Focus on Mammalian Embryogenomics

Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity

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Abstract

Oocyte selection based on glucose-6-phosphate dehydrogenase (G6PDH) activity has been successfully used to differentiate between competent and incompetent bovine oocytes. However, the intrinsic molecular and subcellular characteristics of these oocytes have not yet been investigated. Here, we aim to identify molecular and functional markers associated with oocyte developmental potential when selected based on G6PDH activity. Immature compact cumulus–oocyte complexes were stained with brilliant cresyl blue (BCB) for 90 min. Based on their colouration, oocytes were divided into BCB[−] (colourless cytoplasm, high G6PDH activity) and BCB⁺ (coloured cytoplasm, low G6PDH activity). The chromatin configuration of the nucleus and the mitochondrial activity of oocytes were determined by fluorescence labelling and photometric measurement. The abundance and phosphorylation pattern of protein kinases Akt and MAP were estimated by Western blot analysis. A bovine cDNA microarray was used to analyse the gene expression profiles of BCB⁺ and BCB[−] oocytes. Consequently, marked differences were found in blastocyst rate at day 8 between BCB⁺ (33.1 ± 3.1%) and BCB[−] (12.1 ± 1.5%) oocytes. Moreover, BCB⁺ oocytes were found to show higher phosphorylation levels of Akt and MAP kinases and are enriched with genes regulating transcription (*SMARCA5*), cell cycle (nuclear autoantigenic sperm protein, *NASP*) and protein biosynthesis (*RPS274A* and mRNA for elongation factor 1 α , *EF1A*). BCB[−] oocytes, which revealed higher mitochondrial activity and still nucleoli in their germinal vesicles, were enriched with genes involved in ATP synthesis (*ATP5A1*), mitochondrial electron transport (*FL405*), calcium ion binding (*S100A10*) and growth factor activity (bone morphogenetic protein 15, *BMP15*). This study has evidenced molecular and subcellular organisational differences of oocytes with different G6PDH activity.

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Introduction

In modern animal agriculture, with increasing milk production there is a continuous decline in the fertility of

dairy cows leading to higher economic loss (Macmillan *et al.* 1996). This decline in fertility can be explained by management changes within the dairy industry and also negative genetic correlations between milk production and reproduction. One of the primary mechanisms that depresses fertility in lactating cows is abnormal pre-implantation embryo development, which that may be a result of poor oocyte quality (Snijders *et al.* 2000, Lucy 2007). Oocyte developmental competence is defined as the ability of an oocyte to resume meiosis, to cleave following fertilisation, to develop to the blastocyst stage, to induce a pregnancy and bring offspring to term in a good health (Krisner 2004, Sirard *et al.* 2006). This

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competency is acquired gradually during the course of folliculogenesis as the oocyte grows and its companion somatic cells differentiate (Eppig *et al.* 1994).

Many factors have been shown to affect the oocyte's developmental potential, including follicle size (Lonergan *et al.* 1994), health of the follicle (Blondin & Sirard 1995, Vassena *et al.* 2003), phase of follicular wave (Hagemann 1999, Machatková *et al.* 2004), hormonal stimulation (Blondin *et al.* 2002; for review Sirard *et al.* 2006), maturation environment (Warzych *et al.* 2007; for review Sutton *et al.* 2003), season (Al-Katanani *et al.* 2002, Sartori *et al.* 2002), nutrition (Fouladi-Nashta *et al.* 2007) and age (Rizos *et al.* 2005). Although previous studies support the notion that oocyte competence depends on multiple factors, it remains difficult to draw clear and reliable criteria for oocyte selection.

Morphological assessment of oocytes based on thickness, compactness of the cumulus investment and the homogeneity of the ooplasm (Gordon 2003) is a relatively popular and convenient way of evaluating oocyte quality in practice. However, results derived from this non-invasive approach are often conflicting, largely due to subjectivity and inaccuracy. Morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to bring about full-term pregnancy (Lonergan *et al.* 2003, Coticchio *et al.* 2004, Krisher 2004). With the urgent need for establishing non-invasive and non-perturbing means for oocyte selection, the brilliant cresyl blue (BCB) staining test has been successfully used to differentiate oocytes with different developmental capacity in various species, including pig (Ericsson *et al.* 1993, Roca *et al.* 1998, Wongsrikeao *et al.* 2006), goat (Rodríguez-González *et al.* 2002) and cattle (Alm *et al.* 2005, Bhojwani *et al.* 2007).

During the course of their growth, immature oocytes are known to synthesise a variety of proteins, including glucose-6-phosphate dehydrogenase (G6PDH; Mangia & Epstein 1975, Wassarman 1988). The activity of this protein is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence (Wassarman 1988, Tian *et al.* 1998). BCB is a dye that can be degraded by G6PDH (Ericsson *et al.* 1993, Tian *et al.* 1998); thus, oocytes that have finished their growth phase show decreased G6PDH activity and exhibit cytoplasm with a blue colouration (BCB⁺), while growing oocytes are expected to have a high level of active G6PDH, which results in colourless cytoplasm (BCB⁻).

In our previous studies, it has been shown that oocytes screened based on BCB staining differ in their developmental potential to reach blastocyst stage (Alm *et al.* 2005) and efficiency in utilisation for somatic cell nuclear transfer (Bhojwani *et al.* 2007). Moreover, oocytes screened with BCB staining were reported to differ in various oocyte quality markers like cytoplasmic volume and mitochondria DNA copy number (El-Shourbagy *et al.* 2006). However, little is known about the

molecular and the subcellular characteristics of these oocytes. Therefore, the aim of this study was to characterise these oocytes at the subcellular level (dissolution of nucleoli and mitochondrial activity), molecular level (gene expression profile) and functionally (activity of protein kinase). The results of the present study evidence the prevailing differences of these oocyte groups in relative abundance transcripts and mitochondrial and MAPK activities contributing to their differences in developmental potential.

Results

Chromatin configuration and mitochondrial activity in BCB⁺ and BCB⁻ oocytes

Because of their importance as parameters for oocyte quality, we investigated the status of nuclei and mitochondria in BCB⁺ and BCB⁻ oocytes before *in vitro* maturation (IVM). A larger proportion of oocytes with high G6PDH activity (BCB⁻) were found to be in early stage of diplotene with clear visible nucleoli (Dipl + Nuc) in their germinal vesicle than the BCB⁺ oocytes (Table 1; $P < 0.005$). However, a significantly lower number of a BCB⁻ oocytes was found to be in more progressed diakinesis stage after germinal vesicle breakdown (GVBD) compared with their BCB⁺ counterparts.

To confirm that the fluorescence intensity of the emission light from the fixed MitoTracker-labelled oocytes was stable during the time of storage, a preliminary study was conducted to measure the fluorescence intensity of 40 oocytes in intervals of 7 days during 6 weeks. The measured fluorescence intensity was not influenced by the storage.

The data in Table 1 demonstrate that the fluorescence intensity in the oocytes pre-labelled by the vital mitochondrial-specific probe chloromethyl tetramethylrosamine (CMTM Ros) and measured by fluorescence intensity for 570 nm emission/oocyte is associated with their G6PDH activity ($P < 0.001$). The highest fluorescence intensity/oocyte was found in BCB⁻ oocytes compared with the BCB⁺ ones.

Detection of abundance and phosphorylation of protein kinases Akt and MAP

In order to elucidate the activities of protein kinases that contribute in the regulation of gene expression, we have analysed the abundance and phosphorylation state of the MAPKs ERK1, ERK2 and Akt. As indicated in Fig. 1, the abundance of MAPK and Akt1 was not different between BCB⁺ and BCB⁻ oocytes. In contrast to these observations, BCB⁺ oocytes show a higher phosphorylation of ERK1, ERK2 and Akt at all phosphorylation sites compared with their BCB⁻ counterparts (Fig. 1).

Table 1 Chromatin configuration and mitochondrial activity (fluorescence intensity/oocyte based on vital labelling of metabolic active mitochondria) in brilliant cresyl blue (BCB⁺) and BCB⁻ oocytes (*n*=337).

Oocyte group	Number of oocytes	Chromatin configuration in % $\pm$ S.E.M.							Mitochondrial activity Fluorescence intensity /oocyte in μ A $\pm$ S.E.M.
		Dipl+Nuc	Dipl	CC	Dia	MI	MII	Pyc	
BCB ⁺	169	1.8 $\pm$ 1.0*	17.2 $\pm$ 2.9	49.1 $\pm$ 3.8	20.1 $\pm$ 3.1 [†]	7.1 $\pm$ 2.0	0.6 $\pm$ 0.6	4.1 $\pm$ 1.4	358.4 $\pm$ 18.9
BCB ⁻	168	21.4 $\pm$ 3.1 [†]	16.7 $\pm$ 2.9	42.9 $\pm$ 3.8	8.9 $\pm$ 2.2 [§]	4.2 $\pm$ 1.5	1.2 $\pm$ 0.8	4.7 $\pm$ 1.6	539.1 $\pm$ 19.0 [¶]

*:†, †:§ $P < 0.005$; ||:¶ $P < 0.001$. Dipl+Nuc, diplotene with nucleolus; Dipl, diplotene; CC, condensed chromatin in GV; Dia, diakinesis; MI, metaphase I; MII, metaphase II; Pyc, pycnotic chromatin.

Developmental competence of bovine oocytes depending on their G6PDH status

In order to evaluate the developmental competence of BCB⁺ and BCB⁻ oocytes, developmental phenotypes were assessed until day 8 following IVM, *in vitro* fertilisation (IVF) and *in vitro* culture (IVC). There were no significant differences among the groups in cleavage rate 2 days after IVF. Significant differences ($P < 0.05$) among the groups were observed in blastocyst rate at day 8, where the BCB⁺ oocytes resulted in significantly higher (35.7%) blastocyst rate compared with the BCB⁻ groups (13.2%). In addition, the number of nuclei in the resulting blastocysts was higher for BCB⁺ oocytes compared with the BCB⁻ ones (Table 2).

Genes differentially expressed between BCB⁺ and BCB⁻ oocytes

To identify candidate genes related to oocyte developmental competence, oocytes screened based on G6PDH activity (BCB⁺ and BCB⁻) were analysed using bovine cDNA microarray platform. After LOWESS normalisation of the data, log value of Cy5 total intensities was compared with the log value of Cy3 total intensities for both the target and the respective dye-swap hybridisations. The coefficient of determination was high and consistent between target ($R^2 = 0.98$) and dye-swap ($R^2 = 0.99$) hybridisations.

To obtain a highly confident set of differentially expressed genes, we used a rigorous combination of P values ($P \leq 0.05$) and false discovery rate ($FDR \leq 5\%$). The SAM analysis revealed that a total of 185 genes to be differentially expressed between the BCB⁺ and the BCB⁻ oocytes (with ≥ 1.9 -fold change). Of these, 85 genes were up-regulated (Tables 3 and 4) and 100 were down-regulated (Tables 5 and 6) in BCB⁺ compared with BCB⁻ oocytes. Comparative analysis of the magnitude of differential gene expression between the two oocyte groups showed that, while the up-regulated genes were in the range of 1.9- to 7.8-fold change, down-regulated genes were in the range of 2.0- to 11.5-fold change in BCB⁺ compared with BCB⁻ oocytes.

A combination of hierarchical clustering and heatmap of differentially regulated genes (Fig. 2) was used to show the overall expression pattern of the target genes in replicate hybridisation. The average linkage clustering

analysis revealed the presence of many subgroups within the up- and down-regulated genes (or clusters) sharing similar expression pattern.

Functional classification of target genes

The ontological classification of differentially regulated genes in BCB⁺ versus BCB⁻ oocytes was performed based on the criteria of Gene Ontology Consortium classifications (<http://www.geneontology.org>), which annotates transcripts with regard to their molecular functions. The resulting data were supplemented with additional information from Centre and CowBase at the AgBase database (www.agbase.msstate.edu). The differentially regulated genes between the two groups of oocytes were found to represent genes with known function (57.3%; 106/185), with unknown function (18.4%; 34/185) and novel transcripts (24.3%; 45/185).

Figure 1 Analysis of the abundance and phosphorylation state of the protein kinases Akt (A) and MAPK (B) in BCB differentiated oocytes. Fifty oocytes each, BCB⁺ and BCB⁻, were analysed for the abundance Akt (total Akt) and MAPK (total MAPK) and the phosphorylation state as indicated by Western blotting. As a control, in A and B right panels, the phosphorylation state of MI-stage oocytes (where Akt is the highest phosphorylated) and MII-stage oocytes (where MAPK is the highest phosphorylated) is depicted.

Table 2 Developmental competence of bovine oocytes depending on their glucose-6-phosphate dehydrogenase status (brilliant cresyl blue, BCB⁺, BCB⁻) before *in vitro* maturation (*n*=259).

Oocyte group	Number of oocytes	Cleavage rate day 2 p.IVF in % $\pm$ S.E.M.	Blastocyst rate day 8 p.IVF in % $\pm$ S.E.M.
BCB ⁺	172	78.4 $\pm$ 8.3	33.1 $\pm$ 3.1*
BCB ⁻	87	75.0 $\pm$ 4.7	12.1 $\pm$ 1.5 [†]

*[†]*P*<0.05.

We observed that certain functional annotations were more represented in either BCB⁺ (Fig. 3) or BCB⁻ oocytes (Fig. 4). The BCB⁺ oocytes were found to be enriched with genes related to protein binding (*RALA*), enzymatic activity (*RIOK3*), structural constituent of ribosome (*RPS14*), nucleic acid binding (*H2AFZ*), transcription (*SMARCA5*), ubiquitin-protein ligase activity (*UHRF2*), calmodulin binding (*RGS16*), translation elongation factor activity (*EEF1A1*) and microtubule motor activity (*DYNC1I2*) in BCB⁺ oocytes (Fig. 3). On the other hand, transcripts involved in protein binding (*NLRP2*), ion binding (*NPTX2*), nucleic acid binding (*PAPOLG*), oxidoreductase activity (*PGHS2*), enzymatic activity (*ALOX15*), signal transduction (*LGALS1*), growth factor activity (*BMP15*) and hydrogen ion transporting ATPase activity (*ATP5A1*) were found to be highly abundant in BCB⁻ oocytes compared with BCB⁺ ones (Fig. 4).

Real-time PCR validation

Real-time PCR analysis using a set of samples distinct from those used in microarray experiment validated the mRNA transcript abundance of ten genes (Fig. 5). The relative abundance of the *GAPDH* gene was tested and showed no variability between the samples under investigation. Accordingly, five up-regulated genes namely *EEF1A1*, *ODC1*, *RPS27A*, *NASP* and *SMARCA5* showed higher transcript abundance (*P*≤0.05) in BCB⁺ than BCB⁻ oocytes as observed in array analysis. Similarly, the relative abundance for *ATP5A1*, *FL405*, *S100A10* and *PTTG1* were greater (*P*≤0.05) in BCB⁻ than BCB⁺ oocytes. The transcript abundance for *BMP15* was also confirmed but the differences between the two oocyte groups were not statistically significant.

Discussion

The success of *in vitro* production of bovine transferable blastocysts using oocytes aspirated from slaughterhouse ovaries does not exceed 40–50%. Various studies have shown the quality of the oocyte to be the main determinant of blastocyst rate, while the culture environment affects their quality (Rizos *et al.* 2002, Lonergan *et al.* 2003). Therefore, selection and further use of good quality or developmentally competent oocytes is vital for the success of various embryo technologies. The use of

BCB staining based on the presence of active G6PDH in immature oocytes has proven to be efficient tool to screen developmentally competent or incompetent oocytes for various species including cattle (Alm *et al.* 2005, Bhojwani *et al.* 2007). The present study further evidenced differences in subcellular organisations and transcript abundance between the two oocyte groups.

In terms of biological processes, the expression profiles of BCB⁺ oocytes were markedly different from those of BCB⁻ ones. The majority of expressed genes in BCB⁺ oocytes are associated with regulation of the cell cycle (*NASP*, *MLH1*, *PRC1*, *UHRF2*, *UBE2D3*, *CCNB1*, *MPHOSPH9*, *CETN3*, *ASPM*, *NUSAP1* and *AURKA*), transcription (*SMARCA5*, *ZFP91*, *ZNF519*, *ZNF85*, *HMGN2*, *PA2G4*, *STAT3*, *DNMT1* and *FANK1*) and translation (*EEF1A1*, *RPS27A*, *RPS14*, *RPS15*, *RPS29*, *RPL18A*, *RPL9* and *RPL24*); while BCB⁻ oocytes encoded genes controlling ATP synthesis (*ATP5A1*), mitochondrial electron transport (*FL405*) and calcium ion binding (*S100A10*).

Numerous factors involved in cell cycle regulation have been more recognised in BCB⁺ than BCB⁻ oocytes. Among these cell cycle regulators, a *NASP* was first identified as a nuclear-associated protein in rabbit testis (Welch & O'Rand 1990, Welch *et al.* 1990). This gene has high homology with *Xenopus* histone-binding protein, N1/N2, which is expressed in oocytes (Kleinschmidt *et al.* 1986, Kleinschmidt & Seiter 1988). *NASP* is an H1 histone-binding protein that is cell cycle regulated and occurs in two major forms: t*NASP*, found in gametes, embryonic cells and transformed cells; and s*NASP*, found in all rapidly dividing somatic cells (Richardson *et al.* 2000). Moreover, it was strongly expressed in mouse embryos developed under non-blocking culture conditions in which embryos do not exhibit developmental arrest at the two-cell stage; however, the function of this transcript in early embryonic development remains unknown (Minami *et al.* 2001). *NASP* was one of the genes with increased expression in very fast moving bovine oocytes, which showed higher blastocyst rate compared with the slow groups after dielectrophoretic separation (Dessie *et al.* 2007).

It is not surprising that cell cycle regulator genes category is the one of the largest highly expressed transcripts in BCB⁺ oocytes. The embryo has to divide thrice to reach maternal zygotic transition (MZT) in conditions of very low transcription (Barnes & First 1991). Therefore, the competent oocyte must store enough mRNA coding for cell cycle proteins like *CCNB1* (Tremblay *et al.* 2005) to ensure that these proteins will not be limiting the embryo progression.

Both the assembly of transcriptional machinery and organisation of appropriate chromatin structure are critical for establishing the programme of early mouse development shortly after fertilisation (Sun *et al.* 2007). Changes in chromatin structure are thought to play an

Table 3 Genes up-regulated in brilliant cresyl blue (BCB⁺) compared with BCB⁻ oocytes.

Gene name	Accession no. in GenBank	Fold change	Gene function (biological process)
<i>Homo sapiens</i> zinc finger protein 91 homologue (mouse; ZFP91), transcript variant 1, mRNA	NM_053023	7.8	DNA binding (transcription)
<i>Homo sapiens</i> zinc finger protein 519, mRNA, complete cds (ZNF519)	BC024227	4.4	DNA binding (transcription)
<i>Homo sapiens</i> high-mobility group nucleosomal binding domain 2, mRNA (HMGN2)	BC071707	6.3	DNA binding (transcription)
<i>Homo sapiens</i> DEAD (Asp-Glu-Ala-Asp) box polypeptide 10, mRNA (DDX10)	NM_004398	5.0	RNA binding
<i>Homo sapiens</i> tudor and KH domain containing, mRNA, with apparent retained intron (TDRKH)	BC022467	4.4	RNA binding
TPA_exp: <i>Mus musculus</i> regulator of sex-limitation candidate 2, mRNA, complete cds (Rslcan2)	BK001637	4.4	Nucleic acid binding (transcription)
Bovine mRNA for histone H2A.Z (H2AFZ)	X52318	4.4	DNA binding (chromosome organisation and biogenesis)
<i>Homo sapiens</i> proliferation-associated 2G4, 38 kDa, mRNA, complete cds (PA2G4)	BC007561	4.2	Transcription factor activity
<i>Homo sapiens</i> zinc finger protein 85 (HPF4, HTF1), mRNA (ZNF85)	BC047646	4.0	Transcription factor activity
<i>Bos taurus</i> partial stat3 gene for signal transducer and activator of transcription 3 (STAT3)	AJ620667	4.0	Transcription factor activity
<i>Bos taurus</i> DNA (cytosine 5) methyltransferase 1, mRNA (DNMT1)	NM_182651	3.9	Transcription factor binding
<i>Homo sapiens</i> fibronectin type 3 and ankyrin repeat domains 1, mRNA (FANK1)	BC024189	3.9	Transcription factor binding
<i>Homo sapiens</i> SWI/SNF-related, matrix-associated, actin- dependent (SMARCA5)	NM_003601	6.7	RNA polymerase II transcription factor activity
<i>Homo sapiens</i> ring finger protein 10, mRNA, complete cds (RNF10)	BC016622	3.2	Protein binding
<i>Homo sapiens</i> v-ral simian leukaemia viral oncogene homologue A (ras-related; RALA)	BC039858	3.6	Protein binding (signal transduction)
<i>Homo sapiens</i> related RAS viral (r-ras) oncogene homologue 2, mRNA (RRAS2)	BC013106	3.9	Protein binding (signal transduction)
<i>Homo sapiens</i> cell adhesion molecule with homology to L1CAM (close homologue of L1; CHL1)	NM_006614	3.6	Protein binding (signal transduction)
<i>S. scrofa</i> mRNA encoding G-beta like protein (GNB2L1)	Z33879	2.9	Protein binding (signal transduction)
Canine rab11 mRNA for ras-related GTP-binding protein (RAB11A)	X56388	2.8	Protein binding (plasma membrane to the endosome)
<i>Homo sapiens</i> occluding mRNA (OCLN)	NM_002538	2.6	Protein binding (protein complex assembly)
<i>Canis familiaris</i> occluding 1B mRNA, complete cds (OCLN)	AF246976	2.4	Protein binding
<i>Homo sapiens</i> chaperonin-containing TCP1, subunit 8 (theta), mRNA (CCT8)	BC012584	2.8	Unfolded protein binding (protein folding)
<i>Homo sapiens</i> ADP-ribosylation factor-like 6 interacting protein (ARL6IP1)	BC010281	2.7	Protein binding (protein targeting membrane)
<i>Homo sapiens</i> protein regulator of cytokinesis 1, mRNA (PRC1)	BC003138	2.7	Protein binding (cell cycle)
<i>Homo sapiens</i> nuclear autoantigenic sperm protein (histone-binding) mRNA (NASP)	BT006757	2.6	Hsp90 protein binding (cell cycle, blastocyst development)
<i>Homo sapiens</i> mutL homologue 1, colon cancer, non-polyposis type 2 (<i>E. coli</i>), mRNA (MLH1)	NM_000249	2.4	Protein binding (cell cycle)
<i>Homo sapiens</i> ubiquitin-like, containing PHD and RING finger domains, 2 (UHRF2), mRNA	NM_152896	2.7	Ubiquitin-protein ligase activity (cell cycle)
<i>Homo sapiens</i> ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homologue), (UBE2D3)	BC003395	2.6	Ubiquitin-protein ligase activity (cell cycle)
<i>Mus musculus</i> ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homologue, yeast), mRNA (Ube2d3)	NM_025356	2.6	Ubiquitin-protein ligase activity (cell cycle)
<i>Homo sapiens</i> aurora kinase A, transcript variant 4, mRNA (AURKA)	NM_198435	2.2	Ubiquitin-protein ligase activity (cell cycle)
<i>Bos taurus</i> mRNA sequence (CCNB1)	L26548	2.4	(Regulation of progression through cell cycle)
<i>Homo sapiens</i> M-phase phosphoprotein 9, mRNA (MPHOSPH9)	NM_022782	2.1	(Regulation of progression through cell cycle)
<i>Homo sapiens</i> centrin, EF-hand protein, 3 (CDC31 homologue, yeast), (CETN3)	BC005383	4.1	Calcium ion binding (cell cycle)
<i>Bos taurus</i> isolate Cow1 ASPM mRNA, partial cds (ASPM)	BC010658	2.2	Phosphoprotein phosphatase activity (cell cycle)
<i>Homo sapiens</i> nucleolar and spindle-associated protein 1, mRNA (NUSAP1)	BC011008	2.5	(Establishment of mitotic spindle localisation)
<i>Homo sapiens</i> discs, large homologue 7 (<i>Drosophila</i>), mRNA (DLG7)	AY485424	2.1	Calmodulin binding (cell cycle)
<i>Homo sapiens</i> IQ motif containing GTPase-activating protein 1 (IQGAP1)	NM_003870	2.1	Calmodulin binding (signal transduction)
<i>Homo sapiens</i> regulator of G-protein signalling 16, mRNA (RGS16)	NM_002928	2.6	Calmodulin binding (signal transduction)

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$.

Table 4 Genes up-regulated in brilliant cresyl blue (BCB⁺) compared with BCB⁻ oocytes.

Gene name	Accession no. in GenBank	Fold change	Gene function (biological process)
<i>Homo sapiens</i> regulator of G-protein signalling 2, 24 kDa (RGS2)	NM_002923	2.2	Calmodulin binding (signal transduction)
<i>Bos taurus</i> EF1A mRNA for elongation factor 1 α , complete cds (EEF1A1)	AB060107	2.5	Translation elongation factor activity (translation)
<i>Bos taurus</i> mRNA for elongation factor 1 α (EEF1A1)	AJ238405	2.0	Translation elongation factor activity (translation)
<i>Homo sapiens</i> ribosomal protein S15 mRNA (RPS15)	NM_001018	2.0	Structural constituent of ribosome (translation)
<i>Bos taurus</i> mRNA for similar to ribosomal protein S14, partial cds (RPS14)	AB099089	2.1	Structural constituent of ribosome (translation)
<i>Bos taurus</i> mRNA for similar to ubiquitin-S27a fusion protein (RPS27A)	AB098891	1.9	Structural constituent of ribosome
<i>Bos taurus</i> ribosomal protein S29, mRNA (RPS29)	NM_174804	2.4	Structural constituent of ribosome
<i>Bos taurus</i> ribosomal protein S29 mRNA, complete cds (RPS29)	U66372	1.9	Structural constituent of ribosome
<i>Bos taurus</i> mRNA for similar to ribosomal protein L18a, partial cds, (RPL18A)	AB098916	2.4	Structural constituent of ribosome
<i>Bos taurus</i> mRNA for similar to ribosomal protein L9, partial cds, (RP L9)	AB099048	1.9	Structural constituent of ribosome
<i>Bos taurus</i> ribosomal protein L24 mRNA (RPL24)	NM_174455	2.0	Structural constituent of ribosome (translation)
<i>Bos taurus</i> type 4 mucus-type core 2 (GCNT3)	AY283766	2.3	Glucosyltransferase activity
<i>Homo sapiens</i> asparagine-linked glycosylation 6 homologue (ALG6)	BC001253	2.4	Glucosyltransferase activity (N-linked glycosylation)
<i>Homo sapiens</i> RIO kinase 3 (yeast) transcript variant 1, mRNA (RIOK3)	NM_003831	2.1	Transferase activity (phosphorylation)
<i>Bos taurus</i> S-adenosylmethionine decarboxylase 1 mRNA (AMD1)	NM_173990	2.1	Lyase activity (spermine biosynthetic process)
<i>Homo sapiens</i> tectorin- β mRNA, complete cds (TECTB)	AF312827	1.9	Glycosylphosphatidylinositol anchor binding
<i>Homo sapiens</i> galactokinase 2, mRNA (GALK2)	NM_002044	1.9	Galactokinase activity (galactose metabolic process)
<i>Bos taurus</i> ornithine decarboxylase (ODC1), mRNA	NM_174130	2.1	Ornithine decarboxylase activity (polyamine biosynthetic)
<i>Bos taurus</i> seryl-tRNA synthetase mRNA, complete cds (SARS)	AF297553	2.0	Ligase activity (seryl-tRNA aminoacylation)
<i>Bos taurus</i> mitochondrion, complete genome	AY526085	2.0	Oxidoreductase activity (electron transport)
<i>Homo sapiens</i> degenerative spermatocyte homologue, lipid desaturase (<i>Drosophila</i>), mRNA (DEGS1)	BC000961	2.2	Electron carrier activity (lipid metabolic process)
<i>Homo sapiens</i> tropomyosin 3, mRNA (TPM3)	NM_153649	2.3	Actin binding (cell motility)
<i>Homo sapiens</i> cytoplasmic dynein intermediate chain mRNA, complete cds (DYNC112)	AY037160	2.0	Microtubule motor activity (microtubule movement)
<i>Homo sapiens</i> kinesin family member 20A, mRNA (KIF20A)	BC012999	2.9	Microtubule motor activity (microtubule movement)
<i>Bos taurus</i> zona pellucida glycoprotein 4, mRNA (ZP4)	NM_173975	2.0	Receptor activity (fertilisation)
<i>Homo sapiens</i> tripartite motif-containing 51, mRNA (SPRYD5)	BC005014	2.0	Unknown
<i>Bos taurus</i> p97 protein mRNA (CFDP2)	NM_174800	2.0	Unknown
<i>Homo sapiens</i> haematological and neurological expressed 1, mRNA (HN1)	BC039343	2.0	Unknown
<i>Arabidopsis thaliana</i> T-DNA flanking sequence, left border, clone	AJ521477	2.0	Unknown
<i>Homo sapiens</i> transforming, acidic coiled-coil containing protein 3 (TACC3)	NM_006342	2.3	Unknown
<i>Homo sapiens</i> WW domain containing adaptor with coiled-coil, mRNA (WAC)	BC004258	2.3	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$.

important role in reprogramming gene expression during zygotic genome activation (ZGA) (Schultz & Worrall 1995, Kanka 2003). For example, an apparent increase in histone acetylation accompanies the one- to two-cell transition in the mouse (Sarmiento *et al.* 2004). Chromatin remodelling enzymes belong to the SNF2 family of DNA-dependent ATPases, all of which have a helicase-like ATPase domain (Henikoff 1993). The SWI/SNF ATP-dependent chromatin remodelling complexes are example of these families and SMARCA5 represents one of its members. Mammalian SWI/SNF-related chromatin remodelling complexes regulate transcription and are good candidates for being involved in ZGA in mice (Bultman *et al.* 2006). The expression of *SMARCA1* as another member of this family was increased in eight-cell embryos compared with MII oocytes, which suggest a potential role in regulation of embryonic genome activation (Misirlioglu *et al.* 2006). In addition, the balance of chromatin remodelling factors present in the early cleavage stages can dramatically affect embryo development (Magnani & Cabot 2007). Homozygous *SMARCA4* knockout mouse embryos arrest during pre-implantation development (Bultman *et al.* 2000). Several other subunits of SWI/SNF-related complexes, often referred to as BRG1-associated factors, have also been knocked out and confer periimplantation lethality as well (Klochender-Yeivin *et al.* 2000, Guidi *et al.* 2001). Consistent with this, greater mRNA abundance (6.7-fold change) of the *SMARCA5* transcript was detected in BCB⁺ (with higher developmental competence) when compared with BCB⁻ oocytes. Alterations in the expression of some of genes encoded chromatin regulatory factors in rhesus monkey oocytes of different developmental potentials suggest that the expression of such transcripts could provide useful markers of oocyte quality (Zheng *et al.* 2004).

The bovine oocyte, zygote and embryo have a profound need for protein synthesis. However, the mRNA transcripts for these proteins are not synthesised throughout development, but rather during specific phases (Hyttel *et al.* 2001). In mammals, synthesis of RNA, up to 60–65% of which is ribosomal (rRNA), increases during oocyte growth and reaches a peak at the beginning of follicular antrum formation (Wassarman & Kinloch 1992). This is in accordance with our investigation concerning meiotic configuration in BCB⁻ oocytes (Table 1). These oocytes with insufficient cytoplasmic maturation, under the control of high G6PDH activity, and in the end of oocyte growth showed a proportion of 21.4% with morphological features for rRNA synthesis – nucleoli. In contrast, in BCB⁺ oocytes only a small proportion (1.8%) showed germinal vesicles with nucleoli. This process of nucleolus remodelling in GV-containing oocytes is a marker for the finished r-RNA synthesis for the establishment of sufficient ribosomes for the following protein synthesis during the final oocyte maturation after GVBD. In our

previous studies, we found an increased level in protein synthesis during final oocyte maturation after GVBD, not before (Tomek *et al.* 2002a, 2002b).

Elongation factor 1 α is a component of the eukaryotic translational apparatus and it is also a GTP-binding protein that catalyses the binding of aminoacyl tRNAs to the ribosome (Tatsuka *et al.* 1992). The tRNA carries the amino acid to the ribosome, which is then used in protein synthesis, thereby inferring a crucial role for this factor in the translation process in protein biosynthesis. Acquisition of high developmental capacity in mammalian oocytes is dependent on high rates of RNA and protein synthesis, imprinting processes and biogenesis of organelles such as mitochondria (Eichenlaub-Ritter & Peschke 2002). Consistent with this, oocytes with greater developmental potential (BCB⁺) showed higher mRNA transcript abundance for RPS27A and EEF1A1 that represent members of ribosomal and translation related genes respectively. Collectively, it is possible to conclude that BCB⁺ oocytes have greater stores of cell cycle, transcription and protein biosynthesis transcripts that could be used for resuming meiosis (Tatemoto & Horiuchi 1995) and supporting maternal to zygotic transition (Hyttel *et al.* 2001). This is in accordance with the results obtained with respect to the developmental competence of BCB⁺ and BCB⁻ oocytes (Table 2).

Concerning the activity of cell cycle proteins in oocytes, it has been shown previously that maturing bovine oocytes possess the highest phosphorylation of MAPKs in MII and of Akt in MI stage (Tomek & Smiljakovic 2005, Bhojwani *et al.* 2006). Furthermore, it has been shown that these phosphorylations are tightly correlated with the activities of the kinases. Therefore, from our observations (Fig. 1), it can be concluded that BCB⁺ GV stage oocytes have a higher basal activity regarding MAPK and Akt, which probably positively influences their developmental competence and which is well reflected by corresponding gene expression.

The reduced developmental capacity of early embryonic development has been associated with mitochondrial dysfunction and low ATP in mammalian oocytes and embryos (Keefe *et al.* 1995, Barnett *et al.* 1997, Van Blerkom *et al.* 1998, Van Blerkom 2004). Recently, the amount of mitochondrial DNA and transcripts has been quantified in bovine oocytes and embryos (May-Panloup *et al.* 2005) showing that bovine oocytes that failed to cleave contained significantly lower transcripts implicated in mitochondrial biogenesis. A global down-regulation of mitochondrial transcripts has been reported in human compromised oocytes and embryos (Hsieh *et al.* 2004). In the pig, competent BCB⁺ oocytes contain more copies of mtDNA and are more likely to be fertilised than incompetent BCB⁻ oocytes (El-Shourbagy *et al.* 2006). However, supplementation of BCB⁻ oocytes with mitochondria from BCB⁺ oocytes, and subsequent improved fertilisation outcome, again demonstrates the association between mitochondrial number and fertilisation outcome.

Table 5 Genes down-regulated in brilliant cresyl blue (BCB⁺) compared with BCB⁻ oocytes.

Gene name	Accession no. in GenBank	Fold change	Gene function (biological process)
<i>Homo sapiens</i> neuronal pentraxin II mRNA (NPTX2)	NM_002523	11.5	Calcium ion binding (synaptic transmission)
<i>Bos taurus</i> S100 calcium-binding protein A10 mRNA (S100A10)	NM_174650	11.2	Calcium ion binding
<i>Homo sapiens</i> S100 calcium-binding protein A14 mRNA (S100A14)	NM_020672	10.6	Calcium ion binding
<i>Homo sapiens</i> S100 calcium-binding protein A16, mRNA (S100A16)	BC019099	10.5	Calcium ion binding
<i>Homo sapiens</i> chloride intracellular channel mRNA 1 (CLIC1)	NM_001288	10.4	Chloride ion binding (chloride transport)
Human cysteine-rich intestinal protein mRNA, complete cds (CRIP1)	U58630	9.7	Metal ion binding
<i>Homo sapiens</i> hypothetical protein DKFZp564K0822, mRNA (ECOP)	BC016650	9.5	Signal transducer activity
<i>Homo sapiens</i> basigin long isoform mRNA, complete cds (BSG)	AF548371	9.8	Signal transducer (cell surface receptor linked signal transduction)
<i>Bos taurus</i> mRNA for similar to galactose-binding lectin, partial (LGALS1)	AB099039	6.3	Signal transducer (regulation of apoptosis)
<i>Bos taurus</i> T cell receptor alpha gene, J segments and C region (TCRA)	AY227782	9.1	Transferase activity (apoptosis)
<i>Bos taurus</i> arachidonate 15-lipoxygenase (ALOX15), mRNA	NM_174501	6.3	Lipoxygenase activity (anti-apoptosis)
<i>Homo sapiens</i> , clone IMAGE:4428430, mRNA (PARP12)	BC044660	6.0	Transferase activity (protein amino acid ADP-ribosylation)
<i>Bos taurus</i> conserved helix-loop-helix ubiquitous kinase mRNA (CHUK)	NM_174021	7.4	Transferase activity (immune response)
<i>Homo sapiens</i> fumarate hydratase, mRNA (cDNA clone MGC:15363 (FH))	BC017444	8.6	Fumarate hydratase activity (cell cycle)
<i>Homo sapiens</i> nucleophosmin (nucleolar phosphoprotein B23 numatrin), mRNA (NPM1)	BC016768	7.4	RNA binding (anti-apoptosis)
<i>Homo sapiens</i> poly(A) polymerase gamma (PAPOLG), mRNA	NM_022894	3.7	RNA binding (RNA polyadenylation)
<i>Homo sapiens</i> KIAA0020 mRNA, complete cds (KIAA0020)	D13645	5.9	RNA binding
<i>Homo sapiens</i> maelstrom homologue (<i>Drosophila</i>) mRNA (MAEL)	BC028595	7.3	DNA binding
<i>Homo sapiens</i> zinc finger, BED domain containing 4, mRNA (ZBED4)	NM_014838	7.2	DNA binding
<i>Homo sapiens</i> pituitary tumour-transforming 1, mRNA (PTTG1)	NM_004219	3.7	Transcription factor binding
<i>Homo sapiens</i> centromere protein F, 350/400 kDa (mitosin; CENPF)	NM_016343	7.1	Chromatin binding (G2 phase of mitotic cell cycle)
<i>Mus musculus</i> ADP-ribosylation factor 4 mRNA (Arf4)	NM_007479	6.7	Nucleotide binding
<i>Homo sapiens</i> RAN, member RAS oncogene family, mRNA (RAN)	BC014901	6.7	GTP binding (DNA metabolic process)
<i>Homo sapiens</i> F-box only protein 5 mRNA (FBXO5)	NM_012177	5.4	Protein binding (cell cycle)
<i>Bos taurus</i> BTAB2MDS3 β -2-microglobulin gene, 3' UTR (B2M)	AY325771	4.9	Protein binding (immune response)
<i>Homo sapiens</i> NACHT, leucine-rich repeat and PYD containing 2, mRNA (NLRP2)	BC001039	4.4	Protein binding (apoptosis)
<i>Homo sapiens</i> chromosome 15 open reading frame 23 (C15orf23), mRNA	NM_033286	6.7	Protein binding
<i>Homo sapiens</i> GrpE-like 1, mitochondrial (<i>E. coli</i>), mRNA (GRPEL1)	BC024242	4.6	Unfolded protein binding
<i>Homo sapiens</i> ralA-binding protein 1, mRNA (RALBP1)	BC013126	7.1	Protein binding (signal transduction)
<i>Homo sapiens</i> F-box only protein 34, mRNA (FBXO34)	NM_017943	3.3	Protein transport
<i>Bos taurus</i> non-selenium glutathione phospholipid hydroperoxide (AOP2)	AF090194	2.3	Oxidoreductase activity (response to reactive oxygen species)
<i>Bos taurus</i> prostaglandin G/H synthase-2 mRNA, complete cds (PGHS-2)	AF031698	3.5	Oxidoreductase activity (prostaglandin biosynthetic process)
<i>Homo sapiens</i> retinol dehydrogenase 11 (all-trans and 9-cis), mRNA (RDH11)	BC026274	6.1	Oxidoreductase activity (metabolic process)
<i>Bos taurus</i> NADH dehydrogenase (ubiquinone) 1 α -subcomplex, 7 (NDUFA7)	NM_176658	2.5	Oxidoreductase activity (mitochondrial electron transport)
<i>Bos taurus</i> cytochrome c oxidase subunit VIIa polypeptide 2 (liver; COX7A2)	NM_175807	5.3	Cytochrome c oxidase activity (electron transport)
<i>Bos taurus</i> isolate FL405 mitochondrion, partial genome (FL405)	AY308069	3.1	Oxidoreductase activity (mitochondrial electron transport)
<i>Bos taurus</i> ATP synthase, H ⁺ transporting, mitochondrial F0 complex (ATP5G2)	NM_176613	2.4	Hydrogen ion transporting ATPase activity (ATP synthesis)
<i>Bos taurus</i> ATP synthase, H ⁺ transporting, mitochondrial F1 complex (ATP5A1)	NM_174684	3.8	Hydrogen ion transporting ATPase activity (ATP synthesis)

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$.

Table 6 Genes down-regulated in brilliant cresyl blue (BCB⁺) compared with BCB⁻ oocytes.

Gene name	Accession no. in GenBank	Fold change	Gene function (biological process)
<i>Homo sapiens</i> lectin, galactoside-binding, soluble, 3 (galectin 3), (LGALS3)	BC001120	2.4	Immunoglobulin binding of the IgE isotype
<i>Bos taurus</i> mRNA for StAR protein	Y17259	2.4	Cholesterol binding (regulation of steroid biosynthetic process)
<i>Homo sapiens</i> bone morphogenetic protein 15 precursor gene (BMP15)	AF082350	5.1	Growth factor activity (female gamete generation)
<i>Bos taurus</i> bone morphogenetic protein 15 mRNA, partial cds (BMP15)	AY304484	3.9	Growth factor activity
<i>Bos taurus</i> partial mRNA for bone morphogenetic protein 15 (BMP15)	AJ534391	2.7	Growth factor activity
Bovine mRNA fragment for cytokeratin A (no. 8; KRT8)	X12877	3.1	Structural molecule activity
<i>Homo sapiens</i> calmodulin 2 (phosphorylase kinase, delta) (CALM2)	NM_001743	2.0	Unknown
Human DNA sequence from clone RP11-146N23 on chromosome 9, complete (DENND4C)	AL161909	2.3	Unknown
<i>Bos taurus</i> BAC CH240-454H24 complete sequence	AC150492	2.4	Unknown
Bovine thymus satellite I (1.715 g/ml) DNA	J00037	2.5	Unknown
Bovine satellite DNA fragment	V00121	2.2	Unknown
<i>Homo sapiens</i> chromosome 8 clone CTC-369M3 map 8q24.3, complete sequence	AF186190	2.2	Unknown
<i>Homo sapiens</i> chromosome 16 clone RP11-19H6, complete sequence	AC012175	2.4	Unknown
Dictyostelium discoideum extrachromosomal palindromic rRNA	AY171067	2.0	Unknown
<i>Bos taurus</i> clone rp42-194o5, complete sequence	AC098687	5.1	Unknown
<i>Bos taurus</i> clone RP42-351K5, complete sequence	AC092727	5.6	Unknown
<i>Bos taurus</i> butyrophilin gene, complete cds (BTN1A1)	AF005497	2.2	Unknown
<i>O. aries</i> mRNA for thyroid hormone receptor β 1 (ERBA β 1)	Z68307	5.0	Unknown
<i>Bos taurus</i> DNA for SINE sequence Bov-tA	X64124	2.9	Unknown
<i>Bos taurus</i> X-inactivation centre region, Jpx and Xist genes (XIST)	AJ421481	2.1	Unknown
<i>B. taurus</i> DNA for SINE sequence Bov-2	X64125	2.5	Unknown
<i>Bos taurus</i> clone RP42-400M23, complete sequence	AC090976	2.2	Unknown
<i>Bos taurus</i> clone RP42-221D7, complete sequence	AC136966	2.0	Unknown
<i>Bos taurus</i> clone rp42-513g13, complete sequence	AC107065	2.0	Unknown
<i>Homo sapiens</i> placenta-specific 8, mRNA (PLAC8)	NM_016619	2.7	Unknown
<i>B. taurus</i> cosmid-derived repetitive DNA (clone IDVGA-50; subclone3Rev)	X89421	2.3	Unknown
<i>Homo sapiens</i> chromosome 5 clone CTC-448D22, complete sequence	AC093206	2.2	Unknown
Mouse DNA sequence from clone RP23-44F9 on chromosome 11, complete	AL935275	2.5	Unknown
<i>Mus musculus</i> 11 days embryo gonad cDNA, RIKEN full-length (7030402D04Rik)	AK078561	2.1	Unknown
<i>B. primigenius</i> mRNA for α -cop coat protein	X96768	2.0	Unknown
<i>Homo sapiens</i> G antigen, family C 1, mRNA (PAGE4)	NM_007003	2.0	Unknown
Canis β -galactosides-binding lectin (LGALS3) mRNA, 3' end	L23429	2.4	Unknown
Gallus gallus finished cDNA, clone ChEST201k3	BX950233	2.0	Unknown

Differentially expressed genes were identified by SAM at a false discovery rate (FDR) of $\leq 5\%$ and $P \leq 0.05$.

Figure 2 Hierarchical clustering and heatmap of differentially expressed genes. The red blocks represent up-regulated genes, while the green blocks represent down-regulated genes in BCB⁺ compared with BCB⁻ oocytes. Columns represent individual hybridisations, rows represent individual genes.

Mouse BCB⁺ oocytes gained better cytoplasmic maturity than BCB⁻ oocytes as determined by a higher intracellular glutathione (peroxidase 1) level, fully polarised mitochondrial distribution (most of mitochondria aggregated in the oocyte hemisphere around the MII spindle). In this study, it is remarkable that oocytes with high G6PDH activity (BCB⁻) had an increased level of mitochondrial fluorescence intensity and up-regulation of mitochondrial transcripts (*ATP5A1* and *FL405*) compared with BCB⁺ oocytes. One can speculate that the reason for the higher

fluorescence intensity of labelled mitochondria in BCB⁻ oocytes is likely the increased respiratory activity to provide ATP for still unfinished processes in cytoplasmic maturation. In a recent study, incompetent (BCB⁻) oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors and the oocyte attempts to rescue this during the final stages of maturation. Consequently, oocyte competence in terms of mtDNA replication and composition is not fully synchronised and will result in

Figure 3 Differentially expressed genes in BCB⁺ as classified based on the Gene Ontology Consortium classifications (<http://www.geneontology.org>).

Figure 4 Differentially expressed genes in BCB⁻ as classified based on the Gene Ontology Consortium classifications (<http://www.geneontology.org>).

either failed fertilisation or developmental arrest (Spikings *et al.* 2007). In addition, it could be possible that the higher level of mitochondrial fluorescence intensity in BCB⁻ oocytes may be due to increased oxidative stress in these oocytes. *ATP5A1* is a nuclear-encoded gene whose protein contributes to the overall function of the ATP synthase and it is the universal enzyme for cellular ATP synthesis (Pedersen 1994). It has been reported that null mutations in ϵ -subunit of mitochondrial ATP synthase gene in *Drosophila* lead to embryonic death (Kidd *et al.* 2005). *ATP6V1E1* transcript was up-regulated at two-cell block mouse embryos (Jeong *et al.* 2006). From the above-mentioned facts, it is clear that alterations in mitochondrial distribution, DNA replication, copy number and transcripts may lead to overall dysfunction for the mitochondria and influence the ability of embryos to scavenge free radicals and also induce an oxidative stress response, which contributes to impaired development. It seems also that the competency of oocytes is highly dependent on distinct set of genes mainly regulating transcription, translation, cell cycle, chromatin remodelling and mitochondrial machineries which may interact to fulfil this task.

Overall, this study provides a genome-wide expression profiling of genes that could be associated with functional relevance for the establishment of developmental competence in oocytes. However, further functional investigations based on these data could help to define the exact key regulatory genes controlling oocyte quality, which could be considered as good biomarkers for oocytes with high or low developmental competence.

Materials and Methods

Oocyte recovery and BCB staining

Oocytes aspirated from slaughterhouse ovaries were used for BCB staining. The procedure of BCB staining was done as described in our previous studies (Alm *et al.* 2005, Bhojwani *et al.* 2007). Briefly, a total of 2128 morphologically good quality compact cumulus-oocyte complexes (COCs) were subjected to 26 μ M BCB (B-5388, Sigma-Aldrich) diluted in mDPBS for 90 min at 38.5 °C in humidified air atmosphere. After washing, the stained COCs were examined under stereomicroscope and categorised into two groups according to their cytoplasm colouration: oocytes with any degree of blue colouration in the cytoplasm (BCB⁺;

$n=1167$) and oocytes without visual blue colouration (BCB⁻; $n=961$). From each group, oocytes were used for: analysis of chromatin configuration and mitochondrial activity ($n=337$); detection of abundance and phosphorylation of protein kinases Akt and MAP ($n=500$); investigation of gene expression ($n=1032$) and assessment of *in vitro* development during IVM, IVF and IVC ($n=259$).

In vitro maturation, fertilisation and culture ($n=259$ COCs)

After classification in BCB⁻ and BCB⁺, the COCs were washed twice in maturation medium (TCM 199 supplemented with 20% (v/v) heat-treated fetal calf serum and 10 μ g/ml follicle-stimulating hormone (Ovagen; Auckland, ICP, New Zealand) and then incubated in maturation medium for 24 h at 38.5 °C in 5% CO₂ in air.

After IVM, oocytes were fertilised *in vitro* using frozen-thawed bovine semen. A motile sample of sperm was obtained by swim-up separation based on the method of Lonergan *et al.* (1994). Approximately 0.25 ml cryopreserved semen was layered under 1 ml capacitation base medium (modified Ca²⁺-free Tyrode's medium). Following incubation for 1 h, the uppermost 0.5–0.8 ml of medium containing motile

Figure 5 Quantitative real-time PCR validation of ten differentially expressed genes in BCB⁺ and BCB⁻ oocytes as identified by microarray analysis (A and B). The relative abundance of mRNA levels represents the amount of mRNA compared with the calibrator (with the lowest normalised value). Bars with different superscripts (a and b) are significantly different at $P < 0.05$.

spermatozoa was removed and washed twice with 2–3 ml capacitation base medium followed by centrifugation at 500 g for 7 min. The resulting pellet was measured using an adjustable micropipette. A 50–60 µl aliquot of the swim-up separated spermatozoa were then diluted with an equal volume of capacitation medium containing 200 µg/ml heparin (H 3393). After incubation for 15 min, the suspension was further diluted with capacitation base medium to reduce the concentration of capacitation inductors and to obtain the desired final concentration of spermatozoa for IVF.

After maturation, oocytes were transferred to modified TALP medium and cumulus cells were removed mechanically by gentle pipetting. Five oocytes were placed in a 45 µl droplet of fertilisation medium (TALP; [Lonergan et al. 1994](#)) and 5–8 µl of the final sperm suspension were added to each droplet to have a final concentration of $\sim 1.0 \times 10^6$ motile sperm/ml in the fertilisation droplet. Fertilisation was carried out for 24 h at 38.5 °C under 5% CO₂ in 100% humidified air.

After 20 h coincubation with spermatozoa, presumptive zygotes were denuded and transferred to TCM 199 containing 5% oestrous cow serum. Another 24 h later, the embryos were cultured in synthetic oviductal fluid medium (Minitüb, Tiefenbach, Germany) supplemented with 10% oestrous cow serum and covered with mineral oil. Embryo culture was performed at 38.5 °C in 5% CO₂, 5% O₂ and 90% N₂ and development was evaluated at 48 h (cleavage rate) and at 192 h (day 8; blastocyst rate).

The cleavage rate (number of eggs that had cleaved to the two-cell stage or beyond at 48 h after IVF) and the proportion of blastocysts developing at the end of the 8-day culture period were compared among groups. The number of blastomeres (nuclei) in embryos was determined using the Hoechst staining technique ([Alm & Hinrichs 1996](#)).

Parallel fluorescence labelling of oocytes for the analysis of chromatin configuration and mitochondrial activity (n = 337 COCs)

Oocyte processing

Oocytes were processed for fluorescence labelling of mitochondria according to the procedure described previously for porcine and horse oocytes ([Torner et al. 2004, 2007](#)). Briefly, COCs were incubated for 30 min in PBS containing 3% (w/v) BSA and 200 nM MitoTracker Orange-fluorescent tetramethylrosamine (M-7510; Molecular Probes, Eugene, OR, USA) under culture conditions. The mitochondrial-specific fluorescent and cell-permeant probe MitoTracker Orange (M-7510) is readily sequestered only by actively respiring organelles, depending upon their oxidative activity. Following exposure of COCs to the probe, cumulus cells were mechanically removed from the oocytes by repeated pipetting and subsequent treatment with 3% sodium citrate. The denuded oocytes were washed thrice in pre-warmed PBS without BSA. The oocytes were then fixed for 15 min at 37 °C using freshly prepared 2% (v/v) paraformaldehyde in Hank's balanced salt solution. The thiol-reactive chloromethyl moiety of the probe can react with accessible thiol groups on peptides and proteins of active mitochondria to form an aldehyde-fixable fluorescent conjugate, which is retained after cell fixation

over a period of 8 weeks. Immediately after fixation, the same oocytes were prepared for the further staining of chromatin configuration. They were washed thrice in PBS and then mounted between slide and cover slip in a mixture of Moviol V4-88 (133 mg/ml, Hoechst, Frankfurt, Germany) and *n*-propyl gallate (5 mg/ml, Sigma) containing 2.5 µg/ml bis-benzimide (Hoechst 33342, Sigma) to detect chromatin configuration. The slides were kept for 2–3 weeks at 4 °C in darkness until oocyte analysis.

Oocyte analysis

An epifluorescence microscope (Jenalumar, Carl Zeiss, Jena, Germany) was used for all experiments. First, the chromatin configuration in each oocyte was evaluated under u.v. fluorescence at 410 nm. The chromatin configuration was classified according to the onset of meiotic stages into diplotene with nucleolus (Dipl+Nuc), diplotene (Dipl), condensed chromatin in germinal vesicle (CC), diakinesis (Dia), metaphase I (MI), metaphase II (MII) and degenerated pycnotic chromatin configuration (Pyc).

For subsequent evaluation of mitochondrial activity at $\times 500$ magnification, the emission wavelengths were separated by a 540 nm dichroic mirror followed by further filtering through a 570 nm long pass filter (red emission). Only the labelled mitochondria that were actively respiring were recorded. The fluorescence intensity per oocyte (µA) was measured by the Nikon Photometry System P 100 (Nikon, Düsseldorf, Germany) as described in pig, horse and bovine oocytes ([Torner et al. 2004, 2007](#), [Kuzmina et al. 2007](#)). For measurement of intensity, we placed the whole oocyte (thickness around 20 µm) with the eyepiece of the Photometer head P 100 in a defined area of measurement (same size for all observation). The measured intensity was not influenced by the focus of objectives, e.g. different levels of observation (0–20 nm in the oocyte) led to the same quantitative measurement of emitted fluorescence light, because the Photometer measured all emission light from the whole oocyte in the area of frame. To exclude unspecific or artificial effects of the fluorescence probe, we stored different categories of COCs immediately after recovery in the refrigerator (4 °C) for 5 days. Following staining and fixing with the same protocol as described, we determined only the fluorescence intensity of the oocytes in the same level like background fluorescence. We used the level of amplification for photomultiplier, which allow estimation of the highest and the lowest intensity of light emission in a measurement area of linearly progression. Microscope adjustments and photomultiplier settings were kept constant for all experiments.

Detection of abundance and phosphorylation of protein kinases Akt and MAP (n = 500 COCs)

BCB⁺ and BCB[−] oocytes were analysed for the abundance and phosphorylation state of the MAPKs, ERK1, ERK2 and the protein kinase Akt by Western blotting. The MII stage oocytes (for MAPK) where MAPK shows the highest phosphorylation ([Tomek et al. 2002a](#)) and MI stage oocytes (for Akt) where Akt shows the highest phosphorylation ([Tomek & Smiljakovic 2005](#)) were used as positive controls. Denuded oocytes (50 each) were separated on 10% SDS-gels and transferred to

PVDF membranes as described (Vanselow *et al.* 2006). After blocking the membrane with 5% fat-free dry milk in TTBS, the blot were incubated overnight at 4 °C with antibodies against MAPKs (diluted 1:2500, sc-94; Santa Cruz Biotechnology, Heidelberg, Germany), phospho-MAPK Thr282, Tyr294, Akt1 (diluted 1:1000, CST 9272; Cell Signalling Technologies, Frankfurt a. Main, Germany) phospho-Akt Ser473 (diluted 1:800, CST 9271) and phosphor-Akt Thr308 (diluted 1:800; CST 9275). The blots were washed and incubated with a second HRP-conjugated anti-rabbit IgG (diluted 1:4000) as described previously (Tomek *et al.* 2002a, Bhojwani *et al.* 2006). The bands were visualised with ECL according to the manufacturer's instruction (GE Healthcare, Freiburg, Germany). The experiments were repeated once and representative blots are shown in Fig. 1.

Investigation of gene expression (n = 580 COCs)

From each BCB group, three pools of oocytes (each with 110 oocytes and a total of 330) were used for mRNA isolation and subsequent array hybridisation after removal of cumulus cells. The remaining oocytes from each group were used as independent samples for array results validation using real-time PCR. In this study, a bovine cDNA array (BlueChip v.2 with ~2000 clones or genes; Sirard *et al.* 2005) was used to investigate the gene expression profiles.

Oocyte denudation and storage

The surrounding cumulus cells were removed from the oocytes of each group by treatment with hyaluronidase 1 mg/ml (Sigma) and gentle pipetting in maturation medium. Separation of cumulus cells was carefully checked under a stereomicroscope. Cumulus-free oocytes and the corresponding cumulus cells of each group were washed twice in PBS (Sigma) and snap frozen separately in cryotubes containing 20 µl lysis buffer (0.8% IGEPAL (Sigma), 40 U/µl RNasin (Promega), 5 mM dithiothreitol (Promega)). Finally, samples were stored at –80 °C until RNA extraction.

RNA isolation

mRNA isolation of oocytes and cumulus cells was performed at two different points during the whole experiment. (1) A total of six pools, each containing 110 oocytes, was used for array analysis after amplification. (2) A total of 10 pools, each containing 25 oocytes, was used for real-time validation of array results. The mRNA isolation was performed using Dynabead oligo (dT)₂₅ (DynaL Biotech, Oslo, Norway) according to manufacturer's instructions. Briefly, oocytes in lysis buffer were mixed with 40 µl binding buffer (20 mM Tris–HCl with pH 7.5, 1 M LiCl, 2 mM EDTA with pH 8.0) and incubated at 70 °C for 5 min to obtain complete lysis of the oocytes and to release RNA. Ten microlitres of oligo (dT)₂₅ attached magnetic bead suspension was added to the samples, and incubated at room temperature for 30 min. The hybridised mRNA and magnetic beads were washed thrice using washing buffer (10 mM Tris–HCl with pH 7.5, 0.15 mM LiCl, 1 mM EDTA with pH 8.0). For each sample, cDNA synthesis has been

performed using oligo (dT)₂₃ primer and superscript reverse transcriptase II (Invitrogen) except for samples used in array analysis where the RT was performed using T7 promotor attached oligo d(T)₂₁ primer.

RNA amplification

First- and second-strand cDNA synthesis was carried out as described in our previous study (El-Sayed *et al.* 2006). The resulting double-stranded cDNA was purified and used for *in vitro* transcription using AmpliScribe T7 transcription kit (Epicentre technologies, Oldendorf, Germany) according to manufacturer's instructions. Then, the amplified RNA (aRNA) was purified using RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. Finally, the aRNA was eluted in 30 µl RNase-free water from which 8 µl was taken to estimate the yield, purity of aRNA by gel electrophoresis and u.v. absorbance reading at A260/280 using Ultrospec 2100 pro u.v./Visible Spectrophotometer (Amersham Bioscience).

Labelling and array hybridisation

Minimum information about microarray experiments guidelines were adhered to in the experimental design. Two independent labelling reactions were carried out per aRNA sample pertinent to each biological replicate for dye-swap hybridisations. Accordingly, 3 µg aRNA from each oocyte pool representing each oocyte group (BCB⁺ or BCB[–]) was used as a template in RT reactions incorporating amino-modified dUTPs into the cDNA using the CyScribe Post-Labeling Kit (Amersham Biosciences) as described previously (El-Sayed *et al.* 2006). The aminoallyl-labelled cDNA samples from BCB⁺ and BCB[–] oocytes were differentially labelled indirectly using *N*-hydroxysuccinate-derived Cy3 and Cy5 dyes and incubated for 1.5 h at room temperature in darkness. At the end of incubation, non-reacting dyes were quenched by adding 15 µl of 4 M hydroxylamine solution (Sigma) and incubated for 15 min at room temperature in darkness. To avoid variation due to dye coupling, aRNA samples from the same follicular phase were labelled reversibly either with Cy3 or Cy5 for dye-swap hybridisations. The reaction was then purified with CyScribe GFX purification kit (Amersham Biosciences). Samples were finally eluted in 60 µl elution buffer.

Pre-hybridisation of the slides was performed by placing the array slides into a corning GAPS II slide container as described in El-Sayed *et al.* (2006). Hybridisation and post-hybridisation washes were carried out as previously described elsewhere (Hedge *et al.* 2000) with slight modifications as described in Ghanem *et al.* (2007). Samples that were going to be hybridised on specific array were mixed and dried in speedvac centrifuge (Savant Instruments Inc., Holbrook, NY, USA) and then the pellet was re-suspended in pre-warmed (42 °C) formamide-based hybridisation buffer (15 µl hybridisation buffer (Amersham Bioscience), 30 µl 100% formamide and 15 µl DEPC water). Yeast tRNA (4 mg/ml) and 2.5 µl Cot-human DNA (1 mg/ml; Invitrogen) were used in the reaction in a volume of 2.5 µl each to avoid non-specific hybridisation.

Table 7 Details of primers used for real-time PCR quantitative analysis.

Gene name	GenBank accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
BMP15	AY304484	F: 5'-CTGACGCAAGTGGACACCCTA-3' R: 5'-GACACACGAAGCGGAGTCGTA-3'	60	396
PTTG1	NM_004219	F: 5'-GAAGAGCACCAGATTGCGC-3' R: 5'-GTCACAGCAAACAGGTGGCA-3'	55	194
S100A10	NM_174650	F: 5'-GGATTCTGAGCATATGGGACC-3' R: 5'-GAGCAAGAGGATGCAAGCAATA-3'	55	131
NASP	BT006757	F: 5'-CCTAGAGCTTGCCTGGGATATG-3' R: 5'-TCGTGGGCTTCCAGGTACTG-3'	55	198
SMARCA5	NM_003601	F: 5'-AGTGAACCTTCGCCCATCTTG-3' R: 5'-AGGCTTGTGGATCAGAACTCG-3'	55	194
EEF1A1	AB060107	F: 5'-CCATGGCATATTAGCACTTGGTT-3' R: 5'-GCTTACACCCTGGGTGTGA-3'	55	214
ODC1	NM_174130	F: 5'-CAAAGGCCAAGTTGGTTTTAC-3' R: 5'-CAGAGATGGCTGCACAAAG-3'	55	201
ATP5A1	NM_174684	F: 5'-CTCTTGAGTCGTGGTGTGCG-3' R: 5'-CCTGATGTTGGCTGATAACGTG-3'	55	184
RPS27A	AB098891	F: 5'-TGCAGATTTTCGTGAAGACCCT-3' R: 5'-TTCTTTATCCTGGATCTTGGCC-3'	54	203
GAPDH	BC102589	F: 5'-ACCCAGAAGACTGTGGATGG-3' R: 5'-ACGCCTGCTTACCACCTTC-3'	60	247

Array scanning and data analysis

The slides were scanned using Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA). The GenePix Pro 4.0 software (Axon Instruments) was used to process the images, to find spots, to integrate robot-spotting files and finally to create reports of spot intensity data. The LOWESS normalisation of microarray data was performed using GProcessor 2.0a software (<http://bioinformatics.med.yale.edu/group>). The normalised data were used to calculate intensity ratios of all replicates and to obtain one value per clone. Ratios were finally log₂ transformed and submitted to SAM analysis. Microarray data analysis was performed using SAM (Significance Analysis for Microarray), a free software developed at Stanford University (<http://www-stat.stanford.edu/~tibs/SAM/>). Hierarchical clustering and heatmap of log₂-transformed data for up- and down-regulated genes was generated using PermutMatrix (version 1.8.2) available at (<http://www.lirmm.fr/%7Ecaraux/PermutMatrix/>). Genes expressed equally in both samples were not included in the hierarchical clustering.

Quantitative real-time PCR analysis

To validate microarray results, ten candidate genes were selected for further analysis by real-time PCR (Table 7). Quantitative analysis of cDNA samples was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The cDNA synthesised from BCB⁺ and BCB⁻ samples were subjected to real-time PCR using GAPDH primer to test for any variation in the expression of this internal control gene. The real-time PCR was performed as described in El-Sayed *et al.* (2006). Final quantitative analysis was done using the relative standard curve method and results were reported as the relative expression or *n*-fold difference to the calibrator after normalisation of the transcript amount relative to the endogenous control (Tefaye *et al.* 2004).

Statistical analysis

In all cases, the data of the three independent experiments were statistically analysed; differences of $P \leq 0.05$ were considered to be significant. The data of the Western blots, morphological analysis of oocytes before IVM and results after IVM/IVF/IVC were expressed as means $\pm$ S.E.M. Statistical analysis was done using the SAS system for Windows (release 8.02).

The relative mRNA expression data were analysed using General Linear Model (GLM) of the Statistical Analysis System (SAS) software package version 8.0 (SAS Institute Inc., Cary, NC, USA). Differences in mean values were tested using ANOVA followed by a multiple pairwise comparison using *t*-test.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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