Acta Scientiae Veterinariae, 2013. 41: 1156.

RESEARCH ARTICLE
Pub. 1156

ISSN 1679-9216

Gene Expression and *In Vitro* Nuclear Maturation in Bovine *Cumulus* Oocyte Complexes Maturated in a Medium Supplemented with Bovine Fetal Serum or Bovine Serum Albumin*

Fernanda Araujo de Britto Velho[1,2], Bruna Grandi da Costa[2], Diego Duarte Alcoba[3], Bianca Letícia da Rosa Braga[3], Nathallie Louise Sandi Monroy[3], Alexandre Tavares Duarte de Oliveira[3] & Rui Fernando Felix Lopes[2]

ABSTRACT

***Background*:** The expansion and mucification of granulosa cells of the *cumulus oophorus*-oocyte complex (COC) is observed during the oocyte *in vitro* maturation (IVM) as a result of the intense synthesis of extracellular matrix (ECM) components. These changes in *cumulus* aspect are indicative of maturation and may be influenced by oocyte-related factors and by IVM conditions. The objectives of the present study were (i) to assess the expression of gene transcripts that codify for the proteins hyaluronan synthase-2 (HAS2), link protein 1, connexin 43 and β-actin in bovine *cumulus oophorus*-oocyte complexes (COCs) before and after IVM, and (ii) to determine nuclear maturation rates of oocytes submitted to IVM.
***Materials, Methods & Results*:** Bovine COCs obtained from abattoir-derived ovaries were analyzed and selected for morphological aspects and divided in three experimental groups: G1, COCs submitted to IVM; G2, COCs submitted to IVM in medium supplemented with 10% fetal bovine serum (FBS); and G3, COCs submitted to IVM in medium supplemented with bovine serum albumin (BSA). After extraction of the messenger RNA (mRNA) of COCs, cDNA was extracted and fragments of the gene transcripts were amplified using the reverse transcription (RT) and the polymerase chain reaction (PCR). The RT-PCR products were electrophoresed in agarose gels and amplification intensity was quantified to obtain the relative mRNA abundance. Part of oocytes submitted to IVM medium supplemented with FBS (G2) or BSA (G3) was stained with Hoechst 33342 to assess the nuclear maturation rate by fluorescence microscopy. The results revealed that relative abundances of HAS ($P = 0.000$), link protein 1 ($P = 0.001$), connexin 43 ($P = 0.007$) and β-actin ($P = 0.011$) transcripts differed between COCs submitted to IVM in FBS medium (G2) and COCs not submitted to IVM (G1) or COCs maturated in BSA medium (G3). When COCs submitted to IVM in FBS or BSA media are compared, no statistically significant differences ($P > 0.05$) were observed in meiosis resumption (86.7% and 91.5%, respectively) or in nuclear maturation rates (56.1% and 58.5%).
***Discussion*:** HAS2 is involved in the synthesis of hyaluronic acid (HA) by *cumulus* cells, and plays an important role in ECM expansion and in oocyte competence development. This protein organization of the ECM, formed by the aggregation of HA and proteoglycans, depends on link protein 1; it is also produced by *cumulus* cells and is implicated in COC expansion. Connexin 43 belongs to a protein family that establishes gap junctions that play an important role in the cellular communication and coordinated response processes. The role of gap junctions in bovine oocytes during IVM has been associated with maturation rates and *cumulus* expansion; this expansion of *cumulus* cells is accompanied by changes in the transmembrane channels formed by connexin 43. The higher mRNA expression of the HAS2, link protein 1, connexin 43 and β-actin genes in bovine COCs submitted to IVM in FBS medium, in comparison with COCs before IVM or COCs maturated in BSA medium may be associated with FBS constituents, which would act as transcription factors for these genes during ECM expansion. Although the results obtained allow associating the differential expression of transcripts to the presence of FBS in the IVM medium, the data reveal that meiosis resumption and nuclear maturation apparently were not influenced by the protein supplementation regimens in the IVM medium, supplemented either with FBS or BSA.

Keywords: bovine, *in vitro* maturation, *cumulus oophorus*, gene expression.

Received: 18 June 2013 Accepted: 30 September 2013 Published: 22 October 2013

*Article based upon a thesis submitted by the first author in partial fulfillment of requirements for the Master's degree. [1]Programa de Pós-graduação em Ciências Veterinárias (PPGCV), Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. [2]Laboratório de Biotecnologia Animal Aplicada, Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde (ICBS), UFRGS, Porto Alegre, RS. [3]Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS. CORRESPONDENCE: R.F.F. Lopes [rui.lopes@ufrgs.br - Tel.: +55 (51) 3308 3631]. Departamento de Ciências Morfológicas, ICBS, UFRGS, Rua Sarmento Leite n. 500. CEP 90050-170, Porto Alegre, RS, Brazil.

INTRODUCTION

One of the lasting challenges in reproduction biology is the understanding of the cellular and molecular processes defining oocyte quality. The acquisition of oocyte developmental competence and the role played by the follicle environment in the potential for oocyte development are some aspects that have require further elucidation [9].

The association between the oocyte and *cumulus oophorus* cells, which form the *cumulus oophorus*-oocyte complex (COC) is sustained during oocyte growth, differentiation, maturation and fertilization stages. This communication is essential both for oocyte growth and differentiation and for granulosa cells, allowing the successful oogenesis [3]. Oocyte development competence may be determined by markers expressed by follicle cells that surround it [10]. In this sense, the gene expression profile of *cumulus* cells may reflect the fertilization potential of oocytes selected for *in vitro* maturation (IVM). However, the relationship between expression of specific genes during follicle growth and the respective capacity to define embryo quality have yet to be investigated in more detail [10].

The objectives of this study were (i) to evaluate the transcript expression of genes that codify for the proteins hyalurone synthase 2 (HAS2), link protein 1 (HAPLN1, hyaluronan and proteoglycan link protein 1), connexin 43 (GJA1, gap junction protein, alpha 1, 43 kDa) and β-actin (ACTB, actin, beta) in bovine *cumulus* oocytes complexes (COCs) non-maturated or submitted to IVM in media supplemented with different proteins, and (ii) assess the nuclear maturation rates of oocytes submitted to different IVM conditions.

MATERIALS AND METHODS

The chemicals tested for embryo cultivation used in the preparation of all CCOs manipulation media were purchased from Sigma[1]. The chemicals used in the reverse transcriptase technique (RT) and polymerase chain reaction (PCR) were supplied by Invitrogen[2]. Chemicals acquired from other suppliers are indicated in the text.

In vitro *maturation (IVM) and assessment of nuclear maturation*

Follicles measuring between 2 and 8 mm in diameter were collected by punction from ovaries dissected from bovine females slaughtered in an abattoir. COCs were selected based on a morphological assessment [16] and then transferred to a dish containing the manipulation medium M2 [18].

COCs selected for IVM were divided in groups of 8 to 10 and transferred to 100 µL droplets of TCM 199 medium [17] containing 0.5 µg/mL FSH[3], 0.1 IU/mL LH[4] and a protein source defined individually for each experimental group: 10% fetal bovine serum (FBS)[5] or 4 mg/mL bovine serum albumin (BSA)[6]. IVM was carried out for between 22 h to 24 h in a 5% CO_2 atmosphere and 100% relative humidity at 39ºC.

After IVM, COCs were incubated for 5 min in M2 containing 0.1% hyaluronidase. Then, oocytes were mechanically denuded of *cumulus* cells. Denuded oocytes were transferred to one M2 droplet supplemented with 5 µg/mL Hoechst 33342[1] and incubated for 3 min in a dark chamber. After incubation, oocytes were inspected by fluorescence microscopy under 400 x magnification and sorted according to the following criteria: (i) germinal vesicle (GV), (ii) germinal vesicle breakdown (GVBD), first metaphase plate formation, indicating that the oocyte reached metaphase I (MI), (iii) first polar body (PB) extrusion, indicating metaphase (MII), (iv) degenerated oocytes (DEG) or (v) not observed oocytes (NO). Presence of GVBD, MI or MII was interpreted as meiosis resumption. However, only oocytes presenting PB extrusion (MII) were considered as presenting nuclear maturation.

Extraction of messenger RNA (mRNA) and the RT and PCR techniques

As internal control, 0.1 pg rabbit α-hemoglobin (HBA)[6] mRNA was added to samples containing 8 COCs. Total RNA was extracted using TRIzol™ according to the manufacturer's instructions[6]. Total RNA samples were submitted to specific mRNA capture using a commercially available magnetic separation chemical[7], as previously described [1].

The mRNA isolated from each sample was reversely transcripted in cDNA in a 20 µL final volume solution [1]. The RT reaction was carried out by incubation at 37ºC for 60 min, followed by heating to 95ºC for 10 min. At the end of reverse transcription, cDNA samples of COCs were diluted to a 80 µL final volume completed with ultrapure RNase-free water that samples presented the equivalent to 0.1 COC in each 1 µL cDNA (0.1 COC/µL; COC equivalent).

PCR was conducted in a 20 µL final volume [1] using 10 pmol of each specific primer[6] (except

for link protein 1, for which 20 pmol were used) and 0.1 to 1.0 COC equivalent (1 µL to 12 µL) cDNA of samples, depending on the mRNA investigated, or 1 µL (1 pg/µL) cDNA of rabbit HBA. Negative controls were prepared without cDNA. PCR for the genes investigated and HBA (internal control) was conducted in separate tubes. For that, 0.2 COC equivalent was used for HAS2, 1.2 COC equivalent was used for link protein 1, 0.1 COC equivalent was used for connexin 43, 0.3 COC equivalent was used for β-actin, and 1 µL of the reaction volume (20 µL) for rabbit HBA. Primer sequences and sizes of amplified fragments are shown in Table 1.

PCR was conducted according to the following steps: initial denaturation at 94ºC for 2 min followed by 33 amplification cycles at 94ºC for 1 min, 56ºC for 1 min and 72ºc for 90 s (except for link protein 1, for which 35 cycles were used), and a final extension cycle at 72ºC for 3 min.

The RT-PCR products were electrophoresed in agarose[6] gels 2% in TBE 0.5X buffer (45 mM Trisborate, 1 mM EDTA, pH 8.3) containing 0.5 µg ethidium bromide/mL of gel. Gels were photographed and the amplification intensity obtained was quantified and analyzed using the Scion Image[8] software. Relative mRNA abundance of the genes studies was calculated dividing the intensity of the band of each COC sample by the intensity of the band of rabbit HBA of the corresponding sample.

Experimental design

The relative abundance of HAS2, link protein 1, connexin 43 and β-actin transcripts was compared between bovine COCs not submitted to IVM (G1) and COCs submitted to IVM supplemented with 10% FBS (G2) or 4 mg/mL BSA (G3). Nuclear maturation rate of COCs submitted to IVM was also compared.

Selected COCs were evenly sorted to the three experimental groups. G1 included eight COCs that were not submitted to IVM were collected in 8 µL M2 and stored in a 1.0 mL cone tube containing liquid nitrogen. G2 was formed by eight COCs were submitted to IVM in maturation medium supplemented with 10% FBS. G3 comprised eight COCs were submitted to IVM in a maturation medium supplemented with 4 mg/mL BSA. COCs of G2 and G3 were collected in 80 µL of the respective maturation medium, transferred to 1.0 mL cone tubes and stored in liquid nitrogen. Relative abundance of studied gene transcripts was calculated based on eight repeats (64 COCs).

For COCs used to assess nuclear maturation stage, IVM was carried out in droplets of the maturation medium containing 10 COCs under the same conditions as described for G2 (FBS) and G3 (BSA). Nuclear maturation was evaluated based on 10 repeats.

Statistical analyses

Relative abundance of HAS2, link protein 1, connexin 43 and β-actin transcripts was analyzed using the SPSS 15[9] software. Abundance data were tested for variance homogeneity using the Levene test and then compared by analysis of variance (ANOVA) followed by the multiple comparison test (Tukey). The results of the nuclear maturation evaluation were analyzed using the Chi-square test followed by the exact Fisher test, when necessary. Statistically significance was defined as $P \leq 0.05$.

Table 1. Primer sequences used in the RT-PCR techniques.

Genes	GenBank accession number	Primer sequences	Amplified fragment
HAS2	NM_174079	5' GCTTGACCCAGCATCATCTGTGG 3' CTGGTTTAACCATCTGAGATATT	403bp
HAPLN1	NM_174288	5' GGTCTGTGCAATATCCCATC 3' CCCACTTTAGCAATCTGAGC	232bp
GJA1	NM_174068	5' GGGAAAGAGCGATCCTTACCACACTACCAC 3' CCACCTCCAATGAAACAAAATGAACACCTA	516bp
ACTB	NM_173979	5' GAG AAG CTC TGC TAC GTG GC 3' CCG GAC AGC ACC GTG TTG GC	263bp
HBA	NM_001082389	5' GCAGCCACGGTGGCGAGTAT 3' GTGGGACAGGAGCTTGAAAT	257bp

ACTB (actin, beta): β-actine; GJA1 (gap junction protein, alpha 1, 43kDa): connexin 43; HAPLN1 (hyaluronan and proteoglycan link protein 1): link protein 1; HAS2 (hyaluronan synthase): hyaluronic acid synthase 2; HBA (alpha-hemoglobin): alpha-hemoglobin.

RESULTS

During the manipulation experiments in the collection of oocytes used in the gene expression and nuclear maturation analysis, a higher degree of expansion and mucification of the ECM was observed in COCs submitted to IVM in medium supplemented with FBS, compared with those submitted to IVM in medium supplemented with BSA (data not shown).

HAS2, link protein 1, connexin 43 and β-actin transcripts were detected in all gels submitted to electrophoresis to assess the gene expression profile. The analysis of gels based on band intensity of each cDNA fragment allowed defining a relative abundance value for each sample. Mean and standard deviation of relative abundance of transcripts obtained for 8 bovine COCs of each of the three experimental groups (eight repeats) are presented in Figure 1. Statistically significant difference was observed for the relative abundance of HAS2 (P = 0.000), link protein 1 (P = 0.001), connexin 43 (P = 0.007) and β-actin (P = 0.011) transcripts, between the three experimental groups (Figure 1). The expression of all transcripts was higher in COCs submitted to IVM in medium containing FBS (G2), compared with those not submitted to IVM (G1) or those maturated in BSA (G3).

The assessment of nuclear maturation was carried out in 98 oocytes submitted to IVM in the medium supplemented with FBS (G2) and in 94 oocytes submitted to IVM in the medium containing BSA (G3). Nuclear maturation data of oocytes are shown in Table 2. No statistically significant differences ($P < 0.05$) were observed in the nuclear morphology of oocytes submitted to IVM in the presence of FBS or BSA, for meiosis resumption (86.7% and 91.5%, respectively) or in nuclear maturation rates (56.1% and 58.5%).

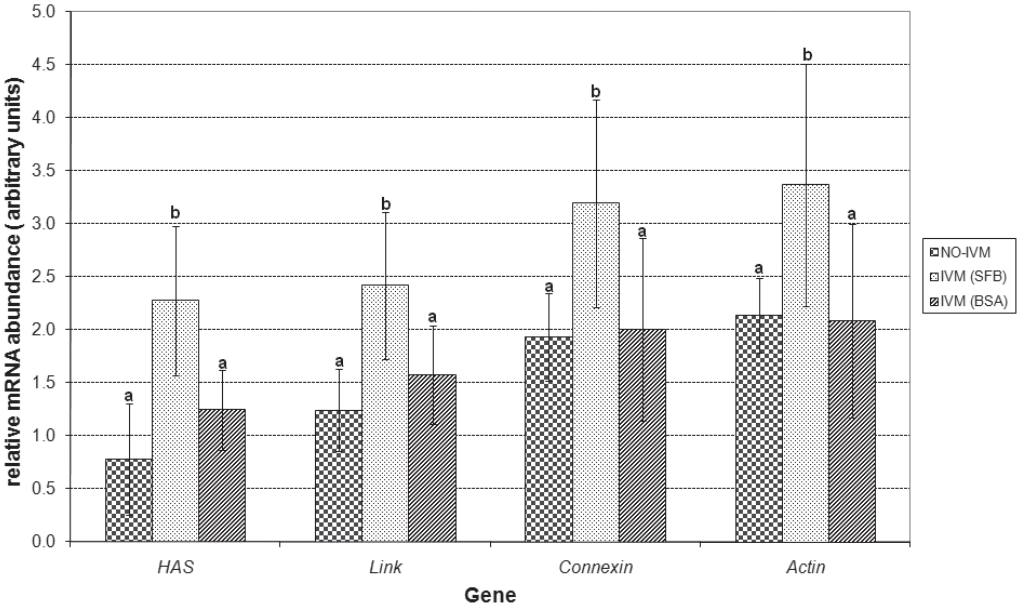

Figure 1. Relative mRNA abundance (mean ± standard deviation) of HAS2, link protein 1, connexin 43 and β-actin transcripts in different bovine COCs. NO-IVM [G1: COCs not submitted to IVM]; IVM (FBS) [G2: CCOs submitted to IVM in a FBS medium]; IVM (BSA) [G3: CCOs submitted to IVM in a BSA medium]. a,b: different letters in each gene represent statistically significant difference between COC groups (ANOVA) [HAS (P = 0.000), link protein 1 (P = 0.001), connexin 43 (P = 0.007) and β-actin (P = 0.011)].

Table 2. Evaluation of meiosis resumption (GVBD, MI and MII) and nuclear maturation (only MII) in bovine oocytes maturated *in vitro* in FBS and BSA media.

Protein source	Oocytes	GV	GVBD	MI	MII	DEG	NO
	n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
FBS	98	1 (1.0)	22 (22.4)	8 (8.2)	55 (56.1)	4 (4.1)	8 (8.2)
BSA	94	3 (3.2)	20 (21.3)	11 (11.7)	55 (58.5)	1 (1.1)	4 (4.3)

GV (germinal vesicle); GVBD (germinal vesicle breakdown); MI (first metaphase plate formation); MII (presence of first polar body extrusion); DEG (degenerated oocyte); NO (not observed oocytes); $P > 0.05$.

DISCUSSION

Research on the expression of genes associated with IVM usually addresses the expression of oocyte transcripts, and essentially discusses the role of *cumulus* cells in gamete development and the processes involved in the interaction between structures [11,19,25]. However, *cumulus* cells are important in the oocyte maturation process. It is known that *in vivo* follicle development, maturation and ovulation are successful only when there is a communication between *cumulus* cells and oocyte [24]. The expansion (or mucification) of the COC plays a role in molecular filtration, which may concentrate signaling molecules produced by the oocyte and *cumulus* cells [7]. In the present study, the relative abundance of mRNA of the genes HAS2, link protein 1, connexin 43 and β-actin differed between COCs before and after IVM, suggesting their importance in the maturation process. Large amounts of hyaluronic acid (HA) and of other proteins are secreted during oocyte maturation, producing the protein ECM associated with the expansion and mucification of COC [12, 20]. The protein HAS2 is implicated in HA synthesis by *cumulus* cells, and is important in the ECM expansion process [11]. Additionally, it has been associated with oocyte developmental competence [7]. The protein organization of the ECM, formed by the aggregation of HA and proteoglycans, depends on link protein 1 (HAPLN1), which is produced by *cumulus* cells [14,23] and is involved also in COC expansion [22,27]. The protein and the mRNA of the connexin 43 protein are present in bovine COCs during IVM [4, 26]. Connexin 43 belongs to a protein family that establishes gap junctions that play an important role in the communication and coordinated response processes between cells. The role of gap junctions for bovine COCs during IVM has been linked with both maturation rates and *cumulus* expansion; the signaling blockade mediated by gap junctions or the reduction of connexin 43 levels lowers maturation rates [26], while the expansion of *cumulus* cells is accompanied by changes in the transmembrane channels formed by connexin 43 [6].

During IVM and subsequent embryo development, important nuclear and cytoplasm reprogramming events take place requiring several chemical compounds that therefore have to be present in the culture medium [5,19]. However, during IVM gene transcription may be affected by a series of supplements to which COCs are exposed. The results show that the expression of all transcripts was higher in COC groups submitted to IVM in a FBS medium, in comparison with COCs before IVM or that were maturated in the BSA medium. Additionally, higher ECM expansion and mucification were observed in the COCs maturated in the FBS medium (data not shown). These results may be associated with components present in FBS, which could act as transcription factors of the genes involved in both the expansion and mucification of the ECM [6,11,22,27].

Additionally, in the present study, although differences were observed in the expression of the genes studied, the nuclear morphology of oocytes after IVM did not differ significantly between COCs maturated in FBS or BSA media, both in terms of meiosis resumption (GVDB, MI and MII) and nuclear maturation (MII). It is known that IVM is a reproduction technology that affords to produce *ex vivo* maturated oocytes, with no need for ovarian gonadotrofins treatments, that involve the removal of COCs from antral follicles and cultivation under standardized conditions until oocytes reach metaphase II [9]. However, even when nuclear maturation takes place, only a small number of these maturated oocytes exhibits full development potential [21]. Cytoplasm maturation also has to be accomplished, at the end of which mRNAs, proteins and transcription factors are stored, which act during maturation, fertilization and the beginning of embryogenesis [8]. Although a considerable number of bovine oocytes, after they were removed from the follicle, spontaneously undergo meiosis resumption, little is known about the requirements for appropriate cytoplasm maturation [4].

When nuclear maturation data are compared with the evaluation of gene expression and ECM expansion and mucification (data not shown), it is observed that COCs maturated in FBS medium presented nuclear maturation rates similar to those shown by COCs submitted to IVM in BSA medium, in spite of the distinctive mucification aspects and of the relative abundance of mRNA of the genes detected in both COC groups. Although it is possible to associate differential expression of transcripts to the presence of FBS in the maturation medium [6,22], nuclear maturation apparently was not influenced by supplementation with protein sources in IVM.

Even though differences were observed in the expression of the transcripts analyzed, gene expression in embryos produced by the IVM protocols adopted was not evaluated in the present study. Some authors understand that higher relative abundance of transcripts in bovine blastocyst produced *in vitro* is linked with the supplementation of the medium with BSA or serum during IVM [19,25], though other researchers have shown that protein supplementation with BSA or serum in the maturation or cultivation medium does not affect the mRNA abundance profiles of embryos [15,17].

Moreover, different genes exposed to the same set of conditions may be induced differently. Studies using bovine embryos [2,17] did not establish any significant difference in mRNA relative abundance for IVM protocols. The reason for this may be the high variation across COCs samples. One possible explanation for the variation in the gene expression profile between samples may be associated with the IVM protocol used. In bovines, the supplementation of the culture medium with serum has been associated with the variation in the *in vitro* responses of embryos [13], of COCs [4] and *cumulus* cells [1]. The data obtained here show that the samples used in the several experimental repeats were homogeneous.

All in all, exposure of bovine COCs to different IVM conditions influenced the expression of HAS2, link protein 1, connexin 43 and β-actin transcripts, showing the increased expression of these transcripts when IVM was conducted in a medium containing FBS. However, IVM in FBS or BSA media did not alter meiosis resumption and nuclear maturation rates.

SOURCES AND MANUFACTURERS

[1]Sigma Chemical Co., St. Louis, MO, USA.

[2]InvitrogenTM, São Paulo, SP, Brazil.

[3]Folltropin®, Vetrepharm, Belleville, ON, Canada.

[4]Chorulon®, Intervet, São Paulo, SP, Brazil.

[5]Nutricell, Campinas, SP, Brazil.

[6]Life Technologies™, Grand Island, NY, USA.

[7]Dynabead mRNA™ DIRECT Micro Kit, DYNAL, Oslo, Norway.

[8]Scion Co., Frederick, MD, USA.

[9]SPSS Inc., Chicago, IL., USA.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1 **Arend F.L., Vieira A.D., Cesaro M.P., Mezzalira A., Oliveira A.T.D. & Lopes R.F.F. 2010.** Expressão gênica das células do *cumulus oophorus* de bovinos após a vitrificação. *Acta Scientiae Veterinariae*. 38(1): 37-46.

2 **Bilodeau-Goeseels S. & Panich P. 2002.** Effects of oocyte quality on development and transcriptional activity in early embryos. *Animal Reproduction Science*. 71(3-4): 143-155.

3 **Buccione R., Schroeder A.C. & Eppig J.J. 1990.** Interactions between somatic cells and germ cells during mammalian oogenesis. *Biology of Reproduction*. 43(4): 543-547.

4 **Calder M.D., Caveney A.N., Smith L.C. & Watson A.J. 2003.** Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation in vitro. *Reproductive Biology and Endocrinology*. 1: 14.

5 **Chanson A., Nocera D., Senn A., De Grandi P. & Germond M. 2001.** Development of a well-defined medium for the *in vitro* maturation of immature bovine cumulus–oocyte complexes. *Journal of Assisted Reproduction and Genetics*. 18(2): 97-105.

6 **Dell'Aquila M.E., Caillaud M., Maritati F., Martoriati A., Gerard N., Aiudi G., Minoia P. & Goudet G. 2004.** Cumulus expansion, nuclear maturation and connexin 43, cyclooxygenase-2 and FSH receptor mRNA expression in equine cumulus-oocyte complexes cultured *in vitro* in the presence of FSH and precursors for hyaluronic acid synthesis. *Reproductive Biology and Endocrinology*. 2: 44.

7 **Dunning K.R., Watson L.N., Sharkey D.J., Brown H.M., Norman R.J., Thompson J.G., Robker R.L. & Russell D.L. 2012.** Molecular filtration properties of the mouse expanded cumulus matrix: controlled supply of metabolites and extracellular signals to cumulus cells and the oocyte. *Biololgy of Reproduction*. 87(4): 89.

8 **Ferreira E.M., Vireque A.A., Adona P.R., Meirelles F.V., Ferriani R.A. & Navarro P.A. 2009.** Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*. 71(5): 836-848.

9 Gilchrist R.B. & Thompson J.G. 2007. Oocyte maturation: Emerging concepts and technologies to improve developmental potential *in vitro*. *Theriogenology*. 67(1): 6-15.

10 Hamel M., Dufort I., Robert C., Gravel C., Leveille M.C., Leader A. & Sirard M.A. 2008. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Human Reproduction*. 23(5): 1118-1127.

11 Irving-Rodgers H.F. & Rodgers R.J. 2005. Extracellular matrix in ovarian follicular development and disease. *Cell and Tissue Research*. 322(1): 89-98.

12 Kimura N., Konno Y., Miyoshi K., Matsumoto H. & Sato E. 2002. Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. *Biology of Reproduction*. 66(3): 707-717.

13 Knijn H.M., Wrenzycki C., Hendriksen P.J., Vos P.L., Herrmann D., Van Der Weijden C.G., Niemann H. & Dieleman S.J. 2002. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*. *Reproduction*. 124(3): 365-375.

14 Kobayashi H., Sun G.W., Hirashima Y. & Terao T. 1999. Identification of link protein during follicle development and cumulus cells cultures in rats. *Endocrinology*. 140(8): 3835-3842.

15 Lazzari G., Wrenzycky C., Herrmann D., Duchi R., Kruip T., Niemann H. & Galli C. 2002. Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biololgy of Reproduction*. 67(3): 767-775.

16 Loos F., Van Vliet C., Van Maurik P. & Kruip T.A. 1989. Morphology of immature bovine oocytes. *Gamete Research*. 24(2): 197-204.

17 Oliveira A.T.D., Lopes R.F.F. & Rodrigues J.L. 2006. Gene expression and developmental competence of bovine embryos produced *in vitro* with different serum concentrations. *Reproduction in Domestic Animals*. 41(2): 129-136.

18 Quinn P., Barros C. & Whittingham D.G. 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human espermatozoa. *Journal of Reproduction and Fertility*. 66(1): 161-168.

19 Sagirkaya H., Misirlioglu M., Kaya A., First N.L., Parrish J.J. & Memili E. 2007. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reproduction Science*. 101(3-4): 225-240.

20 Salustri A., Camaioni A., Di Giacomo M., Fulop C. & Hascall V.C. 1999. Hyaluronan and proteoglycan in ovarian follicles. *Human Reproduction Update*. 5(4): 293-301.

21 Schroeder A.C. & Eppig J.J. 1984. The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. *Developmental Biology*. 102(2): 493-497.

22 Spicer A.P., Joo A. & Bowling Jr. R.A. 2003. A hyaluronan binding link protein gene family whose members are physically linked adjacent to chrondroitin sulfate proteoglycan core protein genes: the missing links. *The Journal of Biological Chemistry*. 278(23): 21083-21091.

23 Sun G.W., Kobayash H. & Tera T. 1999. Expression of link protein during mouse follicular development. *Journal of Histochemistry and Cytochemistry*. 47(11): 1433-1442.

24 Tanghe S., Van Soom A., Nauwynck H., Coryn M. & de Kruif A. 2002. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular Reproduction and Development*. 61(3): 414-424.

25 Vireque A.A., Camargo L.S.A., Serapia R.V., Rosa e Silva A.A.M, Watanabe Y.F., Ferreira E.M., Navarro P.A.A.S., Martins W.P. & Ferriani R.A. 2009. Preimplantation development and expression of Hsp-70 and Bax genes in bovine blastocysts derived from oocytes matured in alpha-MEM supplemented with growth factors and synthetic macromolecules. *Theriogenology*. 71(4): 620-627.

26 Vozzi C., Formenton A., Chanson A., Senn A., Sahli R., Shaw P., Nicod P., Germond M. & Haefliger J.A. 2001. Involvement of connexin 43 in meiotic maturation of bovine oocytes. *Reproduction*. 122(4): 619-628.

27 Wrenzycki C., Herrmann D. & Niemann H. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology*. 68(Suppl 1): s77-s83.