Suppressed expression of genes involved in transcription and translation in *in vitro* compared with *in vivo* cultured bovine embryos

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Abstract

In vivo-derived bovine embryos are of higher quality than those derived *in vitro*. Many of the differences in quality can be related to culture environment-induced changes in mRNA abundance. The aim of this study was to identify a range of mRNA transcripts that are differentially expressed between bovine blastocysts derived from *in vitro* versus *in vivo* culture. Microarray (BOTL5) comparison between *in vivo*- and *in vitro*-cultured bovine blastocysts identified 384 genes and expressed sequence tags (ESTs) that were differentially expressed; 85% of these were down-regulated in *in vitro* cultured blastocysts, showing a much reduced overall level of mRNA expression in *in vitro*- compared with *in vivo*-cultured blastocysts. Relative expression of 16 out of 23 (70%) differentially expressed genes (according to *P* value) were verified in new pools of *in vivo*- and *in vitro*-cultured blastocysts, using quantitative real-time PCR. Most (10 out of 16) are involved in transcription and translation events, suggesting that the reason why *in vitro*-derived embryos are of inferior quality compared with *in vivo*-derived embryos is due to a deficiency of the machinery associated with transcription and translation.

Reproduction (2006) 131 651–660

Introduction

It is estimated that fetal viability is only achieved in 30% of all human conceptions with the greatest loss (50%) occurring prior to the first missed menses and during early stages of implantation (25%) (Edmonds *et al.* 1982). Similarly, cattle experience relatively high rates of embryonic and early fetal mortality (about 40%). Published estimates indicate a fertilization rate of 90% and an average calving rate of about 55%, suggesting an embryonic/fetal mortality of about 35%; it is estimated that 70–80% of the total embryonic loss occurs between days 8 and 16 after insemination (day 16 corresponding to the period of maternal recognition of pregnancy (reviewed by Peters 1996, Ball 1997, Sreenan *et al.* 2001).

The *in vitro* production of bovine embryos is essentially a three-step process involving *in vitro* oocyte maturation, *in vitro* fertilization and *in vitro* culture. Most of the differences in the quality of *in vitro*- compared with *in vivo*-derived embryos are due to the last step, the culture environment for post-fertilization early

embryo development (Rizos *et al.* 2002*b*, 2002*c*, Lonergan *et al.* 2003*a*). It is during this time that the embryo switches from using genes and proteins derived from the maternal genome (in the oocyte) to those resulting from the newly activated embryonic genome (8–16 cell stage in cattle; Memili & First 2000). It is well recognized that bovine embryos derived *in vivo* are of higher quality than those derived *in vitro* (Hasler *et al.* 1995). *In vitro*-produced blastocysts tend to have darker cytoplasm and lower buoyant density (Pollard & Leibo 1994), due to their higher lipid content (Abd El Razek *et al.* 2000), a more fragile zona pellucida (Duby *et al.* 1997), reduced intracellular communication (Boni *et al.* 1999), differences in metabolism (Khurana & Niemann 2000, Thompson 2000) and a higher incidence of chromosome abnormalities (Viuff *et al.* 1999, Lonergan *et al.* 2004). In addition, many differences have been reported at the ultrastructural level (Crosier *et al.* 2000, 2001, 2002, Fair *et al.* 2001, Rizos *et al.* 2002*a*), which reflect some of the differences noted above.

Studies in bovine embryos indicate that many of the differences in quality of *in vitro*- and *in vivo*-derived blastocysts can be related to culture environment-induced changes in mRNA abundance. The post-fertilization embryo culture environment has a dramatic effect on the pattern of gene expression in embryos, which in turn has serious implications for the normality of blastocyst development (Lonergan *et al.* 2003a, Tesfaye *et al.* 2004). This is the case, not only when one compares *in vitro* and *in vivo* culture systems, but also comparing different *in vitro* culture systems (reviewed by Lonergan *et al.* 2003a, Wrenzycki *et al.* 2005).

The oviduct of the ewe has been shown by several authors to be a suitable environment for the development of bovine embryos from the zygote to blastocyst stage. Although not perfect, one advantage of this *in vivo* culture system is the ability to culture large numbers of embryos in a near *in vivo* environment and in a cost-effective manner. While the yield of blastocysts following such *in vivo* culture is not superior to that following culture *in vitro*, the quality of the blastocysts is significantly improved (Galli & Lazzari 1996, Enright *et al.* 2000, Lazzari *et al.* 2002, Rizos *et al.* 2002c, 2003). Blastocysts produced in this way are similar to true *in vivo* embryos in terms of morphology (Fair *et al.* 2001), cryotolerance (Enright *et al.* 2000, Rizos *et al.* 2002a), mRNA expression pattern for select genes (Lazzari *et al.* 2002, Rizos *et al.* 2002c) and pregnancy rate following transfer (Lazzari *et al.* 2002).

Until recently, methods of analysis of differential gene expression in embryos were confined to the use of quantitative real-time PCR (Q-RT-PCR) using primers for specific candidate genes. Such gene-by-gene analysis provides too narrow a view of the potentially complex underlying regulatory networks involved in embryo pre-implantation development. Depending on the species availability, DNA microarray analyses provide a genome-wide perspective by profiling the expression of thousands of genes simultaneously. The aim of the present study was to use this technology to identify a range of mRNA transcripts that are differentially expressed between bovine blastocysts derived from *in vitro* versus *in vivo* culture.

Materials and Methods

Three experiments were performed. In experiment 1, the effect of linear amplification on the relative levels of gene expression in two-cell embryos compared with blastocyst-stage embryos was analysed. Experiment 2 involved the use of microarrays as a discovery tool to identify potentially differentially expressed mRNA transcripts between blastocysts produced by *in vivo* or *in vitro* culture. In experiment 3, Q-RT-PCR was used to confirm differential expression of those transcripts identified in experiment 2 in a third set of embryos.

Experiment 1

In vitro embryo production (IVP)

Immature cumulus oocyte complexes (COCs) were obtained by aspirating surface-visible follicles from ovaries obtained from heifers at a local abattoir. The COCs were then matured and fertilized *in vitro* as previously described (Rizos *et al.* 2002c). At approximately 20 h post-insemination (hpi), presumptive zygotes were denuded by gentle vortexing and washed four times in PBS and twice in synthetic oviduct fluid medium (SOF). Zygotes were then cultured in SOF, supplemented with 10% (v/v) fetal calf serum, for 24 h (to produce two-cell embryos) or for 7 days (to produce blastocyst-stage embryos). Embryos from three separate replicates (from different ovary collection days) were pooled to give a total of 50 two-cell embryos and 50 blastocysts. Samples were snap frozen in liquid nitrogen and stored at -80°C until use.

RNA isolation and amplification

Total RNA was extracted from pools of two-cell embryos and blastocysts using the PicoPure RNA Isolation Kit (catalogue number KIT0204; Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. Following extraction, the RNA samples were DNase-treated (Stratagene, La Jolla, CA, USA) and first-strand cDNA was synthesized incorporating a T7 promoter according to the manufacturer's instructions. Using exogenous primers, double-stranded cDNA was synthesized and then purified using purification columns. A 3.5 μl aliquot of unamplified cDNA was removed from both the two-cell and blastocyst samples for Q-RT-PCR analysis and stored at -20°C until use. The remaining cDNA (12.5 μl) was submitted to one round of amplification using the RiboAmp RNA Amplification Kit (catalogue number KIT0201; Arcturus), which linearly amplifies the mRNA fraction of total RNA using cDNA as template in a T7 RNA polymerase-catalysed amplification reaction. A 3.5 μl aliquot of cDNA was taken following one round of amplification from both the two-cell and blastocyst-stage samples and stored at -20°C until use in Q-RT-PCR. The remainder (12.5 μl) was submitted to a second round of amplification and again a 3.5 μl aliquot of the resulting cDNA was stored at -20°C until use in Q-RT-PCR.

Q-RT-PCR

Q-RT-PCR reactions (MXP3000 Real-Time PCR System; Stratagene) were conducted in triplicate to contrast relative levels of transcripts for two selected genes, ferritin and β -actin, using bovine histone H2AZ as a housekeeping gene (Jeong *et al.* 2005; Table 1). PCR was performed by adding a 4 μl aliquot of each sample (each 3.5 μl sample stored from the amplification reactions was diluted in 40 μl sterile water) to the PCR mix containing gene specific primers and SYBR Green Master Mix (Brilliant Sybr Green QPCR Master Mix; Stratagene). The PCR protocol included

Table 1 GenBank accession number and forward and reverse primer sequences of genes and ESTs for Q-RT-PCR.

Accession number	Gene	Forward primer sequence	Reverse primer sequence
AF540563	Ferritin	GGGTGACCACATAACCAACC	AATATGCATGCACTGCCTTG
AB098974	β -Actin	TTGAACACGGCATCATCACT	GCATAGAGGGAGAGCACAGC
X52318	Histone H2AZ	GGTAAGGCTGGGAAGGACTC	GATGCATTTCCTGCCAATTC
BE588526	Galactose-4'-epimerase (GALE)	CGACGGGAGGTACTGAATGT	ATGGCCTGGACCATCTGTAG
CB461593	Signal-induced proliferation-associated gene 1 (SIPA1)	AGGTGGGCATCTTGTACTGC	ACGTGGTGTAAAGGGAGTGC
CN441852	CCR4-NOT transcription complex, subunit 3	GAGTTCTACCAGCGCTGTC	GGTGAAGCCTTCCTCTTCC
NM_175802	Guanine nucleotide-binding protein (GNB2LI)	GTGCTCTTCGAGGTCACTCC	CGGTTGTGAGAGGAGAAAGC
AF509504	DOT1-like protein (DOT1L)	AGTCTCGCGGTTGAGGTAGA	GCTGCAGTCCCTGTTCACT
NM_005171	Activating transcription factor 1 (ATF1)	GTGGGGAAGTGGGTAGTGAA	AGCTGCCTGAACTGTGGAAC
BF230159	Elongation factor 1 gamma (EEF1G)	TGGATGCTCACTGAAGACG	ACTGGGCCATTTTCTCACAG
NM_174569	Poly(A)-binding protein II (PABPN1)	GGAGGGAGGGTAGTGAAAGG	CTCCAGACCAGGCTACTTCG
NM_019080	NEDD 4 family interacting protein 2 (NDFIP2)	CAGAAACAGAGCGGAAGAG	GTGTGTGGTGTGGGTTTGA
NM_174729	Voltage-dependent anion channel 3 (VDAC3)	CTTCACGCAGAAGTGAACA	ACCTTCAAAGGCCAACACAG
BM251556	Heme-binding protein (HEBP1)	CTGGCTCTGAAGGGAAGTTG	ACTTCTGCACCGGGTATGAC
NM_000160	Homo sapiens glucagons receptor (GCGR)	AACACTGCTGGCTGCTGGT	TCCAGCACTGAATGTTCTCG
NM_015939	BOTL0100009_G06 (9_G06)	TTATCAGTGCCCGCTTCTTT	GCTGAAACGGGAAGATGTGT
BM252092	BOTL0100003XC10R (3XC10R)	GTCCAGGTTCTGCCTCACTC	TGGGTAAAGGAACCACTGGA

an initial step of 95 °C (10 min), followed by 40 cycles of 95 °C (30 s), 56–59 °C (1 min) and 72 °C (30 s). At the completion of cycling, melting curve analysis was carried out to establish the specificity of the amplicons produced. Q-RT-PCR data (C_t values) were analysed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Experiment 2

Blastocyst production

Presumptive zygotes produced *in vitro* as described above were either cultured *in vitro*, in SOF, for 6 days or were cultured *in vivo* following surgical transfer to the ligated ewe oviduct (approximately 100 embryos per oviduct) for 6 days (Rizos *et al.* 2002c). At the end of culture, blastocysts from both groups were snap frozen in liquid nitrogen and stored at –80 °C. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines and in adherence with guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

RNA isolation and amplification

Total RNA was extracted from eight groups of day 7 blastocysts (50 per group; 200 *in vivo* cultured, 200 *in vitro* cultured) and amplified as described above. The resulting aRNA was concentrated using alcohol precipitation and quantified using the Agilent 2100 Bioanalyzer (Agilent, Sandyford, Dublin, Ireland). The final concentration of aRNA was 2.25 µg/µl.

Microarray hybridization

The cDNA microarrays (BOTL-5) used in this experiment contained 3888 total spots with 932 bovine EST clone inserts developed from a normalized bovine total leukocyte (BOTL) cDNA library and an additional

459 amplicons representing additional genes including cytokines, receptors, signal transduction molecules, transcription and growth factors, enzymes, cell cycle regulators and cellular components. We have previously used a predecessor of this array (BOTL-4) to identify genes involved in apoptosis and dominant follicle development during follicular waves in cattle (Evans *et al.* 2004). A list of genes represented on the BOTL microarrays and their sequences can be found at <http://www.nbfgc.msu.edu> under the 'links' section.

Cy3- and Cy5-labelled cDNA probes for use in dual colour microarray hybridizations were created using the Cyscribe Post-Labeling Kit RPN5660 (Amersham). Amino allyl-dUTP (AA-dUTP) was incorporated into the aRNA samples in a cDNA synthesis reaction, catalysed by CyScript reverse transcriptase according to the manufacturer's instructions. The cDNA was then purified from RNA strands and unincorporated nucleotides by ethanol precipitation. cDNA from blastocysts derived from *in vivo* or *in vitro* culture was differentially labelled using *N*-hydroxysuccinimide (NHS)-activated fluorescent Cy3 or Cy5 dyes (Amersham Pharmacia Ltd, Piscataway, NJ, USA). Fluorescent labelling of the cDNA was carried out according to the manufacturer's instructions and was achieved by CyDye NHS esters reacting with the amino allyl groups incorporated into the cDNA first-strand synthesis. Four replicates were carried out; each replicate was comprised of blastocysts derived from a different ovary collection date. *In vivo*-cultured blastocysts in replicates 1 and 2 were labelled with Cy3 fluorescent dye and *in vitro*-cultured blastocysts were labelled with Cy5 fluorescent dye. Blastocysts in replicates 3 and 4 were labelled in the opposite way.

Unincorporated CyDye molecules were removed using purification columns from Qiagen (Min Elute PCR Purification Kit, catalogue number 28004). The two samples within each replicate were combined (i.e. Cy3-labelled, *in vitro*-cultured sample combined with the Cy5-labelled, *in vivo*-cultured sample) and concentrated to 10 µl using

a microcon 30 spin concentrator (Millipore Corp., Bedford, MA, USA). Finally, hybridization buffer (SlideHyb-3, Ambion, TX, USA) was added to give a final volume of 100 μ l.

The hybridization probes were added to the microarray slides, and hybridizations were conducted for 18 h in a commercial microarray hybridization station (HS 400 Tecan, Maennedorf, Switzerland) using a step-down hybridization protocol (65 °C for 3 h, 55 °C for 3 h, 50 °C for 12 h). Following hybridization, cDNA microarrays were washed within the hybridization station (42–50 °C), rinsed once in 2 $\times$ saline sodium citrate and once in ddH₂O and finally dried within the hybridization station. Slides were then scanned using an Axon Genepix 4000B scanner (Molecular Devices Corporation, Union City, CA, USA) and Genepix Pro 5.0 software (Molecular Devices Corporation) was used to process images, find spots, integrate the GAL (gene array list) file and finally to create reports of spot fluorescence intensities and local background fluorescence intensities.

Microarray data analysis

Data were analysed using three different approaches in order to generate a robust data set containing lists of genes/ESTs that were differentially expressed between the two groups of embryos being examined. In the first analysis, data from each replicate were normalized using a robust local regression technique (Cleveland & Grosse 1991) using the LOESS (locally-weighted regression and smoothing scatter plots) procedure of SAS (version 8, SAS Institute Inc., Cary, NC, USA). Normalized data were then back transformed and the intensity for the blastocysts cultured *in vitro* was divided by the intensity for those cultured *in vivo* to give an expression ratio value. The likelihood that the relative expression ratios of the four replicates differed from one (i.e. the same) was determined using Student's *t* test. In the second analysis, the procedure described above was repeated on data that were first background adjusted using Genepix Pro 5.0 software. Again, an expression ratio value was calculated and the likelihood that the values from the four replicates differed from one was determined using Student's *t* test. In the third analysis, a global normalisation method was used where a log ratio value was calculated for Cy3 and Cy5 values adjusted for background fluorescence. These values within each microarray were normalized to the GAPDH housekeeping gene (as there were 76 GAPDH spots on the array) using a single correction factor across that array. The likelihood that the resulting expression ratio values for the replicates differed from one was determined using Student's *t* test.

Mapping of arrayed clones to gene ontology (GO) terms

Arrayed clone sequences were matched to the nearest human and bovine homologs in RefSeq and UniProt by

BLAST searching. For RefSeq homology searches, an e-value threshold of 1×10^{-30} was used for human matches and a more stringent e-value threshold of 1×10^{-100} was used for bovine matches. For UniProt homology searches, an e-value threshold of 1×10^{-30} was used for human searches and 1×10^{-50} for bovine matches. The lower stringency thresholds used in establishing homology to human sequences reflect the evolutionary divergence between human and bovine gene sequences.

GO classifications for clones were obtained via Uniprot and EntrezGene. For UniProt, GO associations for the protein homologs of the arrayed clones were obtained directly from the UniProt database. For Entrezgene, the RefSeq homologs of the arrayed clones were mapped to EntrezGene accessions and from there to GO IDs using the associations in the files gene2refseq and gene2go obtained from the NCBI ftp site (<ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/>). Parent terms of each GO term were obtained by parsing the GO obo flat file (obtained from http://www.geneontology.org/ontology/gene_ontology.obo) using the perl GO parser distributed as part of the Bioperl package (<http://bioperl.org>). For every GO term linked to a clone on the array, the set of parent GO terms were added as new links to the clone. The generic GO slim ontology produced by Mundodi and Ireland was obtained from ftp://ftp.geneontology.org/pub/go/GO_slims/archived_GO_slims/generic.0208. Terms in the full GO were mapped to terms in the slim ontology using the map2slim.pl script included in the Bioperl package to obtain the set of GO slim associations for the clones on the array.

Clones corresponding to the same gene were grouped together. Counts of arrayed genes associated with each GO term and GO slim term were obtained. Representative GO slim terms were selected for inclusion in pie charts. Where GO slim terms were very broad (i.e. applied to a very large proportion of genes), they were excluded in favour of their more specific daughter terms. Conversely, where parent terms had large numbers of daughter terms, each with a small number of associated genes, the parent terms were included and the daughter terms excluded.

Experiment 3

Embryo production and RNA isolation

Day 7 blastocysts were produced from a total of nine replicates for both *in vivo*- and *in vitro*-cultured blastocysts as described in experiment 2 and stored, in pools of 10, at –80 °C until use. RNA was extracted as described above and cDNA was synthesized using SuperScript III Rnase H[–] reverse transcriptase (catalogue number 18080-044; Invitrogen) leaving a final volume of 40 μ l.

Q-RT-PCR analyses were conducted as for experiment 1 on 23 genes of interest, selected on the basis of the lowest *P* value calculated from the combined microarray data analyses. Between 3 and 6 replicates (different ovary collection days) per gene were used in the Q-RT-PCR validation of differential gene expression between *in vivo*- and

in vitro-cultured blastocysts using bovine histone H2AZ as the housekeeping gene. Primer sequences and the gene bank accession numbers of the genes examined are shown in Table 1.

Q-RT-PCR data (C_t values) were analysed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001) to determine the relative level of expression of each mRNA transcript in each sample. Differences in efficiency of cDNA production were corrected for by adjusting expression values (C_t values) of each mRNA according to corresponding H2AZ control/housekeeping gene expression values (C_t values). The relative abundance of the mRNAs between the two populations was calculated by dividing the *in vivo*-cultured blastocyst expression value by the *in vitro*-cultured blastocyst expression (baseline) value, after H2AZ correction, to [Q5] give a fold change value. Relative mRNA expression values for *in vivo*- and *in vitro*-cultured blastocysts were compared using Student's *t* test. Samples were measured in duplicate for each gene of interest, and H2AZ was measured in all samples in every 96-well plate that was subjected to Q-RT-PCR.

Results

Experiment 1. Effects of amplification on relative gene expression differences

When comparing the expression pattern of the same gene (e.g. ferritin) in two different samples (two-cell-stage embryos and blastocysts) the comparative level of expression varied, going from unamplified sample, to one round or two rounds of amplification; however, the relationship between the two samples remained the same, i.e. the level of ferritin transcript abundance was always lower in two-cell embryos compared with day 8 blastocysts (Fig. 1). Results were similar for β -actin expression (Fig. 2).

Figure 1 Effect of linear amplification on the relative expression level of ferritin in two-cell embryos compared with blastocysts.

Figure 2 Effect of linear amplification on the relative expression level of β -actin in two-cell embryos compared with blastocysts.

Experiment 2

A total of 384 genes/ESTs were identified as putatively differentially expressed between the two sample types. The results of the ontology analysis of the differentially expressed genes are shown in Fig. 3. The distribution of these differentially expressed genes across the ontology categories did not differ from the overall distribution of genes on the array. Irrespective of the method of data analysis, the vast majority (approximately 85%) of differentially expressed genes had a reduced expression in *in vitro*-cultured embryos compared with *in vivo*-cultured embryos.

Experiment 3

Q-RT-PCR was carried out using primers for 19 different genes and 4 ESTs, the Genbank accession numbers and primer sequences of which are shown in Table 1 (see Table 1 for abbreviations). Of the 23 transcripts indicated to be differentially expressed in experiment 2, 16 (70%) were verified by RT-PCR to be differentially expressed ($P < 0.05$). Of the genes identified, 7 are involved in transcription and translation (CCR4-NOT, EEF1G, PABPN1, FOXO3A, HMG2, GNB2L1, DOT1L), 2 genes encode or interact with receptors (GCGR, REA) and 3 others encode factors involved in cellular metabolism (GALE, BiKKbeta, HEBP1). The 4 ESTs have similarity to genes involved in transcription, translation and receptors (3XC10R, 13_A08, 9_G06) or have unknown function (6XE03R).

All genes verified as being differentially expressed following Q-RT-PCR had higher expression levels in *in vivo*-cultured blastocysts compared with *in vitro*-cultured blastocysts (Fig. 4) ranging from a 1.40- (6XE03R) to a 6.94- (BiKK beta) fold difference in expression levels.

Figure 3 Ontology classifications of genes identified as being differentially expressed using DNA microarray in *in vivo*-compared with *in vitro*-cultured blastocysts. Some genes are represented in more than one ontology.

Discussion

The data presented here are put in a biological context that potentially explains, at least in part, deficiencies contributing to reduced quality of *in vitro*-produced embryos. Microarray analysis identified 384 genes that differ in expression between *in vivo*- and *in vitro*-cultured blastocysts, 85% of which are underexpressed in *in vitro*-cultured embryos. RT-PCR on 23 of the most differentially

expressed transcripts confirmed down-regulation of 12 genes and 4 ESTs, the majority of which have not been previously characterized in mammalian embryos. Interestingly, 10 of these genes are involved in transcription and translation and we suggest this deficiency compromises the quality of *in vitro*-cultured embryos.

One of the major limiting factors to characterising the global pattern of gene expression in embryos is the limited amount of embryonic RNA available for analysis. Bovine

Figure 4 Level of gene expression in *in vivo*-cultured embryos (filled columns) relative to *in vitro*-cultured embryos (open columns) determined using Q-RT-PCR. * $P < 0.05$; ** $P < 0.01$. See Table 1 for full names of abbreviated genes.

blastocysts contain approximately 5.3 ng total RNA (Bilodeau-Goeseels & Schultz 1997) with a ratio of mRNA to total RNA of about 3.5% (Roozmond 1976, Piko & Clegg 1982). Despite the fact that 50 blastocysts were pooled per biological replicate (giving approximately 9.3 ng mRNA) this was still insufficient RNA for microarray analysis, where quantities in the microgram range are generally used. Linear amplification was used because it has been shown to introduce minimal disruption of mRNA expression patterns (Wang *et al.* 2000, Gomes *et al.* 2003, Jenson *et al.* 2003). We recently examined the fidelity of differential gene expression generated from unamplified versus amplified RNA from the same homogenous starting pools of bovine material using DNA microarrays; the hybridization intensities for each of the spots examined per slide for experiments using amplified versus non-amplified RNA were highly correlated ($R^2 = 0.86$) and the false-positive rate was not affected (Patel *et al.* 2005). Data from the present study (experiment 1) showed that while the fold difference between the same genes in different samples (two-cell embryo and blastocyst) increased as the number of amplifications increased, the expression relationship between the samples was maintained (i.e. if the relative abundance of a transcript was higher in one group before amplification, it remained higher in that group following amplification).

A key factor that facilitates basic cell functioning and thereby embryonic development is the cell's ability to transcribe its DNA to single-stranded RNA and then translate the coding region nucleotide sequence of mRNA into the sequence of amino acids comprising a protein, with tRNA and rRNA providing the apparatus for protein synthesis. Here we have identified seven genes that are involved in transcription and translation, and that may have a crucial role in early embryo development. High mobility group protein 2 (HMG2) is a DNA-binding protein involved in bending DNA for critical steps in DNA replication, transcription and recombination (Fan *et al.* 2002). HMG proteins are a family of chromosomal proteins which act as structural elements of the chromatin and which promote transcription by modifying chromatin conformation (Bianchi & Beltrame 2000, Vigneault *et al.* 2004). Members of the HMG protein family have previously been shown to be expressed throughout oocyte and early bovine development from the germinal vesicle stage to the blastocyst stage in *in vitro*-produced embryos (Vigneault *et al.* 2004). We have also identified the gene for DOT1L protein that is involved in the process of histone methylation, which is important in regulating chromatin dynamics and gene expression (Kouzarides 2002). DOT1L is an histone 3 lysine 79 (H3- K79)-specific methyltransferase and although the function of K79 methylation of histones is unknown, it is suggested that DOT1L regulates telomeric silencing through methylation of this lysine residue (Feng *et al.* 2002). FOXO3A, also known as FKHR-L1, is a member of the FOXO family of Forkhead transcription factors and is regulated by the

phosphoinositide-3-kinase-protein-kinase-B pathway. A number of studies have shown Forkhead transcription factors to have important roles in cell cycle arrest, apoptosis and stress responses *in vitro* (Medema *et al.* 2000, Burgering & Kops 2002, Hosaka *et al.* 2004) and transcriptional control of key metabolic enzymes (O'Brien *et al.* 2001). Its role in reproduction is demonstrated by the fact that FOXO3A-null female mice show age-dependent infertility and abnormal ovarian follicular development (Hosaka *et al.* 2004). The expression of FOXO3A has not been previously described in embryos and the higher relative abundance of FOXO3A *in vivo*- compared with *in vitro*-derived embryos in the present study suggests a role for this family of genes in mammalian embryo development. For the first time we have shown that the gene for CCR4-NOT is more highly expressed in *in vivo*- compared with *in vitro*-cultured embryos (Fig. 4). The CCR4-NOT complex of proteins is central to the regulation of mRNA metabolism. It is involved in several aspects of mRNA formation, including repression and activation of mRNA initiation, control of mRNA elongation and the deadenylation and subsequent degradation of mRNAs (Denis & Chen 2003).

Poly(A) tails are found at the 3' ends of almost all eukaryotic mRNAs. They are bound by two different poly(A)-binding proteins, PABPC in the cytoplasm and PABPN1 in the nucleus. PABPN1, which displayed greater mRNA abundance in *in vivo*-cultured blastocysts, is involved in the synthesis of poly(A) tails, increasing the processivity of poly(A) polymerase and contributing to defining the length of a newly synthesized poly(A) tail (Kuhn & Wahle 2004). The extent of the poly(A) tail at the 3' end of mRNA transcripts is an important regulatory element for determining their stability and control of translation and we have previously demonstrated a clear relationship between the polyadenylation status of certain transcripts and bovine embryo developmental competence (Brevini-Gandolfi *et al.* 2000).

The guanine nucleotide-binding protein gene (GNB2L) is more highly expressed in *in vivo*- compared with *in vitro*-cultured embryos and encodes a receptor for activated C-kinase (RACK1) protein (Wang *et al.* 2003) which recruits activated protein kinase C to the ribosome. This leads to the stimulation of translation through the phosphorylation of initiation factor 6 and, potentially, of mRNA-associated proteins. RACK1 therefore links signal transduction pathways directly to the ribosome, which allows translation to be regulated in response to cell stimuli (Nilsson *et al.* 2004). The relative higher abundance of GNB2L transcripts in *in vivo*-cultured blastocysts compared with *in vitro*-cultured blastocysts may infer a superior translational mechanism in those embryos cultured *in vivo* compared with *in vitro*.

Elongation factor 1 gamma (EEF1G) is a nucleic acid-binding protein and is responsible for the enzymatic delivery of aminoacyl tRNAs to ribosomes. The tRNA carries the amino acid to the ribosome, which is then used in

protein synthesis thereby inferring a crucial role for EEF1G in the translation process in protein biosynthesis. This is the first time that expression of this gene has been identified in bovine embryos. Greater abundance of this transcript in *in vivo*-cultured blastocysts as evidenced here may be important for de novo synthesis of proteins and consequently embryo development. Finally, the unknown EST, 9_G06 has weak similarity to a translation initiation factor in *Saccharomyces cerevisiae* and may play an important role in the steps of protein synthesis via transcription and translation in early developing embryos.

In addition to the genes above that directly modulate transcription and translation, we have identified two genes that are more highly expressed in *in vivo*- compared with *in vitro*- cultured embryos whose products indirectly regulate transcription. The repressor of estrogen receptor activity (REA) is a co-regulator that directly interacts with the estrogen receptor and represses its transcriptional activity. Homozygous REA gene knockout mice do not develop past the embryonic day 9 stage of development, implying a critical role for REA in early development (Park *et al.* 2005). While the EST 3XC10R has no convincing similarity with other known genes, it does have 74% sequence homology to human nuclear receptor co-activator 6 interacting protein (NCOA6IP). The protein encoded by this gene is a transcriptional co-activator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions. Since 8 (and possibly 10, including EST-only genes) of the 16 genes that we have identified as being differentially expressed in *in vivo*- compared with *in vitro*-cultured embryos are associated with transcription and translation, we suggest that the apparent inability (or at least inefficiency) of the transcription–translation machinery to operate properly in *in vitro*- compared with *in vivo*-produced embryos is a major factor in contributing to the phenotypic differences between embryos developing in these two culture environments. In support of this notion, while the expression of connexin 43 (Cx43) transcripts, a gap junction protein involved in cell-to-cell communication, decreased in both *in vivo*- and *in vitro*-cultured embryos between the zygote and 8- to 16-cell stage; levels remained low thereafter in the *in vitro*-cultured embryos but increased significantly in those cultured *in vivo*, such that by the blastocyst stage there was a 10-fold difference in transcript abundance between embryos derived from the two culture environments (Lonergan *et al.* 2003b).

In addition to a number of transcription and translation factors, mentioned above, we have identified a number of genes that are involved in cellular metabolism and processes, and that are more highly expressed in *in vivo*- than *in vitro*-cultured embryos. These genes are the glucagon receptor (GCGR), UDP-galactose 4'-epimerase (GALE), I κ B kinase-beta (IKK-beta), a heme-binding protein and an EST with homology to an adenosine receptor. Glucagon is a key hormone in the regulation of glucose concentrations; its effects are mediated via a membrane receptor

to stimulate an increase in cAMP via G proteins and in turn stimulate protein kinase A (PKA) to phosphorylate many target proteins (Dean & McEntyre 2004). Another gene more highly expressed in *in vivo*- compared with *in vitro*-cultured embryos encodes for heme-binding protein 1 (HEBP1). Heme is an iron and protein complex that serves as the prosthetic group of numerous hemoproteins (e.g. hemoglobin, myoglobin, cytochromes, guanylate cyclase and nitric oxide synthase) and plays an important role in controlling protein synthesis and cell differentiation (Ponka 1999). Heme-binding proteins may enhance, decrease or completely inhibit heme-catalyzed oxidations (Vincent *et al.* 1988) and we suggest that they are important for successful embryo development. Another gene that is up-regulated in *in vivo*- compared with *in vitro*-cultured embryos is bIKK-beta, which is involved in the activation of an inhibitor of NF- κ B kinase (IKK). Activation of the IKK complex is a crucial step in NF- κ B signalling, which in turn has a critical role in the regulation and coordination of a wide range of cellular events such as cell growth, apoptosis and cell differentiation (Rottenberg *et al.* 2002). Another enzyme that is more highly expressed in *in vivo*- than *in vitro*-cultured embryos and that is involved in metabolism is UDP-galactose 4'-epimerase (GALE). GALE functions to interconvert UDP-galactose and UDP-glucose in the final step of the Leloir pathway of galactose metabolism; impairment of human GALE results in the metabolic disorder epimerase-deficiency galactosemia (Walter *et al.* 1999). Finally we have identified two ESTs that are more highly expressed in *in vivo*-than *in vitro*-cultured embryos. The EST 6XE03R has no homology with any previously described gene and the EST 13_A08 has no good sequence homology to other genes but it is moderately similar (74.4%) to the adenosine A2B receptor (*Canis familiaris*). Adenosine, as well as being a building block of many biologically relevant molecules such as ATP and nucleic acids, is an endogenous nucleoside that regulates many physiological processes through the activation of any of its four G-protein-coupled receptor types (Ohta & Sitkovsky 2001). Hence, we have identified five genes that are involved in signalling pathways and/or cellular processes – including metabolism, growth, apoptosis and differentiation – that we suggest are critical factors in determining the success of blastocyst development.

In summary, we have identified 12 genes and 4 ESTs (all except one have not been previously described in embryos) that we suggest are potentially critical in determining the quality of the blastocyst. The majority of these genes (10 of the 16) are involved in the regulation of transcription and translation which are vital processes for the cellular growth and division that is characteristic of embryos during the early stages of development. *In vitro*-cultured embryos are less developmentally competent than *in vivo*-cultured embryos. The suggestion that this is associated with a deficiency in genes controlling transcription in *in vitro*-cultured embryos is further supported by our finding using microarray analysis that 85%

of the differentially expressed genes had lower expression in *in vitro*- than *in vivo*-cultured embryos, showing that the overall level of transcription in *in vitro*-cultured embryos may be much reduced compared with *in vivo*-cultured embryos. This theory is supported by results from a study on gene expression following culture of mouse embryos *in vitro* compared with *in vivo*: the majority of those transcripts that were found to be differentially expressed following culture *in vitro* compared with *in vivo*, were down-regulated in the *in vitro*-cultured embryos (Rinaudo & Schultz 2004).

Understanding the molecular mechanisms underlying the reduced quality of *in vitro*-produced embryos could provide important insights into strategies for overcoming these deficiencies. Moreover, successful strategies could have far-reaching implications, not only for *in vitro* fertilization procedures in several species – including humans – but also for optimising other related technologies such as nuclear transfer induced reprogramming and stem cell procedures.

Acknowledgements

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

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Received 22 October 2005

First decision 22 November 2005

Revised manuscript received 2 January 2006

Accepted 16 January 2006