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Identification of molecular markers for oocyte competence in bovine cumulus cells

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Summary

Cumulus cells (CCs) have an important role during oocyte growth, competence acquisition, maturation, ovulation and fertilization. In an attempt to isolate potential biomarkers for bovine *in vitro* fertilization, we identified genes differentially expressed in bovine CCs from oocytes with different competence statuses, through microarray analysis. The model of follicle size, in which competent cumulus–oocyte complexes (COCs) were recovered from bigger follicles (≥8.0 mm in diameter) and less competent ones from smaller follicles (1–3 mm), was used. We identified 4178 genes that were differentially expressed ($P < 0.05$) in the two categories of CCs. The list was further enriched, through the use of a 2.5-fold change in gene expression as a cutoff value, to include 143 up-regulated and 80 down-regulated genes in CCs of competent COCs compared to incompetent COCs. These genes were screened according to their cellular roles, most of which were related to cell cycle, DNA repair, energy metabolism, metabolism of amino acids, cell signaling, meiosis, ovulation and inflammation. Three candidate genes up-regulated (*FGF11, IGFBP4, SPRY1*) and three down-regulated (*ARHGAP22, COL18A1* and *GPC4*) in CCs from COCs of big follicles (≥8.1 mm) were selected for qPCR analysis. The selected genes showed the same expression patterns by qPCR and microarray analysis. These genes may be potential genetic markers that predict oocyte competence in *in vitro* fertilization routines.

Keywords cattle, gene expression, microarray, oocyte quality

Introduction

Despite great improvements in assisted reproductive technology (ART), the success of *in vitro* embryo production remains relatively low. Most of the oocytes used to produce *in vitro* embryos are recovered from small follicles (<6 mm in diameter), forming a heterogeneous population that must be matured *in vitro*. Since the original *in vitro* maturation (IVM) experiments (Heilbrunn *et al.* 1939), the process by which the most competent oocytes are selected to produce blastocysts remains similar and is still based on morphological aspects of the oocyte cytoplasm and the number of layers and compaction of cumulus cells (CCs) attached to the oocyte surface (Armstrong 2001; Krisher 2003; Lonergan *et al.* 2003; Coticchio *et al.* 2004). However, there is increasing evidence that these morphological criteria are insufficient to precisely distinguish oocytes of high and low competence (Krisher 2003; Lonergan *et al.* 2003; Coticchio *et al.* 2004). Therefore, lack of full competence is a major factor responsible for the lower blastocyst rate when *in vitro* matured oocytes are used for ARTS as compared to the *in vivo* matured oocytes (Dieleman *et al.* 2001; Humblot *et al.* 2005; Assidi *et al.* 2008). Therefore, to improve IVM results and ART outcomes, a means to select the most competent oocytes is of great importance. Despite the accumulation of a great deal of knowledge obtained through IVM research, it is necessary to find more precise parameters to select the best quality oocytes to produce an elevated number of high quality blastocysts.

The competence of the oocyte to produce high-quality blastocysts is acquired gradually during folliculogenesis by cellular and molecular events that provide the oocyte with the capacity to complete meiotic divisions, to be fertilized and to develop into a viable embryo (Fair 2003; Coticchio *et al.* 2004). The information about the molecular events needed for the acquisition of competence that takes place

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during the final steps of folliculogenesis just before ovulation is dispersed and incomplete (Assidi *et al.* 2008; Hamel *et al.* 2008; Bessa *et al.* 2013; Bunel *et al.* 2014; Yerushalmi *et al.* 2014). Most of the data describing the crucial steps of follicular development come from gene expression studies using oocytes with different degrees of competence. Although some differences in gene expression levels are already associated with oocyte developmental competence in cattle (Donnison & Pfeffer 2004; Fair *et al.* 2004; Dode *et al.* 2006; Misirlioglu *et al.* 2006; Mourot *et al.* 2006; Patel *et al.* 2006; Racedo *et al.* 2007; Caixeta *et al.* 2009; Mamo *et al.* 2011; Labrecque *et al.* 2013), these changes cannot be routinely used in ARTs as molecular markers for developmental competence due to invasive methods.

During oogenesis, the somatic cells that surround the oocyte proliferate and differentiate into CCs, which are metabolically coupled to the oocyte, forming the cumulus–oocyte complexes (COCs) (van den Hurk & Zhao 2005). The CCs remain in strong contact with the oocyte, maintained by cytoplasmic bridges called GAP-junctions as well as through justacrine and paracrine signaling networks (Albertini *et al.* 2001; Vozzi *et al.* 2001; Tanghe *et al.* 2002; Webb *et al.* 2002; Gilchrist *et al.* 2004). This intense bidirectional communication between the CCs and the oocyte is maintained during all phases of folliculogenesis and is essential for the acquisition of oocyte competence that is required for blastocyst development (Fair 2003; Gilchrist *et al.* 2004; Assidi *et al.* 2008; Regassa *et al.* 2011). Consequently, CCs may reflect oocyte quality and can be used for oocyte selection. Indeed, the physiological intimacy between the oocyte and CCs makes the latter a valuable source of molecular information, beyond the fact that CCs can be easily sampled before the IVM procedure without compromising oocyte viability (Assou *et al.* 2010; Huang & Wells 2010; Gebhardt *et al.* 2011; Ekart *et al.* 2013; Iager *et al.* 2013; Bunel *et al.* 2015). Therefore, the gene expression profile of CCs may be a valuable source of genetic markers for oocyte competence and have a potential impact on the improvement of several ARTs (Gilchrist & Thompson 2006; Gebhardt *et al.* 2011; Ekart *et al.* 2013; Iager *et al.* 2013; Bunel *et al.* 2015).

An informative model used by many research groups to assess the level of oocyte competence has been follicle size (Donnison & Pfeffer 2004; Lequarre *et al.* 2005; Mourot *et al.* 2006; Caixeta *et al.* 2009; Fagundes *et al.* 2011; Bessa *et al.* 2013; Franco *et al.* 2014). In a previous study, we showed that oocytes obtained from follicles 1–3 mm in diameter are significantly less competent in producing blastocysts than are oocytes obtained from follicles ≥8 mm in diameter (Caixeta *et al.* 2009). In the present study, we used the same model to compare the gene expression profile of more than 23 000 bovine transcripts between the CCs obtained from size-incompetent COCs (1–3 mm) and size-competent COCs (≥8 mm). We found substantial differences in the expression of several gene clusters representing distinct metabolic pathways such as energy metabolism, cell signaling, cell cycle, DNA repair, meiosis and inflammation. Among these gene clusters, we selected six genes from the same biochemical pathway (*ARHGAP22, COL18A1, GPC4, FGF11, IGFBP4, SPRY1*) based on their physiological roles as candidate biomarkers for bovine oocyte competence. We compared the relative expression of these genes in CCs obtained from size-incompetent COCs and size-competent COCs by qPCR. This approach offered several candidate genes for oocyte quality ranking that could be employed for direct selection prior to IVM procedures. A more accurate selection of competent oocytes will certainly improve *in vitro* embryo production in the near future for both human or livestock *in vitro*-produced embryos (Fagundes *et al.* 2011; Gebhardt *et al.* 2011; Ekart *et al.* 2013; Iager *et al.* 2013; Bunel *et al.* 2014, 2015; Franco *et al.* 2013). We aimed to find a marker that could allow us to predict, once COCs are removed from the follicles, which one is capable of becoming a blastocyst. Also, these potential biomarkers can be useful as a tool for the improvement of *in vitro* fertilization (IVF) and embryo culture media in order to increase the production and quality of the blastocysts.

Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich.

Animals

In our study, only ovaries collected at a slaughterhouse were used; therefore, this research was not submitted for ethics committee approval. In Brazil, slaughter of bovines is regulated by the Ministry of Agriculture by law number 7889 from 1989, which regulates the sanitary inspection and industrial production of animal products. The regulation regarding the collection and commercialization of semen is under law number 6446 from 1977, which regulates the inspection and monitoring of industrial semen production from domestic animals.

Cumulus–oocyte complex recovery and storage

Ovaries from crossbred cows (*Bos taurus indicus* × *Bos taurus taurus*) were collected immediately after slaughter and transported to the laboratory in a saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/ml) and streptomycin sulfate (100 mg/ml) at 35–37 °C. The follicles were dissected from the ovarian cortex using scissors, scalpels and tweezers in TCM-199 medium supplemented with Hank's salts and 10% fetal calf serum (GIBCO BRL) at 36 °C. Follicles were measured using a graduated eyepiece (OSM-4; Olympus) and then classified morphologically into two groups according to their diameter: 1.0–3.0 mm or ≥8.0 mm. The criteria used for follicle selection included: (i)

the presence of extensive and fine vascularization; (ii) a shiny and translucent appearance; (iii) after follicular rupture, the presence of granulose cells with a regular and healthy appearance; and (iv) no free-floating particles in the follicular fluid. Only COCs with a homogeneous granulated cytoplasm and at least three layers of compact of CCs were used in the present study. The selected COCs were transferred to a 50-μl droplet of phosphate-buffered saline, and the CCs were mechanically removed by repeated pipetting. After cumulus collection, they were transferred to a 0.2–ml tube and centrifuged twice for 2 min at 700 ***g***. The supernatant was removed, and 2 μl of RNA*later* (Applied Biosystems) was added to the pellet, which was then stored at −20 °C until RNA extraction. For each follicle size group, three replicas of pooled CCs corresponding to 30 oocytes were stored for RNA extraction and subsequent microarray assays, and four independent replicates corresponding to 20 oocytes were stored for RNA extraction for subsequent qPCR assays.

RNA extraction and cDNA synthesis

Total RNA was extracted from pools of CCs using the RNeasy Plus Micro Kit (QIAGEN), according to the manufacturer's instructions. The extracted RNA was subjected to reverse transcription reaction with SuperScript III (200 U/μl; Invitrogen) and Oligo-dT primer (Invitrogen) according to the manufacturer's instructions. After extraction, the RNA samples were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies), and samples with a ratio integrity >9.0 were used for microarray experiments.

Microarray and data analysis

The cDNA synthesis, amplification, biotin labeling, chip hybridizations, chip scanning and data collection were performed by the AFIP-Molecular Core facility (www.afip.com.br/principal.asp) using the Affymetrix microarray platform and the GeneChip Bovine Genome Array (Affymetrix, Inc.), which contains 24 027 bovine probes corresponding to 19 000 unigene clusters. One hundred nanograms of total RNA was amplified with a GeneChip 3′ IVT Express aRNA amplification kit (Affymetrix, Inc.), according to the manufacturer's instructions, to produce enough RNA (1–2 μg) for the hybridizations. Six chips were hybridized with three biological replicates for each treatment (CCs from small and large follicles). The microarray quality controls were performed using distinct platforms including the application of R/AFFY and R/AFFYQCREPORT. The correction algorithm robust multichip average (RMA) was used for normalization, and relative log expression (RLE) and normalized unscaled standard error (NUSE) were used to calculate the relevant statistics (Brettschneider *et al.* 2008). The functional classification of the genes and metabolic pathway analysis were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) software and the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/kegg1.html) database. Probes showing significant fold change in gene expression ($P < 0.05$) were listed, and the metabolic pathway analysis was carried out with the probes indicating a fold change of ≥2.5 (up or down; GEO accession no. GSE65269).

Real-time RT-PCR (qPCR)

Six genes were selected from the microarray experiment according to their biochemical pathway (cell cycle) and their possible role in oocyte competence; these genes included *fibroblast growth factor 11* (*FGF11*), *insulin like growth factor binding protein 4* (*IGFBP4*), *sprouty RTK signaling antagonist 1* (*SPRY1*), *Rho GTPase activating protein 22* (*ARHGAP22*), *collagen type XVIII alpha 1 chain* (*COL18A1*) and *glypican 4* (*GPC4*). Real-time RT-PCR analysis was performed using Fast SYBR Green Master Mix (Applied Biosystems). Reactions were performed in a volume of 25 μl. The PCR cycling conditions were as follows: 95 °C for 20 s; 50 cycles of denaturing at 95 °C for 3 s and annealing at 60 °C for 30 s. Primer sequences, fragment size, annealing temperature and primer concentrations for each gene are listed in Table S1. Reactions were performed in quadruplicate for each gene with an amplification efficiency of >90% for each primer pair. The expression of the endogenous control genes *peptidylprolyl isomerase A* (*PPIA*) (former name: *cyclophilin A*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were quantified, but only *GAPDH* expression was used to normalize target genes due to its greater expression stability than *PPIA*. The relative abundance of each gene was calculated using the ΔΔCt method with efficiency correction (Pfaffl 2001).

Statistical analysis

The microarray data were analyzed using the following R/ANOVA model (Wolfinger *et al.* 2000):

$$Y_{ijkl} = m + T_i + A_{j(i)} + G_k + TG_{ik} + e_{ijk},$$

where Y is the normalized intensity value; m is the general median; A, T and G represent the array principal effect, treatment and gene respectively; TG is the effect of gene and treatment interaction; e is the random error; i is the gene spot intensity and k is the array. The F_S test was applied combining the global data and the specific data for each gene using a shrinkage factor (Cui & Churchill 2002). The false discovery rate was used to adjust the false–positive rate and was fixed at 5%.

The gene expression data collected by qPCR were analyzed using the Mann–Whitney test. All statistical analyses were performed using PROPHET software version

5.0 (BBN Systems and Technologies). Results are presented as the mean ± SEM, with $P < 0.05$ indicating significance.

Results

Microarrays analysis

The microarray hybridizations were within the desirable standard, with a probe hybridization rate over 56% and a low level of background (34–55 points). The RMA Express parameter presented quality limits between 95% and 99% for median and interquartile range and RLE and NUSE values (Fig. 1a). Each of the six arrays were within the quality parameters required for statistical analysis. The array analysis showed 4178 differentially expressed genes ($P < 0.05$), of which 2298 were down-regulated and 1880 were up-regulated in the CCs from large follicles (≥8.0 mm in diameter) compared to CCs from small follicles (1–3 mm in diameter). The fold change in the expression of each gene was plotted on a volcano chart to present the dispersion of gene expression and significance (Fig. 1b).

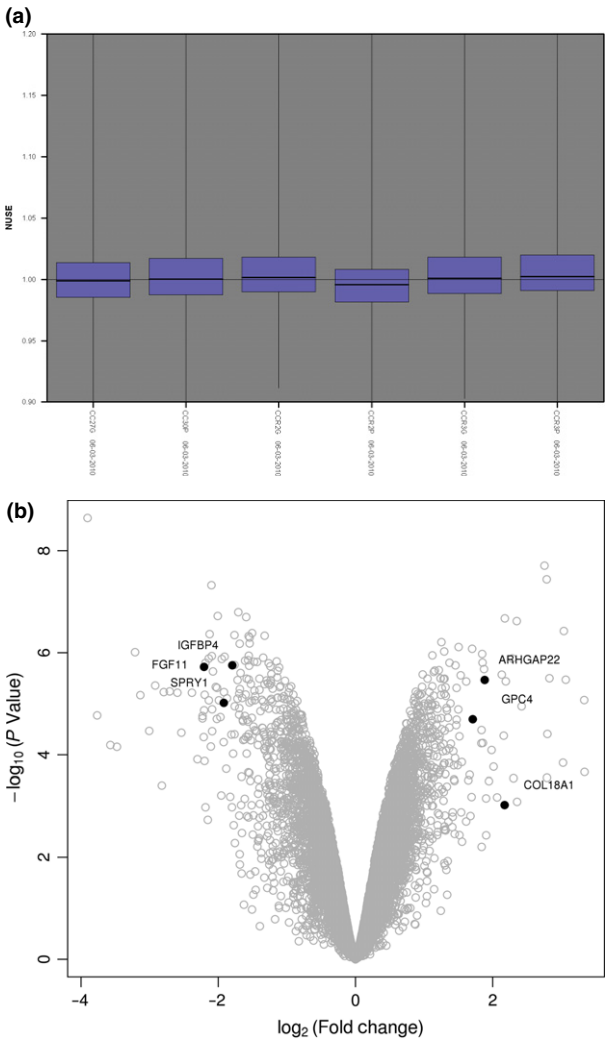

Figure 1 (a) NUSE: normalized unscaled standard errors of the six array chips. (b) Significance dispersion plot of mRNA transcripts from cumulus cells, –log10 (*P*-value), and the fold changes (log2) in gene expression between the small (1–3 mm) and large (≥8.1 mm) follicle groups. The genes used in qPCR analysis are displayed in black.

Annotation of gene function

All differentially expressed genes were classified according to their function using the KEGG database as part of the DAVID software package, and changes in gene expression were presented relative to the CCs from bigger follicles. They grouped into representative pathway categories such as cell cycle, amino acid metabolism, cell signaling, DNA repair, energy metabolism, meiosis and ovulation/inflammation (Table S2). As shown, most of the metabolic pathway categories (cell cycle, cell signaling and energy metabolism) were present in both treatments. Three categories (DNA repair, meiosis and amino acid metabolism) were represented only in the down-regulated expression group, whereas the ovulation/inflammation representative genes were present only in the list of up-regulated genes (Fig. 2). Moreover, 20% and 27% of the genes in the up-regulated and down-regulated groups respectively were annotated as having an unknown function according to DAVID software. A list of differentially expressed genes, including 143 up-regulated and 80 down-regulated genes with a fold change

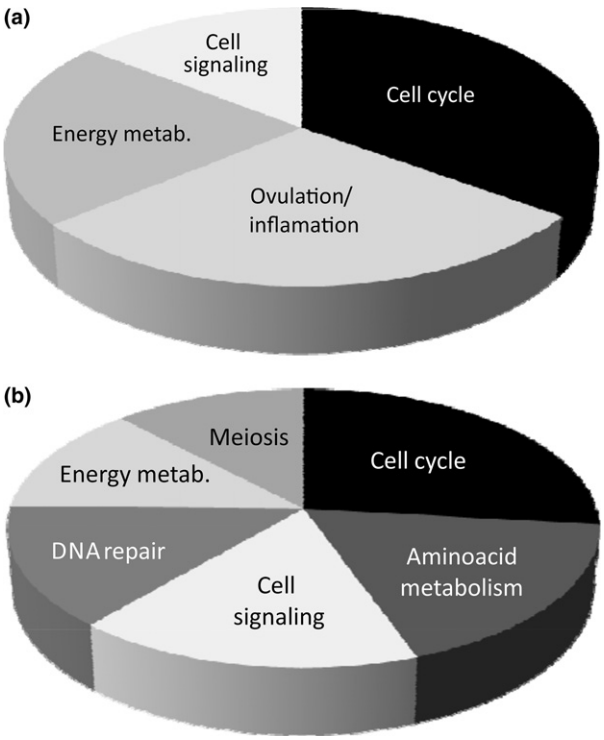

Figure 2 Functional classification of (a) up-regulated and (b) down-regulated genes, according to the KEGG database. The big follicle group (≥8.1 mm in diameter) was used as a reference.

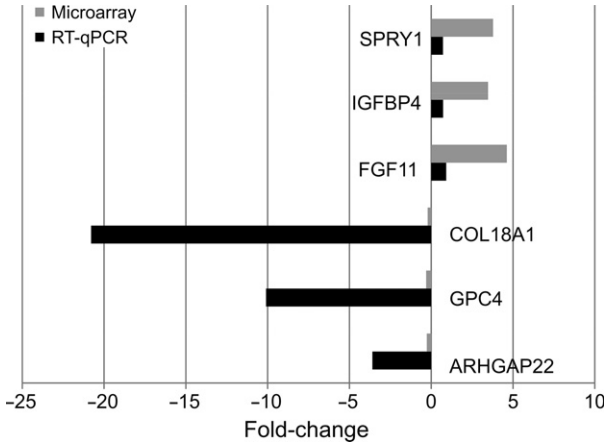

Figure 3 Fold-change values of differential gene expression in cumulus cells (*Bos taurus* × *Bos indicus*) derived from follicles of 1–3 mm ($P < 0.05$) as detected by the microarray (grey bars) or qPCR (black bars). *SPRY1*, *sprouty RTK signaling antagonist 1*; *IGFBP4*, *insulin-like growth factor binding protein 4*; *FGF11*, *fibroblast growth factor 11*; *ARHGAP22*, *Rho GTPase activating protein 22*; *COL18A1*, *collagen type XVIII alpha 1* chain; *GPC4*, *glypican 4*.

interval of ≥2.5 is shown (Tables S3 & S4 respectively). Notably, an elevated number ($n = 56$) of non-annotated genes/probes was present in the two groups. A smaller number of down-regulated genes (with a fold change of ≥2.5) was observed and also showed a lower maximum fold change (10×) compared to the group of up-regulated genes (maximum 15×).

Candidate genes selection and qPCR

Using the list of genes with an expression change of ≥2.5 fold, we selected three up-regulated genes (*FGF11*, *IGFBP4* and *SPRY1*) and three down-regulated genes (*ARHGAP22*, *COL18A1* and *GPC4*) as candidates for biomarkers of COC quality. These six genes were subjectively selected using the physiological function of each gene as the main criterion. The expression change for each of the selected genes was validated by qPCR and matched the pattern seen by microarray analysis (Fig. 3). qPCR was chosen for expression analysis of the selected genes, as it is the most adequate technique to measure the expression of candidate genes during the IVP biopsy routine. *GAPDH* and *PPIA* were used as housekeeping gene controls due to their documented performance as stable controls for cattle oocyte and embryos (Goossens *et al.* 2005; Bettegowda *et al.* 2006; Hatzirodos *et al.* 2014; Luchsinger *et al.* 2014).

Discussion

In the present study, we analyzed the relative abundance of genes potentially involved in oocyte competence using a follicle size model (Caixeta *et al.* 2009). This model is based on the well-established relationship between follicle size and oocyte developmental competence (Lequarre *et al.* 2005; Mourot *et al.* 2006; Caixeta *et al.* 2009). Although attempts to identify potential biomarkers for oocyte quality using the follicle size model have been reported in the literature, they differ from the present study in various aspects. For example, a gene expression profile of the oocyte was not performed in the present study; instead, potential marker genes were screened using CCs as the source, because CCs can be sampled using a noninvasive method. Moreover, a specific bovine microarray was used for the first time as a high-throughput method to analyze the gene expression profile of the crossbred *B. taurus indicus* × *B. taurus taurus* as COC donors, using the follicular size model. The choice of COCs obtained from different-size follicle groups was based on the lower competence of COCs of up to 3 mm in diameter to produce blastocysts compared to COCs from bigger follicles (≥8.0 mm) (Caixeta *et al.* 2009).

The inference of oocyte quality obtained by analyzing the gene expression and physiological state of CCs is based on the metabolic interaction between the oocyte and the CCs through bidirectional communication by GAP-junctions and plasmodesmas projections (Allworth & Albertini 1993; Regassa *et al.* 2011) as well as indirectly by an intense paracrine signaling network (Paulini & Melo 2011). This tight metabolic interdependence between the oocyte and the CCs can be used for an indirect and non-invasive evaluation of oocyte competence prior to IVP (Cillo *et al.* 2007; Feuerstein *et al.* 2007; Assidi *et al.* 2008; Bettegowda *et al.* 2008; Gebhardt *et al.* 2011; Ekart *et al.* 2013; Iager *et al.* 2013; Bunel *et al.* 2014, 2015). In this study, 19 000 bovine unigene clusters were investigated, revealing interesting results and interpretations. Microarray analysis unveiled 4178 differentially expressed genes (Table S5), most of which (54%) were down-regulated in CCs from big follicles (≥8.0 mm). This result is expected, as bigger follicles are prone to ovulation; thus, the gene expression activity should be lower (van Montfoort *et al.* 2008; Assidi *et al.* 2010). According to the KEGG database annotation, the down-regulated genes involve more metabolic pathways (six) than do those that were up-regulated (four) (Fig. 2). Some of the genes are common between the two groups (cell cycle, cell signaling and energy metabolism), and others are exclusive to the down-regulated (meiosis, DNA repair and amino acid metabolism) or up-regulated (ovulation/inflamation) categories. This is consistent with the morphological and physiological events that occur within follicles during growth and maturation, leading to ovulation and demanding intense network signaling to orchestrate cell division and differentiation (Assidi *et al.* 2010). The vigorous division of follicle cells in small follicles may explain the 14% increase in the expression of genes involved with DNA repair activity.

The ovulation/inflammation pathway represents 29% of the genes up-regulated exclusively in CCs from the bigger follicle group. During the final steps of follicular growth, while the COCs are preparing to ovulate, the ovarian

epithelium ruptures, resembling an inflammatory process (Hernandez-Gonzalez *et al.* 2006; Russell & Robker 2006; Richards 2007). Moreover, the immune cells that participate during the inflammatory response are capable of interferon secretion, which has an important role in the physiology of female organs (Brännström *et al.* 1995; Wolf *et al.* 2003; Gérard *et al.* 2004; Bauersachs *et al.* 2008; Asirvatham *et al.* 2009). Several genes related to interferon production were found to be up-regulated in CCs from big follicles in our experiments. Additionally, the data suggest that α-interferon is involved in the differentiation of pre-ovulatory follicles in rats (Lee *et al.* 2009), corroborating our findings.

During maturation, COCs are prepared for radical changes in the source of energy that occurs after ovulation, during fertilization and during initial embryo development (Brison 1999; Thompson *et al.* 2006; Su *et al.* 2007). Therefore, the high expression of genes grouped in the energy metabolism category in both experimental groups is comprehensive. However, amino acids are a relevant source of energy for oocytes during maturation and after ovulation and must be stored during late COC growth phase (Rose-Hellekant *et al.* 1998; Hernandez-Gonzalez *et al.* 2006). Therefore, the intense activity (18%) of genes involved in amino acid metabolism found in CCs from small COCs is also expected. Oocyte maturation is characterized by the completion of the first meiotic division that was held at metaphase II during initial follicle formation (Fair *et al.* 2007). After follicular deviation, the oocyte must be prepared to resume meiotic division, and the CCs must signal to the oocyte to initiate the accumulation of factors involved in the induction of meiosis. In the present study, these factors represent 12% of the genes up-regulated in CCs from growing small follicles.

In agreement with the literature, our data also have identified several genes differentially expressed in CCs derived from follicles of distinct developmental competence, including *FSHR*, *EGFR* and *GHR* (Caixeta *et al.* 2009); *CTSB* (Bettegowda *et al.* 2008); *TNFAIP6*, *TRIB2* and *ERRFI1* (Assidi *et al.* 2010); and *DPP8* (Assidi *et al.* 2011). More recently several articles were published describing the use of CC's gene expression profile as a physiological marker of oocyte competence and quality in cattle (Tesfaye *et al.* 2009; Regassa *et al.* 2011; Bunel *et al.* 2014, 2015) and human (Gebhardt *et al.* 2011; Ekart *et al.* 2013; Iager *et al.* 2013; Xu *et al.* 2015). Despite their differences in the origin of CCs (cattle or human), experimental design and the microarray platform used, some genes are repeatedly present in the lists of differentially expressed in CC from high-competent COCs in several microarray experiments: *HAS2* (Tesfaye *et al.* 2009; Gebhardt *et al.* 2011; Regassa *et al.* 2011; Ekart *et al.* 2013), *FSHR* (Regassa *et al.* 2011; Ekart *et al.* 2013) and *TNFAIP6* (Tesfaye *et al.* 2009; Gebhardt *et al.* 2011). All of them are in our list of differentially expressed genes and can be good candidates for biomarkers of oocyte quality and competence. Several genes showed a similar pattern of expression in our microarrays (*B. indicus* × *B. taurus* crossbred) and in many other microarray experiments (*B. Taurus*) focused on the screening of biomarkers for oocyte competence and quality (Table S6); this corroborates that these genes are really in high correlation with oocyte developmental competence.

Among the differentially expressed genes that were identified through microarray analysis, we selected three genes that are up-regulated in the larger follicle's COCs (*SRY1, IGFBP4, FGF11*) and three genes that are up-regulated in the small follicle's COCs (*COL18A1, GPC4, ARHGAP22*) for qPCR analysis, based on the physiological role and the *P*-value of each candidate gene. All of the selected genes group in the cell cycle category, in which each gene has a distinct and specific role (Table S2), in order to focus on the same metabolic pathway. *ARHGAP22* (*Rho GTPase activating protein 22*) belongs to the Rho-GTPase family, which regulates several aspects of cellular physiology and morphology, such as cell cycle progression, cytoskeleton organization, cell polarity, cell migration and invasion (Ridley 2001; Etienne-Manneville & Hall 2002). *ARHGAP22* stimulates the organization of endothelial cells in capillary tubes during angiogenesis (Katoh & Katoh 2004). Data regarding the expression of *ARHGAP22* in CCs are lacking, but the down-regulation of *ARHGAP22* in CCs of larger follicles may be explained by the decreased requirement for angiogenesis due to the end of intensive follicular growth in preparation for ovulation. The *COL18A1* gene encodes type XVIIIα1 collagen, which is present in the extracellular matrix and is capable of binding and modulating several molecules involved in cell division, polarity and fate, including the Wnt, TGFβ, and FGF families of growth factors (Lin *et al.* 2001; Aricescu *et al.* 2002; Quélard *et al.* 2007). In addition, type XVIII collagen is a component of the basal lamina of the follicle (Irving-Rodgers & Rodgers 2005), and the degradation of collagen fibers is required for follicle rupture during ovulation (Abisogun *et al.* 1988). Therefore, the down-regulation of the *COL18A1* gene is important for the impaired follicle wall resistance that is necessary for its rupture during ovulation. The *GPC4* gene is a member of the glypican family, formed by heparin sulfate proteoglycans that are covalently attached to the cell membrane via a glycosylphosphatidylinositol anchor (De Cat & David 2001; Fico *et al.* 2011). The glypicans play a role in the regulation of FGF, BMP, and GDF signaling as a low-affinity binding receptor that can modulate the interaction between the signaling molecule and its primary receptor (Hagihara *et al.* 2000; Nybakken & Perrimon 2002; Filmus *et al.* 2007; Watson *et al.* 2012). The glypican signaling modulation also mediates the expression of the cumulus-specific matrix genes *HAS2* and *TNFAIP6* (Watson *et al.* 2012). Interestingly, in our analysis *GPC4* (a glypican family member) was down-regulated in CC from big follicles, and the expression of *HAS2* and

TNFAIP6 was increased as expected according the literature data (Watson *et al.* 2012). In humans, the differential expression of *GPC4* in CCs is correlated with the first embryonic cleavage and, consequently, with high embryonic quality (van Montfoort *et al.* 2008). Therefore, we also found *GPC4* to be a good embryo quality marker candidate because its regulation is related to oocyte competence and it mediates the cumulus-specific matrix genes *HAS2* and *TNFAIP6*, all of them differentially expressed in our arrays.

In larger follicles, the expression of up-regulated genes included *IGFBP4*, which encodes one of the main IGF binding proteins in the follicular antrum (Buratini *et al.* 2005). This protein that binds to and sequesters the IGF molecules and modulates follicular deviation and dominance (Fortune *et al.* 2004), probably through its regulatory effect in steroidogenesis (Kwintkiewicz & Giudice 2008). High expression of *IGFBP4* was found in CCs of women with polycystic ovary syndrome (Kwon *et al.* 2010) and can inhibit the steroidogenesis in cumulus, granulosa and teca cells (Cataldo & Giudice 1992; Mason *et al.* 1997). Because IGFs have various roles during all stages of follicle growth and maturation (Fortune *et al.* 2001), it is unclear how the up-regulation of *IGFBP4* in larger follicles (≥8.0 mm) can contribute to the higher competence of oocytes from this group. *FGF11* is also a negative regulator of steroid synthesis and granulose cell survival and is expressed in mouse oocytes and embryos (Zhong *et al.* 2006), but its expression in CCs has not been documented. Despite the extensive literature regarding the FGF family, there is no information about the role of *FGF11* in follicle growth and development. Further investigation is required to determine the role of *FGF11* during follicular development and oocyte competence and its potential use as a genetic marker. The *sprouty RTK signaling antagonist 1* (*SPRY1*) gene belongs to a recently discovered gene family with four members (*SPRY1–4*); it is induced by the FGF family and down-regulates inducers through a negative feedback loop (Faedo *et al.* 2010). The SPRY family members are strictly expressed in mammals in a specific phase of initial embryo development, and their expression pattern resembles that of the FGF family (Felfly & Klein 2012). Down-regulation of *SPRY1* is associated with angiogenesis in endothelial cells and increased cell proliferation, possibly due to the modulation of *p21* and *cyclin D1* expression (Sabatel *et al.* 2009). *FGF2* also induces higher levels of *SPRY2* mRNA in mouse CCs (Sugiura *et al.* 2009) and human luteinic-granulosa cells (Haimov-Kochman *et al.* 2005). The level of *SPRY2* mRNA in bovine granulosa cells is also negatively correlated with oocyte developmental competence in experiments using differential display and suppressive subtractive hybridizations (Robert *et al.* 2001).

In conclusion, we analyzed the expression profile of differentially expressed genes between two distinct populations of bovine CCs associated with oocytes of high and low competence in order to produce healthy IVP embryos. Through these experiments, a list of 4178 significantly expressed genes was generated for use as biomarkers of oocyte competence and IVP embryo quality. The expression of three down-regulated (*COL18A1, GPC4, ARHGAP22*) and three up-regulated (*SPRY1, IGFBP4, FGF11*) candidates was validated by qPCR for use as a marker of oocyte quality and competence for ART. The use of genetic markers from a non-invasive CC biopsy to select the best oocytes has the potential to improve IVF technology for both humans and livestock (Krisher 2003). Also these potential biomarkers can be useful as tools for the improvement of IVF and embryo culture media to increase blastocyst production and quality.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Table S1 Primers used for gene amplification in the qPCR experiment.

Table S2 Gene clustering according to the Kyoto Encyclopedia of Genes and Genomes KEGG) database, using the bioinformatics tool Database for Annotation, Visualization and Integrated Discovery (DAVID).
Table S3 Up-regulated genes with a ≥2.5 fold change, using the big follicle group as the reference.
Table S4 Down-regulated genes with a ≥2.5 fold change, using the big follicle group as the reference.
Table S5 Complete list of differentially expressed genes ($P < 0.05$) of microarray analysis.
Table S6 List of genes differentially expressed in CCs associated with oocyte competence and consistent with the literature.