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Messenger RNAs in metaphase II oocytes correlate with successful embryo development to the blastocyst stage

Fernando Henrique Biase[3,4,6], *Robin Edward Everts*[4,6], *Rosane Oliveira*[4], *Weruska Karyna Freitas Santos-Biase*[2], *Giovana Krempel Fonseca Merighe*[2], *Lawrence Charles Smith*[5], *Lúcia Martelli*[3], *Harris Lewin*[4] *and Flávio Vieira Meirelles*[1]

Faculdade de Zootecnia e Engenharia de Alimentos–Universidade de São Paulo, Pirassununga, São Paulo, Brasil; Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brasil; University of Illinois at Urbana-Champaign, USA; and Faculté de Médicine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Quebec, Canada

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Summary

The mRNAs accumulated in oocytes provide support for embryo development until embryo genomic activation. We hypothesized that the maternal mRNA stock present in bovine oocytes is associated with embryo development until the blastocyst stage. To test our hypothesis, we analyzed the transcriptome of the oocyte and correlated the results with the embryo development. Our goal was to identify genes expressed in the oocyte that correlate with its ability to develop to the blastocyst stage. A fraction of oocyte cytoplasm was biopsied using micro-aspiration and stored for further expression analysis. Oocytes were activated chemically, cultured individually and classified according to their capacity to develop *in vitro* to the blastocyst stage. Microarray analysis was performed on mRNA extracted from the oocyte cytoplasm fractions and correlated with its ability to develop to the blastocyst stage (good quality oocyte) or arrest at the 8–16-cell stage (bad quality oocyte). The expression of 4320 annotated genes was detected in the fractions of cytoplasm that had been collected from oocytes matured *in vitro.* Gene ontology classification revealed that enriched gene expression of genes was associated with certain biological processes: 'RNA processing', 'translation' and 'mRNA metabolic process'. Genes that are important to the molecular functions of 'RNA binding' and 'translation factor activity, RNA binding' were also enriched in oocytes. We identified 29 genes with differential expression between the two groups of oocytes compared (good versus bad quality). The content of mRNAs expressed in metaphase II oocytes influences the activation of the embryonic genome and enables further develop to the blastocyst stage.

Keywords: Blastocyst development, Developmental competence, Oocyte

Introduction

Gene expression in oocytes is regulated and variable throughout oogenesis (Pan *et al.*, 2005). At the final transition from germinal vesicle (GV) to a metaphase II (MII) oocyte, there is a general RNA decay of approximately 30–50% (Biase *et al.*, 2008; Lequarre *et al.*, 2004). Nonetheless, some genes are upregulated during this final oocyte maturation process (Fair *et al.*, 2007; Katz-Jaffe *et al.*, 2009). These maternal gene products are stored in the oocyte and are able to sustain the initial cell cycles of early embryo development. Independent experiments with α-amanitin, an RNA polymerase II inhibitor, have shown that embryos can be cultured *in vitro* until a developmental block occurs at the 2-cell stage in mouse, 4-cell stage in pig and rabbit, 4–8-cell stage in human, 8-cell stage in cow, and 8–16-cell stage in sheep and goat (reviewed by Memili

[1]All correspondence to: Flávio Meirelles. Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos–Universidade de São Paulo, Pirassununga, São Paulo, Brasil. Tel: +55 19 3565 4112; Fax: +55 19 3565 4117. e-mail: meirellf@usp.br
[2]Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos–Universidade de São Paulo, Pirassununga, São Paulo, Brasil.
[3]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto–Universidade de São Paulo, Ribeirão Preto, São Paulo, Brasil.
[4]Department of Animal Sciences, University of Illinois at Urbana-Champaign, USA.
[5]Centre de Recherche en Reproducion Animale, Faculté de Médicine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Quebec, Canada.
[6]Current address: FHB: Institute for Genomic Biology, UIUC, USA; REE: Sequenom Inc., 3595 Johns Hopkins Court, San Diego, CA 92121, USA.

& First, 2000). These observations indicate that the maternal stock of RNA and protein present in the MII oocyte is sufficient to drive early cleavages and that embryo genome activation is likely to be controlled by maternal gene products.

Early embryo development is influenced by the quality of the oocyte. After fertilization or chemical activation, a good quality oocyte will most likely form a blastocyst, whereas a bad quality oocyte is unlikely to complete normal early development. In cattle, experimental models have been employed to characterize developmental oocyte competence based on follicle size (Blondin & Sirard, 1995), age of the oocyte donor (Revel *et al.*, 1995), timing of the first cleavage (Lonergan *et al.*, 1999), and cumulus–oocyte complex morphology (Bilodeau-Goeseels & Panich, 2002). These models have been used to investigate the accumulated maternal RNAs that associate with successful embryo development (Lonergan *et al.*, 2000; Robert *et al.*, 2000; Calder *et al.*, 2001; Donnison & Pfeffer, 2004; Fair *et al.*, 2004a,b; Gutierrez-Adan *et al.*, 2004; Mourot *et al.*, 2006; Patel *et al.*, 2007; Caixeta *et al.*, 2009; Biase *et al.*, 2010; Romar *et al.*, 2011), and have identified approximately 123 genes that might be differentially expressed between developmentally competent and incompetent oocytes. Recently, we proposed a new experimental model that collected a fraction of the ooplasm for experimental analysis, allowing the remaining of the oocyte to be chemically activated and cultured *in vitro.* The developmental competence of the oocytes is then classified based on actual embryo development (Biase *et al.*, 2009). This experimental model has been used to demonstrate that the total amount of the polyadenylated RNA present in MII oocytes does not correlate with developmental competence (Biase *et al.*, 2009).

Results from previous studies suggest that an adequate balance of mRNA abundance for each gene in the oocyte at the moment of fertilization may determine the quality of embryonic development pre-hatching. Even though several studies have characterized RNA accumulation during oogenesis, little information is known of the role these mRNA molecules play in early stages of embryo development. We hypothesized that mRNA species present in *in vitro* matured oocytes are important factors in enabling embryo development from eight cells up to the blastocyst stage in cattle. To test our hypothesis we compared a representative part of the transcriptome of oocytes matured *in vitro* that developed into proper blastocysts with those oocytes that could not sustain embryo development after embryonic genome activation. Our aim was to identify genes in the MII oocyte that were associated with embryo development beyond the maternal–embryonic genome transition.

Material and methods

Oocyte collection, cytoplasm biopsy and embryo culture

The chemicals used for *in vitro* culture were purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada) unless otherwise stated. Oocyte collection and biopsy were conducted as described elsewhere (Biase *et al.*, 2009). Briefly, bovine cumulus–oocyte complexes (COCs) were aspirated from antral follicles of 3–8 mm in diameter. Those COCs that contained compact layers of cumulus cells and homogeneous oocyte cytoplasm were matured *in vitro* (TCM199 medium, Gibco, Burlington, Ontario, Canada) for 20 h in 5% CO_2 and a humidified atmosphere at 38°C. Cumulus cells were removed from oocytes by manual pipetting in hyaluronidase solution (2 mg/ml), and 120 oocytes containing the GV were selected for micromanipulation and cytoplasm collection. Oocytes were placed in cytochalasin B (5 μg/ml) for 10 min before micro-manipulation. A fraction of the cytoplasm (approximately 10–20%) was aspirated from the oocyte on the opposite site of the polar body. The cytoplasm fraction was placed in 2 μl of phosphate-buffered saline that contained polyvinylalcohol (0.1%) and RNase inhibitor (1 U/μl), transferred to 30 μl of Trizol Reagent (Invitrogen, Carlsbad, California, USA) and frozen at –80°C until RNA extraction. The corresponding oocytes were placed individually in a Terasaki plate (Nunc, Inter-Med, Denmark) containing 10 μl of synthetic oviduct fluid (SOF) medium (Tervit *et al.*, 1972). Then, 26 h after the beginning of *in vitro* maturation, oocytes were chemically activated individually using 5 μM of ionomycin in TCM199 medium containing bovine serum albumin (BSA, 0.03 mg/ml) for 5 min following incubation in SOF medium containing 6-dimethylaminopurine (6-DMAP, 2 mM) for 3 h at 38°C in 5% CO_2 and a humidified atmosphere. Embryos were individually cultured *in vitro* (Carolan *et al.*, 1996) on a Terasaki plate (Nunc, Inter-Med, Denmark) that contained 15 μl of SOF medium supplemented with 2% fetal calf serum (FCS) and BSA 0.005 μg/μl per well. The experiment was performed twice. For each replicate, a control group of 25 oocytes was matured *in vitro*, was not micro-manipulated and was chemically activated; the embryos were cultured *in vitro* in groups using the same conditions as the experimental oocytes and embryos but in 100 μl of the supplemented SOF medium under mineral oil.

Embryo development was observed in two stages; cleavage was evaluated at 90 h post activation (hpa), when 10 μl of the medium was replaced with 10 μl of fresh medium, and blastocyst formation was evaluated

Figure 1 Experimental design. Oocytes were matured *in vitro* and 10–20% of the cytoplast was collected through micromanipulation. Oocytes were then chemically activated in individual micro-wells in Terasaki plates. Presumptive zygotes were cultured individually *in vitro* and embryonic development was categorized into two groups: cleavage arrest after the 8-cell stage but before blastocoel formation (*A*) or blastocyst formation (*B*). The cytoplast was frozen and subjected to a microarray analysis to compare the gene expression patterns between oocytes from groups A and B.

190 hpa. The oocytes were classified according to embryo development: 8–16-cell stage embryos that had arrested development before the blastocyst stage (group A); and embryos that formed a blastocyst (group B). Only the oocyte samples that produced embryos in groups A and B were used for microarray experiment, as demonstrated in Fig. 1.

RNA extraction, amplification, labeling and microarray hybridization

Twenty-five cytoplasm fractions from oocytes from group A and 55 cytoplasm fractions from group B were used for the experiment. Each cytoplasm biopsy fraction was kept in a tube with Trizol® Reagent. Five of these tubes from the same oocyte group were pooled to make one biological replicate. Therefore there were respectively five and 11 biological replicates from group A and group B oocytes. Total RNA was extracted with Trizol® Reagent according to the manufacturer's instructions with the addition of 0.1 μg of linear acrylamide (Ambion®, Austin, Texas, USA) at the first step. Antisense oocyte RNA was amplified and labelled using a TargetAmp™ 2-Round Aminoallyl-aRNA Amplification kit 1.0 (Epicentre® Biotechnology, Madison, Wisconsin, USA). One nanogram of reference RNA was also amplified using the same procedure. Three micrograms of aRNA were subjected to integration of either Cy3-ester or Cy5-ester (Amersham, Piscataway, New Jersey USA). The aRNA

from each oocyte pool was combined with reference aRNA and co-hybridized on a 13,257-element bovine oligoarray at 42°C for 40 h (GEO:GPL2853), following successive stringency washes for non-hybridized aRNA removal, essentially as described previously (Everts *et al.*, 2008; Loor *et al.*, 2007). Microarray images were obtained with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA) and processed with GenePix 6.0 software (Molecular Devices, Sunnyvale, CA). Microarray data are available to the community via the NCBI GEO database (GSE29191), following MIAME standards.

Microarray data analysis

Microarray data were imported and analyzed using R programming language (Ihaka & Gentleman, 1996) and the Bioconductor (Gentleman *et al.*, 2004) package Limma (Smyth, 2005). For each microarray, foreground data (median) were subjected to background subtraction (Ritchie *et al.*, 2007), and filtering of the spots for which signal intensity was lower than 300 arbitrary units. The remaining spots were subjected to within-array normalization using print-tip LOESS quantile normalization between arrays (Smyth, 2003). Differential gene expression was assessed using moderated *t*-statistics (Smyth, 2004; Jeanmougin *et al.*, 2010), considering correlation between the duplicate spots (Smyth *et al.*, 2005). Probes with a false discovery rate (FDR; Benjamini & Hochberg, 1995) corrected $P_{(H0)}$-value less than 0.05 and B greater than 0.5 were assumed to be differentially expressed between the two groups tested.

Functional annotation of the genes for the corresponding probes was obtained from Gene Ontology (Ashburner *et al.*, 2000) and Gene Enrichment Analyses were performed using Babelomics v4.1 web tools (Medina *et al.*, 2010).

Validation of microarray result with RT-qPCR

The following genes were selected to validate the microarray result: *IGF2R*, *DDR1*, *DUSP6*, *HERC2*, *NDUFB6*, *NDUFS4*, *PFKFB3*, *SFRS14*, *UQCRH.* Primers were designed for these genes based on reference sequences from the GenBank database (Table 1). Reverse transcription (RT) was performed using 100 ng of amplified RNA and SuperScript® III Reverse Transcriptase (200 U, Invitrogen, Carlsbad, CA), using random hexamers (500 ng), dNTP mix (0.5 mM), RNAseOUT (40 U, Invitrogen, Carlsbad, CA), following manufacturer's instructions for incubation temperature and time, in a final volume of 20 μl. Real-time polymerase chain reactions (PCRs) were set up with one-quarter of the RT reaction volume, using SYBR® GREEN PCR Master Mix (Warrington, UK), following the manufacturer's instructions. PCRs were run on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), following preset cycling parameters, with an annealing temperature of 60°C.

Relative expression values were calculated using the comparative cycle threshold (C_t) approach (Schmittgen & Livak, 2008). Only samples with C_t values bellow 35 were subjected to analysis. The expression values from the samples were quantified relative to *ACTB* and *GAPDH* control genes using the methods presented elsewhere (Hellemans *et al.*, 2007). The significance of the difference of the relative abundance values from two oocyte groups was assessed using a Wilcoxon test (Yuan *et al.*, 2006), performed in R (Ihaka & Gentleman, 1996). Fold changes were calculated for the group of good quality oocytes relative to the bad quality oocytes (calibrator group). Differences with a $P_{(H0)}$-value less than 0.05 were considered to be significant and the gene was inferred to be differentially expressed, unless otherwise stated.

Table 1 Primers used for qPCR

Gene	Sequence (5′→3′)	GenBank accession number
ACTB	CCTGGCACCCAGCACAA AGCGAGGCCAGGATGGA	AY141970
IGF2R	TCTTCTCCTGGCACACCTCT CTCTGAGAGCCCTTTGTGCT	NM_174352
DDR1	CCACCAGCTAGAGGACAAGG CCGATGCACAAAGTTGAGTG	NM_001076012. 2
DUSP6	GAATACGGGCGGCGAGTCGG TGCAGGGCGAACTCGGCTTG	NM_001046195. 1
HERC2	ACCCCGTGTATGCCGCCTCT GACCCCCAGGTTGGCAAGGC	XM_615528. 5
NDUFB6	GCGCCGTCTCTTGCTGCCTT CACAGGCGACACCCTCTGCG	NM_175787. 2
NDUFS4	GCAAGATGGCGGCGGTCTCA GCCAGCCTCCATGTGGAAGTGC	NM_175800. 2
PFKFB3	GAGGAGCATGTGGCCTCTACA AACAGGCTTTCCCTAGCAA	NM_001077837. 1
SFRS14	GTCGCCCCGTGTCCAA AGCTTCTGCTGGGCAAAATC	XM_879722. 4
UQCRH	CGCCTCCGGACATGGGGCTA ACAGAGCTCGAGCCGCTCCC	NM_001034745. 1

Table 2 Oocyte classification and distribution according to the embryo *in vitro* culture outcome[a]

Criteria	Experimental group	Micro-manipulated *N* (%)[b]	Control *N* (%)[b]
Not cleaved		16 (7)	2 (4)
2-cell and arrested		17 (7)	4 (6)
8–16-cell and arrested	Group A	47 (20)	12 (24)
Blastocyst	Group B	71 (30)	17 (34)
Degenerated[c]		89 (37)	15 (30)
Total		240 (100)	50 (100)

[a]Data from two replicates.
[b]Percentages were calculated according to the total of oocytes activated and placed for *in vitro* embryo culture.
[c]Degenerated: oocytes were not classified due to degeneration of the zygote or blastomeres.

Results

The number of oocytes that developed to blastocyst in experimental oocytes and their control non-manipulated counterparts was similar (29.6% vs. 34.0%, $P > 0.05$; Table 2), indicating that the developmental competence of the oocytes was not significantly reduced due to micro-manipulation and ooplasm removal.

Using multiple samples from *in vitro* matured bovine oocytes, we evaluated the relative expression of 4414 probes out of the 13,257 oligonucleotides present on the microarray slide. These probes are equivalent to 4320 annotated genes, from which a subset of 2301 genes have an annotation for biological processes and 2134 genes for molecular function. The 25 categories with the highest number of genes are presented in Fig. 2.

Expressed genes were significantly enriched in the functional categories 'RNA processing', 'translation' and 'mRNA metabolic process' compared with the microarray gene set. Amongst molecular function classes, we observed 'RNA binding' ($N = 282$) as the category with highest number of expressed genes. Along with 'translation factor activity, RNA binding' ($N = 58$), the 'RNA binding' category was also enriched in the set of expressed genes.

Twenty-nine genes were differentially expressed between good and bad quality oocytes (Table 3). Sixteen genes were upregulated in oocytes that sustained early embryo development to blastocyst stage. There was no enrichment of functional or molecular categories in the list of differentially expressed genes (DEG) as compared with either the microarray set of genes or the expressed genes in *in vitro* matured oocytes. Validation of the microarray result was performed by analyzing nine genes and qPCR assays with the same samples used for microarray hybridizations. With the exception of *HERC2*, nine out of the nine genes examined were in agreement with the microarray results (Fig. 3). No fold change was calculated for the genes *PFKFB3* and *IGF2R* because no fluorescence signal was detected from amplification or the C_t values for some samples were higher than 37, therefore above our threshold for data analysis. Thus, these two genes were validated because specific amplification was detected in all samples of good quality oocytes.

Discussion

In this study, we obtained an overall view of the global gene expression pattern of bovine *in vitro* matured oocytes using a small fraction of their cytoplasm. After the removal of the cytoplasm fraction, these oocytes were activated subsequently to examine their ability to undergo early embryonic development *in vitro.* Then, by comparing the transcriptome of oocytes that sustained embryo development to blastocyst stage with those that arrested development before blastula formation, we were able to associate specific gene products in the oocyte to its ability to undergo normal development.

Extraction of a portion of the cytoplasm did not affect the capability of competent oocytes to sustain early embryo development, as shown by the similarity between the blastocyst development ratio among micro-manipulated oocytes compared with the control group. Embryo culture from micro-manipulated oocytes yielded a similar blastocyst rate as observed previously in both individual embryo culture (Carolan *et al.*, 1996) and culture following an oocyte biopsy in microdrops that contained groups of 20 embryos (Biase *et al.*, 2009).

To avoid removing the metaphase spindle, ooplasm samples were aspirated from the opposite site of the polar body. The mammalian oocyte is a polarized

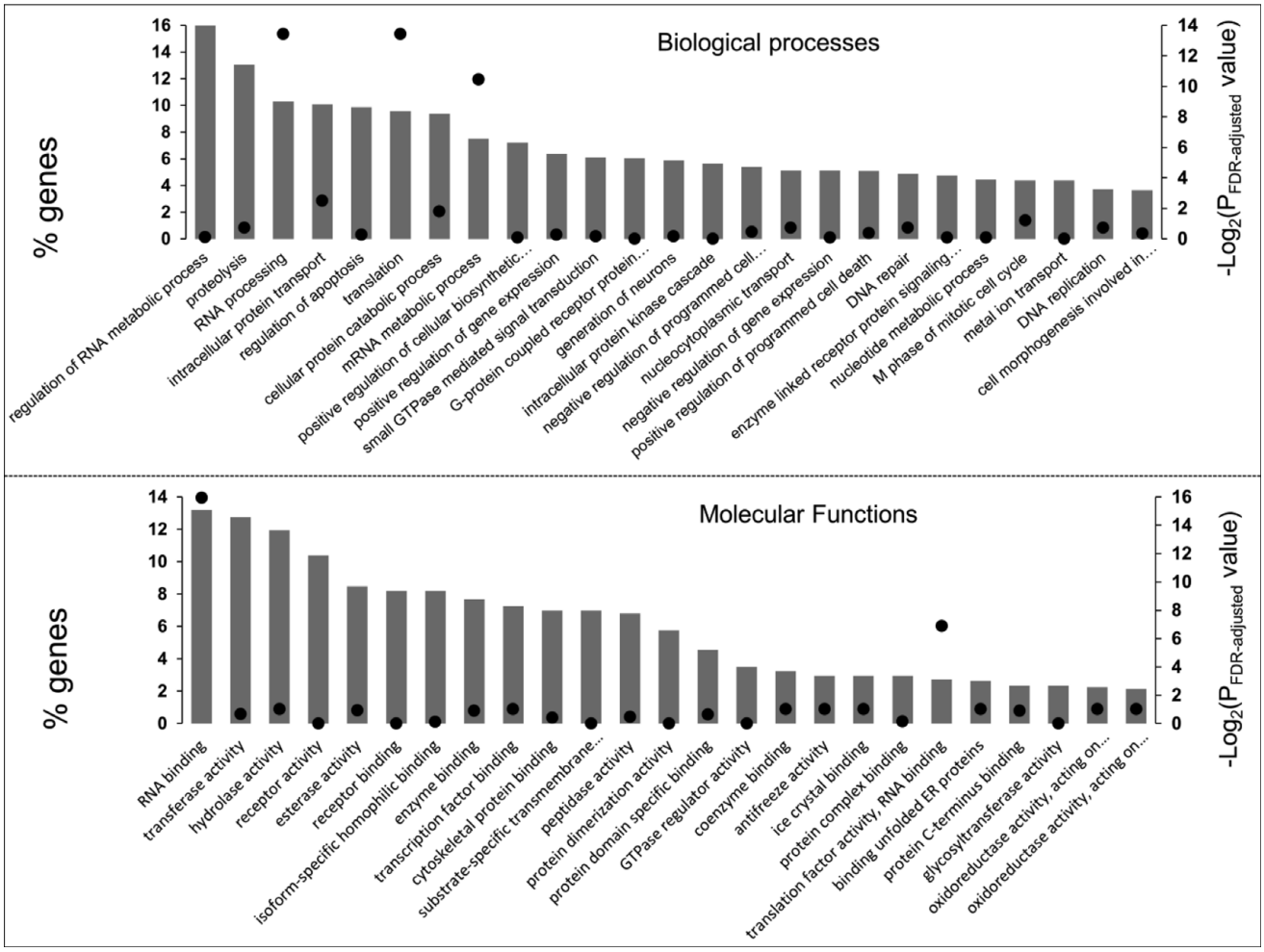

Figure 2 Functional categorization of the genes expressed in ooplasm after *in vitro* maturation. For each chart, vertical bars represent the 25 categories with most number of genes (left axis) while the dots are *P*-values from the functional enrichment analysis (right axis).

cell (Gardner, 2001) and mRNA localization has been demonstrated for different higher eukaryotic organisms (Palacios & St Johnston, 2001). Therefore, it is possible that our sampling was not a full representation of the mRNAs accumulated during oogenesis. Cytoplasm stratification remains a controversial subject in mammalian eggs. The comparison of sister blastomeres from 2-cell murine embryo suggested that transcripts of both blastomeres is very similar (Tang *et al.*, 2011), but some genes have different mRNA quantities between the sister blastomeres (Roberts *et al.*, 2011), a result that suggested that there might be non-uniform distribution of maternal mRNA in the cytoplasm. Nonetheless, as all biopsies were recovered consistently away from animal pole (region containing the metaphase plate), it is likely that the sampled region and its molecular composition were also relatively consistent throughout. Previous studies have identified between approximately 9000–11,000 genes expressed in bovine *in vitro* matured oocytes (Misirlioglu *et al.*, 2006; Fair *et al.*, 2007; Kues *et al.*, 2008), however we quantified the expression for 4320 in the ooplasm fragments, suggesting that the difference may be due to the restricted sampling region, i.e. vegetal pole, chosen for these studies.

The functional characterization of the genes expressed in *in vitro* matured oocytes revealed that a representative portion is dedicated to regulation of transcription and RNA processing and protein synthesis and degradation, corroborating previous findings (Cui *et al.*, 2007; Fair *et al.*, 2007). The RNA and protein synthesis and processing are certainly key functions that mediate activation of the embryonic genome. The presence of genes that function in DNA repair, replication, and the cell cycle in the MII oocyte is also compatible with the need to sustain three cell cycles without major gene transcription. During the initial three cleavages, blastomeres are not susceptible to apoptosis due to different inhibition mechanisms (Brad *et al.*, 2007; Carambula *et al.*, 2009),

Table 3 Differentially expressed genes in good versus bad quality oocytes

Oligo ID	Gene symbol	LogFC[a]	False discovery rate (FDR) *P*-value
OLIGO_07387	*PPP2R1*	0.6644	0.0140
OLIGO_10833	*PFKFB3*	0.6127	0.0016
OLIGO_02939	*MAPKBP*	0.6108	0.0015
OLIGO_08480	*POFUT2*	0.5656	0.0015
OLIGO_01318	*HERC2*	0.5610	0.0010
OLIGO_10148	*SFRS14*	0.4925	0.0015
OLIGO_02767	*ALG9*	0.4766	0.0087
OLIGO_10407	*PSMD3*	0.4646	0.0149
OLIGO_02916	*DDR1*	0.4501	0.0089
OLIGO_12918	*SMC5*	0.4491	0.0140
OLIGO_03648	*IGF2R*	0.4078	0.0195
OLIGO_07919	*TMED9*	0.3940	0.0119
OLIGO_09986	*MRAS*	0.3914	0.0191
OLIGO_09559	*TIMM13*	0.3782	0.0085
OLIGO_06551	*ZMAT2*	0.3733	0.0169
OLIGO_03257	*TMEM163*	0.3251	0.0089
OLIGO_07539	*NDUFB6*	−0.3776	0.0028
OLIGO_07089	*GPRASP2*	−0.3875	0.0169
OLIGO_05922	*UQCRH*	−0.3966	0.0191
OLIGO_09572	*PHF20*	−0.4046	0.0100
OLIGO_08978	*DUSP6*	−0.4411	0.0028
OLIGO_06694	*ENY2*	−0.4411	0.0015
OLIGO_05267	*SENP8*	−0.4467	0.0134
OLIGO_00055	*HADHB*	−0.4509	0.0194
OLIGO_07775	*CDK5RAP3*	−0.4717	0.0087
OLIGO_08925	*NDUFS4*	−0.5241	0.0029
OLIGO_10034	*PRPF4B*	−0.5251	0.0028
OLIGO_02050	*NUPL1*	−0.7252	0.0169
OLIGO_12760	*SNX4*	−0.9410	0.0169

[a]Log(fold change) = Log_2(expression in good quality oocytes) – Log_2(expression in bad quality oocytes).

however we observed expression of genes related to negative ($n = 124$) and positive ($n = 117$) regulation of cell death in our sample of bovine MII oocytes and supporting findings that oocytes accumulate pro- and anti-apoptotic regulators (Dalbies-Tran & Mermillod, 2003; Fair *et al.*, 2007; Fear & Hansen, 2011).

Comparative analysis between oocytes identified 29 DEG between embryos that reached the blastocyst stage and embryos that arrested at the 8–16-cell stage. Of these, 16 genes were more abundant in good quality oocytes and 13 were more abundant in bad quality oocytes when compared with their counterparts. The DEG found in our study have not been previously associated with oocyte quality in cattle, however we found that four genes have been described in studies that compared oocytes from women at different ages (49–51). The rationale of this model is that there is a significant negative correlation between *in vitro* blastocyst developmental ratio and age of women (Janny & Menezo, 1996). Transcripts for *DDR1* were relatively more abundant in GV stage oocytes from younger women (27–35 years) when compared with oocytes from older subjects (37–39 years) (Grondahl *et al.*, 2010). The levels of mRNA for *DUSP6* and *NUFS4* were more abundant in *in vitro* matured oocytes from older women (>40 years) compared with oocytes from younger patients (<32 years) (Steuerwald *et al.*, 2007). In our study, the expression of *PPP2R1B* was more abundant in good quality oocytes, but was also more abundant in oocytes from older women (Steuerwald *et al.*, 2007). We find it worth noting that genes belonging to the same family of *PSMD3*, *TIMM13* and *NDUFS4* were differentially expressed in matured eggs from young and old mice (Hamatani *et al.*, 2004).

We found two receptors that are more abundant in good quality oocytes compared with their bad quality counterparts: *DDR1* and *IGF2R*. The protein DDR1 can interact with type I to IV collagens (Koo *et al.*, 2006) and its activation transduces signals that function towards differentiation, extracellular matrix remodeling and cell cycle control (Vogel *et al.*, 2006). Along with other receptors, the *DDR1* protein may mediate cell communication between oocytes and cumulus cells during maturation, when the synthesis and deposition of collagen type IV increases on the cell surface (Sutovsky *et al.*, 1995).

The other membrane protein differentially expressed was *IGF2R*. Messenger RNA for IGF2R was previously detected in mature human and bovine oocytes (Lighten *et al.*, 1997; Yaseen *et al.*, 2001; Katz-Jaffe *et al.*, 2009; Wang *et al.*, 2009), and IGF2R protein was present in the plasma membrane (Wang *et al.*, 2009), where it may respond to autocrine and paracrine stimulus from the synthesis of IGF2 from oocyte and cumulus cells (Wang *et al.*, 2009). Experiments *in vitro* have shown that oocytes and early developing embryos respond to the presence of *IGF2* in culture medium (Warzych *et al.*, 2007; Wang *et al.*, 2009). The abundance of *IGF2R* mRNA in MII oocytes is altered by *in vitro* maturation. Oocytes cultured *in vitro* showed four-fold fewer transcripts compared with *in vivo* matured oocytes (Katz-Jaffe *et al.*, 2009). Our results support the fact that lower *IGF2R* mRNA expression in mature oocytes result in arrest of early embryonic development. Recent findings of the imprinted status of *IGF2R* in immature, but fully grown competent, oocytes showed one differentially methylated region (DMR) on intron 2 with a methylation profile of 31% on the groups of oocytes analyzed (O'Doherty *et al.*, 2011). We hypothesize that the lower amount of *IGF2R* transcripts in incompetent oocytes may be associated with the alteration in the methylation profile of the DMR in the *IGF2R* gene.

The RNA and proteins accumulated in mature oocytes drive the regulation of gene expression from

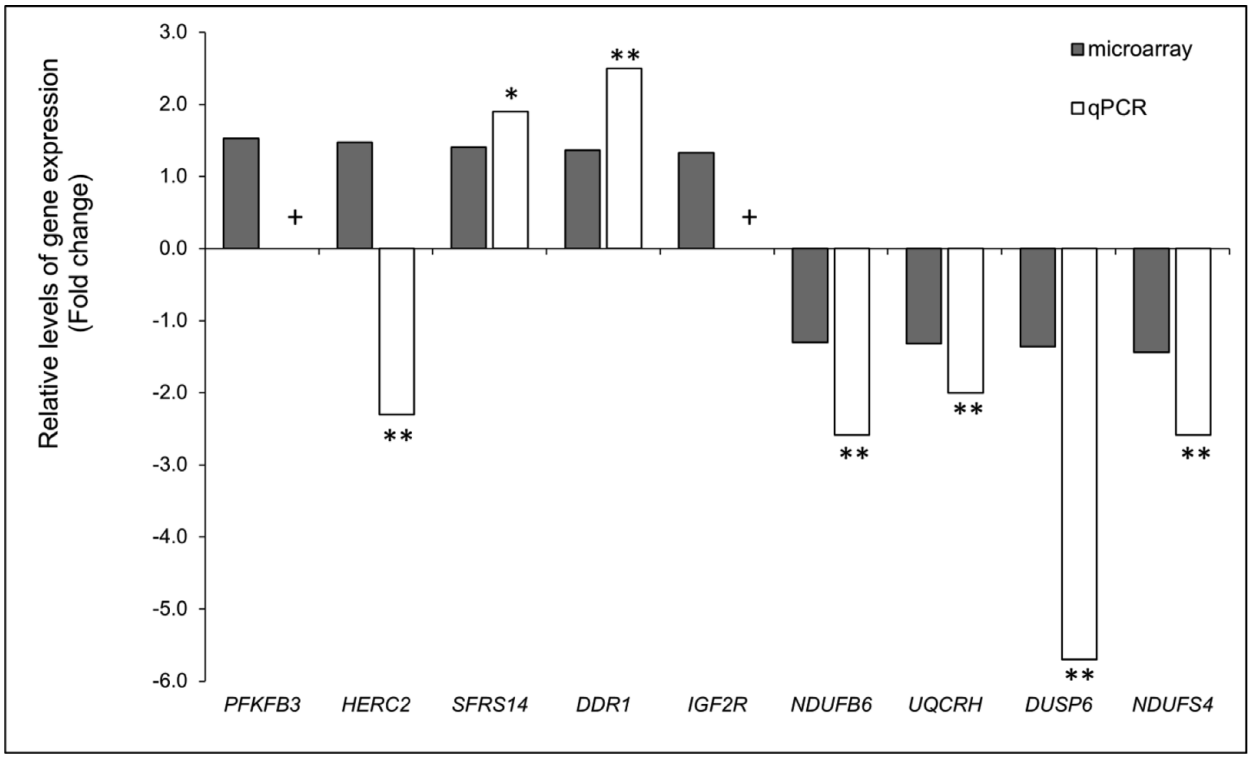

Figure 3 Validation of microarray experiment. $^{**}P < 0.05$, $^{*}P < 0.10$; + No fold change was calculated because fluorescence from no sample (*PFKFB3*) or only one sample (*IGF2R*) was detected from bad quality oocytes.

the embryonic genome and the efficacy of oocyte maturation and may determine the fate of the embryo after the 8–16-cell stage (Memili & First, 2000). Our analysis revealed four DEG that encode for proteins related to gene expression or mRNA metabolic processes: *ALG9*, *SFRS14*, *ENY2*, *PHF20*, *PRPF4B*; and one zinc finger protein: *ZMAT2*. Another group of five genes of similar function were also differentially expressed: *HERC2*, *MRAS*, *TIMM13*, *NUPL1* and *SNX4*. According to gene ontology, the last group of genes code for proteins that function in protein localization and protein transport. The correct location of proteins throughout the ooplasm is important for maintaining the homogeneity of protein segregation among the blastomeres during the early cleavages, cell compaction, and cell differentiation that forms the trophectoderm and inner cell mass (Chen *et al.*, 2010).

In summary, we were able to characterize the transcriptome of *in vitro* matured oocytes from a cytoplast fraction and correlate gene expression with the embryonic development until the blastocyst stage. Using our innovative approach, 29 genes were associated with oocyte quality, some of which had been reported previously in bovine, mouse or human experiments. Genes discovered in the present study are potential markers of mammalian oocyte quality. A better understanding of the gene expression architecture of a developmentally competent oocyte will lead us to create tools to improve fertility rates in *in vitro* fertilization services.

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References

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. & Sherlock, G. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25–9.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B.* **57**, 289–300.

Biase, F.H., Fonseca Merighe, G.K., Santos Biase, W.K., Martelli, L. & Meirelles, F.V. (2008). Global poly(A) mRNA expression profile measured in individual bovine oocytes and cleavage embryos. *Zygote* **16**, 29–38.

Biase, F.H., Martelli, L., Merighe, G.K., Santos Biase, W.K., Miranda, M., Smith, L.C. & Meirelles, F.V. (2009). A retrospective model of oocyte competence: global mRNA

and housekeeping transcripts are not associated with in vitro developmental outcome. *Zygote* **17**, 289–95.

Biase, F.H., Martelli, L., Puga, R., Giuliatti, S., Santos-Biase, W.K., Fonseca Merighe, G.K. & Meirelles, F.V. (2010). Messenger RNA expression of *Pabpnl1* and *Mbd3l2* genes in oocytes and cleavage embryos. *Fertil. Steril.* **93**, 2507–12.

Bilodeau-Goeseels, S. & Panich, P. (2002). Effects of oocyte quality on development and transcriptional activity in early bovine embryos. *Anim. Reprod. Sci.* **71**, 143–55.

Blondin, P. & Sirard, M.A. (1995). Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.* **41**, 54–62.

Brad, A.M., Hendricks, K.E. & Hansen, P.J. (2007). The block to apoptosis in bovine two-cell embryos involves inhibition of caspase-9 activation and caspase-mediated DNA damage. *Reproduction* **134**, 789–97.

Caixeta, E.S., Ripamonte, P., Franco, M.M., Junior, J.B. & Dode, M.A. (2009). Effect of follicle size on mRNA expression in cumulus cells and oocytes of *Bos indicus*: an approach to identify marker genes for developmental competence. *Reprod. Fertil. Dev.* **21**, 655–64.

Calder, M.D., Caveney, A.N., Westhusin, M.E. & Watson, A.J. (2001). Cyclooxygenase-2 and prostaglandin E_2 (PGE_2) receptor messenger RNAs are affected by bovine oocyte maturation time and cumulus–oocyte complex quality, and PGE_2 induces moderate expansion of the bovine cumulus *in vitro*. *Biol. Reprod.* **65**, 135–40.

Carambula, S.F., Oliveira, L.J. & Hansen, P.J. (2009). Repression of induced apoptosis in the 2-cell bovine embryo involves DNA methylation and histone deacetylation. *Biochem. Biophys. Res. Commun.* **388**, 418–21.

Carolan, C., Lonergan, P., Khatir, H. & Mermillod, P. (1996). *In vitro* production of bovine embryos using individual oocytes. *Mol. Reprod. Dev.* **45**, 145–50.

Chen, L., Wang, D., Wu, Z., Ma, L. & Daley, G.Q. (2010). Molecular basis of the first cell fate determination in mouse embryogenesis. *Cell Res.* **20**, 982–93.

Cui, X.S., Li, X.Y., Yin, X.J., Kong, I.K., Kang, J.J. & Kim, N.H. (2007). Maternal gene transcription in mouse oocytes: genes implicated in oocyte maturation and fertilization. *J. Reprod. Dev.* **53**, 405–18.

Dalbies-Tran, R. & Mermillod, P. (2003). Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during *in vitro* maturation. *Biol. Reprod.* **68**, 252–61.

Donnison, M. & Pfeffer, P.L. (2004). Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol. Reprod.* **71**, 1813–21.

Everts, R.E., Chavatte-Palmer, P., Razzak, A., Hue, I., Green, C.A., Oliveira, R., Vignon, X., Rodriguez-Zas, S.L., Tian, X.C., Yang, X., Renard, J.P. & Lewin, H.A. (2008). Aberrant gene expression patterns in placentomes are associated with phenotypically normal and abnormal cattle cloned by somatic cell nuclear transfer. *Physiol. Genomics* **33**, 65–77.

Fair, T., Gutierrez-Adan, A., Murphy, M., Rizos, D., Martin, F., Boland, M.P. & Lonergan, P. (2004a). Search for the bovine homolog of the murine ped gene and characterization of its messenger RNA expression during bovine preimplantation development. *Biol. Reprod.* **70**, 488–94.

Fair, T., Murphy, M., Rizos, D., Moss, C., Martin, F., Boland, M.P. & Lonergan, P. (2004b). Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. *Mol. Reprod. Dev.* **67**, 136–44.

Fair, T., Carter, F., Park, S., Evans, A.C. & Lonergan, P. (2007). Global gene expression analysis during bovine oocyte *in vitro* maturation. *Theriogenology* **68 Suppl 1**, S91–7.

Fear, J.M. & Hansen, P.J. (2011). Developmental changes in expression of genes involved in regulation of apoptosis in the bovine preimplantation embryo. *Biol. Reprod.* **84**, 43–51.

Gardner, R.L. (2001). The initial phase of embryonic patterning in mammals. *Int. Rev. Cytol.* **203**, 233–90.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y. & Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.

Grondahl, M.L., Yding Andersen, C., Bogstad, J., Nielsen, F.C., Meinertz, H. & Borup, R. (2010). Gene expression profiles of single human mature oocytes in relation to age. *Hum. Reprod.* **25**, 957–68.

Gutierrez-Adan, A., Rizos, D., Fair, T., Moreira, P.N., Pintado, B., de la Fuente, J., Boland, M.P. & Lonergan, P. (2004). Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Mol. Reprod. Dev.* **68**, 441–8.

Hamatani, T., Falco, G., Carter, M.G., Akutsu, H., Stagg, C.A., Sharov, A.A., Dudekula, D.B., VanBuren, V. & Ko, M.S. (2004). Age-associated alteration of gene expression patterns in mouse oocytes. *Hum. Mol. Genet.* **13**, 2263–78.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **8**, R19.

Ihaka, R. & Gentleman, R. (1996). R: a language for data analysis and graphics. *J. Comp. Graph. Stat.* **5**, 299–314.

Janny, L. & Menezo, Y. J. (1996). Maternal age effect on early human embryonic development and blastocyst formation. *Mol. Reprod. Dev.* **45**, 31–7.

Jeanmougin, M., de Reynies, A., Marisa, L., Paccard, C., Nuel, G. & Guedj, M. (2010). Should we abandon the *t*-test in the analysis of gene expression microarray data: a comparison of variance modeling strategies. *PLoS One* **5**, e12336.

Katz-Jaffe, M.G., McCallie, B.R., Preis, K. A., Filipovits, J. & Gardner, D.K. (2009). Transcriptome analysis of *in vivo* and *in vitro* matured bovine MII oocytes. *Theriogenology* **71**, 939–46.

Koo, D.H., McFadden, C., Huang, Y., Abdulhussein, R., Friese-Hamim, M. & Vogel, W.F. (2006). Pinpointing phosphotyrosine-dependent interactions downstream of the collagen receptor DDR1. *FEBS Lett.* **580**, 15–22.

Kues, W.A., Sudheer, S., Herrmann, D., Carnwath, J.W., Havlicek, V., Besenfelder, U., Lehrach, H., Adjaye, J. & Niemann, H. (2008). Genome-wide expression profiling reveals distinct clusters of transcriptional regulation

during bovine preimplantation development in vivo. *Proc. Natl. Acad. Sci. USA* **105**, 19768–73.

Lequarre, A.S., Traverso, J.M., Marchandise, J. & Donnay, I. (2004). Poly(A) RNA is reduced by half during bovine oocyte maturation but increases when meiotic arrest is maintained with CDK inhibitors. *Biol. Reprod.* **71**, 425–31.

Lighten, A.D., Hardy, K., Winston, R.M. & Moore, G.E. (1997). Expression of mRNA for the insulin-like growth factors and their receptors in human preimplantation embryos. *Mol. Reprod. Dev.* **47**, 134–9.

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P. & Boland, M.P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* **117**, 159–67.

Lonergan, P., Gutierrez-Adan, A., Pintado, B., Fair, T., Ward, F., Fuente, J.D. & Boland, M. (2000). Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. *Mol. Reprod. Dev.* **57**, 146–52.

Loor, J.J., Everts, R.E., Bionaz, M., Dann, H.M., Morin, D.E., Oliveira, R., Rodriguez-Zas, S.L., Drackley, J.K. & Lewin, H.A. (2007). Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. *Physiol. Genomics* **32**, 105–16.

Medina, I., Carbonell, J., Pulido, L., Madeira, S.C., Goetz, S., Conesa, A., Tarraga, J., Pascual-Montano, A., Nogales-Cadenas, R., Santoyo, J., Garcia, F., Marba, M., Montaner, D. & Dopazo, J. (2010). Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. *Nucleic Acids Res.* **38 Suppl**, W210–3.

Memili, E. & First, N. L. (2000). Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8**, 87–96.

Misirlioglu, M., Page, G.P., Sagirkaya, H., Kaya, A., Parrish, J.J., First, N.L. & Memili, E. (2006). Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **103**, 18905–10.

Mourot, M., Dufort, I., Gravel, C., Algriany, O., Dieleman, S. & Sirard, M.A. (2006). The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol. Reprod. Dev.* **73**, 1367–79.

O'Doherty, A.M., O'Shea, L.C. & Fair, T. (2012). Bovine DNA methylation imprints are established in an oocyte size-specific manner, which are coordinated with the expression of the DNMT3 family proteins. *Biol. Reprod.* **86**, 67.

Palacios, I.M. & St Johnston, D. (2001). Getting the message across: the intracellular localization of mRNAs in higher eukaryotes. *Annu. Rev. Cell Dev. Biol.* **17**, 569–614.

Pan, H., O'Brien, M.J., Wigglesworth, K., Eppig, J.J. & Schultz, R.M. (2005). Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development *in vitro*. *Dev. Biol.* **286**, 493–506.

Patel, O.V., Bettegowda, A., Ireland, J.J., Coussens, P.M., Lonergan, P. & Smith, G.W. (2007). Functional genomics studies of oocyte competence: evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes. *Reproduction* **133**, 95–106.

Revel, F., Mermillod, P., Peynot, N., Renard, J.P. & Heyman, Y. (1995). Low developmental capacity of in vitro matured and fertilized oocytes from calves compared with that of cows. *J. Reprod. Fertil.* **103**, 115–20.

Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A. & Smyth, G.K. (2007). A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23**, 2700–7.

Robert, C., Barnes, F.L., Hue, I. & Sirard, M.A. (2000). Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. *Mol. Reprod. Dev.* **57**, 167–75.

Roberts, R.M., Katayama, M., Magnuson, S.R., Falduto, M.T. & Torres, K.E. (2011). Transcript profiling of individual twin blastomeres derived by splitting two-cell stage murine embryos. *Biol. Reprod.* **84**, 487–94.

Romar, R., De Santis, T., Papillier, P., Perreau, C., Thelie, A., Dell'aquila, M., Mermillod, P. & Dalbies-Tran, R. (2011). Expression of maternal transcripts during bovine oocyte *in vitro* maturation is affected by donor age. *Reprod. Domest. Anim.* **46**, e23–30.

Schmittgen, T.D. & Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* **3**, 1101–8.

Smyth, G. (2003). Normalization of cDNA microarray data. *Methods.* **31**, 265–273.

Smyth, G.K. (2004). Linear models and empirical Bayes methods for assessing differencial expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, 3.

Smyth, G.K. (2005). *Limma: Linear Models for Microarray Data*. Springer: New York, pp. 397–420.

Smyth, G.K., Michaud, J. & Scott, H.S. (2005). Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* **21**, 2067–75.

Steuerwald, N.M., Bermudez, M.G., Wells, D., Munne, S. & Cohen, J. (2007). Maternal age-related differential global expression profiles observed in human oocytes. *Reprod. Biomed. Online* **14**, 700–8.

Sutovsky, P., Flechon, J.E. & Pavlok, A. (1995). F-actin is involved in control of bovine cumulus expansion. *Mol. Reprod. Dev.* **41**, 521–9.

Tang, F., Barbacioru, C., Nordman, E., Bao, S., Lee, C., Wang, X., Tuch, B.B., Heard, E., Lao, K. & Surani, M.A. (2011). Deterministic and stochastic allele specific gene expression in single mouse blastomeres. *PLoS One.* **6**, e21208.

Tervit, H.R., Whittingham, D.G. & Rowson, L.E. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil.* **30**, 493–7.

Vogel, W.F., Abdulhussein, R. & Ford, C.E. (2006). Sensing extracellular matrix: an update on discoidin domain receptor function. *Cell Signal* **18**, 1108–16.

Wang, L.M., Feng, H.L., Ma, Y.Z., Cang, M., Li, H.J., Yan, Z., Zhou, P., Wen, J.X., Bou, S. & Liu, D.J. (2009). Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Anim. Reprod. Sci.* **114**, 99–108.

Warzych, E., Wrenzycki, C., Peippo, J. & Lechniak, D. (2007). Maturation medium supplements affect transcript level of apoptosis and cell survival related genes in bovine blastocysts produced in vitro. *Mol. Reprod. Dev.* **74**, 280–9.

Yaseen, M.A., Wrenzycki, C., Herrmann, D., Carnwath, J.W. & Niemann, H. (2001). Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different in vitro systems. *Reproduction.* **122**, 601–10.

Yuan, J.S., Reed, A., Chen, F. & Stewart, C.N., Jr. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85.