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.


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. 2002b, 2002c, Lonergan et al. 2003a). 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. 2002a), which reflect some of the differences noted above.
Studies in bovine embryos indicate that many of the differences in quality of \textit{in vitro} and \textit{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 \textit{et al.} 2003a, Tesfaye \textit{et al.} 2004). This is the case, not only when one compares \textit{in vitro} and \textit{in vivo} culture systems, but also comparing different \textit{in vitro} culture systems (reviewed by Lonergan \textit{et al.} 2003a, Wrenzycki \textit{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 \textit{in vivo} culture system is the ability to culture large numbers of embryos in a near \textit{in vivo} environment and in a cost-effective manner. While the yield of blastocysts following such \textit{in vivo} culture is not superior to that following culture in \textit{vitro}, the quality of the blastocysts is significantly improved (Galli \& Lazzari 1996, Enright \textit{et al.} 2000, Lazzari \textit{et al.} 2002, Rizos \textit{et al.} 2002c, 2003). Blastocysts produced in this way are similar to true \textit{in vivo} embryos in terms of morphology (Fair \textit{et al.} 2001), cryotolerance (Enright \textit{et al.} 2000, Rizos \textit{et al.} 2002a), mRNA expression pattern for select genes (Lazzari \textit{et al.} 2002, Rizos \textit{et al.} 2002c) and pregnancy rate following transfer (Lazzari \textit{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 \textit{in vitro} versus \textit{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 \textit{in vivo} or \textit{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.

\section*{Experiment 1}

\textbf{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 \textit{vitro} as previously described (Rizos \textit{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 $-80^\circ$C until use.

\textbf{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 \(\mu\)l aliquot of unamplified cDNA was removed from both the two-cell and blastocyst samples for Q-RT-PCR analysis and stored at $-20^\circ$C until use. The remaining cDNA (12.5 \(\mu\)l) 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 \(\mu\)l aliquot of cDNA was taken following one round of amplification from both the two-cell and blastocyst-stage samples and stored at $-20^\circ$C until use in Q-RT-PCR. The remainder (12.5 \(\mu\)l) was submitted to a second round of amplification and again a 3.5 \(\mu\)l aliquot of the resulting cDNA was stored at $-20^\circ$C until use in Q-RT-PCR.

\textbf{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 \(\beta\)-actin, using bovine histone H2AZ as a housekeeping gene (Jeong \textit{et al.} 2005; Table 1). PCR was performed by adding a 4 \(\mu\)l aliquot of each sample (each 3.5 \(\mu\)l sample stored from the amplification reactions was diluted in 40 \(\mu\)l 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.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF540563</td>
<td>Ferritin</td>
<td>GGTGACCACTGACATACCTGT</td>
<td>AATAGTCATGCACCTGCTTG</td>
</tr>
<tr>
<td>AB098974</td>
<td>b-Actin</td>
<td>TTAGAACGCGATCCATCACT</td>
<td>GCATAGAGGAGAAGCAGAC</td>
</tr>
<tr>
<td>X52318</td>
<td>Histone H2AZ</td>
<td>GGTAGGAGATGGCAGACTC</td>
<td>GATGCAATCTCTGCACATTT</td>
</tr>
<tr>
<td>BE588526</td>
<td>Galactose-4-epimerase (GALE)</td>
<td>CGACGGGAGATGCTCAAGGG</td>
<td>ATGCGTGTACCTGCTGTAG</td>
</tr>
<tr>
<td>CB461593</td>
<td>Signal-induced proliferation-associated gene 1 (SIPA1)</td>
<td>AGTGGCCACCTTTGTAGCTGC</td>
<td>AATGCTGTTAAAGGGCTGTGCG</td>
</tr>
<tr>
<td>CN441852</td>
<td>CCR4-NOT transcription complex, subunit 3</td>
<td>GTGCTTCTCAGTACTGATA</td>
<td>CGTGTTGTCAGAGAAAGC</td>
</tr>
<tr>
<td>NM_175802</td>
<td>Guanine nucleotide-binding protein (GNB2LI)</td>
<td>AGTCCTGGGTTGAGTATGAG</td>
<td>AGTGCAGTCCCTGTTGAGC</td>
</tr>
<tr>
<td>AF509504</td>
<td>DOT1-like protein (DOT1L)</td>
<td>GTGGGAAGTGGGTAGTGAA</td>
<td>AGTGCCTTGAACCTGTTGAC</td>
</tr>
<tr>
<td>NM_005171</td>
<td>Activating transcription factor 1 (ATF1)</td>
<td>TCGAGGAGATGGCAGACTC</td>
<td>AATGCTGTTAAAGGGAGCAGC</td>
</tr>
<tr>
<td>BF230159</td>
<td>Elongation factor 1 gamma (EEF1G)</td>
<td>CGACGGGAGATGCTCAAGGG</td>
<td>ATGCGTGTACCTGCTGTAG</td>
</tr>
<tr>
<td>NM_174569</td>
<td>Poly(A)-binding protein II (PABPN1)</td>
<td>AGTCTCGCGGTTGAGGTAGA</td>
<td>GCTGCAGTCCCTGTTCAGTT</td>
</tr>
<tr>
<td>NM_019080</td>
<td>NEDD 4 family interacting protein 2 (NDFIP2)</td>
<td>CTTCAACGAAGTGGAACA</td>
<td>ACTTCAAGGGCCACACAG</td>
</tr>
<tr>
<td>NM_174729</td>
<td>Voltage-dependent anion channel 3 (VDAC3)</td>
<td>GTTCAGCAAGTGGAACA</td>
<td>ACTTCAAGGGCCACACAG</td>
</tr>
<tr>
<td>BM251556</td>
<td>Heme-binding protein (HEBP1)</td>
<td>CTGGCTCTTCGAGGTCACTCC</td>
<td>CGGTTGTCAGAGGAGAAAGC</td>
</tr>
<tr>
<td>NM_000160</td>
<td>Homo sapiens glucagons receptor (GCGR)</td>
<td>AACTACTGCTGGCTGCTGGT</td>
<td>TCCAGCTTGAACCTGTTGAC</td>
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<tr>
<td>NM_021393</td>
<td>GTPase regulator 1 (GTP1)</td>
<td>GTGGGAAGTGGGTAGTGAA</td>
<td>AGTGCCTTGAACCTGTTGAC</td>
</tr>
<tr>
<td>BM252092</td>
<td>AB098974</td>
<td>GGTGACCACTGACATACCTGT</td>
<td>AATAGTCATGCACCTGCTTG</td>
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</tbody>
</table>

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 estimate the specificity of the amplicons produced. Q-RT-PCR data (G_t values) were analysed using the 2^−ΔΔCt 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, Santa Clara, CA, USA). 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 CyScript Post-Labeling Kit RPN5660 (Amersham). Amino allyl-dUTP (AA-dUTP) was incorporated into the aRNA samples in a DNA 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 compared (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 µl.

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 X 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 back-
ground 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 analy-
sis, 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 cul-
tured 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 dif-
furred 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 back-
ground 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 micro-
array 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 $1 \times 10^{-30}$ was used for human
matches and a more stringent e-value threshold of
$1 \times 10^{-100}$ was used for bovine matches. For UniProt hom-
ology searches, an e-value threshold of $1 \times 10^{-30}$ was used
for human searches and $1 \times 10^{-50}$ 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 pro-
tein homologs of the arrayed clones were obtained directly
from the UniProt database. For Entrezgene, the RefSeq
homologs of the arrayed clones were mapped to Entrez-
Gene accessions and from there to GO IDs using the
associations in the files gene2refseq and gene2go obtained
DATA/). Parent terms of each GO term were obtained by
passing 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.gene-
ontology.org/pub/go/GO_slims/archived_GO_slims/ge-
neric.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 associ-
ations 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 blasto-
cysts 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 µl.

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 col-
lection days) per gene were used in the Q-RT-PCR vali-
dation 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 (Ct 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 (Ct values) of each mRNA according to corresponding H2AZ control/housekeeping gene expression values (Ct 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 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).

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 (CCCR4-NOT, EEF1G, PABPN1, FOXO3A, HMG2, GNB2L1, DOT1L), 2 genes encode or interact with receptors (GCCG, 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, T3_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- (βIKK beta) fold difference in expression levels.

![Figure 1](https://example.com/figure1.png)  
Figure 1  Effect of linear amplification on the relative expression level of ferritin in two-cell embryos compared with blastocysts.

![Figure 2](https://example.com/figure2.png)  
Figure 2  Effect of linear amplification on the relative expression level of β-actin in two-cell embryos compared with blastocysts.
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 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.](image)

![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.](image)
blastocysts contain approximately 5.3 ng total RNA (Bilodeau-Goeseels & Schultz 1997) with a ratio of mRNA to total RNA of about 3.5% (Roozemond 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 squared = 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 GNBL2 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), IκB kinase-beta (bIKK-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-κB kinase (IKK). Activation of the IKK complex is a crucial step in NF-κB 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.

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