Zygote **20** (August), pp. 281–290. © Cambridge University Press 2011
doi:10.1017/S0967199411000141 First Published Online 15 April 2011

Differential gene expression and developmental competence in *in vitro* produced bovine embryos

Paula Ripamonte[2], *Lígia Garcia Mesquita*[2], *Sylvia Sanches Cortezzi*[2], *Júlio César de Carvalho Balieiro*[2], *Giovana Krempel Fonseca Merighe*[2], *Yeda Fumie Watanabe*[2], *Alexandre Rodrigues Caetano*[3] *and Flávio Vieira Meirelles*[1]

Universidade de São Paulo, Faculdade de Zootecnia e Engenharia de Alimentos, Departamento de Ciências Básicas, Pirassununga, SP; and EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil

Date submitted: 02.09.10. Date accepted: 31.01.11

Summary

The embryonic developmental block occurs at the 8-cell stage in cattle and is characterized by a lengthening of the cell cycle and an increased number of embryos that stop development. The maternal-embryonic transition arises at the same stage resulting in the transcription of many genes. Gene expression studies during this stage may contribute to the understanding of the physiological mechanisms involved in the maternal-embryonic transition. Herein we identified genes differentially expressed between embryos with high or low developmental competence to reach the blastocyst stage using differential display PCR. Embryos were analysed according to developmental kinetics: fast cleavage embryos showing 8 cells at 48 h post insemination (hpi) with high potential of development (F8), and embryos with slow cleavage presenting 4 cells at 48 hpi (S4) and 8 cells at 90 hpi (S8), both with reduced rates of development to blastocyst. The fluorescence DDPCR method was applied and allowed the recovery of 176 differentially expressed bands with similar proportion between high and low development potential groups (52% to F8 and 48% in S4 and S8 groups). A total of 27 isolated fragments were cloned and sequenced, confirming the expected primer sequences and allowing the identification of 27 gene transcripts. PI3KCA and ITM2B were chosen for relative quantification of mRNA using real-time PCR and showed a kinetic and a time-related pattern of expression respectively. The observed results suggest the existence of two different embryonic genome activation mechanisms: fast-developing embryos activate genes related to embryonic development, and slow-developing embryos activate genes related to cellular survival and/or death.

Keywords: Bovine embryo, DDPCR, Developmental block, Genome activation, Real-time PCR

Introduction

The embryonic pre-implantation transcriptome has been widely studied in many species in an attempt to understand the biological mechanisms that govern this developmental phase (Telford *et al.*, 1990; Schultz, 2002; Kaňka, 2003; Kaňka *et al.*, 2003; Tesfaye *et al.*, 2003; Mamo *et al.*, 2006; Badr *et al.*, 2007; Wrenzycki *et al.*, 2007). In this period, many factors can interfere negatively with the developing bovine embryo causing cleavage to cease. The embryonic developmental block occurs mainly during the fourth, or the transition between the fourth and fifth cell division cycle (Memili & First, 2000).

During the *in vitro* production of bovine embryos approximately 90% of immature oocytes undergo nuclear maturation and 80% undergo fertilization and conclude at least the 2nd cellular cycle (Gordon, 1994; Lonergan *et al.*, 2003). However, only 30–40% of the fertilized oocytes are capable of developing to blastocysts (Lonergan *et al.*, 2003) leading to a large

[1]All correspondence to: F.V. Meirelles. Departamento de Ciências Básicas, Universidade de São Paulo, FZEA, Pirassununga, SP, Brazil., Av. Duque de Caxias Norte 225, Pirassununga, São Paulo, Brasil CEP 13635–900. Tel: +55 19 3565 4112. e-mail: meirellf@usp.br
[2]Universidade de São Paulo, Faculdade de Zootecnia e Engenharia de Alimentos, Departamento de Ciências Básicas, Pirassununga, SP, Brazil.
[3]EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.

proportion of embryos that arrest their development. In general, embryonic development depends on mRNA and protein stocks present in the oocyte at the time of fertilization which are synthesized and stored during oogenesis. The main function of these stocks is to maintain embryo development until Embryonic Genome Activation (EGA) occurs (Schultz, 1993; De Sousa *et al.*, 1998a,b). Embryos that inherit low quality mRNA and protein stocks do not efficiently activate their genome and go into a passive developmental block (Betts & King, 2001). Variation in cleavage timing, fragmentation rates and developmental potential are affected by the quality and quantity of maternally derived mRNAs and proteins (Gutiérrez-Adán *et al.*, 2004; Biase *et al.*, 2008). Although EGA is expected to occur in the 8-cell-stage, there are reports describing genes that are transcribed earlier (Ma *et al.*, 2001). These observations suggest that EGA occurs as a multiple-step event, as opposed to following a single global transcriptional switch (Ma *et al.*, 2001).

In this report we tested the hypothesis that at the moment of major embryonic genome activation, *in vitro*-produced bovine embryos with high and low development potential show different patterns of gene expression. Using fluorescence differential display PCR and real-time PCR we identified genes that are differentially expressed between bovine embryos with high and low developmental competence to reach the blastocyst stage.

Material and methods

In vitro production of bovine embryos

Chemicals were purchased from Sigma Chemical unless otherwise stated.

Bovine embryos were produced after *in vitro* oocyte maturation, fertilization and culture, according to previously published protocols (Bousquet *et al.*, 1999). Briefly, healthy cumulus–oocyte complexes (COCs) with evenly granulated cytoplasms in the oocyte and surrounded by multiple layers of cumulus cells were obtained by aspirating follicles (2–8 mm) from abattoir-derived ovaries, washed with HEPES-buffered Tyrode's medium and cultured for 20 h under mineral oil in 90 µl drops of TCM199 medium with Earles salt solution, glutamine and $NaHCO_3$, supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), sodium pyruvate (22 µg/ml), gentamycin sulfate (50 µg/ml), follicle stimulating hormone (FSH) (0.5 µg/ml), leuteinising hormoneLH (50 µg/ml) and estradiol-17β (1 µg/ml) at 38.5°C in 5% CO_2. Matured oocytes were fertilized with motile sperm from a single Nellore bull recovered by centrifugation for 30 min at 700 *g* in a Percoll discontinuous density gradient (2 ml 45% Percoll over 2 ml 90% Percoll) in 90 µl drops under mineral oil of TALP medium supplemented with heparin (10 µg/ml), sodium pyruvate (22 µg/ml), gentamycin (50 µg/ml), fatty acid-free BSA (6 mg/ml) and PHE solution (2 µM penicillamine, 1 µM hypotaurine and 0.25 µM epinephrine) at 38.5°C, in 5% CO_2.

At approximately 10 hours post insemination (hpi), presumptive zygotes were denuded by pipetting and co-cultured in modified CR2 medium supplemented with 10% (v/v) FBS and granulosa cells coming from the maturation dish (Watanabe *et al.*, 1999). At 48 hpi, embryos were analysed and those with fast cleavage divisions and high development potential (F8), and embryos with slow cleavage divisions (S4), showing 8 and 4 cells respectively, were recovered from culture, snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. The remaining embryos with slow cleavage divisions and reduced rates of development to blastocyst (S8), showing 0–3 cells at 48 hpi were maintained in culture until 90 hpi when embryos with 8 cells were collected and frozen as described above.

RNA extraction and reverse transcription

Total RNA was extracted from groups of 50 embryos of each category (F8, S4 and S8) with the RNeasy® Protect Mini Kit (Qiagen), according to manufacturer's instructions and eluted in 30 µl RNase-free water. Nine microlitres of total RNA from each embryo group was reversely transcribed in 20 µl reactions containing 1 µl SuperScriptIII™ (200 U/µl; Invitrogen™), 1 µl of an anchor primer (5 µM) 5′-AATACGACTCACTATAGT(T)12NN-3′ (NN = GC, GA or GG; Table 1), 2 µl dNTPs (2.5 mM each), 2 µl dithiothreitol (DTT) (0.1 M), 1 µl RNaseOUT® recombinant ribonuclease inhibitor (40 U/µl; Invitrogen™) and 4 µl 5× first strand buffer. Reactions were carried out at 42°C for 15 min, 50°C for 50 min and 70° for 15 min for enzyme inactivation.

Fluorescent differential display analysis

Differential display PCR analysis was performed according to previously published protocols (Ripamonte *et al.*, 2005). Briefly, RNA was reverse-transcribed using different anchor primers with the following structure: 5′-T7 tail-T_{12}-NN-3′, where NN was GC, GA or GG (Table 1). The resulting cDNAs were then PCR-amplified with different 10-mers and a TAMRA-labelled T7 primer. Each sample was PCR-amplified in triplicate. Amplified fragments were separated in 6.5% polyacrylamide gels for 5 h at 1500 V. After electrophoresis, gels were scanned with a Fuji FLA3000 fluorescence scanner and fragments were visualized with Image Reader FLA-3000 Series and

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

Table 1 Primers used in the fluorescent DDPCR experiments

Primer	Sequence 5′→ 3′	Primer	Sequence 5′→ 3′
Universal T7 rev	AATACGACTCACTATAGT TAMRA	ARP9 fwd	TAAGACTAGC
AP1 rev	AATACGACTCACTATAGT$_{(12)}$GA	ARP10 fwd	GATCTCAGAC
AP2 rev	AATACGACTCACTATAGT$_{(12)}$GC	ARP11 fwd	ACGCTAGTGT
AP3 rev	AATACGACTCACTATAGT$_{(12)}$GG	ARP12 fwd	GGTACTAAGG
ARP1 fwd	CGACTCCAAG	ARP13 fwd	GTTGCACCAT
ARP2 fwd	GCTAGCATGG	ARP14 fwd	TCCATGACTC
ARP3 fwd	GACCATTGCA	ARP15 fwd	CTTTCTACCC
ARP4 fwd	GCTAGCAGAC	ARP16 fwd	TCGGTCATAG
ARP5 fwd	ATGGTAGTCT	ARP17 fwd	CTGCTAGGTA
ARP6 fwd	TACAACGAGG	ARP18 fwd	TGATGCTACC
ARP7 fwd	TGGATTGGTC	ARP19 fwd	TTTTGGCTCC
ARP8 fwd	TGGTAAAGGG	ARP20 fwd	TCGATACAGG

AP, anchor primer; ARP, arbitrary primer.

Image Gauge software (Fuji Photo Film Co.). The gel was dried at 80°C and rinsed with ddH_2O repeatedly to completely remove urea residues. Fragments differentially amplified in all triplicates in at least one of the embryo groups were precisely excised from the dried polyacrylamide gel with a scalpel, eluted in 80 µl of ultrapure water at 80°C for 10 min and stored at –80°C until analysis.

Cloning and sequencing of different amplified fragments

Each excised fragment was re-amplified with an unlabelled T7 primer and the corresponding arbitrary primer. Re-amplifications were carried out in 25 µl reactions containing 0.5 µl of a solution containing the eluted DDPCR fragment, 2.5 µl 10× *Taq* polymerase buffer, 4 µl dNTPs (2.5 mM each), 3.25 µl unlabelled T7 (5 µM), 3.25 µl arbitrary primer (5 µM), 0.2 µl *Taq* DNA polymerase (5 U/µl; Invitrogen™) and 1.5 µl $MgCl_2$ (50 mM). PCR cycling conditions were: 95°C for 2 min, followed by 30 cycles of 92°C for 15 s, 42°C for 30 s and 72°C for 2 min, with a final step of 72°C for 10 min. Re-amplified fragments were cloned into the TOPO TA Cloning® vector (Invitrogen™) following the manufacture's instructions and sequenced with BigDye dideoxy terminator chemistry (Applied Biosystems). The sequences were submitted to BLAST analysis (BlastN, nr database, December 2007) for identification at the GenBank website.

Real-time PCR quantification

For quantitative analysis of individual genes, mRNA was extracted from embryos of each category (F8, S4 and S8) with QuickPrep Micro mRNA Purification kit (Amersham). Messenger RNA obtained from each embryo group was then amplified with MessageAmp aRNA kit (Ambion®), according to manufacturer's instructions and described in the literature (Kralj *et al.*, 2009). Briefly, double-stranded cDNA was synthesized with an oligo(dT) primer bearing a T7 promoter which was then used in an *in vitro* transcription procedure with T7 RNA polymerase. A second round of amplification, using different primers, was done to generate additional aRNA. cDNA from each embryo group was then generated as follows: 9 µl of amplified RNA was reversely transcribed in 20 µl reactions containing 1 µl SuperScriptIII™ (200U/µl; Invitrogen™), 1 µl poly(A) primer (5 µM), 2 µl dNTPs (2.5 mM each), 2 µl DTT (0.1 M), 1 l RNaseOUT® recombinant ribonuclease inhibitor (40 U/µl; Invitrogen™) and 4 µl 5× first strand buffer. Reactions were carried out at 42°C for 15 min, 50°C for 50 min and 70°C for 15 min for enzyme inactivation.

To determine the relative abundance of transcripts, real-time quantitative PCR was performed in an ABI Prism® 7500 (Applied Biosystems) using TaqMan™ technology (Applied Biosystems). Primers and probes were designed by Assay-by-Design (Applied Biosystems; Table 2). Prior to the quantification analysis, standard curves were constructed for both transcript

Table 2 Primers used for real-time PCR

Primer	Sequence 5′→3′
PI3K fwd	CCCAAGAATGCACAAAGACAAGA
PI3K rew	GCCGAATAGCTAGATAAGCCTTGTA
PI3K probe	FAM-CTGAAACCTCTCAAATTC-NFQ
ITM2B fwd	GTCCCAGAGTTTGCAGATAGTGA
ITM2B rew	GGAATCACATAGCACTTATCCAGGTT
ITM2B probe	FAM-CATGACTTCAACAAGAAACT-NFQ
18S rRNA[a]	Hs99999901_s1

[a]Assay identifier provided by Applied Biosystems.

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

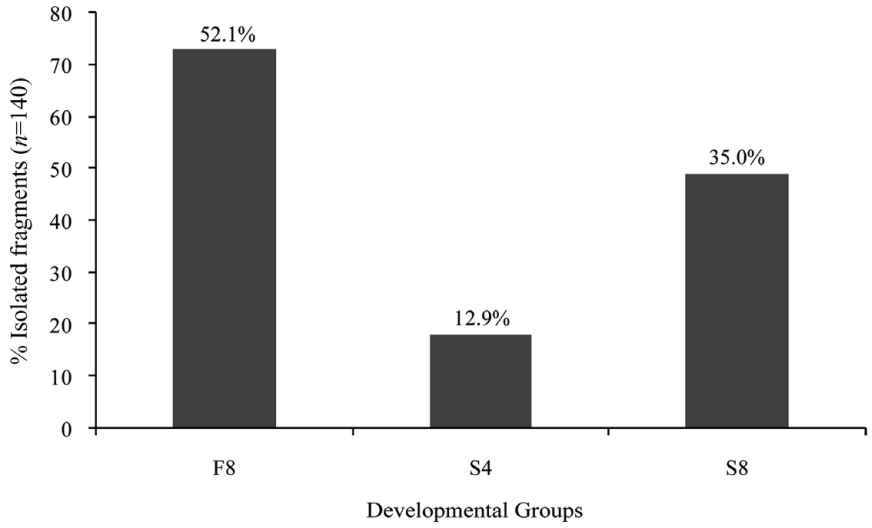

Figure 1 Proportion of differentially amplified fragments isolated using differential display PCR in *in vitro*-produced bovine embryos. F8: 8-cell stage at 48 hpi; S4 and S8: embryos that reached the 4-cell and 8-cell stage at 48 and 90 hpi, respectively.

targets phosphoinositide-3-kinase, catalytic, alpha polypeptide (PI3KCA) and integral membrane protein 2B (ITM2B), and endogenous control 18S ribosomal RNA (18S rRNA), using five serial dilutions of cDNA from F8 embryos. The cycling threshold (C_t) mean values were used to estimate amplification efficiency (E) given by $E = 10^{(-1/\text{slope})-1}$ and all of them were >0.99. Each reaction was performed in duplicate. Three replicates of RNA amplified samples from three different embryos groups (F8, S4 and S8) were evaluated. All reactions were carried out in 25 µl reactions containing TaqMan™ PCR Universal Master Mix (Applied Biosystems) and PCR cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Relative mRNA expression levels were estimated based on the relative standard curve method suggested by the instrument manufacturer. Results are shown as relative expression or fold difference to the calibrator sample (i.e., F8 embryo) after normalization of transcript amount to the endogenous control ($2^{-\Delta\Delta Ct}$ Method; Livak & Schmittgen, 2001).

Statistical and gene ontology analysis

Expression level means and standard errors were estimated using least squares method in Proc GLM on S.A.S. v9.1.3 (SAS Institute Inc.). In instances in which no differences were observed among all three embryo groups, data were analysed using the same SAS procedure described above considering time of culture (F8 and S4 with 48 hpi vs. S8 with 90 hpi) or development kinetics (fast-developing F8 embryos vs. slow-developing S4 and S8 embryos). Gene ontology (GO) analysis was performed for categorizing differentially expressed sequences, including biological process, molecular function and cellular component using EASE, the Expression Analysis Systematic Explorer, v2.0 (Huang *et al.*, 2007).

Results

The fluorescence differential display PCR method was performed using 60 primer combinations (three anchor and 20 random primers) generating a total of 140 potential differentially amplified fragments, ranging in size from 150 bp to 1.5 kb. Fifty-two per cent of fragments were isolated from F8 embryos, while 35% and 13% were derived from S8 and S4, respectively (Fig. 1). From these, a total of 27 bands were selected for further analysis by cloning and sequencing. BLAST searches of the nr database with the resulting sequences showed hits for 33 different gene transcripts possibly related to mammalian/bovine embryonic developmental processes (Table 3). In some instances, we observed two to four heterogeneous sequences resulting from the same isolated DDPCR band therefore, sequencing of more than one colony was necessary for precise identification and characterization of the putative differentially expressed mRNAs. To categorizing the differentially transcript genes we used the EASE tool and a list of over-represented GO terms in our groups embryos was showed in Fig. 2. Several GO categories were found and over-represented in biological processes were related to physiological, cellular and metabolic process. Catalytic activity and binding, cellular and intracellular were over-represented terms for molecular function and cellular component, respectively.

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

Table 3 Description of DDPCR fragments that were isolated, cloned and sequenced

Seq no.	Best BLAST Hit (GenBank Acc. no.)	% Identity	Embryos		
			F8	S4	S8
1	*Bos taurus* phosphoinositide-3-kinase, catalytic, alpha polypeptide, mRNA, PI3KCA, NM_174574.1	83	–	√	–
2	*Bos taurus* optineurin, mRNA, OPTN, NM_001034602.1	99	√	–	–
3	*Bos taurus* nucleolar and spindle associated protein 1, mRNA, NUSAP1, NM_001046571	99	√	–	–
4	*Bos taurus* similar to cyclin I, mRNA, CCNI, NM_001083373.1	98	–	√	–
5	PREDICTED: *Bos taurus* similar to integral membrane protein 2B, transcript variant 1, mRNA, ITM2B, NM_001035093.1	99	√	–	√
6	*Bos taurus* integrator complex subunit 12, mRNA, INTS12, NM_001075753	98	–	–	√
7	PREDICTED: *Bos taurus* similar to transcription factor TZP, transcript variant 1, mRNA, TZP, XM_608043.3	99	√	–	–
8	*Bos taurus* similar to COPG2 protein, mRNA, COPG2, NM_001082448.1	99	√	√	–
9	*Bos taurus* T cell receptor gamma V5–1, complete sequence, TCRV5–1, AY644518.1	80	–	–	√
10	*Bos taurus* ribosomal protein L10a, mRNA, complete cds, RPL10A, BC102653.1	98	√	–	√
11	*Bos taurus* solute carrier family 25, member 40, mRNA, SLC25A40, NM_001076299.1	100	–	√	–
12	*Bos taurus* abhydrolase domain containing 11, mRNA, ABHD11, NM_001034372.1	97	–	√	–
13[a]	*Bos taurus* ADP-ribosylation factor-like 6 interacting protein 1, ARL6IP1, NM_001078157.1	97	–	–	√
14[a]	*Bos taurus* TFG TRK-fused gene, mRNA, complete cds, TFG, BC104525.2	99	–	–	√
15	*Bos taurus* chromosome 6 open reading frame 25, mRNA, C6orf25, NM_001078081.1	99	√	–	–
16	*Bos taurus* Coatomer gamma-2 subunit, mRNA, COP2-γ, XM_591934	100	√	√	–
17	*Bos taurus* glutamate-cysteine ligase catalytic subunit, GCLC, AY957499.1	94	√	√	–
18	*Bos taurus* eukaryotic translation elongation factor 1 alpha 1, mRNA, EEF1A1, complete cds, BC142302.1	98	√	–	–
19	*Bos taurus* ER degradation enhancer, mannosidase alpha-like 2, mRNA, EDEM2, NM_001101085.1	74	√	–	–
20	*Bos taurus* Cyclin y-like 1, mRNA, CCNYL1, XM_618363.3	98	√	–	–
21	*Bos taurus* similar to Tumor necrosis factor receptor superfamily member 7 precursor (CD27L receptor) (T-cell activation antigen CD27) (T14) (MGC148404), mRNA, NM_001082434.1	84	–	–	√
22	*Bos taurus* Xist X (inactive)-specific transcript, Xist genes, XIST, AJ421481.1	76	–	–	√
23	*Bos taurus* peroxiredoxin 1, mRNA, complete cds, PRDX1, BC148009.1	100	√	–	–
24	*Bos taurus* endoplasmic reticulum aminopeptidase 2, mRNA, ERAP2, BC149475.1	93	√	–	–
25	*Bos taurus* uroplakin 1B, mRNA, UPK1B, NM_174482.2	92	√	–	–
26	*Bos taurus* T-cell receptor alpha gene, J segments and C region, TCRA, NW_001501855.1	84	√	–	–
27	*Bos taurus* partial stat5A gene, exons 5–19, STAT5A, AJ237937.1	100	√	–	–
28[a]	*Bos taurus* ribosomal protein S15A, mRNA, RPS15A, NM_001037443	99	–	–	√
29	*Homo sapiens* integrator complex subunit 12, mRNA complete cds, INTS12, BC014442.1	89	√	–	–
30[a]	*Bos taurus* similar to oligonucleotide/oligosaccharide-binding fold containing 1, mRNA, OBFC1, NM_001076849	97	√	–	–
31	*Bos taurus* ankyrin repeat domain 17, mRNA, ANKRD17, XM_585174	99	√	√	–
32	*Bos taurus* thioredoxin domain containing 9, mRNA, TXNDC9, NM_001076149	100	√	–	–
33	*Homo sapiens* HIN1-like cysteine protease (HIN1L) pseudogene on chromosome 12, HIN1L, NG_005318	87	√	–	–

[a]Sequences obtained from the same gel fragment after cloning in TA vector.

PI3KCA (Craddock *et al.*, 1999) and ITM2B (Fleischer *et al.*, 2002a,b) were chosen from the sequenced fragments for mRNA quantification by real-time PCR. PI3KCA was initially isolated in S4 embryos but the relative expression of PI3KCA did not differ when comparing S4 with S8 embryos ($p > 0.05$). However, comparisons between slow and fast-developing embryos showed higher PI3KCA expression levels in

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

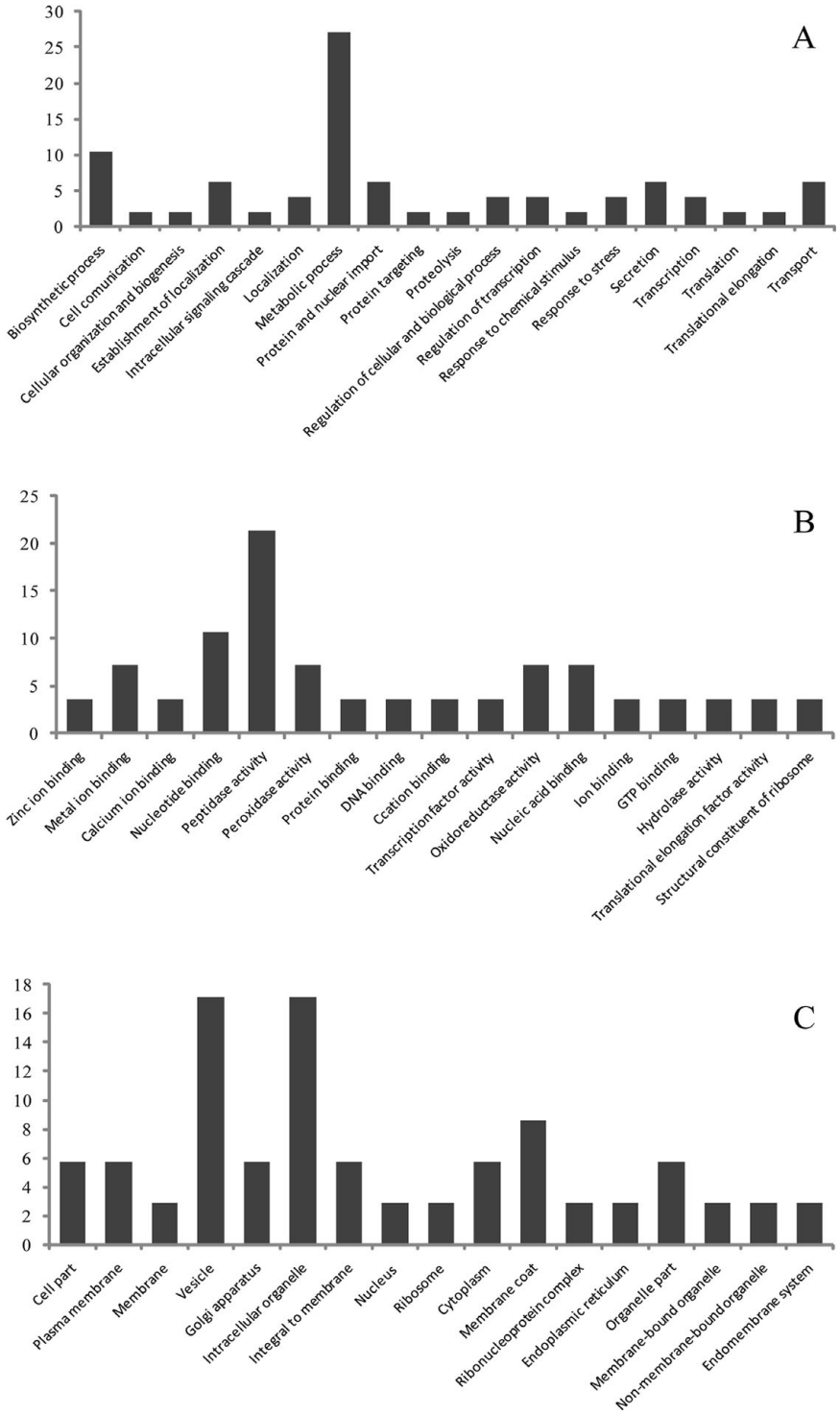

Figure 2 Selected Gene Ontology (GO) categories over-represented in bovine embryos identified by DDPCR. (*A*) Biological process; (*B*) molecular function, and (*C*) cellular component.

embryos with lower developmental potential ($p < 0.01$; Fig. 3). ITM2B was originally isolated from F8 and S8 embryos. However, the relative expression levels were ~40-fold higher in S8 than in F8 embryos while S4 embryos did not differ from F8 embryos (Fig. 4).

Discussion

Many studies have reported correlations between COC morphology, 1st polar body extrusion or first cleavage timing and age of the oocyte donor with

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

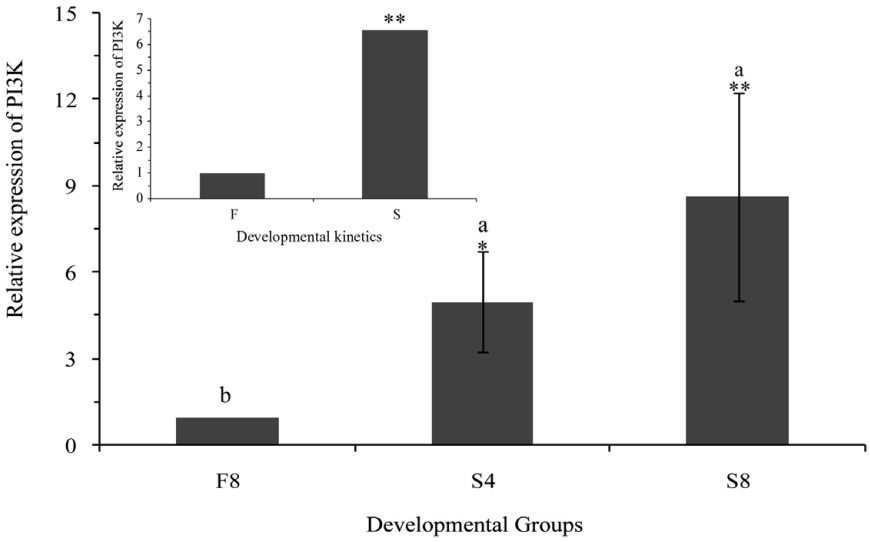

Figure 3 [a, b]Relative gene expression levels estimated by real-time PCR for PI3KCA in bovine embryos with high (F8) or low (S4 and S8) developmental potential. Detail: relative gene expression of PI3KCA by developmental kinetics in fast (F) and slow (S) bovine embryos. $^{*}p < 0.05$ and $^{**}p < 0.01$, $n = 3$.

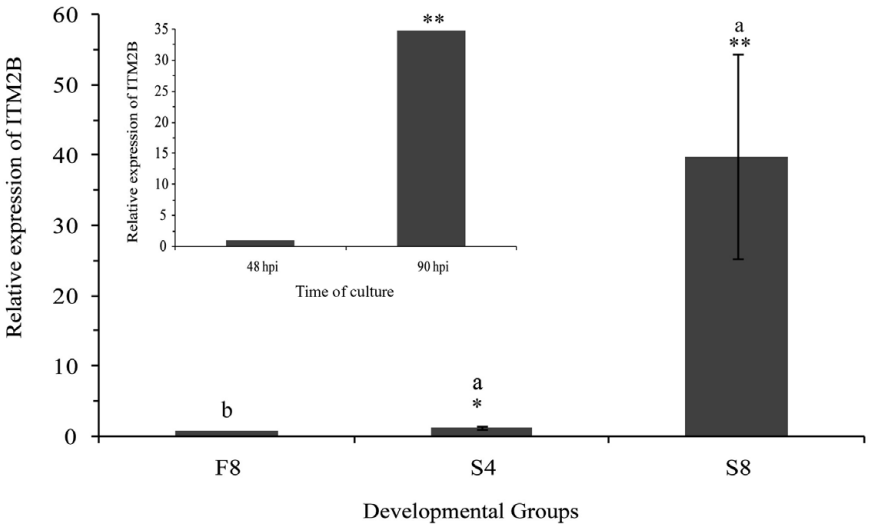

Figure 4 [a, b]Relative gene expression estimated by real-time PCR for ITM2B in bovine embryos with high (F8) or low (S4 and S8) developmental potential. Detail: relative gene expression of ITM2B by time of culture in fast (F) and slow (S) bovine embryos. $^{*}p < 0.05$ and $^{**}p < 0.01$, $n = 3$.

developmental competence of pre-implantation embryos (Pavlok *et al.*, 1992; Lonergan *et al.*, 1994; Brevini *et al.*, 2002; Lonergan *et al.*, 2003; Meirelles *et al.*, 2004). Additionally, possible interactions of these factors with culture systems have also been reported (Niemann & Wrenzycki, 2000, 2003; Watson *et al.*, 2000; Natale *et al.*, 2001; Gutiérrez-Adán *et al.*, 2004). When considered jointly, these findings strongly indicate that the oocyte's inherited mRNA and protein stocks indeed play a major role in the early pre-cleavage embryonic development, and that early developing embryos interact with the development environment to express this potential.

DDPCR has been successfully used to identify new or rare gene transcripts when amounts of available RNA samples are limited, as is the case with preimplantation embryo models (Natale *et al.*, 2001; Kaňka *et al.*, 2003; Li *et al.*, 2003; Tesfaye *et al.*, 2003). In the present study we used the fluorescence DDPCR method previously described by Ripamonte *et al.* (2005) to identify messages putatively differentially expressed among three *in vitro*-produced bovine embryos with high- and low-development potential. The strategy proved to be very successful as we recovered hundreds of putative fragments differentially expressed. Using gene ontology analysis

https://doi.org/10.1017/S0967199411000141 Published online by Cambridge University Press

the main identified terms were related to embryo functions such as physiological process, metabolism, development, cell proliferation, cell cycle and death, and these terms reflect the biological condition of embryo groups.

Differential expression of anti- and pro-apoptotic genes in oocytes and early-developing embryos has been previously shown to be associated developmental potential (Jurisicova *et al.*, 1998; Dalbiés-Tran & Mermillod, 2003). Genes previously shown to be involved in apoptotic mechanisms, like STAT5 (Debierre-Grockiego, 2004), PI3KCA (Craddock *et al.*, 1999) and ITM2B (Fleischer *et al.*, 2002a,b), were recovered in the present study. Considering the importance of apoptotic processes in determining early-embryo survival, PI3KCA and ITM2B were chosen for further study.

In both cases different results were observed between the DDPCR and the real-time PCR quantification. These variations may be generated by the artifacts of similar sizes fragments creating bands with heterogeneous cDNA as revealed by cloning and sequencing of bands recovered fragments.

PI3KCA is responsible for regulation of various biological processes such as growth, survival, proliferation and cellular metabolism (Fruman, 1998; Craddock *et al.*, 1999; Wymann *et al.*, 2003). PI3KCA is also associated with anti-apoptotic signaling in a large number of cell types (Craddock *et al.*, 1999). In our experiments, PI3KCA was isolated from S4 embryos and is expressed at higher levels in slow-developing embryos, which have also been shown to have lower developmental competence and therefore are usually destined to stop developing (Lonergan *et al.*, 1999; Gutierréz-Adán *et al.*, 2004). Considering PI3KCA's previously describe role as an anti-apoptotic molecule, we hypothesize that slow-developing embryos may be over-expressing this gene in an attempt to avoid going into developmental block.

Conversely, ITM2B has been described as a pro-apoptotic regulator that shares the BH3 domain present in Bcl-2 gene family members (Deleersnijder *et al.*, 1996; Fleischer *et al.*, 2002a, 2004; Choi *et al.*, 2003; Van Den Plas & Merregaert, 2004;). ITM2B induces mitochondrial membrane permeability promoting release of cytochrome *c* and activation of caspase-9 and -3 during apoptosis (Fleischer *et al.*, 2002b). Furthermore, Fleischer *et al.* (2002a) suggest that ITM2B contributes to the establishment of mitochondrial PTP complexes (Permeability Transition Pore) and, as other Bcl-2 family members, it is involved in membrane pore formation. In both cases, ITM2B expression results in mitochondrial membrane potential loss and consequently induction of programmed cell death pathways. The higher expression level of ITM2B observed in S4 and S8 embryos may indicate that expression of embryonic genes is necessary to drive non-competent embryos to developmental block. Therefore, (partial) EGA is required even in early-developing embryos that are designated to die.

The majority of the differentially expressed transcripts isolated in the study were present in embryos with high development potential (F8), which is expected as a consequence of the activation of the embryo's genome. Our findings are consistent with the notion that embryos with high development potential strongly activate genes responsible for survival. However, several differentially expressed messages were isolated from low developmental potential embryos (S8), which according to the interpretation of the current literature are not supposed to undergo EGA (Bettegowda *et al.*, 2008). Moreover, to differentially express genes related to cell death, embryos with low development potential also overexpress genes responsible for survival. These findings lead to the hypothesis that at the moment of EGA, slow developing embryos overexpress survival genes as a countermeasure to attempt to evade undergoing full developmental block and the consequential death.

Studies using DNA fragmentation assays as indicators of the onset of apoptosis (Singh *et al.*, 1988; Gavrieli *et al.*, 1992;Takahashi *et al.*, 2000) verified that blastocysts from S8 embryos have higher DNA damage and nuclear fragmentation in comparison with F8 embryos (Garcia, 2004, unpublished data). These observations indicate that apoptosis is indeed occurring at a higher rated in slow-developing embryos.

In conclusion, our data suggest there are two different EGA mechanisms that may come into play in early-developing *in vitro*-produced bovine embryos: on one hand, genes responsible for embryonic development are activated in high-development potential embryos, while on the other hand genes responsible for survival/death are activated in low-development potential embryos. Therefore, it is tempting to propose that the developmental block is rather an active event, and that the embryo genome activation at a partial or specific level is required to trigger the 8-cell developmental arrest.

Acknowledgements

The authors thank FAPESP for financial support (Grant 99/12351–3) and for providing a scholarship to P. Ripamonte (Grant 02/02532–5).

References

Badr, H., Bongioni, G., Abdoon, A.S.S., Kandil, O. & Puglisi, R. (2007). Gene expression in the *in vitro*-produced

preimplantation bovine embryos. *Zygote* **15**, 355–367.

Betts, D.H. & King, W.A. (2001). Genetic regulation of embryo death and senescence. *Theriogenology* **55**, 171–91.

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

Bousquet, D., Twagiramungu, H., Morin, N., Brisson, C., Carboneau, G. & Durocher, J. (1999). *In vitro* embryo production in the cow: an effective alternative to the conventional embryos production approach. *Theriogenology* **51**, 59–70.

Brevini, T.A.L., Lonergan, P., Cillo, F., Francisci, C., Favetta, L.A., Fair, T. & Gandolfi, F. (2002). Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol. Reprod. Dev*. **63**, 510–7.

Choi, D.K., Yoo, K.W., Hong, S.K., Rhee, M., Sakaki, Y. & Kim, C.H. (2003). Isolation and expression of Napor/CUG-BP2 in embryo development. *Biochem. Biophys. Res. Commun*. **305**, 448–54.

Craddock, B.L., Orchiston, E.A., Hinton, H.J. & Welham, M.J. (1999). Dissociation of apoptosis from proliferation, protein kinase B activation, and BAD phosphorylation in interleukin-3-mediated phosphoinositide 3-kinase signaling. *J. Biol. Chem*. **274**, 10633–40.

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

Debierre-Grockiego, F. (2004). Anti-apoptotic role of STAT5 in haematopoietic cells and in the pathogenesis of malignancies. *Apoptosis* **9**, 717–28.

De Sousa, P.A., Caveney, A., Westhusin, M.E. & Watson, A.J. (1998a). Temporal patterns of embryonic gene expression and their dependence on oogenetic factors. *Theriogenology* **49**, 15–28.

De Sousa, P.A., Westhusin, M.E. & Watson, A.J. (1998b). Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. *Mol. Reprod. Dev*. **49**, 119–30.

Deleersnijder, W., Hong, G., Cortvrindt, R., Poirier, C., Tylzanowski, P., Pittois, K., Van Marck, E. & Merregaert, J. (1996). Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. Molecular cloning and characterization of a gene belonging to a novel multigene family of integral membrane proteins. *J. Biol. Chem*. **271**, 19475–82.

Fleischer, A., Ayllon, V., Dumoutier, L., Renault, J.C. & Rebollo, A. (2002a). Proapoptotic activity of ITM2Bs, a BH3-only protein induced upon IL-2-deprivation which interacts with Bcl-2. *Oncogene* **21**, 3181–9.

Fleischer, A., Ayllon, V. & Rebollo, A. (2002b). ITM2Bs regulate apoptosis by inducing loss of mitochondrial membrane potential. *Eur. J. Immun*. **32**, 3498–3505.

Fleischer, A. & Rebollo, A. (2004). Induction of p53-independent apoptosis by the BH3-only protein ITM2Bs. *FEBS Lett*. **557**, 283–7.

Fruman, D.A., Meyers, R.E. & Cantley, L.C. (1998). Phosphoinositide kinases. *Annu. Rev. Biochem*. **67**, 481–507.

Garcia, S.M. (1994). *Bloqueio e morte celular programada em embriões bovinos: efeitos da fonte de suplementação protéica e da cinética do desenvolvimento*. Dissertação de Mestrado, FZEA/USP.

Gordon, I. (1994). *Laboratory Production of Cattle Embryos* 1st edn. Cambridge University Press.

Gavrieli, Y., Sherman, Y. & Bem-Sasson, S.A. (1992). Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol*. **119**, 493–501.

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

Huang, D.W., Sherman, B.T., Tan, Q., Kir, J., Liu, D., Bryant, D., Guo, Y., Stephens, R., Baseler, M.W., Lane, H.C. & Lempicki, R.A. (2007). DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists *Nucl. Acid Res*. **35**, W169–75.

Jurisicova, A., Latham, K.E., Casper, R.F., Casper, R.F. & Varmuza, S.L. (1998). Expression and regulation of genes associated with cell death during murine preimplantation embryo development. *Mol. Reprod. Dev*. **51**, 243–53.

Kaňka, J. (2003). Gene expression and chromatin structure in the pre-implantation embryo. *Theriogenology* **59**, 3–19.

Kaňka, J., Bryova, A., Duranthon, V., Oudin, J.-F., Peynot, N. & Renard, J.P. (2003). Identification of differentially expressed mRNAs in bovine preimplantation embryos. *Zygote* **11**, 43–52.

Kralj, J.G., Player, A., Sedrick, H., Munson, M.S., Petersen, D., Forry, S.P., Meltzer, P., Kawasaki, E. & Locascio, L.E. (2009). T7-based linear amplification of low concentration mRNA samples using beads and microfluidics for global gene expression measurements. *Lab. Chip* **7**, 917–24.

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

Lonergan, P. (1994). Growth of preimplantation bovine embryos. *Acta Vet. Scand*. **35**, 307–20.

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

Lonergan, P., Gutiérrez-Adán, A., Fair, T. & Boland, M.P. (2003). Effect of culture environment on embryo quality and gene expression – experience from animal studies. *Reprod. Biomed. Online* **7**, 657–63.

Ma, J., Svoboda, P., Schultz, R.M. & Stein, P. (2001). Regulation of zygotic gene activation in the preimplantation mouse embryo: global activation and repression of gene expression. *Biol. Reprod*. **64**, 1713–21.

Mamo, S., Sargent, C.A., Affara, N.A., Tesfaye, D., El-Halawany, N., Wimmers, K., Gilles, M., Schellander, K. & Ponsuksili, S. (2006). Transcript profiles of some developmentally important genes detected in bovine oocytes and *in vitro*-produced blastocysts using RNA

amplification and cDNA microarrays. *Reprod. Dom. Anim.* **41**, 527–34.

Meirelles, F.V., Caetano, A.R., Watanabe, Y.F., Ripamonte, P., Carambula, S.F., Merighe, G.K. & Garcia, S.M. (2004). Genome activation and developmental block in bovine embryos. *Ann. Reprod. Sci.* **82–83**, 13–20.

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

Natale, D.R., De Sousa, P.A., Westhusin, M.E. & Watson, A.J. (2001). Sensitivity of bovine blastocyst gene expression patterns to culture environment assessed by differential display RT-PCR. *Reproduction* **122**, 687–93.

Niemann, H. & Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: implications for subsequent development. *Theriogenology* **53**, 21–34.

Niemann, H. & Wrenzycki, C. (2003). Epigenetic reprogramming in early embryonic development: effects of in-vitro production and somatic nuclear transfer. *Reprod. Biomed. Online* **7**, 649–56.

Nozawa, K., Casiano, C.A., Hamel, J.C., Molinaro, C., Fritzler, M.J. & Chan, E.K. (2002). Fragmentation of Golgi complex and Golgi autoantigens during apoptosis and necrosis. *Arthritis Res.* **4**, R3.

Pavlok, A., Lucas-Hahn, A. & Niemann, H. (1992). Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol. Reprod. Dev.* **31**, 63–7.

Ripamonte, P., Merighe, G.K., Watanabe, Y.F., Caetano, A.R. & Meirelles, F.V. (2005). Development and optimization of a fluorescent differential display PCR system for studying bovine embryo development *in vitro*. *Genet. Mol. Res.* **4**, 726–33.

Schultz, R.M. (1993). Regulation of zygotic gene activation in the mouse. *Bioessays* **15**, 531–8.

Schultz, R.M. (2002). The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum. Reprod. Update* **8**, 323–31.

Telford, N.A., Watson, A.J. & Schultz, G.A. (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol. Reprod. Dev.* **26**, 90–100.

Tesfaye, D., Ponsuksili, S., Wimmers, K., Gilles, M. & Schellander, K. (2003). Identification and quantification of differentially expressed transcripts in *in vitro*-produced bovine preimplantation stage embryos. *Mol. Reprod. Dev.* **66**, 105–14.

Van Den Plas, J. & Merregaert, D. (2004). *In vitro* studies on Itm2a reveal its involvement in early stages of the chondrogenic differentiation pathway. *Biol. Cell.* **96**, 463–70.

Watanabe, M.R., Franceschini, P.H., Dayan, A., Galerani, M.A.V., Vila, R.A., Lôbo, R.B. & Watanabe, Y.F (1999). Ultrasound guided oocytes recovery in Nellore cows and *in vitro* embryo production. *Theriogenology* **51**, 438.

Watson, A.J., De Sousa, P., Caveney, A., Barcroft, L.C., Natale, D., Urquhart, J. & Westhusin, M.E. (2000). Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number and apoptosis. *Biol. Reprod.* **62**, 355–64.

Wrenzycki, C., Herrmann, D. & Niemann, H. (2007). Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology* **68**, 77–83.

Wymann, M.P., Zvelebil, M. & Laffargue, M. (2003). Phosphoinositide 3-kinase signalling—which way to target? *Trends Pharmacol. Sci.* **24**, 366–76.