions
244 were 94°C for 3 min followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for
245 10 s and 10 s of fluorescence acquisition. Each pair of primers was tested to obtain
246 efficiencies close to 1. The comparative cycle threshold (Ct) method was used to
247 quantify expression levels (Schmittgen & Livak 2008). In each cycle, fluorescence
248 was acquired at a temperature higher than the melting temperature of primer
249 dimers to avoid primer-dimer artifacts (specific for each product, 76–86°C). The
250 threshold cycle or the cycle during the log-linear phase of the reaction at which
251 fluorescence increased above background was determined for each sample.
252 Within this region of the amplification curve, a difference of one cycle is equivalent
253 to a doubling of the amplified PCR product. According to the comparative Ct
254 method, the ΔCt value was determined by subtracting the endogenous control Ct
255 value (mean for *H2AFZ* and *ACTB)* for each sample from each gene Ct value of
256 the sample. To calculate ΔΔCt, the highest treatment ΔCt value (i.e. the treatment
257 with the lowest target expression) was used as a constant to subtract from all other
258 ΔCt sample values. Fold changes in the relative gene expression of the target were
259 calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen 2001).
260
261 **Statistical analysis**

262 All statistical tests were performed using SigmaStat (Jandel Scientific, San Rafael,
263 CA, USA) and InfoStat (Infostat 2015, http://www.infostat.com.ar) statistical
264 softwares. Data for cleavage rates, blastocyst yields, nuclear maturation, CG
265 distribution patterns, and relative mRNA abundance were compared by one-way
266 analysis of variance (ANOVA). When they showed normality, significant differences
267 between the mean values were determined (LSD Fisher´s test, $p<0.05$). In
268 addition, t-test was also applied to pairwise comparisons.
269
270

271 **Results**

272 **Developmental competence of bovine oocytes *in vitro* matured in the**
273 **presence of uPA or amiloride**

274 The results on cleavage and embryo development are shown in Figure 1.
275 Supplementation of uPA to IVM medium did not affect cleavage rate compared to
276 control and DMSO groups (85.7±4.1% vs. 80.9±1.4% and 83.2±2.1% respectively),
277 while supplementation of amiloride it decreased significantly (28.5±5.2%).
278 Blastosyst yield on day 7 was similar for uPA and control groups (18.6±1.5% and
279 17.4±4.2%) but significantly lower to DMSO and amiloride (8.0±2.6% vs.
280 1.3±1.0%). Similarly, for days 8 and 9 blastocyst yield was no different for uPA,
281 control and DMSO groups (Day 8: 21.9±0.4%; 24.4±2.3%; 18.1±3.4% and Day 9:
282 26.5±3.4%; 25.2±2.9%; 21.0±3.8%, respectively) while it was significantly
283 decreased for amiloride group (Day 8: 5.3±2.2% and Day 9: 6.0±1.9%).

284

285 **Nuclear maturation**

286 Nuclear maturation was unaffected by the presence of 10 nM uPA. Similar MII
287 rates were recorded for the uPA and control groups (62.7±10.7%, vs. 62.7±8.2%;
288 respectively). However, the addition of amiloride to the IVM medium, led to a
289 drastic increase in number of oocytes with intact GV (Figure 2) indicating that a
290 higher percentage of oocytes remained arrested or had not initiated meiosis
291 (83.3±6.6% vs. 14.9±8.0% in DMSO group, $p<0.05$).

292

293 **Cortical granules distribution patterns**

294 No significant differences in CG distributions were observed between the uPA and
295 control groups (type III CG distribution observed in 32.9±7.0% vs. 51.9±16.5%
296 respectively; Figure 3). Interestingly, significant differences were detected between
297 amiloride and control groups. As shown in Figure 3, most oocytes matured in the
298 presence of amiloride showed a type I CG distribution pattern compared to oocytes
299 in the control DMSO group (75.0±6.0% vs. 14.4±10.9%, respectively), while no
300 oocytes displayed a type III CG distribution (0.0% vs. 25.4±5.8%, respectively;
301 $p<0.05$). The presence of oocytes with type II CG distribution pattern was also

302 lower under amiloride treatment (25.0±1.6) than with uPA supplementation
303 (31.0±11.3), DMSO group (60.2±10.4) and control (29.5 ±10.4).
304

305 **Effect of uPA and amiloride on gene expression levels in oocytes and**
306 **cumulus cells**

307 The presence of uPA in the IVM medium only affected the expression of *SOD2* in
308 oocytes while no differences were observed in CC. The relative abundance of
309 *SOD2* in oocytes was higher in the uPA group than control group ($p<0.05$) (Figure
310 4 A).

311 In contrast, the addition of amiloride during *in vitro* maturation affected the
312 expression levels of several genes. Compared with controls, *GPX1* and *GJA1* were
313 up-regulated while *CCNB1* and *BMP15* were down-regulated in oocytes (Figure 5
314 A), and *BAX*, *BCL2*, *TP53*, *SHC1, TJP1*, *GJA1* and *CCNB1* were up-regulated,
315 while *SOD2*, *SERPINE1* and *SERPINA5* were down-regulated in CC (Figure 5 B).
316
317

318 **Discussion**

319 The role played by the plasminogen activation system in the initial steps of
320 mammalian reproduction has been addressed by many researchers who have tried
321 to elucidate its implication in the processes of gametogenesis (Liu *et al.* 1986, Liu
322 *et al.* 2013), fertilization (Huarte *et al.* 1993, Mondejar *et al.* 2012), early embryonic
323 development (Aflalo *et al.* 2005) and implantation (Whiteside *et al.* 2001). In the
324 present study, we sought to determine whether uPA activity could affect the quality
325 of oocytes used for *in vitro* embryo production, their developmental competence
326 and the expression of candidate genes in oocytes and CC after IVM.

327 According to our findings, excess uPA during IVM did not affect oocyte
328 maturation nor embryo development. In a preliminary experiment two different
329 concentrations of uPA were evaluated, 10 and 50 nM. No significant differences
330 were detected in the percentages of cleavage (78.0±5.5 in control; 86.2±3.8 with
331 uPA 10 nM and 77.2±1.1 with uPA 50 nM) nor blastocyst rate (23.9±2.6 in control;
332 28.7±3.1 with uPA 10 nM and 20.8±0.8 with uPA 50 nM). Then, we decided to use
333 10 nM of uPA as previously reported (Dumler *et al.* 1994, García *et al.* 2014).
334 Given that inhibitors of the plasminogen activation system exist in bovine COCs
335 (Bieser *et al.* 1998, García *et al.* 2016), these could both *in vivo* and *in vitro* control
336 the proteolytic activity of the system, diminishing the activity of exogenous uPA.
337 Therefore, an excess of uPA activity could be controlled by inhibitors that maintain
338 the balance of proteolytic activity initiated by uPA. This is in agreement with what
339 has been described by other authors (Krania *et al.* 2015a), who analyzed the effect
340 of the inclusion of exogenous uPA in the *in vitro* bovine embryo culture medium.
341 They found that it does not have any effect on embryo yield and/or quality. These
342 authors highlight the importance to the balance of activity between activators and
343 inhibitors in a highly regulated manner, to ensure the correct embryo development.
344 In the same extend during *in vitro* oocyte maturation, a well-balanced proteolytic
345 activity must be of the importance to assure the oocyte developmental
346 competence. To examine whether endogenous uPA is important for *in vitro*
347 maturation, we blocked the catalytic activity of uPA present in the female gamete
348 using amiloride. Our results indicate that amiloride inhibits the maturation of bovine

349 oocytes at the nuclear level as well at cytoplasmic level, taking into account the CG
350 distribution. We thus inferred that the proteolytic activity of uPA is required for the
351 processes of bovine oocyte maturation and cumulus cell expansion, in agreement
352 with observations in mouse COCs (Lu *et al.* 2013). The main function of uPA is to
353 generate plasmin through activation of plasminogen near the plasma membrane of
354 cells that express uPAR (Blasi & Sidenius 2010). The presence of uPAR and
355 plasminogen has been established in the female bovine gamete (Mondèjar *et al.*
356 2012, García *et al.* 2016), indicating that plasmin generation in bovine COCs could
357 involve uPA bound to its receptor at the plasma membrane of the oocyte and
358 cumulus cells. By blocking uPA activity using amiloride, we observed that plasmin
359 generation mediated by uPA is needed to ensure COC maturation. It has been
360 reported that plasmin increases maturation rates when added 18 h after *in vitro*
361 maturation without affecting embryonic developmental rates (Papanikolaou *et al.*
362 2008). However, plasmin's mechanism of action during oocyte maturation remains
363 to be established.

364 To gain insight into the molecular mechanisms affected by the presence of
365 uPA or amiloride during IVM, we examined, in both oocytes and cumulus cells, the
366 expression of candidate genes related to apoptosis (*BAX, BCL2, TP53, SHC1*), cell
367 junctions (*GJA1*, *TJP1*), cell cycle (*CCNB1*), oxidative stress (*SOD2, GPX1*),
368 oocyte quality (*BMP15*, *GDF9*) and serpin protease inhibitors (*SERPINE1,*
369 *SERPINA5*). Although a limited number of genes were analyzed in this study,
370 several changes were registered under different treatments carried out during
371 COCs *in vitro* maturation, suggesting that certain cellular processes are affected,
372 especially in the presence of amiloride. We observed that uPA does not provoked
373 changes in the expression levels of these genes in cumulus cells, while in oocytes,
374 only *SOD2* was up-regulated, suggesting that uPA could protect against oxidative
375 stress (Bermejo-Alvarez *et al.* 2010, Dovolou *et al.* 2014). In contrast, the presence
376 of amiloride modified the expression of several of the genes examined in both
377 oocytes and cumulus cells.

378 In oocytes matured in the presence of amiloride, *GJA1*, related to cell
379 junctions was up regulated respect to oocytes corresponding to the DMSO control

380 group, indicating increased gap junctions between oocyte and cumulus cells. On
381 the contrary, *CCNB1*, involved in the regulation of cell cycle and *BMP15* as a
382 mechanism promoting the developmental competence of the oocytes was down
383 regulated. The reduced expression of this gene has been reported to impair
384 germinal vesicle breakdown (Sánchez & Smitz 2012). A higher *CCNB1* mRNA
385 level has been linked to the greater activity of mitosis-promoting factor (Bermejo-
386 Alvarez *et al.* 2010). *CCNB1* translation regulates oocyte meiosis resumption
387 (Levesque & Sirard 1996), and an abundance of its mRNA has been correlated
388 with developmental competence in goats (Anguita *et al.* 2008). In our study, the
389 down-regulation of *CCNB1* could indicate the reduced capacity of the oocyte to
390 resume meiosis when matured in the presence of amiloride.

391 In cumulus cells, the apoptosis-related genes (*BAX*, *BCL-2*, *TP53* and
392 *SHC1*) were up regulated when amiloride was added to the IVM medium.
393 However, the *BAX/ BCL2* ratio was not significantly modified. Besides, it is known
394 that BMP15 serves to maintain a low level of cumulus cell apoptosis (Hussein *et al.*
395 2005). Interestingly, amiloride down-regulated oocyte *BMP15* possibly reducing its
396 antiapoptotic effect. These results suggest a tendency of cumulus cells to initiate
397 apoptosis. Nevertheless, in these cells *CCNB1* was up-regulated. The molecular
398 mechanism whereby amiloride affects apoptosis and/or proliferation of cumulus
399 cells requires clarification. The expression of *TJP1* and *GJA1* involved in gap
400 junction connections was markedly up-regulated in cumulus cells. Studies have
401 shown that *GJA1*-mediated gap junction communication regulates oocyte meiosis
402 resumption, and that lower levels of *GJA1* in cumulus cells are beneficial for oocyte
403 maturation (Fair 2003, Edry *et al.* 2006). Indeed, this gene has been proposed as a
404 potential marker of oocyte maturation (Li *et al.* 2015). Accordingly, the up-
405 regulation of *TJP1* and *GJA1* by amiloride noted here could be related to the
406 observed inhibition of oocyte maturation.

407 We also detected the down-regulation of *SOD2* in cumulus cells exposed to
408 amiloride. This could reflect a certain vulnerability of COCs to oxidative stress,
409 thereby influencing the subsequent developmental competence of the oocyte
410 (Combelles *et al.* 2010). *SERPINE1* and *SERPINA5* were down-regulated. Both

411 genes code for serine protease inhibitors. *SERPINA5* has been described as one
412 of the most over-expressed genes in cumulus cells after *in vivo* and IVM (Salhab *et*
413 *al.* 2013, Blaha *et al.* 2015). This protein plays a role in the regulation of ECM
414 degradation, coagulation, fibrinolysis, wound healing, and fertility (Suzuki 2008,
415 Meijers & Herwald 2011). In the present study, the down-regulation of *SERPINE1*
416 and *SERPINA5* observed in cumulus cells treated with amiloride suggests the
417 altered balance of ECM remodeling affecting cumulus cell expansion.

418 Although the mechanism of amiloride action in bovine COCs has not been
419 evaluated, its action as inhibitor of the uPA proteolytic activity would be responsible
420 of this effect, as suggested by Lu *et al.* (2013). Even though the principal action of
421 uPA is to proteolytically activate plasminogen to plasmin, several molecules could
422 be substrate of plasmin or uPA itself, such as certain members of
423 metalloproteinases (Zhao *et al.* 2008). Recent studies demonstrated that uPA is
424 able to activate the epithelial sodium channel (ENaC), by the proteolysis of γ ENaC
425 (Ji *et al.* 2015), a known target of amiloride (Kashlan *et al.* 2005). This is in
426 agreement with the dual effects of amiloride, since it can produce directly the
427 ENaC inhibition and indirectly through its novel effects on uPA activity with the
428 consequent attenuation of posttranslational ENaC activation, proposed by other
429 authors (Svenningsen *et al.* 2015, Warnock 2015, Zachar *et al.* 2015). Taking into
430 account all these evidences, it is probable that a regulated proteolytic activity of
431 uPA is necessary to ensure the quality of oocyte maturation process and that
432 amiloride disrupt this equilibrium.

433 In conclusion, although uPA supplementation during *in vitro* maturation did
434 not affect oocyte maturation or early embryo production, blockage of endogenous
435 uPA activity by amiloride unveiled an important role for uPA in bovine oocyte
436 maturation. Further work is needed to clarify the biological and molecular
437 mechanisms whereby amiloride is able to impair successful *in vitro* maturation.

438

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446

447 **Declaration of interest**

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

450

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1 **Legends to Figures**

2

3 **Figure 1.** Effect of uPA or amiloride supplementation during IVM on embryo
4 development in bovine. After IVM, presumptive zygotes were *in vitro* cultured in
5 synthetic oviductal fluid (control: n=129; uPA: n=205; DMSO: n=137; amiloride:
6 n=209). Bars represent cleavage rates (48 h.p.i.) and blastocyst rates recorded at 7
7 (D7), 8 (D8) and 9 (D9) days p.i. Values are reported as the mean ± S.E.M. [a, b, c]
8 Different letters indicate significant differences according to ANOVA ($p<0.05$).

9

10 **Figure 2:** Nuclear maturation rates recorded for bovine oocytes *in vitro* matured in
11 the presence of uPA or amiloride (control: n=55; uPA=41; DMSO: n=59; amiloride:
12 n=53 oocytes analysed). Values are reported as the mean ± S.E.M. [a,b] Different
13 letters indicate significant differences between the controls and treatment groups
14 according to ANOVA ($p<0.05$). GV: germinal vesicle; MI: metaphase I; MII:
15 metaphase II.

16

17 **Figure 3:** Cortical granules distribution patterns recorded from bovine oocytes *in*
18 *vitro* matured in the presence of uPA or amiloride (control: n=39; uPA=28; DMSO:
19 n=39; amiloride: n=35 oocytes analysed). Values are reported as the mean ±
20 S.E.M. [a, b] Different letters indicate significant differences between the controls and
21 experimental groups according to ANOVA ($p<0.05$).

22

23 **Figure 4.** Relative mRNA transcription of selected genes in bovine oocytes (A) and
24 cumulus cells (CC, B) after *in vitro* maturation in the presence of uPA. Genes
25 analyzed were related to apoptosis (*BAX, BCL2, TP53, SHC1*), cell junctions
26 (*GJA1, TJP1*), oxidative stress (*GPX1, SOD2*), cell cycle (*CCNB1*), oocyte quality
27 (*GDF9, BMP15*), serpins (*SERPINE1, SERPINA5*). Data are expressed relative to
28 the means recorded for the housekeeping genes *ACTB* and *H2AFZ.* Values are
29 reported as the mean ± S.E.M. Significant differences between the control and uPA
30 treatment groups ($p<0.05$) in *SOD2* mRNA relative abundance are indicated as (*).

31

32 **Figure 5.** Relative mRNA transcription of selected genes in bovine oocytes (A) and
33 cumulus cells (CC, B) after *in vitro* maturation in the presence of amiloride. Genes
34 analyzed were related to apoptosis (*BAX, BCL2, TP53, SHC1*), cell junctions
35 (*GJA1, TJP1*), oxidative stress (*GPX1, SOD2*), cell cycle (*CCNB1*), oocyte quality
36 (*GDF9, BMP15*), and serpins (*SERPINE1, SERPINA5*). Data are expressed relative
37 to the means recorded for the housekeeping genes *ACTB* and *H2AFZ.* Values are
38 reported as the mean ± S.E.M. Significant differences between control and
39 amiloride treatment groups are indicated as (*) for each analyzed gene ($p<0.05$).

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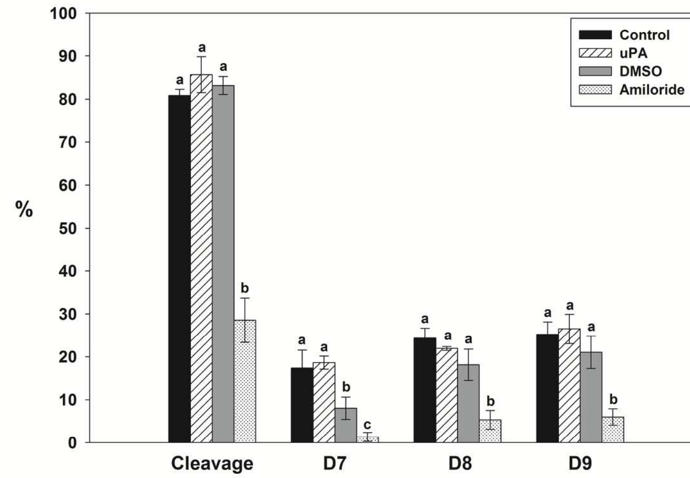

Figure 1: Effect of uPA or amiloride supplementation during IVM on embryo development in bovine. After IVM, presumptive zygotes were *in vitro* cultured in synthetic oviductal fluid (control: n=129; uPA: n=205; DMSO: n=137; amiloride: n=209). Bars represent cleavage rates (48 h.p.i.) and blastocyst rates recorded at 7 (D7), 8 (D8) and 9 (D9) days p.i. Values are reported as the mean ± S.E.M. [a, b, c] Different letters indicate significant differences according to ANOVA ($p<0.05$).

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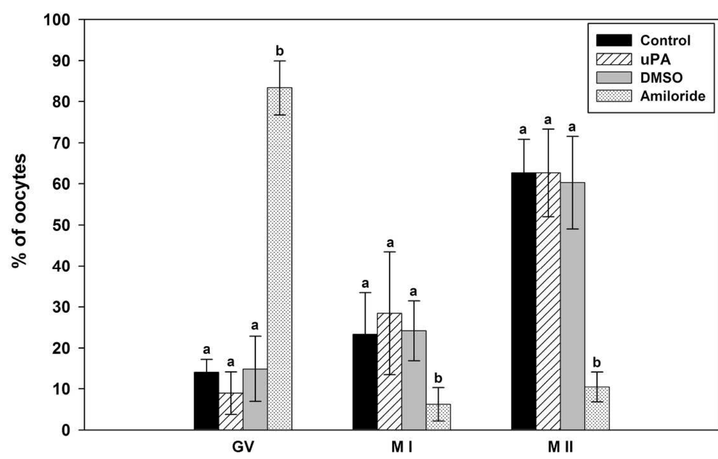

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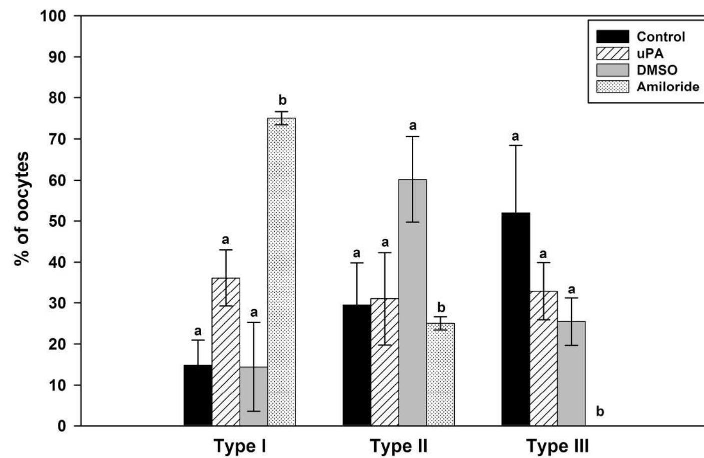

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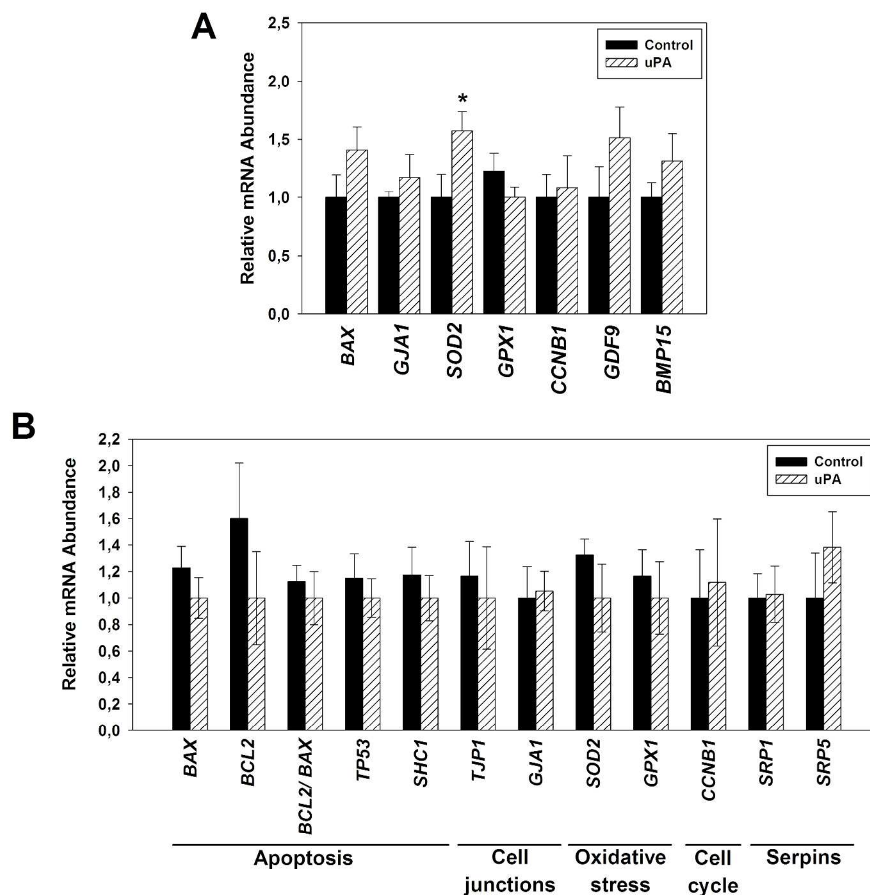

Figure 4: Relative mRNA transcription of selected genes in bovine oocytes (A) and cumulus cells (CC, B) after in vitro maturation in the presence of uPA. Genes analyzed were related to apoptosis (*BAX, BCL2, TP53, SHC1*), cell junctions (*GJA1, TJP1*), oxidative stress (*GPX1, SOD2*), cell cycle (*CCNB1*), oocyte quality (*GDF9, BMP15*), serpins (*SERPINE1, SERPINA5*). Data are expressed relative to the means recorded for the housekeeping genes *ACTB* and *H2AFZ*. Values are reported as the mean ± S.E.M. Significant differences between the control and uPA treatment groups ($p<0.05$) in *SOD2* mRNA relative abundance are indicated as (*).

308x296mm (300 x 300 DPI)

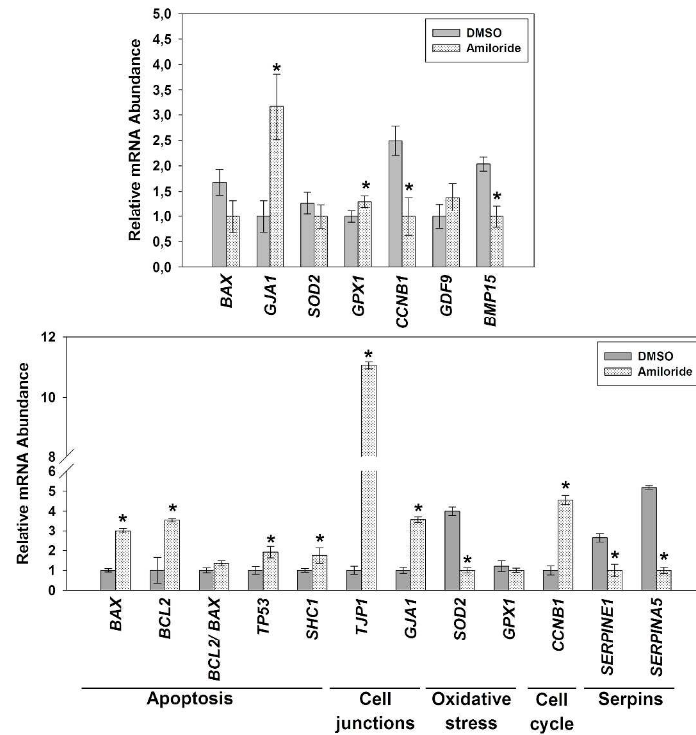

Figure 5: Relative mRNA transcription of selected genes in bovine oocytes (A) and cumulus cells (CC, B) after in vitro maturation in the presence of amiloride. Genes analyzed were related to apoptosis (*BAX, BCL2, TP53, SHC1*), cell junctions (*GJA1, TJP1*), oxidative stress (*GPX1, SOD2*), cell cycle (*CCNB1*), oocyte quality (*GDF9, BMP15*), and serpins (*SERPINE1, SERPINA5*). Data are expressed relative to the means recorded for the housekeeping genes *ACTB* and *H2AFZ*. Values are reported as the mean ± S.E.M. Significant differences between control and amiloride treatment groups are indicated as (*) for each analyzed gene ($p<0.05$).

355x265mm (300 x 300 DPI)

Table 1. Primers used for RT-qPCR

Gene symbol	Gene name		Primer sequence (5' - 3')	Fragment size (bp)	GenBank access No.
ACTB	Actin, beta	Forward	GAGAAGCTCTGCTACGTCG	264	AF191490.1
		Reverse	CCAGACAGCACCGTGTTGG		
BAX	BCL2-Associated X Protein	Forward	CTGGAGCAGGTGCCTCAGGA	300	NM_001166486.1
		Reverse	ATCTCGAAGGAAGTCCAGCGTC		
BCL2	B-Cell CLL/Lymphoma 2	Forward	GGAGCTGGTGGTTGACTTTC	517	BC147863.1
		Reverse	CTAGGTGGTCATTCAGGTAAG		
BMP15	Bone Morphogenetic Protein 15	Forward	ATCATGCCATCATCCAGAACC	72	NM_001031752.1
		Reverse	TAAGGGACACAGGAAGGCTGA		
CCNB1	Cyclin B1	Forward	TGGGTCGGCCTCTACCTTTGCACTTC	332	NM_001045872.1
		Reverse	CGATGTGGCATACTTGTTCTTGATAGTCA		
GDF9	Growth differentiation factor 9	Forward	AGCGCCCTCACTGCTTCTATAT	80	NM_174681.2
		Reverse	TTCCTTTTAGGGTGGAGGGAA		
GJA1	Gap junction protein, alpha 1 (former *CX43*)	Forward	TGCCTTTCGTTGTAACACTCA	142	NM_174068.2
		Reverse	AGAACACATGAGCCAGGTACA		
GPX1	Glutathione Peroxidase 1	Forward	GCAACCAGTTTGGGCATCA	116	NM_174076.3
		Reverse	CTCGCACTTTTCGAAGAGCATA		

H2AFZ	H2A histone family, member Z	Forward	AGGACGACTAGCCATGGACGTGTG	209	NM_174809
		Reverse	CCACCACCAGCAATTGTAGCCTTG		
SCH1	SHC (Src Homology 2 Domain Containing) Transforming Protein 1	Forward	GTGAGGTCTGGGGAGAAGC	334	NM_001075305
		Reverse	GGTTCGGACAAAGGATCACC		
SERPINA5	Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 5	Forward	TGGAAAATGGCCTGAAGGAA	74	NM_176646
		Reverse	ATAAAGCTCAAGCCGCCTCTT		
SERPINE1	Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 1	Forward	CAGGCGGACTTCTCCAGTTTT	77	NM_174137
		Reverse	ACCTCAATCTTCACCTTCTGCAG		
SOD2	Superoxide Dismutase 2, Mitochondrial (former *MnSOD*)	Forward	GCTTACAGATTGCTGCTTGT	101	S67818.1
		Reverse	AAGGTAATAAGCATGCTCCC		
TJP1	Tight Junction Protein 1	Forward	AATCATCCGACTCCTCGTCG	255	XM_010817146.1
		Reverse	CCCAAACACAGCGCGTAAAA		
TP53	Tumor Protein P53	Forward	CTCAGTCCTCTGCCATACTA	364	NM_174201.2
		Reverse	GGATCCAGGATAAGGTGAGC		