Effects of Kisspeptin-10 on in vitro proliferation and Kisspeptin receptor (Kiss-1R) expression in primary epithelial cell cultures isolated from bovine placental cotyledons of embryos/fetuses at the first trimester of pregnancy

NA Martino[1*], A Rizzo[1], F Pizzi[2], ME Dell'Aquila[3#], RL Sciorsci[1#]

Running title: Bovine placental cotyledon cell cultures and Kisspeptin-10

[1]Veterinary Clinics and Animal Production Unit – Dipartimento dell'Emergenza e Trapianti D'Organo (DETO), Università degli Studi di Bari Aldo Moro, Bari, Italy.

[2]Istituto di Biologia e Biotecnologia Agraria (IBBA) Consiglio Nazionale delle Ricerche (CNR), Milan, Italy

[3] Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica (DBBB), Università degli Studi di Bari Aldo Moro, Bari, Italy.

*Correspondence to: nicmartino@libero.it

[#]These two authors contributed equally

ABSTRACT

Kisspeptins (Kps) and Kiss-1 receptor (Kiss-1R) expression have been reported in the placenta and a possible involvement of the Kiss-1R/Kps system in regulating trophoblast invasion and proliferation has been hypothesized. The aim of the present study was to investigate whether Kiss-1R activation by Kisspeptin 10 (Kp-10) could modulate *in vitro* proliferation and progesterone (P_4) secretion of bovine primary placental cell lines isolated from cotyledons of foetuses in the first pregnancy trimester. The involvement of Kiss-1R in the cell responses observed was also analyzed. Uteri from cows at the first trimester of pregnancy were obtained from local abattoirs. Fetal cotyledon fragments were digested with collagenase in Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM) and cell lines were isolated. After being characterized for epithelial polygonal morphology, presence of binucleate cells, male gender and expression of cytokeratin and zona occludens 2, cell lines were cultured in a LG-DMEM based expansion medium in the presence of 0.01, 0.1, 1 and 10 µM Kp-10. Control cells were cultured in the absence of Kp-10. Cell population doubling time was evaluated for each culture passage (P) from P1 to P10. Cells were tested for Kiss-1R mRNA expression analysis by Real Time RT-PCR and culture media were analyzed for P_4 concentration by radioimmunoassay. Kp-10 modulated *in vitro* proliferation of epithelial cell lines isolated from cotyledons recovered from bovine fetuses in the first trimester of pregnancy. Inhibitory (line A) or stimulatory (line B) effects of Kp-10 on cell proliferation were found in different cell lines and observed cell responses were found to be related to Kiss-1R mRNA levels. Inhibition of cell proliferation matched with not significant variation of Kiss-1R expression, whereas stimulation of cell proliferation was found to be related to Kiss-1R up-regulation. In both cell lines, no effect of Kp-10 on P_4 secretion was found at any tested concentration. These results lead to the conclusion that the Kiss-1R/Kps system is involved in the regulation of cell proliferation of bovine placental cotyledon cell lines isolated at the first trimester of pregnancy but, at this gestational stage, it may be not involved in modulating placental P_4 secretion.

Key Words Bovine placental cotyledon cell line, *in vitro* proliferation, Kiss-1R, Kisspeptin-10, progesterone secretion.

1. Introduction

Kisspeptins (Kps) are a family of peptides derived from the primary translation product of the Kiss-1 gene, which was identified and designated in 1996 [1]. The main Kiss-1 gene product is a 145 amino acid polypeptide which, by proteolytic processing, results in shorter Kps, with 54 (Kp-54; metastin), 14 (Kp-14), 13 (Kp-13) or 10 (Kp-10) amino acids, respectively. All small peptides share a common carboxyl-terminal amidated 10-aa sequence which is enough to efficiently bind the Kp receptor (Kiss-1R) and induce its biological activity [2]. Kiss-1R is a G protein-coupled receptor also known as GPR54 and transduces the kps signal through Gq/11-α subunits of heterotrimeric G proteins. The activation of phospholipase C (PLC), upon Kiss-1R stimulation, induces the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and subsequent mobilization of calcium (Ca^{2+}) ions from intracellular stores. In addition, the rise of PIP_2 hydrolysis following Kp stimulation, leads to diacylglycerol (DAG) formation and, thereby, protein kinase C (PKC) activation [3,4]. In turn, activated PKC is thought to cause phosphorylation of mitogen-activated protein kinases (MAPKs), such as ERK1/2 and p38, which are involved in several biological processes, such as pituitary [5,6,7,8] and luteal hormone secretion [9], neuroendocrine function and the regulation of cell proliferation and migration [4,10].

The expression of Kiss-1R and Kp has been demonstrated in a variety of human tissues. They are mainly expressed in the placenta, with much lower expression levels in ovary, testis, liver, pancreas, small intestine and brain e.g., hypothalamus and pituitary gland [4,6,7,11,12,13]. The Kiss-1 gene was originally identified as a human metastasis suppressor gene; however, Kps have emerged as important gatekeepers of key aspects of reproductive maturation and function, from sexual differentiation of the brain and puberty onset to adult regulation of gonadotropin secretion and the

metabolic control of fertility [4,14]. Kisspeptins and Kiss-1R play fundamental roles in pregnancy establishment. Saadeldin et al. [15], in fact reported that porcine oocyte maturation and oocyte maternal gene expression (MOS, GDF9 and BMP15), blastocyst formation rate, blastocyst hatching and blastocyst total cell count increased significantly when oocytes were cultured in medium containing Kp [15]. Moreover, the highest expression of Kps and Kiss-1R in trophoblastic cells during the first trimester in humans and, at embryonic day 12.5 in rats, coincides with the peak of trophoblastic invasion which is critical for pregnancy establishment. In addition, the fact that Kp-10 inhibits the migration and invasion of trophoblastic cells provides further support to the notion that Kps may play a role in controlling trophoblastic invasion and migration during human [3,16, 17] and rat placentation [18]. The invasion of extravillous trophoblasts into the uterine wall is of crucial importance for placental and fetal development, and its deregulation has been implicated in a wide spectrum of abnormal pregnancies [3]. Reduced expression of Kiss-1 and increased expression of Kiss-1R at both mRNA and protein levels have been involved in the pathogenesis of pre-eclampsia [14]. In humans, decreased Kp expression in trophoblasts is associated with recurrent pregnancy loss and Kp has been hypothesized as engaging the regulation of decidual natural killer cell infiltration [19]. To date, no studies have been performed on the role of Kps on placental cell proliferation and progesterone (P_4) secretion.

In the present study, we evaluated the effects of Kp-10 addition in culture media on proliferation and P_4 secretion of bovine placenta cotyledon cell lines isolated from fetuses in the first trimester of pregnancy. The involvement of Kiss-1R in observed cell responses was investigated by analyzing the relative abundance variations of Kiss-1R mRNA by quantitative Real Time RT-PCR.

2. Materials and methods

2.1. *Chemicals and Drugs*

All chemicals were purchased from Sigma-Aldrich (Milan Italy) unless otherwise indicated.

2.2. *Tissue collection*

Uteri from pregnant cows in the 2nd or 3rd month of gestation were retrieved at a local slaughterhouse within 20 min after slaughter, kept at 4°C in phosphate-buffered saline (PBS) with penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin (0.25 mg/mL) solution during transport to the cell culture laboratory and processed immediately. Prior to incision of the uterine wall, the site was cleaned with PBS supplemented with antibiotics and antimycotic. Placentomes were put in a sterile beaker and immediately placed under the laminar flow hood for subsequent processing. They were manually separated into fetal cotyledon and maternal caruncle. Depending on the size of each tissue, a 1–2 cm^3 piece of fetal cotyledon was dissected into small pieces (2x2 mm) before enzymatic digestion.

2.3. *Isolation of cells from cotyledons*

Fetal cotyledon fragments were collected in falcon tubes and washed two times in PBS supplemented with antibiotics and antimycotic at 300 g for 5 min, and then digested with 0.75 mg⁄ml collagenase in Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM) at 37 °C for 4h, until each fragment was completely digested. After incubation, collagenase activity was stopped with LG-DMEM supplemented with 10% Fetal Calf Serum (FCS), the suspension was filtered through 80µm filters (Millipore, Milan, Italy), centrifuged at 300 g for 10 min and cells were collected from the pellet.

2.4. *Cell culture and expansion*

Cells were cultured in expansion medium consisting of LG-DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphoterycin solution, 2 mM L-glutamine and 10 ng/ml Epidermal Growth Factor (EGF), as reported in previous studies carried out

by our research unit of extra fetal tissues [20,21]. The number of viable cells was counted by the trypan blue dye exclusion test, using a Bürker chamber. For culture maintenance, cells were plated at a density up to 1 x 10^5 cells/cm^2 and incubated at 38.5 °C in a humidified atmosphere (95%) with 5% CO_2. The medium was replaced for the first time after 72 h to remove non-adherent cells, and then changed either twice per week or according to the experimental design.

2.5. Cell line characterization

2.5a Cell morphology

The epithelial origin of primary placental cotyledonary cell lines was assessed following the criteria by Hambruch et al., 2010 [22]. Morphologically, these cells form monolayers composed of approximately polygonal cells, clearly distinguishable from maternal caruncular epithelial cells which displayed curl-like morphology within the colonies (Hambruch et al., 2010). For assessing the presence of binucleate cells, 1x10^4 cells were seeded onto cover slips and cultured, in the conditions described above, until 50% confluence was reached. Cells were fixed with 2% paraformaldehyde in PBS for 10 min, stained with 2.5 μg/ml Hoechst 33258 in 3/1 glycerol/PBS solution and mounted onto slides in the same solution. Evaluation of cell morphology, by phase contrast microscopy, and nuclear chromatin, by epifluorescence microscopy, were performed under a Nikon Eclipse 600 (x 400 magnification) epifluorescence microscope. Stainings were carried out in triplicates.

2.5b XY-chromosome specific PCR

After thawing, cell lines underwent genomic DNA extraction using the Genelute genomic DNA kit (Sigma-Aldrich, Milan, Italy) following the protocol recommended by the manufacturers. The tissue was disrupted by liquid nitrogen and, before going through the extraction procedure,

incubated with proteinase K overnight at 54 °C, whereas cell samples were centrifuged and then incubated with lysis buffer supplemented with proteinase K for 10 min at 72 °C. As positive control, for the presence of Y-chromosome, genomic DNA extracted from a skin sample collected at a local slaughterhouse from a male calf was used. As Y-chromosome negative control, bovine genomic DNA isolated from ovarian surface epithelial cells was used. The sequence length polymorphisms between bovine amelogenin X and amelogenin Y genes (AMLX/Y) were used as markers for sexing the bovine fetal cells, as previously described in other bovine cell systems [23]. The oligonucleotide sequences of used primers (Primm S.R.L., Milano, Italy) were (forward) 5′-CAGCCAAACCTCCCTCTGC-3′ and (reverse) 5′-CCCGCTTGGTCT TGTCTGTTGC-3'. This primer set was designed to amplify a single fragment of 280 bp on the X-chromosome (female cells) and two fragments of 280 and 217 bp on X and Y chromosomes, respectively (male cells). Therefore, our sexing method was used to confirm the presence of male fetus cells in our samples. The PCR reaction mixture (50 μL/tube) consisted of 1.0 U Taq DNA polymerase, 5x Taq buffer, 0.2 mM of each dNTPs, 1 μM specific primers, and 100 ng template DNA. The PCR conditions consisted of an initial denaturing cycle at 94 °C for 5 min followed by 37 cycles of 94 °C for 45 s., 64.7 °C for 45 s., 72 °C for 30 s. and, after the last cycle, samples were kept at 72 °C for 5 min for the final extension, and then the PCR procedure was completed. The PCR products were analyzed by electrophoresis in 2 % (w/v) agarose gel and visualized under UV light after ethidium bromide staining. The analysis was carried out in triplicates.

2.5c Gene expression study

2.5.c1 Total RNA extraction

The RNeasy kit (QIAGEN, Milan, Italy) was used to extract total RNA. After thawing, cells were washed in PBS and then disrupted in Lysis Buffer RLT and homogenized at room temperature. A 70% ethanol solution was then added to the cell lysate, thus creating conditions that promoted

selective binding of RNA to the RNeasy membrane. The lysate was transferred to an RNeasy spin column and centrifuged for 15 s at 10000 rpm. The column was washed twice with washing buffer and finally, the total RNA was eluted in 30 µl of RNase-free water and stored at -80°C. The total amount of RNA of each sample was measured with the BioPhotometer plus (Eppendorf, Milan, Italy).

2.5.c2 Reverse Transcription PCR

The High Capacity cDNA Reverse Transcription kit (LifeTechnologies, Monza, Italy) was used to convert RNA to cDNA. After thawing, 2 µg of total RNA of each samples were added to 2 µL 10X RT Buffer, 0.8 µL 25X dNTP Mix, 2 µL RT Random Primers, 1 µL M-MLV Reverse Transcriptase, 1 µL RNase Inhibitor, nuclease-free H2O for a total volume of 20 µl and then mixed gently and centrifuged briefly. Reaction tubes were incubated at 10°C for 10 min, then at 37°C for 120 min and finally at 85°C for 5 min. Specific bovine cDNAs of Cytokeratin (CK), Tight junction protein 2 (zona occludens 2, ZO-2) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified by PCR, using the primers as shown in Table 1. PCR conditions were as follows: 95 °C for 2 min, 35 cycles of denaturation at 94 °C (45 s), annealing at 60 °C for GAPDH and CK and 56 °C for ZO-2 (45 s) and extension at 72 °C (45 s), final extension at 72 °C for 5 min. PCR products were separated using a 2% agarose gel and visualized by ethidium bromide staining. The analysis was carried out in triplicates.

2.6. *Proliferation study*

The proliferation study was carried out on placenta cotyledon cell lines from fetuses in the first trimester of pregnancy. Gestational age was determined on the basis of fetal body length [24]. In order to calculate doubling time (DT, indicative of the proliferation rate), cells were seeded at a density of 1000 cell/cm^2 in expansion medium containing 0.01, 0.1, 1 or 10 µM of human Kp-10 (Sigma M2816). Cell cultures performed in the absence of Kp-10 were used as controls. Culture

passages were performed every 3 days and Kp-10 was added at each passage (P). Cells of each well were detached using 0.05% trypsin/0.02% EDTA in PBS and were counted, by dilution (1:1) in Trypan blue, with Bürker's chamber. The DT data were calculated by using the following formula: CD = ln(Nf/ Ni)/ln 2 and DT = CT/CD, where DT is the cell-doubling time, CD is the cell-doubling number, and CT is the cell culture time. The proliferation rate was calculated from each passage, where Nf is the final number of cells and Ni the initial number of cells. The total number of live cells was obtained for each passage and DT values were calculated from P1 to P10.

2.7. *Placenta cells and culture media cryopreservation*

At the end of each passage, cells were divided into two parts, one of which was used for the proliferation study, as described above, and the other was suspended in a crypreservation medium consisting of 40% LG-DMEM, 50% FCS and 10% Dimethyl sulfoxide (DMSO) and stored at -80 °C, whereas the culture media for each passage and culture condition were removed and stored in cryotubes at -80 °C for P_4 radioimmunoassay. After thawing, cells were used for molecular analysis.

2.8. *Progesterone measurements*

Frozen culture media were lyophilized (freeze-dried) and then the P_4 levels were measured by the use of a chemiluminescent immunoassay kit (LKPG1, MEDICAL SYSTEM, Genoa, Italy). The assay detection range was 0.2–100 ng/mL.

2.9. *Real Time PCR*

Real Time PCR was performed using Real Time TaqMan® technology and analyzed on the automated StepOne System (Applied Biosystems, Monza, Italy). All TaqMan bovine primers and probes were purchased from Life Technologies. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Bt03210913_g1) gene assay was inventoried by Life Technologies. For the specific

bovine Kiss-1R, primers and probes were properly designed, across exon-exon junction, using the Primer Express 3.0 software (Applied Biosystem) based on NCBI Bos Taurus available sequences or on mammal multi-aligned sequences. Kiss-1R Real Time PCR primers: forward primer: 5'-GCGGTCACGGACTTAACGTT -3'; reverse primer: 5'- CACCGAGACCTGCTGGATGT -3'; Kiss-1R hybridization probe: -5'TGTGCTGCGTGCCCT-3'. The TaqMan® MGB (minor groove-binder) hybridization probe was labeled with a reporter dye (6-carboxy-fluorescein, FAM) on the 5' nucleotide and a quenching dye with NFQ (non fluorescent quencher) on the 3' nucleotide where MGB hyper-stabilized duplexes with complementary DNA. Samples were run in duplicates on Microamp fast optical 48-well reaction plate (Life Technologies) where twenty-microliter reactions for each well contained: 10 µL TaqMan gene expression Master Mix 2X (Life Technologies), 1 µL 800 nM Primers and 250 nM Probe, 1-4 µL cDNA and RNase Free H_2O. Cycle parameters were: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were collected using the StepOne Software and relative quantification was performed using a comparative method after determining the Ct (threshold cycle) values for the reference endogenous control (GADPH) and the target gene in each sample set, according to the $2^{-\Delta\Delta Ct}$ method, as described by the manufacturer. Changes in mRNA expression levels were calculated after normalization to GAPDH. The program calculates the ΔCt and the ΔΔCt with the formulas below: ΔCt = Ct_Mean(GAPDH) – Ct_Mean(Kiss-1R); ΔΔCt = ΔCt – ΔCt_Mean, so that the gene expression level = $2^{-\Delta\Delta Ct}$. Changes in gene expression were reported as percentage changes relative to controls.

2.10. *Statistical analysis*

Cell proliferation (doubling time) data were analyzed by using the General Linear Model procedure (SAS™ package v. 9.1, SAS Institute, Cary, NC) considering line, passage and Kp-10 concentration as fixed effects. Least-Square means (LS-means) ± standard error (SE) are shown. Within-passage Kp-10 concentration effects on DT data were evaluated by one-way ANOVA

(GraphPad Prism Software 5.0) followed by Dunnett's multiple comparison post-hoc test and means ± standard deviations (SD) are shown. Relative abundance of Kiss1R mRNA and P_4 levels were analyzed by the GLM procedure and LS means ± SE are shown. Values with $P<0.05$ were considered significantly different.

3. Results

3.1. *Bovine primary placenta cotyledon cell cultures*

The proliferation study was carried out on two placenta cell lines, namely line A, which was isolated from a fetus in the 2nd month of gestation (thus having a body length of 8 cm), and line B, which was isolated from a fetus in the 3rd month of pregnancy (with a body length of 12 cm). Upon *in vitro* culture, primary cotyledon cell lines formed monolayers composed of polygonal epithelial cells (Fig. 1 A, B). Morphologically, they were clearly distinguishable from maternal caruncular epithelial cells which display curl-like morphology as shown in previous studies [22]. Characterisation of both cell lines via epifluorescence revealed the presence of larger binucleated cells (Fig. 1 C, D). The presence of a Y-chromosome specific sequence in both cell lines confirmed their fetal origin, as reported in previous studies [23, literature cited in ref. 23]. By gel electrophoresis of PCR products amplified from the two cell lines, two fragments of 280 and 217 bp, indicating the presence of X- and Y-chromosome specific sequences, were observed (male pattern; Fig. 1, E). The expression study demonstrated that both cell lines express CK and ZO-2 (Fig. 1, F), as revealed by gel electrophoresis of PCR products amplified from mRNA extracted from the two cell lines, showing specific bands for CK (152 bp) and ZO-2 (224 bp).

3.2. *Effects of Kp-10 on in vitro proliferation of bovine placental cotyledon cell lines*

Significant "cell line" effect was observed, as line A showed significantly higher DT values than line B (LS means ± SE: 1.69±0.12 versus 1.32±0.12 for lines A and B, respectively; GLM procedure: $P<0.05$). Significant "cell passage" effect was detected in both cell lines and the two cell

lines behaved differently. Line A showed a slower DT at P1 which then settled into a faster rate from P2 to P10 (GLM procedure: significance level ranging from $P<0.05$ to $P<0.001$; Fig. 2, panel A); whereas line B had a steady DT until P9 which then increased at P10 (GLM procedure: significance level ranging from $P<0.05$ to $P<0.001$; Fig. 2, panel B). As for the "within-line Kp-10 concentration effect", no effect of Kp-10 addition was found in either cell line at any tested concentration (Table 2, GLM procedure: not significant). Instead, by analyzing the "within-passage Kp-10 concentration effect", it was found that, within each specific cell passage, the two cell lines responded differently to Kp-10 addition (One-way ANOVA followed by Dunnett's post-hoc test). Specifically, in line A, a stimulatory effect of Kp-10 was found during early cell passages (P1 and P2 with significance level ranging from $P<0.05$ to $P<0.01$), followed by a switch to an inhibitory effect at P3 and from P5 to P10 (significance level ranging from $P<0.05$ to $P<0.001$; Fig. 2 panel A'). In line B, a stimulatory effect was observed during intermediate passages (P3, P4, P6, P7; significance level up to $P< 0.01$; Fig. 2, panel B') whereas no effects were observed in other passages. In both cell lines, cell viability did not change with passage number and was not affected by Kp-10 treatment (no significant differences, data not shown).

3.3. *Effects of Kp-10 on Kiss-1R mRNA relative abundance of bovine placental cotyledon cell lines*

Based on the results of the proliferation study, the involvement of Kiss-1R on observed cell response was analyzed at mRNA level by quantitative Real Time RT-PCR. In each cell line, relevant passages representative of the intermediate phase (P3 to P9) were selected for molecular analysis, due to their higher and more stable proliferation rate. Among them, those showing inhibition (P7 and P9 for cell line A), switching (P4 for cell line A) or stimulation (P3, P4 and P6 for cell line B) of cell proliferation upon Kp-10 addition were used. In cell line A, no significant differences were found in Kiss1R mRNA relative abundance, upon Kp-10 addition, at any examined passage and concentration (Fig. 3, panel A; GLM procedure: not significant). In cell line B, the Kiss-1R transcript level significantly increased upon Kp-10 stimulation at P3 and P6 (Fig. 3,

311 panel B, GLM procedure). At P3, the relative abundance was doubled in cells treated with 0.01 μM
312 Kp-10 and increased approximately 2.5-fold in cells treated with 10 μM Kp-10 ($P<0.05$) and at P6,
313 the Kiss-1R transcript level was approximately 6-fold higher in cells treated with 0.01 μM Kp-10
314 ($P<0.001$) and 3-fold higher in cells treated with 0.1 and 10 μM Kp-10 ($P<0.01$; Fig. 3, panel B).
315
316 3.4. *Effect of Kp-10 on P_4 secretion in bovine placental cells*
317 The effects of kp-10 on P_4 secretion in *in vitro* cultured placenta cotyledon cell lines were analyzed
318 in order to elucidate the role of Kp-10 on the secretion of this pregnancy supporting hormone. In
319 preliminary tests performed in media of cells cultured in control conditions, P_4 levels were found to
320 be below minimal detectable values (less than 0.20 ng/ml). For this reason, media were lyophilized
321 and used for single determinations. Data from culture media collected at a representative early (P2),
322 intermediate (P4) and late cell passage (P10) were averaged. In both cell lines, P_4 production was
323 not affected by Kp-10 addition at any tested concentration and LS mean values ranged from 0 to 2.9
324 ng/ml (GLM procedure: not significant; Fig. 4).
325
326 **4. Discussion**
327 The prime purpose of this study was to establish primary cultures of cell lines from bovine placental
328 cotyledons, which may provide a valuable tool for the study of bovine feto-maternal interactions.
329 These cell lines may be useful as *in vitro* models to elucidate the roles of factors involved in the
330 regulation of bovine placenta development and function, such as the Kiss-1R/Kps system, and to
331 establish new pregnancy maintenance strategies for practical application in bovine reproductive
332 management. Moreover, the bovine placenta stands as an ideal model for the study of factors
333 involved in human placenta development and function due to the similar nine-month pregnancy
334 duration, comparable to that in humans, despite the known differences in placenta distribution
335 (cotyledonary versus discoid) and structure (syndesmochorial versus hemochorial). In the present
336 study, the isolation of bovine placental cotyledonary cell lines was confirmed by the epithelioid

polygonal morphology, presence of binucleate cells, presence of the Y-chromosome, and the simultaneous expression of cytokeratin and zonula occludens 2 as trophoblast-specific markers (Fig. 1) [22]. To our knowledge, this is the first study reporting a proliferation study through 10 consecutive passages of bovine placental cotyledon cell lines, which provides useful indications of their behavior in *in-vitro* cultures.

After having successfully isolated and expanded epithelioid cotyledon cell lines, we decided to determine whether - in these cell lines - Kiss-1R activation, by means of its endogenous ligand Kp-10, could modulate their *in vitro* proliferative activity. Kiss-1R expression in human placenta [16,17,25] as well as in animal models [18,26] has been reported and a possible involvement of the Kiss-1R/Kps system in regulating trophoblast invasion and proliferation has been hypothesized [3,16,17,27]. In the present study, cell line effect and cell passage effect were observed, due to the use of primary cell lines isolated from bovine placenta of fetuses in the first trimester of pregnancy. In detail, line A showed high DT value at P1, followed by increased cell proliferation rate, maybe due to gradual adaptation of the cell line to *in vitro* culture conditions (Fig. 2, panel A), whereas cell line B showed a prolonged phase of high proliferation rate and inhibition of cell proliferation in late passages, perhaps due to loss of cell viability (Fig. 2, panel B). Taking into account these effects, the effects of Kp-10 concentration were first evaluated by grouping data from all passages, which showed no effects of Kp-10 addition at any tested concentration (Table 2). However, this analysis turned out to be very weak, as very low R-square values were found in both cell lines (R-square value = 0.004035 and 0.000971 for cell line A and B, respectively), indicating that the effects of Kp-10 are masked by greater effects related to cell line and cell passages. After excluding cell line and cell passage effects, we demonstrated using within-cell passage analysis that Kp-10 modulates *in vitro* proliferation in these cells. The two analyzed cell lines behaved differently upon Kp-10 addition, with inhibition of cell proliferation prevailing in line A, isolated from embryos/fetuses during the 2nd month of gestation (Fig. 2, panel A') and stimulation of cell proliferation prevailing

in line B, isolated from embryos/fetuses at the 3rd month of pregnancy (Fig. 2, panel B'). To our knowledge, this is the first study in which Kp-10 has been reported as having a stimulatory effect on cell proliferation, as we observed in line B. In previous studies, analogously with its originally identified antimetastatic role [1], Kp was reported as having inhibitory effects in cell proliferation [28-32], although the biological mechanisms remains to be further elucidated. It has already been clarified that the activation of Kiss-1R leads to phosphorylation of different MAPKs, which might contribute to the antimetastatic and/or antiproliferative effects of kisspeptins (Kps, [28-30]). However, the subset of intracellular kinases activated upon kisspeptin stimulation appears to be, at least partially, dependent on the cellular context [4], and maybe on the cell type isolated from different tissues.

The Kp-10 effects observed on bovine primary placenta cotyledon cell lines were not dose-dependent. Previous studies refer to the variable effects of Kp-10 addition, depending on cell type, whether primary or established or embryonic or cancer cell line, and the nature of Kiss-1R expression, whether endogenous or artificially-induced. Kotani et al., 2001 [29] reported inhibition of cell proliferation in CHO-K1 cells transfected and expressing rat or human GPR54 (Kiss-1R) and treated with 1 μM Kp-10. Cho et al., 2009 [30] reported no dose-dependent effect of Kp-10 tested at concentrations ranging from 1.0 to 100 μM on cell proliferation of human umbilical vein endothelial cells (HUVEC) and prostate cancer cells (PC-3). Huma et al., 2013 [31] reported a dose-dependent inhibitory effect of Kp-10 on cell proliferation of Rhesus Monkey derived embryonic stem cells (R366.4) at concentrations ranging from 0.1 to 100 nM. Ziegler et al., 2013 [32] reported no effects in breast cancer cell lines (such as MDA-MB-231; MDA-MB-435s; HCC 1806 and MCF-7) expressing the receptor endogenously but significant inhibition of cell proliferation in transfected neuronal cells overexpressing Kiss-1R. To the best of our knowledge, no data have been reported to date on the effects of Kp-10 addition on cell proliferation of primary placenta cell lines. Primary cell lines isolated from placenta cotyledons recovered in the first trimester of pregnancy in

the bovine model can be considered as a physiological model for studying the role of the Kiss1-R/Kp-10 system in trophoblastic growth and invasion at the early stages of pregnancy other than immortalized trophoblastic cell lines, as also reported in a recent study by Francis et al., 2014 on human trophoblastic cells isolated in the first trimester of pregnancy [33].

In order to identify the reasons for different *in vitro* effects of Kp-10 on the proliferative activity of the two cell lines, we considered it important to assess whether Kp-10 stimulation could differently modify Kiss-1R expression. Thus, we investigated the effects of Kp-10 on the relative abundance of mRNA in the two cell lines. Specifically, in cell line A, in which a prevailing inhibition of cell proliferation was found upon Kp-10 stimulation, the Kiss-1R transcript level did not change in Kp-10 treated cells compared with control conditions. In cell line B, in which a stimulatory effect on cell proliferation was observed upon Kp-10 addition, the relative abundance of Kiss-1R significantly increased in Kp-10 treated cells compared with controls. At the present time, these findings can only be interpreted in the light of results from previous studies. In human [3,16,17,30] and rat placenta [18], it has been reported that Kiss-1R mRNA expression increases significantly during pregnancy, with distinct spatial and temporal variations. Indeed, the two cell lines analyzed came from embryos/fetuses at the 2^{nd} and the 3^{rd} month of gestation, respectively. The recent study by Ziegler et al., 2013 [33], evidencing a connection between the anti-proliferative effect of Kp-10 and Kiss-1R overexpression, suggests possible correlations between Kiss-1R expression status in the bovine primary placenta cotyledon cell lines used in the present study and observed proliferative and molecular cell responses. Further studies are needed to verify this hypothesis. The modulation of the Kiss-1R mRNA transcription in bovine cotyledons raises the hypothesis that the Kiss-1R/Kps system plays a pivotal role in embryo/placenta development and reinforces its suggested role in the regulation of trophoblast proliferation. Although Kp is already known to play a key role in the first trimester of pregnancy in humans [3], to our knowledge, detailed information about cell proliferation and related underlying mechanisms during the first trimester of bovine placenta

formation have not been reported to date. Our data lead us to hypothesize that, in line B, kp-10 addition significantly up-regulated kiss-1R expression and, perhaps consequently, increased cell proliferation whereas, in line A, kp-10 had no effect on the relative abundance of Kiss-1R mRNA. Possible explanations for Kiss-1 R involvement in the observed inhibitory effect on cell proliferation in line A could only be hypothesized as being related to: 1) receptor desensitization, even not associated with its mRNA transcription down-regulation; 2) different signaling pathways elicited by the ligand, such as transactivation of other receptors, as reported by Roseweir et al., 2012 [17]; 3) different functional status of Kiss-1R in placental cells recovered at early gestational age. The qRT-PCR analysis performed in this study, using bovine primary placental cotyledonary cell lines, is of particular interest because the results may show more precisely what would happen in native tissue, compared with the use of immortalized cells which have been shown to have a differential gene expression to primary cells brought about by the immortalization process [17].

As a further approach to elucidate the role of the Kiss-1R/Kps system on bovine cotyledon placenta cell lines, we investigated the effects of Kp-10 on the P_4 secretory activity of these cells. Although considerable efforts have been addressed to unravelling the major role of Kps as regulatory factors for the neurons producing gonadotropin-releasing hormone, which stimulates pituitary gonadotropin secretion [4,6,10], the isolation and identification by mass spectrometry of Kps from human placenta [4] opens the way for investigating their effects on P_4 secretion. Previous studies were reported on the effects of kp-10 on P_4 secretion in ovarian cell systems involved in P_4 production. In chicken *in vitro* cultured granulosa cells, Kp-10 was found to stimulate P_4 secretion in a dose-dependent manner and depending on follicle developmental stage [34]. In rat *in vitro* cultured luteal cell lines, kp-10 was found to stimulate P_4 secretion via the ERK1/2 mitogen-activated protein kinase signalling pathway and by increasing the synthesis of three key steroidogenic enzymes, such as StAR, CYP11A and 3-beta-HSD [9]. To our knowledge, no studies have been reported to date on the effects of Kp-10 on P_4 secretion of placenta cell lines in any species and at any stage of

pregnancy and this is the first study reporting the effects of Kp-10 addition on P_4 secretion in bovine primary placenta cotyledon cell lines. The ability of fetal cotyledon to secrete P4 is documented in early as well as in late pregnancy [35] even though P_4 concentration in cotyledons increased sharply in later stages from the 7^{th} to the 9^{th} month [36]. In the present study, in supernatants of culture media of both cell lines, P_4 was found at basal levels, ranging from 0 to 2.9 ng/ml, but its secretion was not influenced by Kp-10 addition. The P_4 values found in the present study can be compared with those reported in previous studies in other P_4 cell systems after Kp-10 stimulation. Xiao et al., 2011 [34] in chicken granulosa cell lines found values within 0.8 ng/ml or within 8.5 ng/ml, depending on follicle size after stimulation with Kp-10 at concentrations ranging from 10 nM to 1 μM. On investigating rat luteal cells, Peng et al., 2013 [9] found a higher range of P_4 values (ranging from 45 to 80 ng/ml) after stimulating with Kp-10 at concentrations comprised between 40 to 160 nM. Our data lead us to conclude that the Kiss1R/Kps system may not be involved in modulating P_4 synthesis/secretion in bovine placenta cotyledon cell lines isolated in early gestational age. Rather, at this stage, it could be involved in initiating the signalling pathways leading to P_4 synthesis which could become more consistent/relevant in later stages of pregnancy. Further studies are needed to elucidate this hypothesis.

Taken together, these results lead to the conclusion that the Kiss-1R/Kps system could play a role in the fine developmental regulation of bovine placental cotyledonal cell proliferation in the first trimester of pregnancy, but it may not yet be competent enough to activate P_4 secretion. In the cell line isolated from an embryo/fetus at the 2^{nd} month of pregnancy, inhibition of cell proliferation matching with non inducible Kiss-1R gene expression, as revealed by not significant variation of Kiss-1R expression was observed; whereas in the cell line isolated from an embryo/fetus at the 3rd month of pregnancy, stimulation of cell proliferation associated with inducible gene expression, as revealed by Kiss-1R up-regulation,was found. This study contributes to elucidate the role exerted by the Kiss-1R/Kps system in bovine placenta development and function. Primary cultures of

epithelial cell lines from bovine placental cotyledons provide a valuable tool for the study of the role of the Kiss-1R/Kps system in feto-maternal interactions in bovines and could be used in translational research programs aimed at human reproductive medicine.

AUTHOR'S ROLES

RLS, NAM, AR and MED designed the study. NAM conducted most of the experimental procedures used in the study, i.e. the kinetic/proliferation, the expression study and data analysis and drafted the manuscript. AR performed tissue selection and setting up and P_4 determinations. NAM and FP performed data analysis and statistics. MED, FP and RLS supervised experiments and critically revised the manuscript. All authors have read and approved the manuscript.

ACKNOWLEDGEMENTS The authors thank Dr. Michele Derosa e Gaetano Carriello (Murgia Carni; Noicattaro, Bari) for kindly providing pregnant bovine uteri used in this study. Dr. NA Martino was supported by the project ONEV (Omica e Nanotecnologie applicate agli Esseri Viventi per la diagnosi di malattie) MIUR PONa3_00134 – n.254/R&C 18/05/2011. The authors would also like to thank Anthony Green for kindly revising the English of the manuscript.

CONFLICTS OF INTEREST: None.

References

[1] Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. KiSS-1, a novel human malignant melanoma metastasis suppressor gene. J Natl Cancer Inst 1996;88(23):1731-7.

[2] Roa J, Navarro VM and Tena-Sempere M. Kisspeptins in Reproductive Biology: Consensus Knowledge and Recent Developments. Biol Reprod 2011;85:650–60.

[3] Hiden U, Bilban M, Knöfler M, Desoye G. Kisspeptins and the placenta: regulation of trophoblast invasion. Rev Endocr Metab Disord 2007;8:31–9.

[4] Pinilla L, Aguilar E, Dieguez C, Millar RP and Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev 2012;92:1235–1316.

[5] Suzuki S, Kadokawa H, Hashizume T. Direct kisspeptin-10 stimulation on luteinizing hormone secretion from bovine and porcine anterior pituitary cells. Animal Reprod Sci 2008;103:360–5.

[6] Kadokawa H, Suzuki S, Hashizume T. Kisspeptin-10 stimulates the secretion of growth hormone and prolactin directly from cultured bovine anterior pituitary cells. Animal Reprod Sci 2008;105:404–8.

[7] Ezzat AA, Saito H, Sawada T, Yaegashi T, Goto Y, Nakajima Y et al. The role of sexual steroid hormones in the direct stimulation by Kisspeptin-10 of the secretion of luteinizing hormone, follicle-stimulating hormone and prolactin from bovine anterior pituitary cells. Animal Reprod Sci 2010;121:267–72.

[8] Saito H, Sawada T, Yaegashi T, Goto Y, Jin J, Sawai K et al. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. Anim Sci J 2012;83(6):487-92.

[9] Peng J, Tang M, Zhang BP, Zhang P, Zhong T, Zong T et al. Kisspeptin stimulates progesterone secretion via the Erk1/2 mitogen-activated protein kinase signaling pathway in rat luteal cells. Fertil Steril 2013;99(5):1436-43

[10] Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 2009;15:203–12.

[11] Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 2001;411(6837):613-7.

[12] Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, et al. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? Endocrinology 2006;147:4852–62.

[13] Cejudo Roman A, Pinto FM, Dorta I, Almeida TA, Hernandez M, Illanes M et al. Analysis of the expression of neurokinin B, kisspeptin, and their cognate receptors NK3R and KISS1R in the human female genital tract. Fertil Steril 2012;97:1213–9.

[14] Cartwright JE, Williams PJ. Altered placental expression of kisspeptin and its receptor in pre-eclampsia. J Endocrinol 2012;214(1):79-85.

[15] Saadeldin IM, Koo A OJ, Kang JT, Kwon DK, Park SJ, Kim SJ et al. Paradoxical effects of kisspeptin: it enhances oocyte in vitro maturation but has an adverse impact on hatched blastocysts during in vitro culture. Reprod Fertil Dev 2012;24:656–68.

[16] Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C et al. Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci 2004;15;117(Pt 8):1319-28.

[17] Roseweir AK, Katz AA, Millar RP. Kisspeptin-10 inhibits cell migration in vitro via a receptor-GSK3 beta-FAK feedback loop in HTR8SVneo cells. Placenta 2012; 33(5): 408-15.

[18] Mark PJ, Jones ML, Lewis JL, Waddell BJ, Smith JT. Kiss1 and Kiss1r mRNA expression in the rat placenta: Changes with gestational age and regulation by glucocorticoids. Placenta 2013;34(8):657-62.

[19] Park DW, Lee SK, Hong SR, Han AR, Kwak-Kim J, Yang KM. Expression of Kisspeptin and its receptor GPR54 in the first trimester trophoblast of women with recurrent pregnancy loss. Am J Reprod Immunol 2012;67(2):132-9.

[20] Martino NA, Lange-Consiglio A, Cremonesi F, Valentini L, Caira M, Guaricci AC et al. Functional expression of the extracellular calcium sensing receptor (CaSR) in equine umbilical cord matrix size-sieved stem cells. PLoS One 2011:17;6(3):e17714.

[21] Filioli Uranio M, Valentini L, Lange-Consiglio A, Caira M, Guaricci AC, L'Abbate A et al. Isolation, proliferation, cytogenetic, and molecular characterization and in vitro differentiation potency of canine stem cells from foetal adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. Mol Reprod Dev 2011;78(5):361-73.

[22] Hambruch N, Haeger JD, Dilly M, Pfarrer C. EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via Ras and MAPK. Placenta 2010;31:67-74.

[23] Divar MR, Sharifiyazdi H, Kafi M. Application of polymerase chain reaction for fetal gender determination using cervical mucous secretions in the cow. Vet Res Commun 2012;36:215–20.

[24] Noakes D, Parkinson TJ and England GCW. In Arthur's Veterinary Reproduction and Obstretics, Eighth Edition. London: Saunders WB; 2001.

[25] Horikoshi Y, Matsumoto H, Takatsu Y, Ohtaki T, Kitada C, Usuki S, et al. Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. J Clin Endocrinol Metab 2003;88:914e9.

[26] Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T et al. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. Biochim Biophys Acta 2004;1678:102e10.

[27] Reynolds RM, Logie JJ, Roseweir AK, McKnight AJ, Millar RP. A role for kisspeptins in pregnancy: facts and speculations. Reproduction 2009;138:1–7.

[28] Castano JP, Martinez-Fuentes AJ, Gutierrez-Pascual E, Vaudry H, Tena-Sempere M, Malagon MM. Intracellular signaling pathways activated by kisspeptins through GPR54: do multiple signals underlie function diversity? Peptides 2009;30:10–5.

[29] Kotani M, Detreux M, Vandenbogaerde A, Communi D, Vanderwidden JM, Le Poul E et al. The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 2001;276:34631-36.

[30] Cho SG, Yi Z, Pang X, Yi T, Wang Y, Luo J et al. Kisspeptin-10, a Kiss1-derived decapeptide, inhibits tumor angiogenesis by suppressing Sp1-mediated VEGF Expression and FAK/Rho GPTase activation. Cancer Res 2009;69:7062-70.

[31] Huma T, Wang Z, Rizak J, Ahmad F, Shahab M, Ma YY et al. Kisspeptin-10 modulates the proliferation and differentiation of the Rhesus Monkey derived Stem Cell line: R366.4 The Scientific World Journal 2013, Article ID 135470 7 pages. http://dx.doi.org/10.1155/2013/135470.

[32] Ziegler E, Olbrich T, Emons G, Grundker C. Antiproliferative effects of kisspeptin-10 depends on artificial GPR54 (KISS1R) expression level. Oncology Reports 2013;29:549-544.

[33] Francis VA, Abera AB, Matjila M, Millar RP, Katz AA. Kisspeptin regulation of genes involved in cell invasion and angiogenesis in first trimester human trophoblast cells. PLoS One 2014;9:e99680-90.

[34] Xiao Y, Yingdong N, Yanbing H, Jing W, Grossmann R, Zhao R. Effects of kisspeptin-10 on progesterone secretion in cultured chicken ovarian granulosa cells from preovulatory (f1-f3) follicles. Peptides 2011;32:2091–97.

[35] Izhar M, Pasmanik M, Shemesh M. Bovine placental progesterone synthesis: comparison of first and second trimesters of gestation. Biol Reprod 1992;46(5):846-52.

[36] Tsumagari S, Kamata J, Takagi K, Tanemura K, Yosai A, Takeishi M. 3 beta-Hydroxysteroid dehydrogenase activity and gestagen concentrations in bovine cotyledons and caruncles during gestation and parturition. J Reprod Fertil. 1994;102(1):35-9.

Figure legends

Figure 1. Characterization of primary epithelial cell lines isolated from bovine placental cotyledons of embryos/fetuses at the first trimester of pregnancy. Photomicrographs of cell lines exhibiting

polygonal epithelial morphology by phase contrast microscopy (A, B for cell line A and B, respectively; Scale bars: 10µm) and presence of larger binucleated cells (white arrows) by epifluorescence microscopy (C, D for cell line A and B, respectively; Scale bars: 20µm). Presence of a Y-chromosome specific sequence confirming the fetal origin of cell lines: gel electrophoresis of PCR products amplified from genomic DNA isolated from the two cell lines, A and B (E). In lanes 1 and 2, corresponding to cell lines A and B, respectively, two fragments of 280 and 217 bp are shown (male pattern). A single fragment of 280bp is evident in the Y-chromosome negative control (female pattern, lane 3) whereas two fragments are shown in the Y-chromosome positive control (lane 4). Lane M: 100bp markers. CK and ZO-2 expression study: gel electrophoresis of PCR products amplified from mRNA extracted from the two cell lines, A (F, lane 1, 2 and 3) and B (F, lane 4, 5 and 6). Bovine cotyledonary cells show specific bands for CK (152 bp, lane 2 and lane 5) and ZO-2 (224 bp, lane 3 and lane 6). GAPDH (197 bp, lane 1 and 4) was used as a housekeeping gene. Lane 7 shows the negative PCR control. Lane M: 100bp markers.

Figure 2. Effects of Kp-10 on cell proliferation (Doubling Time, DT) of primary epithelial cell lines isolated from bovine placental cotyledons. Cell passage effect (panel A and panel B for cell line A and B, respectively). Results are Least Square means ± SE of 20 data points (4 data points for 5 conditions; GLM procedure). Line A (panel A) shows a higher DT at P1 compared with all other passages and then a faster proliferation rate from P2 to P10; line B (panel B) has a steady DT until P9, which then significantly increases at P10. In panels A and B, each passage is compared with all other passages; each symbol indicates a specific passage and crescent symbol numbers indicate increasing levels of significance (i.e. P1: *; * $P<0.05$; ** $P<0.01$;*** $P<0.001$). Within-passage Kp-10 concentration effect (panel A' and panel B' for cell line A and B, respectively): results are means ± SD of 4 data points analyzed by one-way ANOVA followed by Dunnett's post-hoc test. In line A (panel A'), stimulatory effect of Kp-10 can be seen at P1 and P2, followed by a switch to inhibitory effect at P3 and from P5 to P10; in Line B (panel B'), stimulatory effect is

650 visible at P3, P4, P6 and P7 whereas no effects can be seen in other passages. In panel A' and B',
651 bars with different letters indicate statistically significant differences: a,b $P<0.05$; a,c $P<0.01$; a,d
652 $P<0.001$.
653
654 **Figure 3**. Effects of Kp-10 on Kiss-1R mRNA relative abundance of primary epithelial cell culture
655 isolated from bovine placental cotyledons analyzed by quantitative Real Time RT-PCR. Data were
656 analyzed using the General Linear Model procedure (see Materials and Methods). In cell line A
657 (panel A), no statistical significance on Kiss-1R expression levels, upon Kp-10 addition, was found
658 in cells at any examined passage. In cell line B (panel B), Kiss-1R transcription was significantly
659 up-regulated upon Kp-10 stimulation in P3 and P6. For each sample, data (Least Square means ±
660 SE of two independent experiments in duplicate, Kiss-1R average Ct) were normalized relatively to
661 the abundance of GAPDH mRNA (endogenous control) and normalized values were compared
662 among groups. Bars with different letters indicate statistically significant differences: a,b $P<0.05$;
663 a,c $P<0.01$; a,d $P<0.001$.
664
665 **Figure 4.** Within-line Kp-10 concentration effects on progesterone secretion of primary epithelial
666 cell culture isolated from bovine placental cotyledons. Results are Least Square means ± SE of 3
667 data points (GLM procedure). Values from an early (P2), an intermediate (P4) and a late passage
668 (P10) were averaged. In both cell lines, Kp-10 had no effect on P_4 secretion at any tested
669 concentration.
670
671
672
673
674
675

Table 1. Sequence of primers used for PCR

Name	Accession number		Sequence	Product lenght
CK	NM_001033610.1	Forward: Reverse:	5'TGGAAGGGCTGACTGATGAG 3' 5'CTTGACCTCAGCGATGATGC 3'	152 pb
ZO-2	NM_001102482.1	Forward: Reverse:	5'GGATATTTGTGGCCGGCATT 3' 5'ATGAATCCCCTCTGCCACAA 3'	224 pb
GAPDH	NM_001034034	Forward: Reverse:	5'GTCTTCACTACCATGGAGAAGG-3' 5'TCATGGATGACCTTGGCCAG 3'	197 pb

Table 2. Within-line Kp-10 concentration effect on cell proliferation of primary epithelial cell lines from bovine placental cotyledons, expressed as DT values.

Cell line	Kp-10 concentration (μM)	Least Square (LS) means ± standard error (SE)
line A	0 (CTRL)	1.37±0.12
	0.01	1.30±0.11
	0.1	1.30±0.12
	1	1.33±0.11
	10	1.23±0.12
line B	0 (CTRL)	1.31±0.11
	0.01	1.29±0.11
	0.1	1.34±0.11
	1	1.34±0.11
	10	1.35±0.11

For each cell line and Kp-10 concentration, data are LS means ± SE of DT values from 40 data points (4 data points for 10 passages). The overall F test of the model is not significant (F = 0.19; p 0.9427), indicating that the model containing only within-line Kp-10 concentration effect does not accounts for a significant amount of the variation in DT.

Figure 1

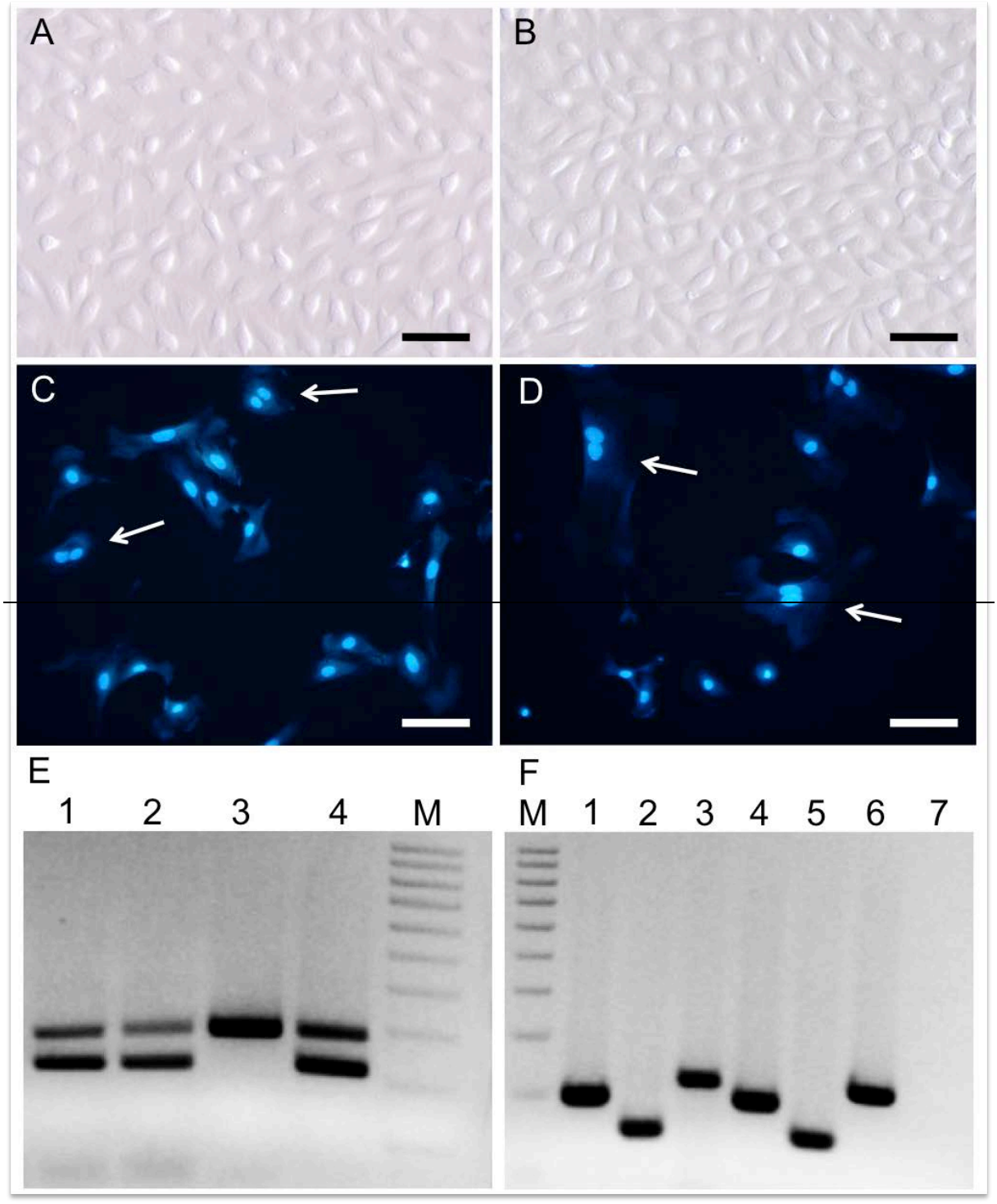


Figure 2

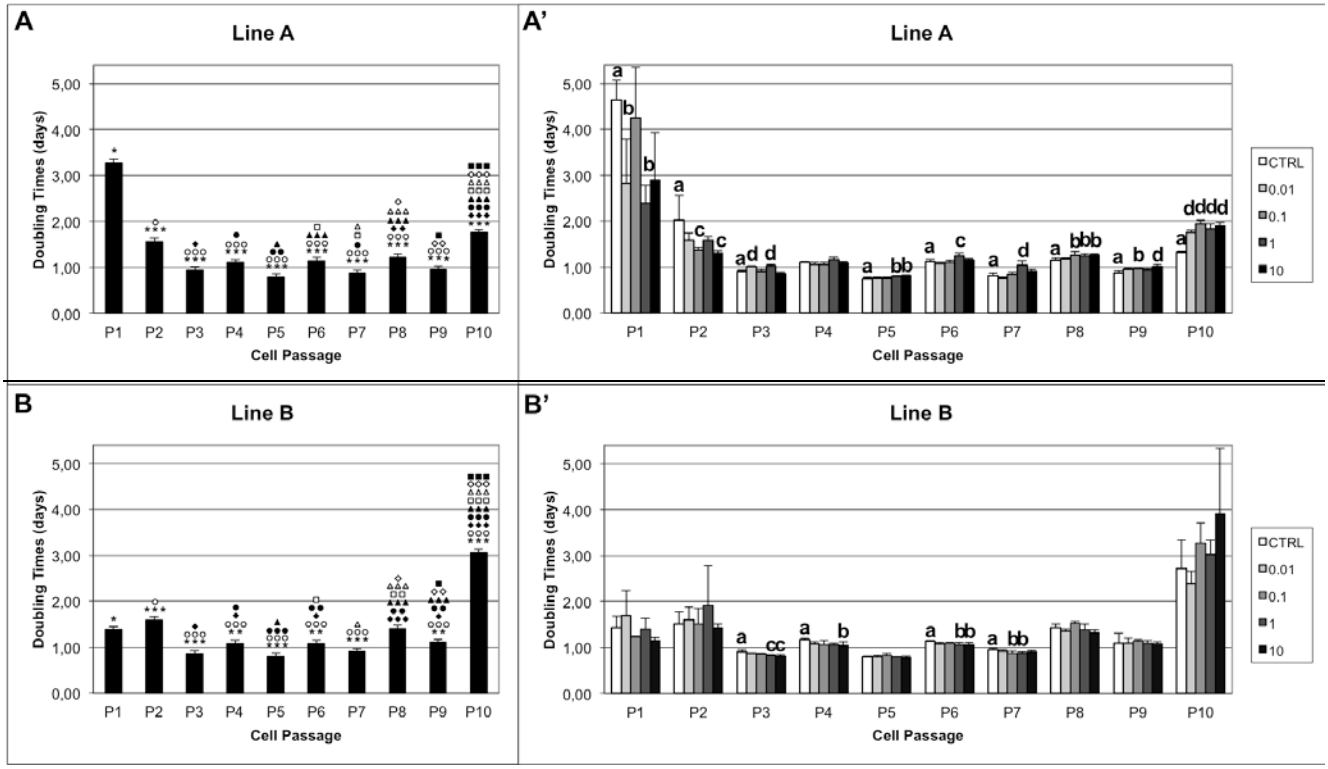
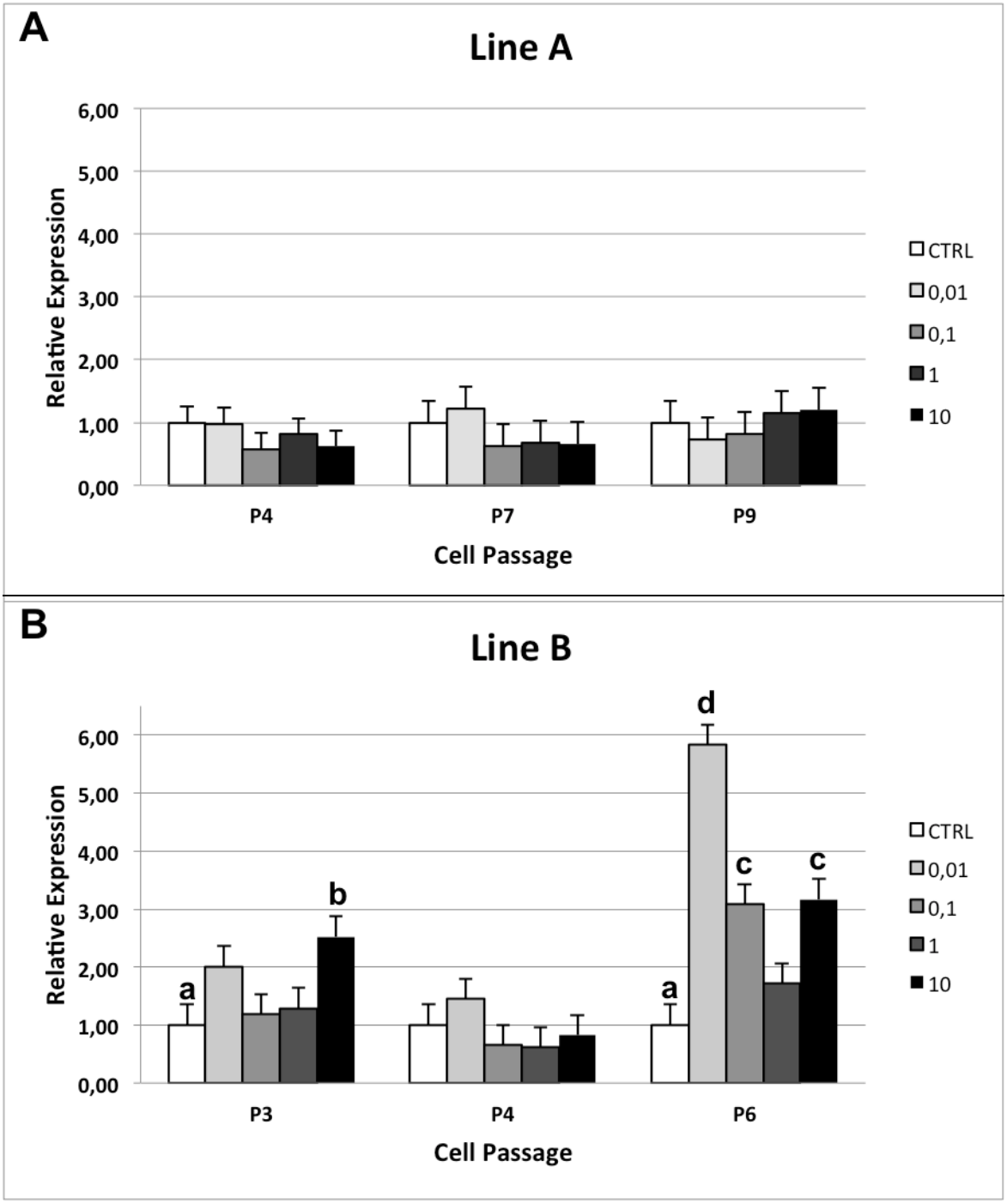
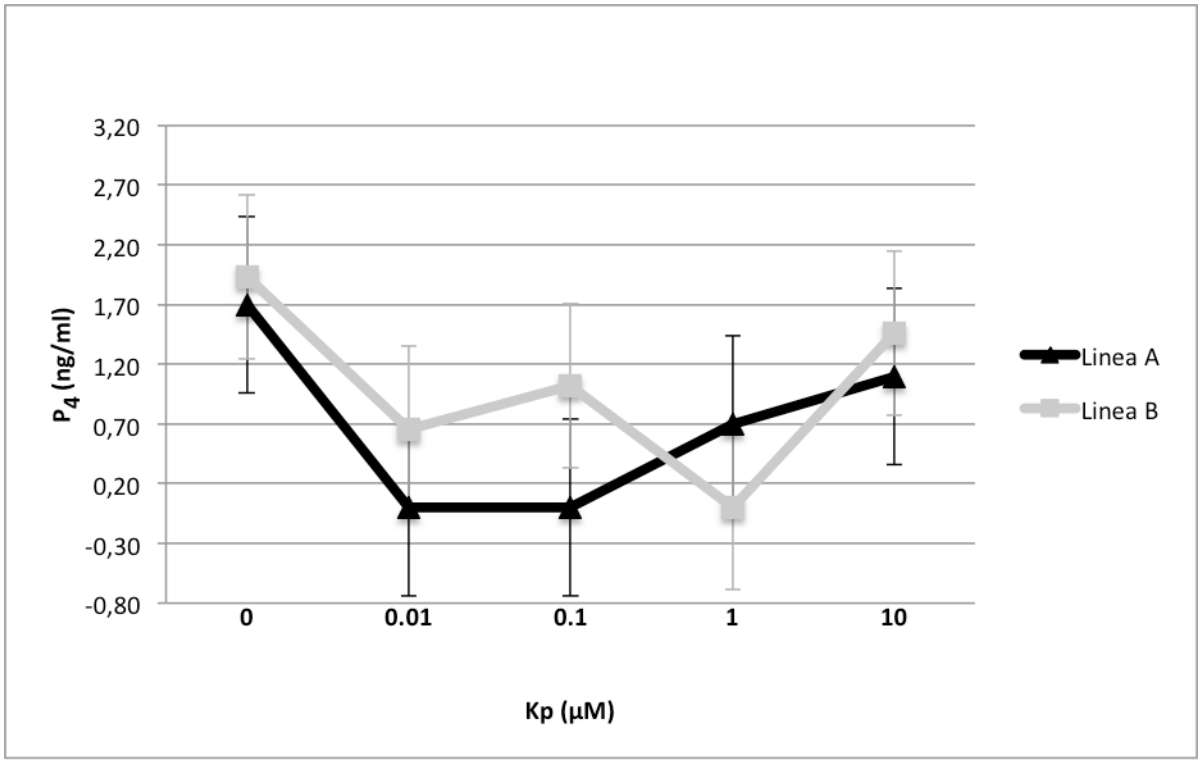

A
Line A
A'
Line A
B
Line B
B'
Line B
Doubling Times (days)
Cell Passage
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
CTRL
0.01
0.1
1
10

Figure 3


A
Line A
Relative Expression
6,00
5,00
4,00
3,00
2,00
1,00
0,00
P4
P7
P9
Cell Passage
CTRL
0,01
0,1
1
10
B
Line B
Relative Expression
6,00
5,00
4,00
3,00
2,00
1,00
0,00
a
b
a
d
c
c
P3
P4
P6
Cell Passage
CTRL
0,01
0,1
1
10

738 Figure 4



739

Supplementary file 1:

Within-passage Kp-10 concentration effect on cell proliferation of primary epithelial cell line from bovine placental cotyledons.

Line A

Cell passage	Kp-10 concentration (μM)				
	0 (CTRL)	0.01	0.1	1	10
P1	4.64 ± 0.43 **a**	2.82 ± 0.97 **b**	4.25 ± 1.11	2.39 ± 0.39 **b**	2.89 ± 1.03
P2	2.02 ± 0.55 **a**	1.59 ± 0.15	1.38 ± 0.05 **c**	1.59 ± 0.07	1.31 ± 0.04 **c**
P3	0.91 ± 0.02 **a**	1.01 ± 0.02 **d**	0.91 ± 0.05	1.03 ± 0.02 **d**	0.86 ± 0.02
P4	1.11 ± 0.02	1.06 ± 0.04	1.06 ± 0.03	1.16 ± 0.05	1.09 ± 0.03
P5	0.75 ± 0.03 **a**	0.77 ± 0.01	0.77 ± 0.02	0.80 ± 0.01 **b**	0.80 ± 0.03 **b**
P6	1.12 ± 0.05 **a**	1.08 ± 0.02	1.11 ± 0.04	1.24 ± 0.06 **c**	1.15 ± 0.03
P7	0.82 ± 0.06 **a**	0.77 ± 0.01	0.83 ± 0.05	1.04 ± 0.10 **d**	0.90 ± 0.04
P8	1.15 ± 0.05 **a**	1.18 ± 0.03	1.26 ± 0.07 **b**	1.25 ± 0.05 **b**	1.26 ± 0.03 **b**
P9	0.88 ± 0.03 **a**	0.95 ± 0.04	0.97 ± 0.01 **b**	0.94 ± 0.03	1.00 ± 0.05 **d**
P10	1.33 ± 0.02 **a**	1.76 ± 0.04 **d**	1.94 ± 0.07 **d**	1.83 ± 0.11 **d**	1.89 ± 0.08 **d**

Line B

Cell passage	Kp-10 concentration (μM)				
	0 (CTRL)	0.01	0.1	1	10
P1	1.43 ± 0.26	1.70 ± 0.54	1.22 ± 0.03	1.40 ± 0.23	1.15 ± 0.07
P2	1.51 ± 0.27	1.60 ± 0.27	1.51 ± 0.34	1.91 ± 0.87	1.43 ± 0.08
P3	0.90 ± 0.04 **a**	0.87 ± 0.00	0.85 ± 0.02	0.82 ± 0.02 **c**	0.81 ± 0.03 **c**
P4	1.16 ± 0.05 **a**	1.08 ± 0.04	1.07 ± 0.08	1.06 ± 0.04	1.04 ± 0.08 **b**
P5	0.80 ± 0.01	0.80 ± 0.04	0.82 ± 0.04	0.79 ± 0.01	0.78 ± 0.02
P6	1.14 ± 0.02 **a**	1.08 ± 0.03	1.08 ± 0.02	1.06 ± 0.04 **b**	1.06 ± 0.05 **b**
P7	0.95 ± 0.03 **a**	0.92 ± 0.03	0.87 ± 0.05 **b**	0.88 ± 0.03 **b**	0.90 ± 0.04
P8	1.43 ± 0.07	1.36 ± 0.04	1.52 ± 0.05	1.39 ± 0.12	1.32 ± 0.07
P9	1.09 ± 0.22	1.10 ± 0.11	1.14 ± 0.03	1.09 ± 0.07	1.08 ± 0.04
P10	2.72 ± 0.62	2.39 ± 0.26	3.27 ± 0.44	3.03 ± 0.31	3.92 ± 1.42

Data are means ± standard deviations. One-way ANOVA followed by Dunnett's post hoc test.

For Kp-10 concentration effect: comparisons treated versus CTRL (between columns): a,b $P<0.05$; a,c $P<0.01$; a,d $P<0.001$.

No passage effect (between lines comparisons) is represented in this table, as it is shown in Figure 2.