Zygote **24** (August), pp. 624–633. © Cambridge University Press 2016
doi:10.1017/S0967199415000672 First Published Online 17 February 2016

In vitro maturation alters gene expression in bovine oocytes

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Date submitted: 12.02.2015. Date revised: 20.10.2015. Date accepted: 29.10.2015

Summary

Gene expression profiling of *in vivo*- and *in vitro*-matured bovine oocytes can identify transcripts related to the developmental potential of oocytes. Nonetheless, the effects of *in vitro* culturing oocytes are yet to be fully understood. We tested the effects of *in vitro* maturation on the transcript profile of oocytes collected from *Bos taurus indicus* cows. We quantified the expression of 1488 genes in *in vivo*- and *in vitro*-matured oocytes. Of these, 51 genes were up-regulated, whereas 56 were down-regulated (≥2-fold) in *in vivo*-matured oocytes in comparison with *in vitro*-matured oocytes. Quantitative real-time polymerase chain reaction (PCR) of nine genes confirmed the microarray results of differential expression between *in vivo*- and *in vitro*-matured oocytes (*EZR*, *EPN1*, *PSEN2*, *FST*, *IGFBP3*, *RBBP4*, *STAT3*, *FDPS* and *IRS1*). We interrogated the results for enrichment of Gene Ontology categories and overlap with protein–protein interactions. The results revealed that the genes altered by *in vitro* maturation are mostly related to the regulation of oocyte metabolism. Additionally, analysis of protein–protein interactions uncovered two regulatory networks affected by the *in vitro* culture system. We propose that the differentially expressed genes are candidates for biomarkers of oocyte competence. *In vitro* oocyte maturation can affect the abundance of specific transcripts and are likely to deplete the developmental competence.

Introduction

Cumulus–oocyte complexes (COCs) are dependent on adequate gene expression to initiate and to undergo oocyte maturation (meiotic progression) and embryonic development (Labrecque *et al.*, 2013; Li *et al.*, 2013). The mechanisms by which oocytes acquire competence to develop up to the blastocyst stage are still not fully understood. There is evidence that the acquisition of competence is correlated with RNA and protein molecules processed and stored during growth and maturation periods (Ferreira *et al.*, 2009; Caixeta *et al.*, 2013). To enable the storage and the convenient use of the molecules stored in oocytes, several mechanisms should act efficiently (Gandolfi & Gandolfi, 2001; Tomek *et al.*, 2002). Some transcripts have already been associated with oocyte developmental competence (Caixeta *et al.*, 2009; Katz-Jaffe *et al.*, 2009; Biase *et al.*, 2010; Kanka *et al.*, 2012; Bessa *et al.*, 2013; Biase *et al.*, 2014), and those results support the hypothesis that specific RNAs or proteins produced during oogenesis contribute to oocyte competence (Sirard *et al.*, 2006).

It is estimated that during embryogenesis about 5000–10,000 genes are simultaneously expressed in oocytes with a high level of control (Niemann *et al.*, 2007). The transcripts for key transcription factors represent a small number of copies and the ones that encode most of the structural proteins may represent approximately 2% of the mRNA pool (Yu *et al.*, 2002). Approximately 10–20% of total RNA consists of

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polyadenylated mRNAs (Niemann *et al.*, 2007), which are associated with oocyte developmental competence (Pocar *et al.*, 2001; Biase *et al.*, 2008; Biase *et al.*, 2010).

The molecular mechanisms that govern oocyte competence are mostly still unknown. However, some oocyte-specific genes have been described revealing their importance in promoting embryogenesis (Katz-Jaffe *et al.*, 2009; Biase *et al.*, 2010; Belli *et al.*, 2013; Bessa *et al.*, 2013; Biase *et al.*, 2014). The profiling of gene expression in oocytes during maturation may help us understand the regulation of oocyte competence to mature and to sustain embryo development during the first two cleavages (Fair *et al.*, 2007). It also promotes the identification of molecular markers for oocyte developmental potential. Nonetheless, most studies have addressed this subject in taurine subspecies (*Bos taurus taurus*). Here, we performed microarray-based transcriptome analyses of *in vivo*- and *in vitro*-matured bovine oocytes collected from *Bos taurus indicus* cows in order to enrich our knowledge of genes involved in the acquisition of oocyte competence.

Materials and methods

Estrous synchronization and superovulation protocols

Eight Nelore cows (crossbred) with good body condition and in reproductive age were synchronized on random days of the estrous cycle (D0) by intramuscular (IM) application of 2 mg estradiol benzoate RIC-BE (Tecnopec) and with placement of a bovine intravaginal progesterone device (Schering) for 8 days. On the fourth day (D4) follicle-stimulating hormone (FSH) treatment (Follitropin-V, Vetrepharm) was initiated with decreasing doses (80, 60, 40 or 20 mg FSH – IM) during 4 consecutive days. Simultaneously with the last FSH application (D7), cows received 0.150 mg Prolise (D-cloprostenol, IM), a $PGF_{2\alpha}$ analogue (Tecnopec), and after 36 h (D8), the animals were separated randomly into two groups of four animals for oocyte collection.

One group ($n = 4$) was designated for collection of immature oocytes at the germinal vesicle stage (GV). The intravaginal progesterone device was removed from the cows at D8 and oocyte collection was performed by ultrasound-guided follicular aspiration ovum pick up (OPU) so that they could be matured *in vitro*.

The second group ($n = 4$) was designated for collection of *in vivo*-matured (MII) oocytes. The intravaginal progesterone device was removed from the cows at D8, 25 mg luteinizing hormone (LH) (Lutropin-V, Vetrepharm, IM) was administered and we performed OPU 22–24 h later for collection of MII oocytes. OPU was performed three times in the same animals at intervals of approximately 80 days between the synchronizations.

Oocytes selection criteria

During the three collections for each group, we selected only those follicles with diameter greater than 8 mm for OPU. Cumulus–oocyte complexes collected at GV stage were used for *in vitro* maturation if the oocyte presented homogeneous cytoplasm and at least two compact layers of cumulus cells. Cumulus–oocyte complexes collected at MII phase were used for further procedures if the oocyte presented homogeneous cytoplasm and several layers of expanded cumulus cells.

The procedures involving animal handling were approved by the Ethics Committee of the University of São Paulo – School of Animal Sciences and Food Engineering.

In vitro maturation of GV oocytes

Oocytes collected at GV phase were matured *in vitro* for 22 h in TCM-199 medium (Sigma) supplemented with 10% bovine fetal serum (Sigma), 5.0 μg/ml LH, 0.5 μg/ml FSH, 200 μM pyruvate (Sigma), and 50 μg/ml gentamicin (Sigma). *In vitro* maturation culture was carried out in 100 μl droplets (20–25 oocytes in each droplet) under mineral oil at 38.5°C and an atmosphere of 5% CO_2 in air.

RNA extraction and amplification

For each of the three replicates, we selected 50 *in vitro*-matured and 50 *in vivo*-matured oocytes presenting the first polar body after removal of cumulus cells. The oocytes were pooled and stored at −80°C in calcium- and magnesium-free phosphate-buffered saline (PBS) with 0.1% polyvinyl alcohol (PVA) and 100 U/ml RNase inhibitor (Invitrogen). RNA extraction from oocytes was performed using RNeasy Protect Mini Kit (Qiagen) following the manufacturer's recommendations. Total RNA (~10 ng) was used as template for mRNA amplification with the SuperScript RNA Amplification Kit (Invitrogen) following the manufacturer's recommendations and oligo$(dT)_{12–18}$ as primers. Samples of amplified mRNA (mRNAa) were assayed in a Bioanalyzer 2100 equipment to assess quality and integrity using the RNA 6000 LabChip kit, following the manufacturer's recommendations (Agilent Technologies).

Probe labelling and microarray hybridization

Hybridization probes from the mRNAa were prepared by reverse transcription followed by the incorporation of Cy3 or Cy5 fluorophores according to the

https://doi.org/10.1017/S0967199415000672 Published online by Cambridge University Press

recommendations of CyScribe Post-Labelling Kit and CyScribe GFX Purification (GE Healthcare). cDNA labelled with Cy3 or Cy5 was measured in a NanoDrop 2000 spectrophotometer. Hybridizations were performed on microarray slides (BLO Plus (GPL9176)), containing oligonucleotides (70-*mer*) representing 8400 bovine genes. This long oligo set includes 10 bovine control genes and 10 Stratagene Alien Genes spotted multiple times on the array. Approximately 400 ng of labelled cDNA was hybridized to the microarray, following the dye-swap schema with two technical replicates. Thus, for each of the three biological replicates, we hybridized four slides, composing 12 slides for the experiment. Hybridization was carried in an automated station (Tecan HS400) for 6 h at 42°C, for 6 h at 35°C and for 6 h at 30°C, followed by three washes in 2× sodium chloride and sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS) at 37°C, three washes in 0.1× SSC, 0.1% SDS at 30°C and other three washes in 0.1× SSC at 25°C.

Data collection and analysis

The array images were digitalized by GenePix 4000B (Axon Instruments). The images were compiled using Imagene 5.0 (BioDiscovery), followed by the identification of the points of fluorescence and by background reading.

Raw intensities were normalized by the Lowess local regression using the LIMMA computational package according to procedures recommended for dye-swap labelling (Smyth & Speed 2003; Smyth 2005). The data obtained from the array spots were filtered and processed in order to eliminate poor quality, saturated or low fluorescence intensity spots relative to the background. Following data normalization, spots with intensity two-fold or greater than the background were considered for downstream analysis. Student's *t*-test was used to assess the statistical significance between the gene expression data generated from two experimental groups. Genes were inferred as differentially expressed between *in vivo*- and *in vitro*-matured oocytes if fold change was ≥2 and *P*-value <0.05.

The list of differentially expressed genes (DEG) was queried for biological processes potentially affected by *in vitro* maturation of oocytes using DAVID Bioinformatics Resources (v6.7, (Huang *et al.*, 2009)). The probabilities of significance were adjusted for multiple hypotheses testing using false discovery rate (FDR) (Benjamini & Yekutieli 2001), and a Gene Ontology term was assumed enriched if FDR < 0.1. The DEGs were overplayed on the topology of a protein–protein network according to the human and mouse BioGRID (v.3.2) database (Chatr-Aryamontri *et al.*, 2013) The network was built by expanding one protein interaction from each gene. The putative protein–protein network with DEGs in oocytes was visualized in Cytoscape (Shannon *et al.*, 2003).

Validation of the microarray results

In order to validate the microarray, cDNA was synthesized from the mRNAa used for the preparation of probes. The nine genes with the greatest difference in expression between *in vivo*- and *in vitro*-matured oocytes and known to be associated with the physiology of oocyte maturation were chosen for validation. Five of those genes were up-regulated (*EZR, EPN1, PSEN2, FST,* and *IGFBP3*), and four genes were down-regulated (*RBBP4, STAT3, FDPS, IRS1*) in *in vivo*-matured oocytes. Primers and probes for TaqMan Gene Expression Assays were designed by the manufacturer (*EZR* (Bt03223252_m1), *EPN1* (Bt03233436_g1), *PSEN2* (Bt03237484_m1), *FST* (Bt03259671_m1) and *IGFBP3* (Bt03223808_m1), *RBBP4* (Bt03230465_g1), *STAT3* (Bt03259866_g1), *FDPS* (Bt03216346_g1), Applied Biosystems). The exception was *IRS1* whose primers (GGCAGATCTGGATAATCGGT, AATGGAAGCCACAGAGGACT) and probe (CGG-ACTCACTCTGCGGGCAC) were made to order.

Reverse transcription was performed with the SuperScript II kit, following the manufacturer's recommendations (Invitrogen) and oligo(dT)$_{12–18}$ as primers. The real-time PCR reactions were set up according to the TaqMan PCR Master Mix Kit (Applied Biosystems). Real-time PCR data were normalized relative to H2A histone family gene, member Z (*H2AFZ,* Bower *et al.*, 2007) and fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). We used the *in vivo*-matured oocytes as calibrator sample. The ΔCTs were used as input for analysis of variance (BioEstats 5.0) (Ayres *et al.*, 2007) to assess the significance of differential gene expression between the two groups (Yuan *et al.*, 2006). Differential gene expression between *in vivo*- or *in vitro*-matured oocytes was assumed significant when $P < 0.05$.

Results

Genes expressed in bovine oocytes matured *in vivo* and *in vitro*

Our experiment yielded 1488 genes quantified with two-fold or greater intensity than the background. Among these, 51 genes were up-regulated (≥2-fold) in *in vivo*-matured oocytes compared with *in vitro* counterparts (Table 1). By comparison, 56 genes were

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Table 1 Genes up-regulated in *in vivo*-matured oocytes compared with *in vitro* counterparts

Symbol	Gene	Accession no.	Ratio (*in vivo*/*in vitro*)
EZR	ezrin	NM_174217.2	5.74
LAP3	leucine aminopeptidase 3	NM_174098.3	4.59
EPN1	epsin 1	NM_001038670.1	4.51
CFB	complement factor B	NM_001040526.1	4.12
GAP43	growth associated protein 43	NM_203358.2	3.99
ATHL1	acid trehalase-like	XM_589347.6	3.95
SMARCC1	SWI/SNF, actin	XM_002707780.2	3.88
COL13A1	collagen, type XIII, alpha 1	NM_001105433.1	3.84
MED29	mediator complex subunit 29	NM_001080316.2	3.77
MMAB	methylmalonic aciduria cblB type	NM_001079632.1	3.70
LCK	lymphocyte-specific protein tyrosine k	NM_001034334.1	3.66
AP2A2	adaptor-related protein 2, α 2	NM_001075702.1	3.57
SPHK2	sphingosine kinase 2	XM_002695198.2	3.51
RPLP2	ribosomal protein, large, P2	NM_174788.4	3.46
EPHB3	EPH receptor B3	NM_001192796.1	3.33
PLK1	polo-like kinase 1	NM_001038173.2	3.32
MRPS10	mitochondrial ribosomal prot. S10	NM_001035314.2	3.30
PSEN2	presenilin 2	NM_174440.4	3.28
DGKA	diacylglycerol kinase, alpha 80kDa	NM_001077860.1	3.27
FST	follistatin	NM_175801.3	3.25
PIK3R6	phosphoinositide-3-kinase, R6	NM_001102028.2	3.21
NADK	NAD kinase	NM_001034445.1	3.15
COX6A2	cytochrome c oxidase sub. 6A2	NM_174522.2	2.90
RPS6KB2	ribosomal protein S6 K, 70kDa, 2	NM_001205582.1	2.78
DYNLL1	dynein, light chain, LC8-type 1	NM_001003901.1	2.73
ETFB	electron-transfer-flavoprotein, β	NM_001038582.1	2.67
NUMA1	nuclear mitotic apparatus protein 1	NM_001205746.1	2.66
ADIPOQ	adiponectin, C1Q collagen	NM_174742.2	2.63
PTPRCAP	protein tyrosine phosphatase	NM_001046618.1	2.50
NGFRAP1	nerve growth factor receptor	NM_001163777.2	2.50
TACC3	transforming, acidic coiled-coil	NM_001100305.2	2.47
LTBP3	latent transforming growth factor b	NM_001192738.1	2.47
IGFBP5	IGF-binding protein 5	NM_001105327.2	2.37
STARD13	StAR-related lipid transfer	NM_001192070.1	2.37
POLR2E	polymerase (RNA) II, 25kDa	NM_001038093.2	2.35
UBE2B	ubiquitin-conjugating enzyme E2B	NM_001037459.2	2.30
FSD1	fibronectin type III and SPRY domain	NM_001081518.1	2.30
CFDP1	craniofacial development protein 1	NM_174268.1	2.26
PHF19	PHD finger protein 19	NM_001192715.1	2.25
PLOD3	procollagen-lysine, 2-oxoglutarate	NM_001193255.1	2.21
IGFBP3	IGF-binding protein 3	NM_174556.1	2.20
G6PC3	glucose 6 phosphatase, catalytic, 3	NM_183364.3	2.20
KEAP1	kelch-like ECH-associated protein 1	NM_001101142.1	2.16
ITGA11	integrin, alpha 11	XM_002690525.2	2.16
MYF6	myogenic factor 6 (herculin)	NM_181811.1	2.15
STK39	serine threonine kinase 39	NM_001075826.1	2.12
ARFRP1	ADP-ribosylation factor related	NM_001037607.1	2.11
WDR5	WD repeat domain 5	NM_001105475.2	2.11
SAP30L	SAP30-like	NM_001191372.1	2.05
POLRMT	polymerase (RNA) mitochondrial	NM_001205551.1	2.04
GHR	growth hormone receptor	NM_176608.1	2.02

down-regulated (≥2-fold) in *in vivo*-matured oocytes (Table 2). Among these 107 DEGs genes, 25 genes were annotated to the enriched Gene Ontology (GO) biological process 'negative regulation of cellular process' (FDR < 0.1, Table 3). Noticeably, several of these 25 DEGs are also possibly associated with regulation of metabolic processes (FDR < 0.2, Table 3). Further inspection of these 25 DEGs demonstrated

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Table 2 Genes down-regulated in *in vivo*-matured oocytes compared to *in vitro* counterparts

Symbol	Gene	Accession no.	Ratio (*in vivo*/*in vitro*)
IRS1	insulin receptor substrate-1	XM_003585773.2	0.17
H3F3A	H3 histone, family 3A	NM_001014389.2	0.20
SUCLG2	succinate-CoA ligase, GDP form	NM_001034639.1	0.21
CNOT7	CCR4-NOT transcription complex	NM_001034312.1	0.22
SUPT3H	suppressor of Ty 3 homolog	NM_001105008.1	0.22
RDH10	retinol dehydrogenase 10	NM_174734.2	0.23
CXCR5	chemokine C-X-C motif) R5	NM_001011675.1	0.24
RBBP7	retinoblastoma binding protein 7	NM_001034638.1	0.24
GOPC	Golgi associated PDZ and coiled-coil	NM_001206157.1	0.24
LGTN	ligatin	BT021884.1	0.25
BAZ1A	bromodomain adjacent to zinc finger	NM_001192940.1	0.25
RBBP4	retinoblastoma binding protein 4	NM_001077013.2	0.25
HMGB2	high-mobility group box 2	NM_001037616.1	0.26
HDAC2	histone deacetylase 2	NM_001075146.1	0.26
LRRC1	leucine rich repeat containing 1	NM_001205469.1	0.26
RBP4	retinol binding protein 4	NM_001040475.2	0.26
TMSB4X	thymosin beta 4, X-linked	NM_001002885.1	0.27
GBP5	guanylate binding protein 5	NM_001075746.1	0.27
TPX2	TPX2, microtubule-associated, targeting protein for Xklp2	NM_001098898.1	0.27
NDUFA1	NADH dehydrogenase 1 alpha	NM_175794.2	0.27
KIAA1310	KIAA1310 ortholog	NM_001099172.1	0.28
FLNC	filamin C, gamma	NM_001206990.1	0.28
STAT3	transducer and activator of transcription	NM_001012671.2	0.28
CALML5	calmodulin-like	NM_001098049.2	0.28
LMAN1	lectin, mannose-binding, 1	NM_001098943.2	0.28
HSPA9	heat shock 70 protein 9 (mortalin)	NM_001034524.1	0.29
KAT2A	K (lysine) acetyltransferase 2A	NM_021078.2	0.29
HPRT1	hypoxanthine phosphoribosyltransferase	NM_001034035.2	0.31
DEF6	differentially expressed in FDCP 6	NM_001098994.1	0.32
YIF1A	Yip1 interacting factor homolog A	NM_001034269.2	0.32
RASA1	RAS p21 protein activator 1	NM_174449.2	0.32
SFT2D1	SFT2 domain containing 1	NM_001034551.1	0.35
SLC7A14	solute carrier family 7, n14	NM_001077992.2	0.35
MOBKL2A	MOB1, Binder kinase activator	BT021734.1	0.36
CCNB1	cyclin B1	NM_001045872.1	0.36
NR1H2	nuclear receptor subfamily 1, H2	NM_001014883.1	0.37
PATZ1	POZ and AT hook containing zinc	NM_001191197.1	0.37
SNRPF	small nuclear ribonucleoprotein F	NM_001195027.1	0.37
DEDD	death effector domain containing	NM_001034643.2	0.37
KNG1	kininogen 1	NM_175774.3	0.37
ATF2	activating transcription factor 2	NM_001081584.2	0.38
SLC34A2	solute carrier family 34, n2	NM_174661.2	0.38
SOX18	SRY sex region Y)-box 18	NM_001075789.1	0.40
SNRPE	small nuclear ribonucleoprotein E	NM_001083459.2	0.42
MID1	midline 1	NM_001192822.1	0.43
NCOA1	nuclear receptor coactivator 1	NM_001206215.1	0.43
IDUA	iduronidase, alpha-L	XM_002688446.2	0.43
CTNNB1	catenin beta 1	NM_001076141.1	0.44
FLRT3	fibronectin leucine rich transmembrane	NM_001192674.1	0.44
SEPHS1	selenophosphate synthetase 1	NM_001075316.1	0.45
TSSC1	Tumor suppressing subtransferable	NM_001191328.1	0.45
FDPS	farnesyl diphosphate synthase	NM_177497.2	0.46
GGT7	gamma-glutamyltransferase 7	NM_001076401.1	0.47
SLC2A8	solute carrier family 2 member 8	NM_201528.1	0.47
TMEM59L	transmembrane protein 59-like	NM_001075301.1	0.49
NPHP1	nephronophthisis 1	NM_001105332.1	0.49

Table 3 Top 10 GO biological processes associated with DEG between *in vivo*- and *in vitro*-matured oocytes

Category	Term	*P*-value	FDR	Gene symbols
GO:0048513	Organ development	0.0003	0.0566	*RBP4, HMGB2, FST, ITGA11, HPRT1, CTNNB1, EZR, CXCR5, PATZ1, SOX18, CALML5, RASA1, IDUA, GHR, MYF6, SPHK2, COL13A1, TACC3, IRS1, STAT3, CCNB1, SMARCC1, LCK, PSEN2, GAP43, IGFBP5*
GO:0048523	Negative regulation of cellular process	0.0003	0.0566	*RBP4, HMGB2, DEDD, FST, CTNNB1, NR1H2, DYNLL1, GOPC, RASA1, HSPA9, MYF6, KNG1, RBBP4, SPHK2, RBBP7, MID1, IRS1, ADIPOQ, STAT3, HDAC2, PSEN2, CFDP1, TMSB4X, IGFBP3, IGFBP5*
GO:0031324	Negative regulation of cellular metabolic process	0.0014	0.1280	*MYF6, HMGB2, DEDD, FST, RBBP7, ADIPOQ, STAT3, CTNNB1, NR1H2, HDAC2, DYNLL1, PSEN2, IGFBP3, IGFBP5*
GO:0009892	Negative regulation of metabolic process	0.0029	0.1280	*MYF6, HMGB2, DEDD, FST, RBBP7, ADIPOQ, STAT3, CTNNB1, NR1H2, HDAC2, DYNLL1, PSEN2, IGFBP3, IGFBP5*
GO:0031325	Positive regulation of cellular metabolic process	0.0030	0.1280	*MYF6, HMGB2, HPRT1, CNOT7, ADIPOQ, IRS1, STAT3, CTNNB1, NR1H2, CCNB1, NCOA1, HDAC2, PLK1, SMARCC1, GHR*
GO:0048522	Positive regulation of cellular process	0.0035	0.1280	*MYF6, KNG1, RBP4, HMGB2, SPHK2, DEDD, CNOT7, HPRT1, ADIPOQ, IRS1, STAT3, CTNNB1, NR1H2, CCNB1, NCOA1, HDAC2, DYNLL1, PLK1, SMARCC1, LCK, PSEN2, NGFRAP1, IGFBP3, GHR*
GO:0043434	Response to peptide hormone stimulus	0.0044	0.1280	*SLC2A8, RBP4, UBE2B, IRS1, STAT3, GHR*
GO:0045185	Maintenance of protein location	0.0045	0.1280	*EZR, GOPC, TMSB4X, TACC3*
GO:0009893	Positive regulation of metabolic process	0.0046	0.1280	*MYF6, HMGB2, HPRT1, CNOT7, ADIPOQ, IRS1, STAT3, CTNNB1, NR1H2, CCNB1, NCOA1, HDAC2, PLK1, SMARCC1, GHR*
GO:0031323	Regulation of cellular metabolic process	0.0047	0.1280	*SUPT3H, HMGB2, DEDD, FST, KEAP1, HPRT1, CNOT7, ATF2, CTNNB1, DGKA, NR1H2, DYNLL1, MED29, PATZ1, SOX18, SAP30L, RASA1, GHR, KAT2A, MYF6, RBBP4, SPHK2, RBBP7, IRS1, ADIPOQ, STAT3, CCNB1, NCOA1, PHF19, BAZ1A, HDAC2, PLK1, SMARCC1, PSEN2, IGFBP3, GAP43, IGFBP5*

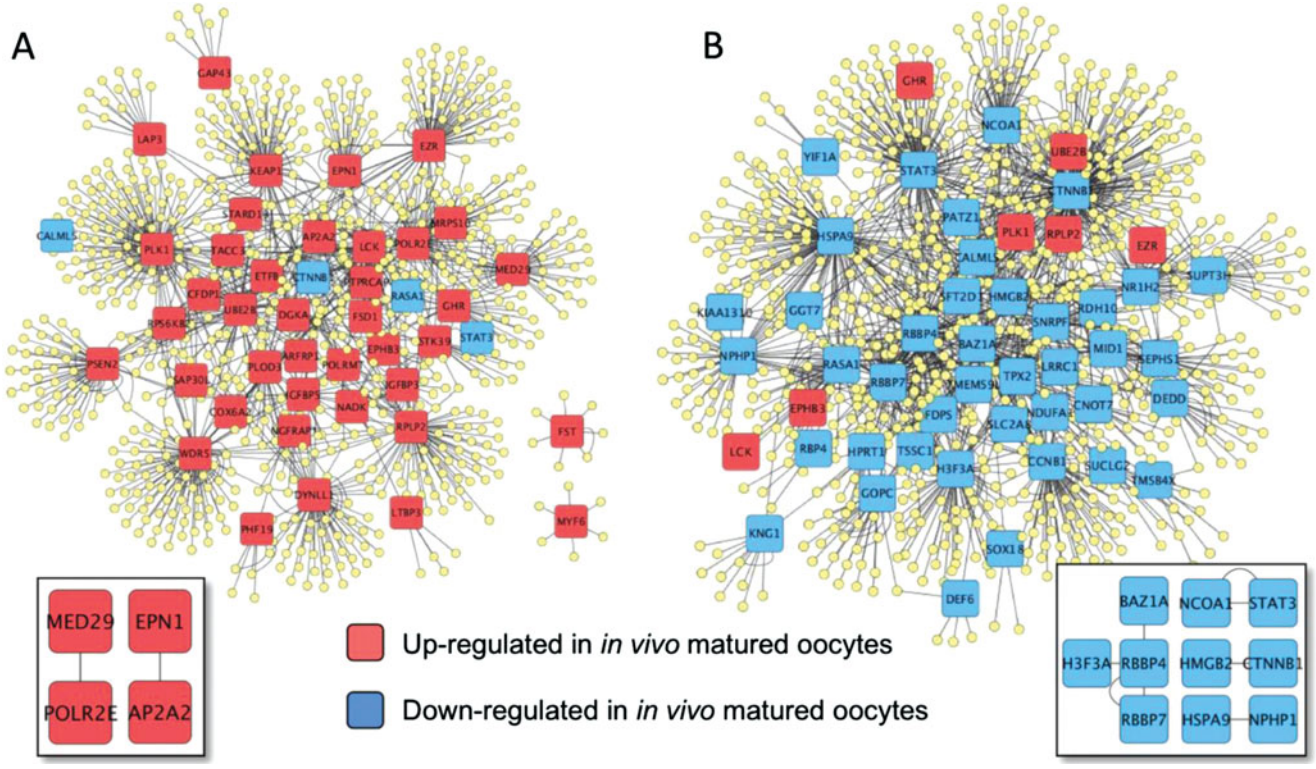

Figure 1 Regulatory protein–protein network of genes affected by the *in vitro* culture of oocytes. (*A*) Genes up-regulated in *in vivo*-matured (MII) oocytes collected *in vivo.* (*B*) Genes down-regulated in MII oocytes collected *in vivo.* Differentially expressed genes (DEGs) are marked in red or blue, and yellow depicts the proteins potentially interacted with DEGs. The insets highlight the direct connection between proteins whose coding genes are differentially regulated by *in vitro* maturation.

that nine of them are involved in regulation of transcription (*CTNNB1*, *DEDD*, *FST*, *HMGB2*, *HDAC2*, *MYF6*, *NR1H2*, *RBBP7*, *STAT3*).

Next, we searched for altered gene expression that would affect interacting proteins. Most DEGs were part of a protein–protein network. Forty out of 51 genes up-regulated in *in vivo*-matured oocytes composed a protein interaction network (Fig. 1*A*), four of those genes identified two pairs of direct protein-protein interaction (Fig. 1*A*, inset). Two of those genes are associated with the transcription complex, namely: polymerase (RNA) II (DNA directed) polypeptide A and the Mediator Complex Subunit 29. By comparison, 41 of the 56 genes up-regulated in *in vitro*-matured oocytes composed another protein–protein network (Fig. 1*B*), 10 of which identified direct protein interactions (Fig. 1*B*, inset). It is noteworthy that we found four genes associated with chromatin remodelling factors that were positively modulated by *in vitro* culture, namely: H3 histone, family3A, retinoblastoma binding protein 4 and 7, and bromodomain adjacent to zinc finger domain, 1A.

Confirmation of differentially expressed genes by real-time PCR

We used real-time PCR to validate our microarray results. The nine genes tested were differentially expressed in *in vivo*-matured oocytes (*EZR*, *EPN1*, *IRS1*, *FDPS*, *FST*, *IGFBP3*, *PSEN2*, *RBBP4*, *STAT3*) compared with *in vitro*-matured oocytes in the two analyses, microarray and RT-PCR ($P < 0.05$. Fig. 2).

Discussion

We determined whether the *in vitro* maturation process affected the gene expression of oocytes collected from *Bos taurus indicus* cows. With our investigation, we showed that 107 genes have altered expression due to the *in vitro* maturation system. Functional annotation of the data suggests that dysfunctional gene expression is not random and mostly affected the metabolism of oocytes. Inspection of the GO annotation of the genes suggests that one of the metabolic processes highly affected is the regulation of RNA synthesis.

https://doi.org/10.1017/S0967199415000672 Published online by Cambridge University Press

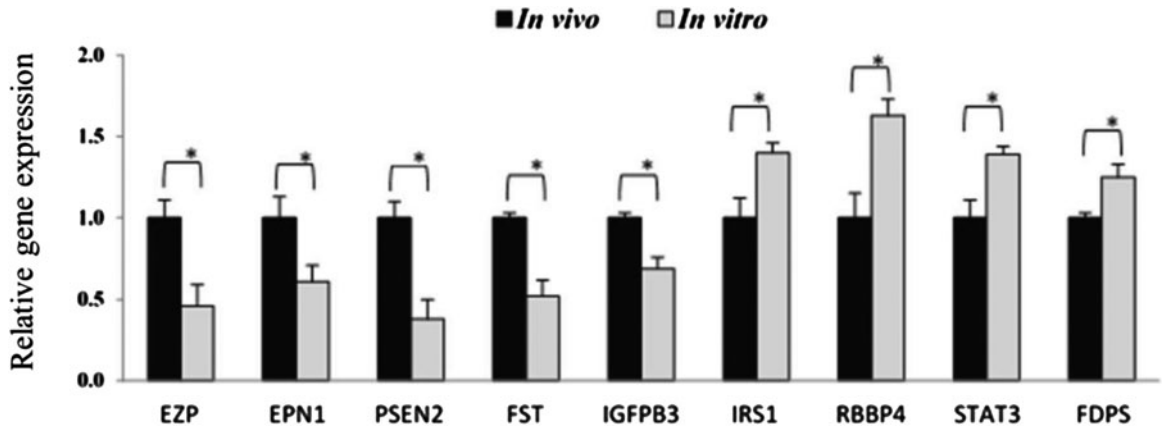

Figure 2 Relative expression of transcripts in *in vivo*- and *in vitro*-matured oocytes. Significant differences ($P < 0.05$) between groups (*in vivo* versus *in vitro*) are denoted by an asterisk. The results of three replicates are shown.

Corroborating our results, 21 of the DEGs were previously shown to be associated with oocyte developmental competence, 13 of those genes were up-regulated in *in vivo*-matured oocytes (*DGKA* (Beltman *et al.*, 2010), *GHR* (Caixeta *et al.*, 2009), *FST* (Bonnet *et al.*, 2011), *HMGB2* (Corcoran *et al.*, 2007), *TACC3* (Hao *et al.*, 2002), IGFBP3 (Sawai 2009), *EZR* (Heng *et al.*, 2011), *KEAP1* (Powell *et al.*, 2010), *SMARCC1* (Lisboa *et al.*, 2012), *PLK1* (Sun *et al.*, 2012), *NGFRAP1* (Jiang *et al.*, 2010), *NUMA* (Kolano *et al.*, 2012) and *EPN1* (Liu & Zheng, 2009)) and eight of them were up-regulated in *in vitro*-matured oocytes [*IRS-1* (Yamamoto-Honda *et al.*, 1996), *STAT3* (Mohammadi-Sangcheshmeh *et al.*, 2011), *CCNB1* (Liu *et al.*, 2012), *RBBP4* and *RBBP7* (Gasca *et al.*, 2008), *ATF2* (Vigneault *et al.*, 2009), *TPX2* (Brunet *et al.*, 2008) and *HDAC2* (Caixeta *et al.*, 2013)]. This observation supports our approach and analysis. Most importantly, we showed 86 new potential biomarkers associated with oocyte competence. Further investigation will be required to conclusively demonstrate that the transcription of these 86 genes are specifically altered in oocytes collected from *B. taurus indicus* and matured *in vitro*.

Functional analysis of the DEGs revealed that 37 genes were annotated to 'regulation of cellular metabolic process', which was previously shown to be important for the maturation of oocytes (Fair *et al.*, 2007; Katz-Jaffe *et al.*, 2009). Interestingly, 26 (of the 37) DEGs were also functionally related to 'negative regulation of cellular process', and those genes are potentially important for cytoplasmic maturation (Ferreira *et al.*, 2009), and developmental potential of the oocytes. The dysregulation of a metabolic process such as the synthesis of RNA, due to *in vitro* maturation, is likely to affect the transcription during cleavage stages of development (Smith *et al.*, 2009) and alter cleavage kinetics during embryo development (Knijn *et al.*, 2003).

Our results of the transcriptome analysis were further supported by protein–protein interactome. The formation of protein–protein networks composed of the majority of genes either up-regulated (Fig. 1*A*) or down-regulated (Fig. 1*B*) regulated in *in vivo*-matured oocytes strongly suggests biological co-regulation of such genes in MII oocytes. Interestingly, we observed subsets of DEGs whose protein may form regulatory complexes (Fig. 1, insets). Two examples of gene co-expression and protein–protein interaction are potentially associated with gene regulation. First, the transcripts of *MED29* and *POLR2E* are up-regulated in *in vivo*-matured oocytes, where this protein complex may function in the elongation phase of transcription (Takahashi *et al.*, 2011). Second, we found the complex formed around the retinoblastoma binding protein 4 (RBBP4) in the genes up-regulated in *in vitro*-matured oocytes. The abnormal abundance of this complex may contribute to negative regulation of genes important for embryo development (Wolffe *et al.*, 2000). These results showed that the *in vitro* culture system also disturbs the regulation of oocyte's gene expression at the transcriptional level.

In summary, we established the transcript profile of *in vivo*- and *in vitro*-matured oocytes of *Bos taurus indicus* cows using microarray technology. Our experiment allowed us to uncover genes potentially involved in the control of oocyte competence. In light of our results, we suggest that the harmonious function of metabolism and regulation of gene expression is pivotal for the acquisition of oocyte developmental competence. The identification of potential competence markers will be useful for developing better

https://doi.org/10.1017/S0967199415000672 Published online by Cambridge University Press

in vitro culture conditions to allow the oocyte to adequately obtain competence.

Acknowledgements

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 05/58702–4 and 05/59694-5) and Universidade de São Paulo (USP/FZEA) for financially supporting the project.

References

Ayres, M., Ayres, J.M., Ayres, D.L. & Santos, A.S. (2007). *BioEstat 5.0. Statistical Applications in Biological and Medical Sciences*. Sociedade Civil Mamirauá, Brasília, CNPq, 138 pp.

Belli, M., Cimadomo, D., Merico, V., Redi, C.A., Garagna, S. & Zuccotti, M. (2013). The NOBOX protein becomes undetectable in developmentally competent antral and ovulated oocytes. *Int. J. Dev. Biol.* **57**, 35–9.

Beltman, M.E., Forde, N., Furney, P., Carter, F., Roche, J.F., Lonergan, P. & Crowe, M.A. (2010). Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on day 7 after insemination. *Reprod. Fertil. Dev.* **22**, 987–99.

Benjamini, Y. & Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. *Annals Stat.* **29**, 1165–88.

Bessa, I.R., Nishimura, R.C., Franco, M.M. & Dode, M.A.N. (2013). Transcription profile of candidate genes for the acquisition of competence during oocyte growth in cattle. *Reprod. Domest. Anim.* **48**, 781–9.

Biase, F.H., Fonseca Merighe, G.K., Santos-Biase, W., 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., 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.

Biase, F.H., Everts, R.E., Oliveira, R., Santos-Biase, W.K., Fonseca Merighe, G.K., Smith, L.C., Martelli, L., Lewin, H. & Meirelles, F.V. (2014). Messenger RNAs in metaphase II oocytes correlate with successful embryo development to the blastocyst stage. *Zygote* **22**, 69–79.

Bonnet, A., Bevilacqua, C., Benne, F., Bodin, L., Cotinot, C., Liaubet, L., Sancristobal, M., Sarry, J., Terenina, E. & Martin, P. (2011). Transcriptome profiling of sheep granulosa cells and oocytes during early follicular development obtained by laser capture microdissection. *BMC Genomics* **12**, 417.

Bower, N.I., Moser, R.J., Hill, J.R., Lehnert, S.A. (2007). Universal reference method for real-time PCR gene expression analysis of preimplantation embryos. *Biotechniques* **42**, 199–206.

Brunet, S., Dumont, J., Lee, K.W., Kinoshita, K., Hikal, P., Gruss, O.J., Maro, B. & Verlhac, M.-H. (2008). Meiotic regulation of TPX2 protein levels governs cell cycle progression in mouse oocytes. *PLoS One* **3**, e3338.

Caixeta, E.S., Ripamonte, P., Franco, M.M., Junior, J.B. & Dode, M.A.N. (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.

Caixeta, E.S., Sutton-McDowall, M.L., Gilchrist, R.B., Thompson, J.G., Price, C.A., Machado, M.F., Lima, P.F. & Buratini, J. (2013). Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during *in vitro* maturation of bovine cumulus–oocyte complexes. *Reproduction* **146**, 27–35.

Chatr-Aryamontri, A., Breitkreutz, B.J., Heinicke, S., Boucher, L., Winter, A., Stark, C., Nixon, J., Ramage, L., Kolas, N., O'Donnell, L., Reguly, T., Breitkreutz, A., Sellam, A., Chen, D., Chang, C., Rust, J., Livstone, M., Oughtred, R., Dolinski, K. & Tyers, M. (2013). The BioGRID interaction database: 2013 update. *Nucleic Acids Res.* **41**, D816–23.

Corcoran, D., Rizos, D., Fair, T., Evans, A.C. & Lonergan, P. (2007). Temporal expression of transcripts related to embryo quality in bovine embryos cultured from the two-cell to blastocyst stage *in vitro* or *in vivo*. *Mol. Reprod. Dev.* **74**, 972–977.

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

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A. & Navarro, P.A.A.S. (2009). Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71**, 836–48.

Gandolfi, T.A. & Gandolfi, F. (2001). The maternal legacy to the embryo: cytoplasmic components and their effects on early development. *Theriogenology* **55**, 1255–76.

Gasca, S., Pellestor, F., Assou, S., Loup, V., Anahory, T., Dechaud, H., De Vos, J. & Hamamah, S. (2008). Identifying new human oocyte marker genes: a microarray approach. *Reprod. Biomed. Online* **14**, 175–83.

Hao, Z., Stoler, M.H., Sen, B., Shore, A., Westbrook, A., Flickinger, C.J., Herr, J.C. & Coonrod, S.A. (2002). TACC3 expression and localization in the murine egg and ovary. *Mol. Reprod. Dev.* **63**, 291–9.

Heng, S., Cervero, A., Simon, C., Stephens, A.N., Li, Y., Zhang, J., Paule, S., Rainczuk, A., Singh, H. & Quinonero, A. (2011). Proprotein convertase 5/6 is critical for embryo implantation in women: regulating receptivity by cleaving EBP50, modulating ezrin binding, and membrane-cytoskeletal interactions. *Endocrinology* **152**, 5041–52.

Huang, D.W., Sherman, B.T. & Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nat. Protoc.* **4**, 44–57.

Jiang, J.-Y., Xiong, H., Cao, M., Xia, X., Sirard, M.-A. & Tsang, B.K. (2010). Mural granulosa cell gene expression associated with oocyte developmental competence. *J. Ovarian Res.* **3**, 6.

Kanka, J., Nemcova, L., Toralova, T., Vodickova-Kepkova, K., Vodicka, P., Jeseta, M. & Machatkova, M. (2012). Association of the transcription profile of bovine oocytes

and embryos with developmental potential. *Anim. Reprod. Sci.* **134**, 29–35.

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.

Knijn, H.M., Gjørret, J.O., Vos, P.L., Hendriksen, P.J., van der Weijden, B.C., Maddox-Hyttel, P. & Dieleman, S.J. (2003). Consequences of *in vivo* development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. *Biol. Reprod.* **69**, 1371–8.

Kolano, A., Brunet, S., Silk, A.D., Cleveland, D.W. & Verlhac, M.-H. (2012). Error-prone mammalian female meiosis from silencing the spindle assembly checkpoint without normal interkinetochore tension. *Proc. Natl. Acad. Sci.* **109**, E1858–67.

Labrecque, R., Vigneault, C., Blondin, P. & Sirard, M.A. (2013). Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol. Reprod. Dev.* **80**, 428–40.

Li, Y., Ray, D. & Ye, P. (2013). Identification of germ cell-specific genes in mammalian meiotic prophase. *BMC Bioinform.* **14**, 1–13.

Lisboa, L.A., Bordignon, V. & Seneda, M.M. (2012). Immunolocalization of BRG1–SWI/SNF protein during folliculogenesis in the porcine ovary. *Zygote* **20**, 243–8.

Liu, Z. & Zheng, Y. (2009). A requirement for epsin in mitotic membrane and spindle organization. *J. Cell Biol.* **186**, 473–80.

Liu, W., Yin, J., Zhao, G., Yun, Y., Wu, S., Jones, K.T. & Lei, A. (2012). Differential regulation of cyclin B1 degradation between the first and second meiotic divisions of bovine oocytes. *Theriogenology* **78**, 1171–81.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–8.

Mohammadi-Sangcheshmeh, A., Held, E., Ghanem, N., Rings, F., Salilew-Wondim, D., Tesfaye, D., Sieme, H., Schellander, K. & Hoelker, M. (2011). G6PDH-activity in equine oocytes correlates with morphology, expression of candidate genes for viability, and preimplantative *in vitro* development. *Theriogenology* **76**, 1215–26.

Niemann, H., Carnwath, J.W. & Kues, W. (2007). Application of DNA array technology to mammalian embryos. *Theriogenology* **68**, S165–77.

Pocar, P., Brevini, T.A.L., Perazzoli, F., Cillo, F., Modina, S. & Gandolfi, F. (2001). Cellular and molecular mechanisms mediating the effects of polychlorinated biphenyls on oocyte developmental competence in cattle. *Mol. Reprod. Dev.* **60**, 535–41.

Powell, M.D., Manandhar, G., Spate, L., Sutovsky, M., Zimmerman, S., Sachdev, S.C., Hannink, M., Prather, R.S. & Sutovsky, P. (2010). Discovery of putative oocyte quality markers by comparative ExacTag proteomics. *PROTEOMICS – Clinical Applications* **4**, 337–51.

Sawai, K. (2009). Studies on gene expression in bovine embryos derived from somatic cell nuclear transfer. *J. Reprod. Dev.* **55**, 11–6.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. & Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498–504.

Sirard, M.-A., Richard, F., Blondin, P. & Robert, C. (2006). Contribution of the oocyte to embryo quality. *Theriogenology* **65**, 126–36.

Smith, S.L., Everts, R.E., Sung, L.Y., Du, F., Page, R.L., Henderson, B., Rodriguez-Zas, S.L., Nedambale, T.L., Renard, J.P., Lewin, H.A., Yang, X., Tian, X.C. (2009). Gene expression profiling of single bovine embryos uncovers significant effects of *in vitro* maturation, fertilization and culture. *Mol. Reprod. Dev*. **76**, 38–47.

Smyth, G.K. (2005). Limma: linear models for microarray data. In *Bioinformatics and computational biology solutions using R and Bioconductor* (R. Gentleman, V. Carey, W. Huber, R. Irizarry & S. Dudoit, eds), pp. 397–420. Springer.

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

Sun, S.-C., Liu, H.-L. & Sun, Q.-Y. (2012). Survivin regulates Plk1 localization to kinetochore in mouse oocyte meiosis. *Biochem. Biophys. Res. Commun.* **421**, 797–800.

Takahashi, H., Parmely, T.J., Sato, S., Tomomori-Sato, C., Banks, C.A., Kong, S.E., Szutorisz, H., Swanson, S.K., Martin-Brown, S., Washburn, M.P., Florens, L., Seidel, C.W., Lin, C., Smith, E.R., Shilatifard, A., Conaway, R.C. & Conaway, J.W. (2011). Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92–104.

Tomek, W., Torner, H. & Kanitz, W. (2002). Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes *in vitro*. *Reprod. Domest. Anim.* **37**, 86–91.

Vigneault, C., McGraw, S. & Sirard, M.-A. (2009). Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine *in vitro* embryogenesis. *Reproduction* **137**, 13–21.

Wolffe, A.P., Urnov, F.D. & Guschin, D. (2000). Co-repressor complexes and remodelling chromatin for repression. *Biochem. Soc. Trans.* **28**, 379–86.

Yamamoto-Honda, R., Honda, Z.I., Ueki, K., Tobe, K., Kaburagi, Y., Takahashi, Y., Tamemoto, H., Suzuki, T., Itoh, K. & Akanuma, Y. (1996). Mutant of insulin receptor substrate-1 incapable of activating phosphatidylinositol 3-kinase did not mediate insulin-stimulated maturation of *Xenopus laevis* oocytes. *J. Biol. Chem.* **271**, 28677–81.

Yu, J., Hecht, N.B. & Schultz, R.M. (2002). RNA-binding properties and translation repression *in vitro* by germ cell-specific MSY2 protein. *Biol. Reprod.* **67**, 1093–8.

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