Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments

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Abstract

This study was conducted to investigate the gene expression profile of *in vivo*-derived bovine embryo biopsies based on pregnancy outcomes after transferring to recipients. For this, biopsies of 30–40% embryos were taken from grade I blastocysts (International Embryo Transfer Society Manual) and the remaining 60–70% of the intact embryos were transferred to recipients. Frozen biopsies were pooled into three distinct groups based on the pregnancy outcome after transferring the corresponding parts, namely those resulting in no pregnancy (NP), pregnancy loss (PL), and calf delivery (CD). Array analysis revealed a total of 41 and 43 genes to be differentially expressed between biopsies derived from blastocysts resulting in NP versus CD and PL versus CD respectively. Genes regulating placental development and embryo maternal interaction (*PLAC8*) were found to be upregulated in embryo biopsies that ended up with CD. Embryo biopsies that failed to induce pregnancy were enriched with mitochondrial transcripts (*FI405*) and stress-related genes (*HSPD1*). Overall, gene expression profiles of blastocysts resulting in NP and CD shared similar expression profiles with respect to genes playing significant roles in preimplantation development of embryo. Finally, comparing the transcript signatures of *in vivo*- and *in vitro*-derived embryos with developmental competence to term revealed a similarity in the relative abundance of 18 genes. Therefore, we were able to present a genetic signature associated with term developmental competence independent of the environmental origin of the transferred blastocysts.

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Introduction

Pregnancy loss (PL) due to early embryonic mortality is considered as a major reproductive problem in all mammalian species. In cattle, early embryonic loss between days 8 and 18 accounts for ~40% of all PL (Ayalon 1978, Diskin & Sreenan 1980). Similarly, the vast majority of human embryos cultured *in vitro* at fertility centers worldwide fail to develop *in vivo* following transferring to the uterus (Dobson *et al.* 2004) with implantation rates of only 20% (Ebner *et al.* 2001). However, in contrast to the bovine embryos, human embryos were transferred back to the oocyte donors and were transferred to the uterus at earlier stages of development. To date, the underlying molecular mechanisms regulating early embryo loss in both humans and cows are generally unknown. In addition, studies on embryo biology remain ethically challenging

in humans. Therefore, other species like cows could be used as an experimental model to understand the molecular mechanisms related to embryonic developmental competence.

The impact of early embryonic mortality is not only a major obstacle to the cattle-breeding industry but also hinders the application of reproductive technologies in mammals such as *in vitro* embryo production and somatic cell nuclear transfer. Early embryonic mortality is caused by intrinsic defects within the embryo, an inadequate maternal environment, asynchrony between embryo and mother, or failure of the mother to respond appropriately to embryonic signals. However, the extent and regulation of altered gene expression during preimplantation development are likely to be critical for later development of the conceptus (Brison & Schultz 1997). Using specific culture environments to produce mice or bovine embryos did not only result in altered

gene expression of transcripts related to metabolism and growth but also resulted in altered conceptus and fetal development following transfer (Khosla *et al.* 2001, Lazzari *et al.* 2002).

Many studies examined differences in the expression of developmentally important genes (Rizos *et al.* 2002, Knijn *et al.* 2005, Corcoran *et al.* 2007) or global gene expression profile of *in vitro*-produced bovine embryos compared with their *in vivo* counterparts (Smith *et al.* 2005, 2009, Corcoran *et al.* 2007). These studies confirmed that there are alterations in gene expression of embryos developed under different culture conditions, which result in reduced quality to be associated with fetal and neonatal abnormalities (Farin *et al.* 2006) subsequently. Such losses are commonly associated with IVF procedures in human and livestock species of the agriculture industry (Imakawa *et al.* 2004).

It is assumed that essential mechanisms for embryo implantation must be supported by redundant pathways to ensure the conception of new offspring. Mamo *et al.* (2006) showed the differences in gene expression profiles of oocytes and embryos that differed in their developmental potential. However, the establishment of direct connections between embryonic gene expression profile and subsequent developmental potential remained challenging. So better knowledge of gene expression patterns and levels during early embryonic development would yield insights into the molecular pathways controlling early development (Khurana & Niemann 2000) to improve success of embryo culture and, in particular, to develop precise criteria for assessing the health of the early mammalian embryo (Watson *et al.* 1999).

Recently, we showed that transcriptome analysis of biopsies taken from *in vitro*-produced bovine blastocysts based on pregnancy outcome after transfer could help to identify transcripts directly related to embryonic developmental potential (El-Sayed *et al.* 2006). Similarly, we also reported that transcriptome analysis of biopsies taken from *in vivo*-produced bovine blastocysts could be used as an indicator for further development (Salilew-Wondim *et al.* 2010). However, direct comparison of both studies is not possible because different array platforms were used to identify differentially regulated genes as well as different synchronization protocols of recipient heifers. Nevertheless, comparing the genetic signatures of *in vivo*- and *in vitro*-derived blastocysts with respect to developmental competence, if analyzed by the same techniques and experimental design, could open a new window to understand the molecular mechanisms related to embryonic developmental competence. Therefore, we aimed to extend that work (El-Sayed *et al.* 2006) with *in vivo*-derived embryos by using the same techniques and the same experimental design. Consequently, in this study, we aimed to compare the gene expression profile of embryo biopsies taken from *in vivo*-produced bovine blastocysts depending on the

pregnancy outcome after embryo transfer. Finally, our superordinate goal was to compare the signatures of *in vivo*- and *in vitro*-derived blastocysts related to term development to identify common signatures closely related to developmental competence.

Results

Pregnancy outcomes after embryo transfer

In the present experiment, 49 *in vivo* day 7 blastocysts were biopsied. Thirty-eight of these blastocysts (77.7%) subsequently re-expanded and were transferred to recipients. Of the total recipients that received biopsied and re-expanded blastocysts, 36.8% (14/38) were classified as non-pregnant, while the remaining 63.1% (24/38) were classified as pregnant confirmed by ultrasound-guided pregnancy examination performed at day 28. Nine recipients (23.6%) were found to be pregnant at day 28 after estrus but no longer at day 42, which were classified as PL group. The remaining 15 recipients (39.5%, 15/38) resulted in calf delivery (CD).

Genes differentially regulated between embryo biopsies resulting in no pregnancy and CD

To obtain a high-confident set of differentially regulated genes, we used a rigorous combination of *P* values ($P < 0.05$) and false discovery rate ($FDR \leq 5\%$). In addition, we set the cut-off value of fold change as ≥ 1.5 . Based on these criteria, 41 genes were found to be differentially expressed. Biopsies corresponding to counterparts resulting in no pregnancy (NP) showed upregulation of 20 transcripts whereas 21 transcripts were found to be downregulated (Fig. 1A).

These differentially expressed genes (Table 1) were found to represent genes with known function ($n = 30$) as well as unknown function ($n = 11$). All differentially regulated transcripts were functionally classified based on the criteria of Gene Ontology Consortium classifications (<http://www.geneontology.org>). The resulting data were supplemented with additional information from various tools available in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Accordingly, certain groups of genes were up- or downregulated in the NP group compared with the CD group: biopsies that ended up with NP were found to be enriched with transcripts related to tumor necrosis factor receptor binding (*TNF*), protein binding (*HSPD1*), RNA binding (*PA2G4*), calcium ion binding (*S100A10* and *S100A14*), and oxidoreductase activity (*ND1*, *FL405*, and *AKR1B1*). Transcripts related to protein binding (*KRT8* and *ELOVL1*), DNA binding (*H3F3B* and *H2FA*), structural constituent of ribosome (*RPLP0*), placenta specific (*PLAC8*), growth factor activity (bone morphogenetic protein 15 (*BMP15*)), and signal transducer activity (*RGS2*) were found to be highly expressed in

Figure 1 Venn diagram showing common differentially expressed genes between no pregnancy, pregnancy loss, and calf delivery both in *in vivo*- and *in vitro*-derived embryos (A). List of common genes up- or downregulated in no calf delivery compared with calf delivery group both in *in vivo*- and *in vitro*-derived embryos (B).

embryos resulting in CD. RT-qPCR confirmed the expression profile of *S100A10*, *PLAC8*, *KRT8*, *BMP15*, *HSPD1*, *RGS2*, and *FL405* to be in agreement with microarray results considering biopsies of NP and CD groups (Fig. 2).

Genes differentially regulated between embryo biopsies resulting in PL and CD

Using criteria of *P* values ($P < 0.05$) and cutoff fold change ≥ 1.5 , 43 genes were found to be differentially regulated between embryo biopsies resulting in PL (pregnancy at day 28 but resorption until day 42) compared with embryo biopsies resulting in CD

(Table 2). In summary, 20 transcripts were upregulated and 23 transcripts were downregulated in biopsies of the PL group compared with biopsies resulting in CD (Table 2). These transcripts represent genes with known function ($n = 36$) as well as genes with unknown function ($n = 7$). Based on their functional annotation, genes involved in oxidoreductase activity (*FL405* and *FL396*), structural constituent of ribosome (*RPS15A* and *RPL3*), ATPase inhibitor activity (*ATPIF1*), protein binding (*CD9* and *HSPD1*), calcium ion binding (*S100A10*), and nucleotide binding (*EEF1A1*) were upregulated in embryo biopsies resulting in PL. However, biopsies resulting in CD were enriched with transcripts regulating signal transducer activity (*RGS2*), cell redox homeostasis

Table 1 List of genes up- and downregulated in blastocyst biopsies resulting in no pregnancy compared with calf delivery.

Gene name	GenBank accession no.	Fold change	Gene function (biological process)
<i>Genes upregulated in blastocyst biopsies resulting in no pregnancy compared with calf delivery (n=20)</i>			
<i>Homo sapiens</i> S100 calcium-binding protein A14 (<i>S100A14</i>), mRNA	NM_020672	2.7	Calcium ion binding
<i>Bos taurus</i> S100 calcium-binding protein A10 mRNA (<i>S100A10</i>)	NM_174650	2.4	Calcium ion binding
<i>Homo sapiens</i> S100 calcium-binding protein A16, mRNA (cDNA clone)	BC019099	1.6	Calcium ion binding
<i>Homo sapiens</i> heat-shock 60 kDa protein 1 (chaperonin), mRNA (cDNA) <i>HSPD1</i>	BC002676	2.1	Protein binding (response to stress)
<i>Bos taurus</i> clone RP42-351K5, complete sequence	AC092727	1.5	Unknown
<i>Mus musculus</i> cysteine and glycine-rich protein 2, mRNA (cDNA clone) (<i>CSRP2</i>)	BC012663	1.6	Metal ion binding (cell differentiation)
<i>Homo sapiens</i> TGFB-induced factor (TALE family homeobox) (<i>TGIF</i>)	NM_003244	1.9	Transcription factor activity (transcription)
<i>Homo sapiens</i> proliferation-associated 2G4, 38 kDa, mRNA (cDNA clone) (<i>PA2G4</i>)	BC007561	1.5	RNA binding (cell cycle arrest)
<i>Mus musculus</i> 11 days embryo gonad cDNA, <i>RIKEN</i> full-length (<i>7030402D04Rik</i>)	AK078561	1.8	Unknown
<i>Bos taurus</i> EF1A mRNA for elongation factor 1 α , complete cds (<i>EEF1A1</i>)	AB060107	1.7	Nucleotide binding
<i>Homo sapiens</i> RNA-binding motif protein 16 (<i>RBM16</i>), mRNA	XM_376547	1.5	Unknown
<i>Homo sapiens</i> TFIIA- α/β -like factor transcript variant (<i>GTF2A1L</i>)	NM_006872	1.8	RNA polymerase II transcription factor (transcription)
Bovine ATP synthase inhibitor protein mRNA, complete cds (<i>ATPIF1</i>)	M22559	2.1	ATPase inhibitor (negative regulation of cellular metabolic)
<i>Homo sapiens</i> split hand/foot malformation (ectrodactyly) type (<i>SHFM1</i>)	NM_006304	1.9	Peptidase activity (proteolysis)
<i>Bos taurus</i> isolate FL405 mitochondrion, partial genome (<i>FL405</i>)	AY308069	2.7	Oxidoreductase activity (mitochondrial electron transport)
<i>Bos taurus</i> isolate 65 NADH dehydrogenase subunit 1 (<i>ND1</i>), NADH	AF490528	3.1	Oxidoreductase activity (mitochondrial electron transport)
<i>Homo sapiens</i> lectin, galactoside-binding, soluble, 3 (galectin 3), (<i>LGALS3</i>)	BC001120	4.2	IgE binding
<i>Homo sapiens</i> hypothetical protein LOC57821, mRNA (cDNA clone) (<i>C1orf114</i>)	BC008389	2.5	Unknown
<i>Bos indicus</i> tumor necrosis factor- α (<i>TNF</i>) gene, complete cds	AF011927	1.8	Tumor necrosis factor receptor binding (immune response)
<i>Bos taurus</i> <i>AKR1B1</i> aldo-keto reductase family 1, member B1	M31463	1.9	Oxidoreductase activity (oxidation reduction)
<i>Genes downregulated in embryo biopsies resulting in no pregnancy compared with calf delivery (n=21)</i>			
<i>Homo sapiens</i> placenta-specific 8 (<i>PLAC8</i>), mRNA	NM_016619	1.8	Unknown
Bovine mRNA fragment for cytokeratin A (no. 8) (<i>KRT8</i>)	X12877	1.5	Protein binding (response to other organism)
Bovine mRNA for histone H2A.Z (<i>H2FAZ</i>)	X52318	1.9	DNA binding (nucleosome assembly)
<i>Homo sapiens</i> H3 histone, family 3A (<i>H3F3A</i>)	BT020962	2.5	DNA binding (nucleosome assembly)
<i>Bos taurus</i> mitochondrial RNA, similar to 16S rRNA, clone: <i>ORCS13962</i>	AB099147	3.3	Unknown
<i>Bos taurus</i> BTAB2MDS3 β 2-microglobulin (<i>B2M</i>) gene, 3'-UTR	AY325771	2.1	Unknown
Ovaries mRNA for thyroid hormone receptor β 1	Z68307	1.7	Unknown
<i>Homo sapiens</i> tubulin α 6, mRNA (cDNA clone MGC:19827 (<i>TUBA1C</i>))	BC011790	1.6	Nucleotide binding (microtubule-based movement)
<i>Homo sapiens</i> mutL homolog 1, colon cancer, non-polyposis type 2 (<i>MLH1</i>)	NM_000249	2.1	ATP binding (cell cycle)
<i>Homo sapiens</i> elongation of very long chain fatty acids (FEN1/Elo2) (<i>ELOVL1</i>)	NM_022821	1.5	Protein binding (fatty acid biosynthetic process)
<i>Homo sapiens</i> bone morphogenetic protein 15 precursor (<i>BMP15</i>) gene	AF082350	2.4	Growth factor activity (female gamete generation)
Human DNA sequence from clone RP5-1080B10 on chromosome 20. Contains	AL096769	3.3	Unknown
<i>Bos taurus</i> prostaglandin G/H synthase 2 (<i>PTGS2/PGHS2</i>) mRNA, complete cds	AF031698	1.7	Oxidoreductase activity (electron transport)
<i>Mus musculus</i> acetylcholinesterase (Ache) and D5Ert655e genes	AF312033	2.2	Structural constituent of ribosome (translation)
<i>Bos taurus</i> acidic ribosomal phosphoprotein P0 mRNA, partial cds (<i>RPLP0</i>)	AB098748	1.8	Acetylcholinesterase activity
<i>Bos taurus</i> vacuolar ATPase subunit F (<i>VATF</i>) mRNA, complete cds	U43176	2.3	ATP synthase activity, rotational mechanism (ATP synthesis)
<i>Homo sapiens</i> KIAA0893 protein, mRNA (cDNA clone MGC:26714)	BC034964	1.9	Unknown
<i>Homo sapiens</i> mRNA for KIAA1052 protein, partial cds (<i>CEP164</i>)	AB028975	2.1	Protein binding
<i>Bos taurus</i> zona pellucida glycoprotein 3 (sperm receptor) (<i>ZP3</i>), mRNA	NM_173974	1.6	Protein binding (binding of sperm to zona pellucida)
<i>Homo sapiens</i> regulator of G-protein signaling 2, 24 kDa (<i>RGS2</i>), mRNA	NM_002923	2.5	Signal transducer activity (cell cycle)
<i>Rattus norvegicus</i> similar to cofactor required for Sp1 (<i>RGD1560170</i>)	XM_228713	2.2	Unknown

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

Figure 2 RT-qPCR validation of seven differentially expressed genes between no pregnancy, pregnancy loss, and calf delivery groups. Bars with different superscript letters (a, b, c) indicate significantly different amounts of mRNA within the same gene at $P \leq 0.05$.

(TXN), DNA binding (*H2F2Z*), placenta specific (*PLAC8*), growth factor activity (*BMP15*), and protein binding (*KRT8*). From those differentially expressed genes, the expression profile of two upregulated (*FL405* and *S100A10*) and three downregulated genes (*BMP15*, *KRT8*, and *PLAC8*) were validated using RT-qPCR, and the result showed similar expression patterns in both expression-profiling methods (Fig. 2).

Genes differentially regulated between embryo biopsies resulting in aggregate no CD and CD

RT-qPCR validation of three downregulated genes (*BMP15*, *KRT8*, and *PLAC8*) and two upregulated genes (*FL405* and *S100A10*) for NP, PL, and CD groups showed similar values for NP and PL groups without significant differences in relative abundance (Fig. 2). In contrast, relative abundance of all five transcripts was significantly different for CD group ($P < 0.05$) compared with NP group as well as for PL group (Fig. 2).

Since the biopsies of NP and PL did not result in CD, differentially expressed genes of these two groups were considered as no CD signatures (Tables 3 and 4). Therefore, the pooled results from these groups were compared with the expression profile of embryo biopsies resulting in CD. Accordingly, the results showed 18 genes to be differentially regulated in common in NP and PL groups (Fig. 1A and Table 3). However, 23 genes were identified to be differentially regulated exclusively in the NP group whereas 25 genes were differentially regulated exclusively in the PL group (Table 4). Considering all 66 genes, no gene showed a contradictory trend of gene expression considering NP and PL groups as shown in Tables 3 and 4. Since corresponding biopsies of NP as well as PL did not result in CD, differentially expressed genes of these two groups were therefore combined with an aggregate no CD signature (Tables 3 and 4).

Genes differentially regulated between aggregate no CD and CD for in vitro- and in vivo-derived embryos

To investigate whether *in vivo*-derived embryos resulting in CD or no CD could have similar gene expression compared with *in vitro*-derived embryos of the same developmental competence, we compared the results of this study with those of our previous study focusing on *in vitro* embryo biopsies (El-Sayed *et al.* 2006). In both studies, we identified 21 genes (Fig. 1B) that are differentially regulated between biopsies resulting in no CD compared with CD for both *in vivo*- and *in vitro*-derived embryos. Forty-five genes were differentially regulated exclusively for *in vivo*-derived embryos (19 upregulated and 26 downregulated) and 81 genes were differentially regulated exclusively for *in vitro*-derived embryos (64 upregulated and 17 downregulated). Of these 21 commonly differently expressed genes between no CD and CD, 18 showed the same trend of gene expression for both *in vivo*- and *in vitro*-derived embryo signatures (Fig. 1A). Twelve genes were found to be commonly upregulated and six genes were found to be downregulated (Fig. 1A). Only two differentially expressed genes (*KRT8* and *RPLO*) did not show the same trend and only one gene (*COQ7*) showed a contradictory trend as shown in Fig. 1B.

Discussion

Analysis of gene expression patterns in early embryos using cDNA microarray provides insight into the function of gene regulatory networks leading to unravel the molecular mechanisms underlying developmental abnormalities (Adjaye *et al.* 2004). Therefore, we focused on searching for genes that regulate the developmental competence of embryos to term. Hence, similar to our previous study (El-Sayed *et al.* 2006) focused on *in vitro* embryo biopsies, in this study, we identified gene expression differences between *in vivo*-derived blastocysts resulting in CD and NP or PL. For instance, blastocysts that ended up in NP were enriched with genes related to TNF, protein binding (*HSPD1*), RNA binding (*PA2G4*), calcium ion binding (*S100A10* and *S100A14*), and oxidoreductase activity (*ND1*, *FL405*, and *AKR1B1*). Otherwise, transcripts related to protein binding (*KRT8* and *ELOVL1*), DNA binding (*H3F3B* and *H2FA*), structural constituent of ribosome (*RPLP0*), placenta specific (*PLAC8*), growth factor activity (*BMP15*), and signal transducer activity (*RGS2*) were found to be downregulated in embryos resulting in NP compared with CD. Biopsies that ended up in PL were found to be enriched with genes involved in oxidoreductase activity (*FL405* and *FL396*), structural constituent of ribosome (*RPS15A* and *RPL3*), ATPase inhibitor activity (*ATPIF1*), protein binding (*CD9* and *HSPD1*), calcium ion binding (*S100A10*), and nucleotide binding (*EEF1A1*). Otherwise, transcripts regulating

Table 2 List of genes up- and downregulated in embryo biopsies resulting in pregnancy loss compared with calf delivery.

Gene name	GenBank accession no.	Fold change	Gene function (biological process)
<i>Genes upregulated in embryo biopsies resulting in resorption compared with calf delivery (n=20)</i>			
<i>Bos taurus</i> isolate FL405 mitochondrion, partial genome (FL405)	AY308069	1.9	Oxidoreductase activity (mitochondrial electron transport)
<i>Bos taurus</i> isolate FL396 mitochondrion, partial genome	AY308068	1.7	Oxidoreductase activity (mitochondrial electron transport)
<i>Homo sapiens</i> pituitary tumor-transforming 1 (PTTG1), mRNA	NM_004219	1.5	Transcription factor activity (transcription)
<i>Homo sapiens</i> heat shock 60 kDa protein 1 (chaperonin), (HSPD1)	BC002676	1.7	Protein binding (response to stress)
<i>Bos taurus</i> mRNA for similar to ribosomal protein S15a, partial cds, (RPS15A)	AB099015	2.0	Structural constituent of ribosome (translation)
<i>Bos taurus</i> mRNA for similar to ribosomal protein L3, partial cds, (RPL3)	AB099068	1.6	Structural constituent of ribosome (translation)
<i>Bos taurus</i> T cell receptor α (TCRA) gene, J segments and C region	AY227782	3.5	Unknown
<i>Homo sapiens</i> lectin, galactoside-binding, soluble 3, (LGALS3)	BC001120	2.1	IgE binding
<i>Bos taurus</i> X-inactivation center region, Jpx and Xist genes	AJ421481	1.8	Unknown
<i>Homo sapiens</i> leukocyte receptor cluster (LRC) member 9, mRNA (cDNA)	BC015921	1.9	Nucleic acid binding (RNA metabolic process)
<i>Homo sapiens</i> RNA polymerase II termination factor mRNA, (TTF2)	NM_003594	2.1	DNA binding (transcription)
<i>Homo sapiens</i> S100 calcium binding protein A14 (S100A14), mRNA	NM_020672	2.0	Calcium ion binding
<i>Mus musculus</i> cysteine and glycine-rich protein 2, mRNA (cDNA clone) (CSR2P2)	BC012663	2.5	Metal ion binding (cell differentiation)
<i>Homo sapiens</i> TGFB-induced factor (TALE family homeobox) (TGIF)	NM_003244	1.7	Transcription factor activity (transcription)
<i>Homo sapiens</i> B-cell translocation gene 4 (BTG4), mRNA	NM_017589	2.0	(cell cycle arrest)
<i>Mus musculus</i> 11 days embryo gonad cDNA, RIKEN full-length (7030402D04Rik)	AK078561	2.3	Unknown
<i>Bos taurus</i> EF1A mRNA for elongation factor 1 α , complete cds (EEF1A1)	AB060107	1.5	Nucleotide binding
Bovine ATP synthase inhibitor protein mRNA, complete cds (ATPIF1)	M22559	1.9	ATPase inhibitor (negative regulation cellular metabolic)
<i>Bos taurus</i> S100 calcium-binding protein A10 mRNA (S100A10)	NM_174650	2.8	Calcium ion binding
<i>Bos taurus</i> CD9 molecule (CD9), mRNA	NM_173900	1.6	Protein binding (negative regulation of cell proliferation)
<i>Genes downregulated in embryo biopsies resulting in resorption compared with calf delivery (n=23)</i>			
<i>Homo sapiens</i> estrogen-related receptor γ , mRNA (cDNA clone) (ESRRG)	BC064700	2.8	Protein binding (positive regulation of transcription)
<i>Homo sapiens</i> regulator of G-protein signaling 2, 24 kDa (RGS2), mRNA	NM_002923	2.3	Signal transducer activity (cell cycle)
<i>Bos taurus</i> β -actin (ACTB) mRNA, complete cds	AY141970	1.8	Nucleotide binding
Bovine mRNA fragment for cytokeratin A (no. 8) (KRT8)	X12877	3.7	Protein binding (response to other organism)
Bovine mRNA for histone H2A.Z (H2FAZ)	X52318	2.5	DNA binding (nucleosome assembly)
<i>Bos taurus</i> histone H4.1 mRNA, complete cds (H4)	AF001288	1.5	DNA binding (nucleosome assembly)
<i>Bos taurus</i> mRNA for similar to ribosomal protein L18a, partial cds (RPL18a)	AB098916	1.6	Structural constituent of ribosome (translation)
<i>Homo sapiens</i> mitochondrial ribosomal protein L33 (MRPL33), nuclear	NM_004891	1.5	Structural constituent of ribosome (translation)
<i>Homo sapiens</i> elongation of very long chain fatty (ELOVL1)	BC000618	1.6	Protein binding (fatty acid biosynthetic process)
<i>Homo sapiens</i> coenzyme Q7 homolog, ubiquinone (yeast), mRNA (cDNA) (COQ7)	BC003185	1.7	Oxidoreductase activity (protein metabolic process)
<i>Homo sapiens</i> lipoma HMGIC fusion partner-like 3 (LHFPL3), mRNA	NM_199000	1.7	Unknown
<i>Homo sapiens</i> zinc finger protein 85 (TFP85), mRNA (cDNA clone)	BC047646	2.1	Transcription factor activity (transcription)
<i>Homo sapiens</i> testis mitotic checkpoint BUB3 (BUB3) mRNA, complete cds	AF047473	1.5	Protein binding (mitotic cell cycle spindle assembly)
<i>Bos taurus</i> thioredoxin mRNA, complete cds (TXN)	AF104105	2.4	(cell redox homeostasis)
<i>Homo sapiens</i> NANOS1 mRNA, complete cds	AF275269	1.9	RNA binding (regulation of translation)
<i>Homo sapiens</i> cyclin B3 (CCNB3), transcript variant 2, mRNA	NM_033671	1.7	(cell cycle)
<i>Homo sapiens</i> placenta-specific 8 (PLAC8), mRNA	NM_016619	2.0	Unknown
<i>Bos taurus</i> mitochondrial RNA, similar to 16S rRNA, clone: ORCS13962	AB099147	1.8	Unknown
<i>Homo sapiens</i> bone morphogenetic protein 15 precursor (BMP15) gene	AF082350	2.1	Growth factor activity (female gamete generation)
<i>Bos taurus</i> acidic ribosomal phosphoprotein PO mRNA, partial cds (RPLP0)	AF013214	1.9	Structural constituent of ribosome (translation)
<i>Homo sapiens</i> aurora kinase A (AURKA)	BC027464	2.4	Kinase activity (cell cycle)
Oocyte expressed protein homolog (dog) (<i>Homo sapiens</i>) (OOEP)	BC024931	2.1	Unknown
<i>Bos taurus</i> prostaglandin G/H synthase 2 (PTGS2/PGHS2) mRNA, complete cds	AF031698	1.5	Oxidoreductase activity (electron transport)

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

Table 3 Commonly expressed transcripts of biopsies resulting in no pregnancy or pregnancy loss relative to biopsies that resulted in calf delivery.

Gene	Gene function	<i>In vivo</i> biopsies		
		No pregnancy	Pregnancy loss	No calf delivery
<i>ELOVL1</i>	Protein binding and synth.	↓	↓	↓
<i>KRT8</i>	Protein binding and synth.	↓	↓	↓
<i>RPLP0</i>	Protein binding and synth.	↓	↓	↓
<i>EEF1A1</i>	Protein binding and synth.	↑	↑	↑
<i>HSPD1</i>	Protein binding and synth.	↑	↑	↑
<i>H2FAZ</i>	DNA binding	↓	↓	↓
<i>BMP15</i>	Growth factor	↓	↓	↓
<i>S100A10</i>	Ion binding	↑	↑	↑
<i>S100A14</i>	Ion binding	↑	↑	↑
<i>CSRP2</i>	Ion binding	↑	↑	↑
<i>PTGS2</i>	Metabolism/oxidative stress	↓	↓	↓
<i>LGALS3</i>	Immune response	↑	↑	↑
<i>FL405</i>	Mitochondrial transcripts	↑	↑	↑
<i>ATPIF1</i>	Mitochondrial transcripts	↑	↑	↑
<i>RGS2</i>	Cell cycle	↓	↓	↓
<i>ORCS13962</i>	Unknown	↓	↓	↓
<i>RIKEN</i>	Unknown	↑	↑	↑
<i>PLAC8</i>	Unknown	↓	↓	↓

signal transducer activity (*RGS2*), cell redox homeostasis (*TXN*), DNA binding (*H2F2Z*), placenta specific (*PLAC8*), growth factor activity (*BMP15*), and protein binding (*KRT8*) were downregulated in embryos resulting in PL compared with CD.

Interestingly, the signatures of *in vivo*-derived embryos resulting in NP and PL showed high similarities in our study. The RT-qPCR validation of five selected transcripts (*FL405*, *S100A10*, *BMP15*, *KRT8*, and *PLAC8*) showed similar trends and amounts for embryos of NP and PL groups, however, being significantly different compared with the CD group, that is, in great accordance with our previous study dealing with *in vitro*-derived embryos (El-Sayed *et al.* 2006). Accordingly, comparison of the expression of 18 genes differentially expressed in NP and PL groups relative to the CD group showed the same trend for all 18 genes. Moreover, no contradictory expression trends were observed for 66 genes differentially expressed either for NP or for PL compared with the CD group. For example, embryo biopsies resulting in CD showed upregulation of *RGS2* as well as *KRT8* compared with both NP and PL groups. *RGS2*, a regulator of G-protein signaling family member (Zmijewski *et al.* 2001), has been reported to be involved in regulating the intracellular calcium mobilization and T-cell proliferation at the maternal–fetal interface during embryo implantation (Huang *et al.* 2003). Higher gene expression has already been reported for bovine zygotes with superior developmental competence (Salilew-Wondim *et al.* 2007). Keratins like *KRT8* are among the first cytoskeleton genes differentially expressed in the trophectoderm (TE) of mouse blastocysts (Brulet *et al.* 1980, Jackson *et al.* 1980, Oshima *et al.* 1983) and mediate cellular proliferation and

differentiation during conceptus development (Blomberg *et al.* 2005). Mice deficient for *KRT8* caused embryonic lethality (Baribault *et al.* 1993) due to loss of contact between maternal and embryonic tissues resulting in early abortion (Hesse *et al.* 2000). According to the results of this study and that of our previous study (El-Sayed *et al.* 2006), we suggest that embryos that result in NP or PL are tending to have similar gene expression patterns. Indeed, embryos being viable until day 25, for example, would have been classified as NP group whereas embryos dying at day 29 would have been classified as PL group due to the fact that first pregnancy check was performed at day 28 in this study. Consequently, we aggregated the signatures of biopsies classified as NP and PL groups to an aggregate no CD signature being contrary to CD.

The results of this study were compared with our previous results (El-Sayed *et al.* 2006) to look into common molecular signatures of *in vivo*- and *in vitro*-derived embryos related to developmental competence. Accordingly, we identified 21 genes differentially regulated between embryos of no CD and CD groups both in *in vivo*- and *in vitro*-derived embryos (Fig. 1). Thus, the results of this study and the previous study (El-Sayed *et al.* 2006) evidenced that embryos developed under different culture conditions (*in vivo* and *in vitro*) have some similarities in their transcript abundances. That observation seems to be contradictory to numerous studies reporting differences in global gene expression profile between *in vivo*- and *in vitro*-derived embryos (Smith *et al.* 2005, 2009, Corcoran *et al.* 2007) as well as differences in terms of selected developmentally important genes (Rizos *et al.* 2002, Knijn *et al.* 2005, Corcoran *et al.* 2007). In contrast to these studies, however, our study includes subpopulations of embryos with variable developmental competence into account, which might give a more objective picture.

Of these 21 genes, 18 showed the same expression trend for *in vivo*- and *in vitro*-derived embryos comparing no CD with CD respectively. For example, *TXN* was found to be upregulated in biopsies of embryos cultured both *in vitro* and *in vivo* resulting in CD compared with those leading to no CD. *TXN* had been identified as so-called ‘early pregnancy factor’ (Clarke *et al.* 1991, Clarke 1992). The biological functions of *TXN* include activation of some transcription factors, anti-apoptotic function, and proliferation (Hirota *et al.* 1997, Powis *et al.* 1997, 1998, Arrigo 1999, Arner & Holmgren 2000). *TXN* helps the embryo to resist against oxidative stress (Kobayashi-Miura *et al.* 2002). For instance, supplementation of the culture medium with thioredoxin was found to improve blastocyst formation, cell numbers, and incidence of apoptosis of *in vitro*-produced embryos of pigs (Ozawa *et al.* 2006), cattle (Bing *et al.* 2003), and mice (Nonogaki *et al.* 1991). Lopata *et al.* (2001) demonstrated the importance of

Table 4 Transcripts not commonly expressed in biopsies resulting in no pregnancy or pregnancy loss relative to biopsies that resulted in calf delivery.

Gene	Gene function	In vivo biopsies		
		No pregnancy	Pregnancy loss	No calf delivery
<i>PTTG1</i>	Transcription factors		↑	↑
<i>TGIF</i>	Transcription factors	↑		↑
<i>GTF2A1L</i>	Transcription factors	↑		↑
<i>TPF85</i>	Transcription factors		↓	↓
<i>CEP164</i>	Protein binding and synth.	↓		↓
<i>ZP3</i>	Protein binding and synth.	↓		↓
<i>ESRRG</i>	Protein binding and synth.		↓	↓
<i>RPL18a</i>	Protein binding and synth.		↓	↓
<i>MRPL33</i>	Protein binding and synth.		↓	↓
<i>BUB3</i>	Protein binding and synth.		↓	↓
<i>RPL3</i>	Protein binding and synth.		↑	↑
<i>RPS15A</i>	Protein binding and synth.		↑	↑
<i>COQ7</i>	Protein binding and synth.		↓	↓
<i>CD9</i>	Protein binding and synth.		↑	↑
<i>TUBA1C</i>	Nucleotide binding	↓		↓
<i>ACTB</i>	Nucleotide binding		↓	↓
<i>LRC</i>	Nucleotide binding		↑	↑
<i>TTF2</i>	DNA binding		↑	↑
<i>TGIF</i>	DNA binding		↑	↑
<i>H3F3A</i>	DNA binding	↓		↓
<i>H4</i>	DNA binding		↓	↓
<i>PA2G4</i>	RNA binding	↑		↑
<i>NANOS1</i>	RNA binding		↓	↓
<i>S100A16</i>	Ion binding	↑		↑
<i>TXN</i>	Metabolism/oxidative stress		↓	↓
<i>AKR1B1</i>	Metabolism/oxidative stress	↑		↑
<i>VATF</i>	ATP synthesis	↓		↓
<i>TNF</i>	Immune response	↑		↑
<i>TCRA</i>	Immune response		↑	↑
<i>FL396</i>	Mitochondrial transcripts		↑	↑
<i>NADH</i>	Mitochondrial transcripts	↑		↑
<i>SHFM1</i>	Peptidase activity	↑		↑
<i>ACHE</i>	Acetylcholinesterase activity	↓		↓
<i>CCNB3</i>	Cell cycle		↓	↓
<i>AURKA</i>	Cell cycle		↓	↓
<i>BTG4</i>	Cell cycle		↑	↑
<i>MLH1</i>	Cell cycle	↓		↓
<i>RP42-351K5</i>	Unknown	↑		↑
<i>JPX</i>	Unknown		↑	↑
<i>RP5-1080B10</i>	Unknown	↓		↓
<i>B2M</i>	Unknown	↓		↓
<i>C1orf114</i>	Unknown	↑		↑
<i>OOEP</i>	Unknown		↓	↓
<i>MGC:26714</i>	Unknown	↓		↓
<i>LHFPL3</i>	Unknown		↓	↓
<i>RGD1560170</i>	Unknown	↓		↓
<i>RBM16</i>	Unknown	↑		↑
<i>THRB1</i>	Unknown	↓		↓

thioredoxin expression in cytotrophoblast cells during human embryo implantation.

Six genes with the same expression trend for *in vivo*- and *in vitro*-derived embryos comparing no CD with CD groups, respectively, had also identical expression trends for NP and PL groups (*HSPD1*, *H2FAZ*, *BMP15*, *PTGS2/PGHS2/COX2*, *PLAC8*, *FL405*). These genes may be ideal candidates for further functional analysis for the establishment of comprehensive physiological models of early embryonic mortality. For example, heat-shock 60 kDa protein 1 (*HSPD1*) are among the first proteins

produced during embryogenesis (Bensaude *et al.* 1983, Liu *et al.* 2004) and their main function is to protect cellular proteins during stress and act by anti-apoptotic action (Betts & King 2001, Edwards *et al.* 2001). Heat-shock genes prevent apoptosis induced by a variety of stressors including oxidative stress (Yamagishi *et al.* 2002) and *TNF* (Soto *et al.* 2003, Loureiro *et al.* 2007), which was found to be also upregulated in embryos resulting in no CD in this study. In addition to *HSPD1*, others including *PTGS2/PGHS2/COX2*, *BMP15*, and *PLAC8* were also enriched in embryo biopsies resulting in CD. For instance, prostaglandin G/H synthase 2 (*PTGS2*), which was also enriched in biopsies derived from blastocysts resulting in CD, is involved in the process of blastocyst implantation and plays important roles in embryo to uterus interaction processes (Snabes & Harper 1984). Charpigny *et al.* (1997) showed that *PTGS2* protein was localized only in trophoblast cells of ovine embryos. This may suggest that *PTGS2/PGHS2/COX2* is a potential candidate for blastocyst competency. *PLAC8* was found to be upregulated in embryo biopsies resulting in CD in *in vitro*- and *in vivo*-derived embryos. That result is in line with several studies (Galaviz-Hernandez *et al.* 2003, Klein *et al.* 2006), suggesting that *PLAC8* has a potential role for placental development as well as for the embryo maternal interface. *BMP15*, an important oocyte-derived growth factor that is required for normal folliculogenesis and female fertility of mammals (Juengel *et al.* 2002), was highly enriched in embryo biopsies that ended up with CD. That is in line with the observation of Wu *et al.* (2007) who showed that human oocytes from follicles with relatively high *BMP15* levels had a better developmental potential than those from follicles with relatively low *BMP15* levels. Moreover, mutation of *BMP15* has been found to be associated with hypergonadotropic ovarian failure in women (Di Pasquale *et al.* 2004). Embryo biopsies resulting in CD also over-expressed *H2FAZ*, a gene related to histone modification. Aberrations in transcript levels of histone-modifying genes before and after embryonic genome activation had been reported to contribute to the poor quality of cloned embryos (Santos *et al.* 2003, Nowak-Imialek *et al.* 2008, Shao *et al.* 2008).

According to the high signature similarities, we could imagine that *in vitro*-derived bovine embryos bearing term developmental competence do not differ largely compared with *in vivo*-derived embryos with term developmental competence. We speculate that differences between embryos of different developmental competence (CD versus no CD) are larger than those between embryos of different sources (*in vivo* versus *in vitro*). Whether embryos of both origins with developmental competence to term are similar in an absolute manner or in more a relative manner should be the topic of further studies. Nevertheless, our RT-qPCR validation results as well as the fact that some of the genes present in the commonly expressed signature had been reported

to be affected by the environment previously (Hoelker *et al.* 2009) indicate a relative similarity. However, gene expression was only investigated as average for a pool of blastocysts in that study. Whether environment affects gene expression of all embryos similarly or differently for subpopulations of embryos, for example, according to developmental competence, has not been evaluated in that study.

Taken together in the present work, we identified genetic signatures of *in vivo*-derived embryos correlated with developmental competence to term. Embryos that result in NP or PL were difficult to distinguish with respect to gene expression profile. Moreover, a subpopulation of *in vitro*-derived blastocysts, namely those with developmental competence to term, expressed a genetic signature similar to that of *in vivo*-derived embryos with term developmental competence for a set of genes. Further gene expression analysis with larger numbers of genes of *in vitro*- and *in vivo*-derived embryos that ended up with similar phenotypes and further functional analysis of these genes will help in the establishment of comprehensive physiological models of early embryonic mortality and will provide the basis to devise new strategies with the aim of enhancing embryonic survival for human and bovine ARTS.

Materials and Methods

Experimental design

In vivo-derived blastocysts were flushed from superovulated heifers. After collection, blastocysts were split in two proportions: ~60–70% of the entire embryos were transferred to synchronized recipients and the remaining 30–40% were stored frozen until CD. After checking the outcome of the transferred embryos, the corresponding counterparts were pooled into three groups, namely, I) NP, II) PL, and III) CD. These groups were subsequently subjected to gene expression analysis to identify differentially expressed genes related to developmental competence. Gene expression patterns were compared with embryo biopsies (group I versus group III and group II versus group III). An overview of the experimental design is shown in Fig. 3.

In vivo embryo production

All experimental animals were handled according to the animal protection law of Germany. To collect *in vivo*-derived blastocysts, Simmental heifers ($n=12$) of similar age (24 months) were maintained under identical conditions compared with our previous study (El-Sayed *et al.* 2006). Heifers were synchronized as described by El-Sayed *et al.* (2006). Briefly, heifers were synchronized by i.m. injection of 500 mg cloprostenol (PGF_{2α}, Estrumate; Essex Tierarznei, Munich, Germany) twice within 11 days (Fig. 4). Two days after the administration of PGF_{2α}, all animals received 0.02 mg GNRH (Receptal; Intervet, Boxmeer, The Netherlands). Twelve days after the second GNRH injection, heifers received the first of

eight consecutive FSH injections over 4 days in decreasing doses (in total 300–400 mg FSH according to body weight; University of Liege, Belgium). PGF_{2α} was administered 72 and 84 h after the initial FSH injection. Finally, 48 h after the first administration of PGF_{2α}, ovulations were induced by injecting 0.02 mg GNRH simultaneously with the first of a total of two artificial inseminations within a 24 h interval. The first day of insemination was defined as day 0.

Figure 3 Overview of the overall experiment. Bovine blastocysts were split, the smaller proportions (30–40%) were stored frozen, and the parts consisting of 60–70% were transferred to recipients. Following this, the stored embryo biopsies were pooled according to the pregnancy outcome as no pregnancy, pregnancy loss, and calf delivery groups for gene expression analysis.

Figure 4 Synchronization of donor animals by intramuscular injection of PGF_{2α}, twice within 11 days. Two days after administration of PGF_{2α}, all animals received GNRH. Twelve days after the second GNRH injection, heifers received the first of eight consecutive FSH injections over 4 days. PGF_{2α} was administered 72 and 84 h after the initial FSH injection. Finally, 48 h after the first administration of PGF_{2α}, ovulations were induced by injecting GNRH simultaneously with the first of a total of two artificial inseminations (AI) within a 24 h interval.

Blastocyst recovery and embryo biopsy

The blastocysts were collected on day 7 after insemination. Embryo flushing catheter (CH15, Wörrlein, Ansbach, Germany) was fixed in the uterine horn and subsequently embryos were flushed out by draining each uterine horn with 500 ml PBS. All the flushed blastocysts were assessed under a stereo microscope for their quality. Only blastocysts scored as grade I according to the Manual of the International Embryo Transfer Society (IETS) were used for biopsy performed using a Beaver microplate (Minitüb, Tiefenbach, Germany) and were fixed to a micromanipulator under an inverse microscope (Leica Camera, Solms, Germany). In this procedure, 30–40% of blastocysts containing both inner cell mass and TE cells were taken for biopsy, and the remaining 60–70% were cultured *in vitro* for 2 h to allow re-expansion before transferring to recipients. Only re-expanded biopsied blastocysts were transferred to recipients and the corresponding biopsies were snap frozen in cryo-tubes containing minimal amounts of lysis buffer (0.8% Igepal (Sigma), 40 U/ml RNasin (Promega), 5 mM dithiothreitol (DTT; Promega)). All frozen biopsies were stored at -80°C until used for RNA isolation and subsequent transcriptome profiling.

Embryo transfer and pregnancy monitoring

Simmental heifers of similar age (24 months) were used as recipients for embryo transfer. Estrus was synchronized by administration of PGF_{2α} (2 ml Estrumate; Fa. Essex, München, Germany) followed by a second administration 11 days later. Single re-expanded and blastocoel-formed biopsied blastocysts were transferred to recipients by non-surgical standard procedures at day 7 of the estrous cycle. Following transfer, pregnancy monitoring was done at days 28 and 42. While those recipients that were not pregnant confirmed by ultrasonography at day 28 were classified as the NP group and those that were pregnant at day 28 but lost their pregnancy between days 28 and 42 were classified as PL group. Embryo biopsies that sustained pregnancy to term and delivering live calves were classified as CD group.

RNA isolation

Total RNA was isolated using PicoPure RNA isolation kit (MDS Analytical Technologies GmbH, Ismaning, Germany) according to manufacturer's instructions, and RNA isolation was performed using I) a total of nine pools, therefore three for each category, each containing three embryo biopsies, to be used for array analysis after amplification. II) RNA of these pools was enhanced with the RNA extracted from additional biopsies of the same group, which were not used for array analysis (two biopsies per each of the three pools for CD group, one biopsy per each of the three pools for NP group, and no additional biopsy for PL group) to perform RT-qPCR. Briefly, embryo biopsies using lysis buffer were mixed with 100 µl extraction buffer and incubated at 42°C for 30 min to get complete lysis of the embryo biopsies and to release RNA. The embryo extract was loaded onto a pre-conditioned purification column and centrifuged to allow for the RNA to bind to the spin column. On-column DNA digestion using RNase-free DNase (Qiagen GmbH) was performed to remove any genomic DNA contamination. Following subsequent washing, the RNA was eluted with 12 µl RNase-free water.

RNA amplification

First- and second-strand cDNA synthesis were carried out as described in our previous studies (El-Sayed *et al.* 2006). Briefly, 1 µl of 20 µM T7 oligo d(T)21 primer (TCTAGTCGACGGC-CAGTGAATTGTAATACGACTCACTATAGG-GCG(T)21) was added to total RNA of embryo biopsies and co-incubated for 3 min at 70°C and placed immediately on ice for 2 min. Eight microliters of RT mix (1× first-strand buffer, 0.3 mM dNTP, 0.1 mM DTT, 10 U RNase inhibitor (Promega), and 200 U Superscript II RT (Invitrogen)) were added to the 11 µl mRNA sample. The reaction was incubated at 42°C for 90 min and terminated by heating at 75°C for 15 min. The second-strand synthesis and global PCR amplification were carried out using degenerated oligonucleotides primer (DOP) and PCR master kit (Roche Diagnosis). For this, 20 µl first-strand cDNA product, 40 µl of 2× DOP PCR master mix, 1 µl DOP primer to the final concentration, 1 µl of 20 µM T7 oligo d(T)21 primer, and 16 µl

water were properly mixed. The reaction was heated at 95 °C for 5 min to activate the polymerase, followed by cycle of denaturation at 95 °C for 30 s and annealing at 30 °C for 90 s. Subsequently, the temperature was increased at the rate of 0.2 °C/s until it reached 72 °C and incubated for 3 min at this temperature. Once the second-strand synthesis was completed, global PCR amplification was continued for the rest of the ten cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 3 min, and final extension at 72 °C for 7 min. Ten cycles of amplification were found to be optimum to keep representation of the mRNA population after *in vitro* transcription (Mamo *et al.* 2006). The cDNA was purified and used for *in vitro* transcription using AmpliScribe T7 transcription kit (Epicentre Technologies, Oldendorf, Germany) according to manufacturer's instruction. Briefly, 2 µl of 10× reaction buffer, 4 µl dNTP (100 mM each of ATP, CTP, GTP and UTP), 2 µl DTT, and 2 µl of T7 RNA polymerase were added to 10 µl purified cDNA, mixed well, and incubated at 42 °C for 3 h. At the end of incubation, 1 µl DNase has been added and incubated at 37 °C for 30 min. Then, the amplified RNA (aRNA) was purified using RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. Finally, the aRNA was eluted in 30 µl RNase-free water.

Aminoallyl labeling and dye coupling

Aminoallyl labeling and dye coupling was performed as described previously. Briefly, Minimum Information About Microarray Experiments (MIAME) guidelines were adhered to our experimental design. Three independent labeling reactions (including dye-swaps, Cy3, and Cy5) were carried out per aRNA sample pertaining to each biological replicate. Accordingly, 3 µg aRNA from each embryo biopsy pool representing each group was used as template in RT incorporating amino-modified dUTPs into the cDNA using the CyScribe Post-Labeling Kit (Amersham Biosciences). The aRNA, anchored oligo(dT), and random nanomer primers were co-incubated at 70 °C for 5 min followed by incubation for 10 min at

room temperature. Then, 10 µl reaction mix (containing 4 µl of 5× first-strand buffer, 2 µl of 0.1 M DTT, 1.5 µl dNTP mix, 1.5 µl aminoallyl dUTP, and 1 µl CyScript reverse transcriptase) was added to the RNA primer mix and incubated at 42 °C for 90 min. At the end of this reaction, 2 µl of 2.5 M NaOH was added to hydrolyze any rest of mRNA and incubated at 37 °C for 15 min. The aminoallyl-labeled cDNA samples were purified using CyScribe GFX purification kit (Amersham Biosciences) after adding 10 µl of 2 M HEPES. The purified aminoallyl-labeled cDNA samples were then eluted in 60 µl of 0.1 M sodium bicarbonate. The triplicate cDNA samples of each embryo biopsy group were differentially labeled indirectly using *N*-hydroxysuccinate-derived Cy3 and Cy5 dyes and incubated for 1.5 h at room temperature in dark. At the end of incubation, non-reacting dyes were quenched by adding 15 µl of 4 M hydroxylamine solution (Sigma) and incubated for 15 min at room temperature in darkness. To avoid variation due to dye coupling, aRNA samples from the same group were labeled reversibly either with Cy3 or with Cy5 for dye-swap hybridizations. The reaction was then purified with CyScribe GFX purification kit (Amersham Biosciences). Samples were finally eluted in 60 µl elution buffer.

Probe hybridization

Probe hybridization was performed as described previously (El-Sayed *et al.* 2006). Briefly, pre-hybridization of the slides was performed by placing the array slides into a corning GAPS II slide container and incubating in warm (55 °C) pre-hybridization buffer (5× SSC, 0.1% SDS (Sigma), and 1% BSA (Roche)) for 20 min. Following pre-hybridization, slides were rinsed for 1 min in boiling RNase-free water to denature probes and to wash unbound cDNA from the slide surface. The slides were immediately immersed in RNase-free water at room temperature and then isopropanol each for 1 min. Then, the slides were dried by centrifugation at 2000 *g* (370 r.p.m.) for 2 min.

Hybridization and post-hybridization washes were carried out as described previously (El-Sayed *et al.* 2006). The samples

Table 5 Details of the primers used for quantitative real-time PCR analysis.

Gene name	GenBank accession number	Primer sequences	Product size (bp)
<i>S100A10</i>	NM_174650	F: 5'-GGATTCTGAGCATATGGGACC-3' R: 5'-GAGCAAGAGGATGCAAGCAATA-3'	131
<i>GAPDH</i>	BC102589	F: 5'-ACCCAGAAGACTGTGGATGG-3' R: 5'-ACGCCTGCTTCACCACTTC-3'	247
<i>PLAC8</i>	NM_016619	F: 5'-CGGTGTTCCAGAGGTTTTCC-3' R: 5'-ACTGACCGTCTGACCGTAGAA-3'	163
<i>KRT8</i>	X12877	F: 5'-CACCAGTTCGAAGCCTGTGG-3' R: 5'-TCAGGTCTCCTGTGCAGATGC-3'	176
<i>BMP15</i>	AY304484	F: 5'-CTGACGCAAGTGACACCCTA-3' R: 5'-GACACACGAAGCGGAGTCGTA-3'	396
<i>HSPD1</i>	BC002676	F: 5'-ACTGTACTGGCAGCTCTAT-3' R: 5'-ATTGTAGCAACCTGAGCAAT-3'	172
<i>RPLP0</i>	AB098748	F: 5'-GAATCCCATATCCTCGTCCGA-3' R: 5'-TTGCTGAAAAGGTCAAGGCCT-3'	162
<i>RGS2</i>	NM_002923	F: 5'-GCAGTTGTAAGCAGCCACTTG-3' R: 5'-ATATTGAATTCTGGCTGGCCTG-3'	193
<i>FL405</i>	AY308069	F: 5'-GGTCTCTTTACCTCTCCAC-3' R: 5'-CTACGTTGAAGCCCGAGACT-3'	169

to be hybridized on specific array were mixed and dried in speedvac (SPD111v-230) and then centrifuged (Savant, Holbrook, NY 11741-4306 USA). Then, the pellet was re-suspended in pre-warmed (42 °C) formamide-based hybridization buffer (15 µl hybridization buffer (Amersham Bioscience), 30 µl of 100% formamide and 15 µl diethylpyrocarbonate (DEPC) water (1 ml of 0.1% diethylpyrocarbonate to 1000 ml distilled water)). To this, 2.5 µl yeast tRNA (4 mg/ml) and 2.5 µl Cot-human DNA (1 mg/ml; Invitrogen) were added to avoid non-specific hybridization. The pellet was denatured at 95 °C for 5 min, centrifuged briefly, and hybridized to the array. The arrays were covered with glass coverslips (ROTH, Karlsruhe, Germany) and placed and fixed well in the hybridization cassette (TeleChem International, Inc., Sunnyvale, CA, USA). The cassette was incubated in a hybridization chamber (GFL, Dülmen, Germany) at 42 °C for 16 h. After hybridization, slides were washed twice with 2 × SSC-0.1% SDS buffer for 5 min at 42 °C, then once with 1 × SSC, 0.2 × SSC, and 0.1 × SSC for 5 min each at room temperature. Finally, the slides were rinsed with RNA-free water and then with isopropanol for one min each and centrifuged at 2000 g (370 r.p.m.) for 2 min.

Array scanning and data analysis

Array scanning and data analysis were performed as described previously (El-Sayed *et al.* 2006). Briefly, slides were scanned using Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA). The GenePix Pro 4.0 software (Axon Instruments) was used to process the images, to find spots, to integrate robot-spotting files, and finally to create reports of spot intensity data. The LOWESS normalization of microarray data was performed using GPRocessor 2.0a software (<http://bioinformatics.med.yale.edu/group/>). The normalized data were used to calculate intensity ratios of all replicates and to obtain one value per clone. Ratios were finally log₂ transformed and submitted to significance analysis for microarray (SAM) analysis. Microarray data analysis was performed using SAM – free software developed at Stanford University (<http://www-stat.stanford.edu/~tibs/SAM/>).

RT-qPCR analysis

To validate microarray results, eight candidate genes were selected due to their high fold change or because of their differential expression both in this and in our recent study (El-Sayed *et al.* 2006) for further analysis by RT-qPCR (Table 5). Quantitative analysis of cDNA samples was performed as described previously (El-Sayed *et al.* 2006) using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The cDNA was synthesized from independent embryo biopsies of all groups. RT-qPCR was first performed with all samples to test for any variation in the expression of this internal control gene (*GAPDH*). After confirming that there were no significant differences in the relative abundance of *GAPDH* between samples, all transcripts were quantified using independent RT-qPCR runs. Standard curves were generated for both the target and the endogenous control genes using serial dilution of plasmid DNA (10¹ to 10⁸ molecules). The RT-qPCR was performed in 20 µl reaction volume containing 10 µl iTaq

SYBR Green Supermix with ROX (Bio-Rad). During each RT-qPCR reaction, samples from the same cDNA source were run in duplicate to control the reproducibility of real-time results. A universal thermal cycling parameter (10 s at 50 °C, 10 min at 95 °C, 15 s at 95 °C for 45 cycles, and 60 s at 60 °C) was used to quantify each gene of interest. At the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60 °C and taking measurements at every 7 s interval until the temperature reached 95 °C. Final quantitative analysis was performed using the relative standard curve method, and relative gene abundance of NP and PL groups were reported as *n*-fold difference compared with the CD group. Since gene expression of CD group served as control, relative expression of genes for CD group was set to 1.0.

Statistical analysis

The gene expression analysis for studied genes was performed based on the standard curve method. The relative expression data were analyzed using general linear model (GLM) of the Statistical Analysis System (SAS) software package version 8.0 (SAS Institute, Inc., Cary, NC, USA). Differences in mean values were obtained using ANOVA followed by a multiple pairwise comparison using the Tukey test. Differences of *P* < 0.05 were considered as significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Adjaye J, Herwig R, Herrmann D, Wruck W, Benkhalha A, Brink TC, Nowak M, Carnwath JW, Hultschig C, Niemann H *et al.* 2004 Cross-species hybridisation of human and bovine orthologous genes on high density cDNA microarrays. *BMC Genomics* **28** 83. (doi:10.1186/1471-2164-5-83)
- Arner ES & Holmgren A 2000 Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry* **267** 6102–6109. (doi:10.1046/j.1432-1327.2000.01701.x)
- Arrigo AP 1999 Gene expression and the thiol redox state. *Free Radical Biology & Medicine* **27** 936–944. (doi:10.1016/S0891-5849(99)00175-6)
- Ayalon N 1978 A review of embryonic mortality in cattle. *Journal of Reproduction and Fertility* **54** 483–493. (doi:10.1530/jrf.0.0540483)
- Baribault H, Price J, Miyai K & Oshima RG 1993 Mid-gestational lethality in mice lacking keratin 8. *Genes and Development* **7** 1191–1202. (doi:10.1101/gad.7.7a.1191)
- Bensaude O, Babinet C, Morange M & Jacob F 1983 Heat shock proteins, first major products of zygotic gene activity in mouse embryo. *Nature* **305** 331–333. (doi:10.1038/305331a0)
- Betts DH & King WA 2001 Genetic regulation of embryo death and senescence. *Theriogenology* **55** 171–191. (doi:10.1016/S0093-691X(00)00453-2)

- Bing YZ, Hirao Y, Takenouchi N, Che LM, Nakamura H, Yodoi J & Nagai T 2003 Effects of thioredoxin on the preimplantation development of bovine embryos. *Theriogenology* **59** 863–873. (doi:10.1016/S0093-691X(02)01158-5)
- Blomberg LA, Long EL, Sonstegard TS, Van Tassell CP, Dobrinsky JR & Zuelke KA 2005 Serial analysis of gene expression during elongation of the peri-implantation porcine trophectoderm (conceptus). *Physiological Genomics* **20** 188–194. (doi:10.1152/physiolgenomics.00157.2004)
- Brisson DR & Schultz RM 1997 Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. *Biology of Reproduction* **56** 1088–1096. (doi:10.1095/biolreprod56.5.1088)
- Brulet P, Babinet C, Kemler R & Jacob F 1980 Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. *PNAS* **77** 4113–4117. (doi:10.1073/pnas.77.7.4113)
- Charpigny G, Reinaud P, Tamby JP, Creminon C & Guillomots M 1997 Cyclooxygenase-2 unlike cyclooxygenase-1 is highly expressed in ovine embryos during the implantation period. *Biology of Reproduction* **57** 1032–1040. (doi:10.1095/biolreprod57.5.1032)
- Clarke FM 1992 Identification of molecules and mechanisms involved in the 'early pregnancy factor' system. *Reproduction, Fertility, and Development* **4** 423–433. (doi:10.1071/RD9920423)
- Clarke FM, Orozco C, Perkins AV, Cock I, Tonissen KF, Robins AJ & Wells JRE 1991 Identification of molecules involved in the 'early pregnancy factor' phenomenon. *Journal of Reproduction and Fertility* **93** 525–539. (doi:10.1530/jrf.0.0930525)
- Corcoran D, Rizos D, Fair T, Evans AC & Lonergan P 2007 Temporal expression of transcripts related to embryo quality in bovine embryos cultured from the two-cell to blastocyst stage *in vitro* or *in vivo*. *Molecular Reproduction and Development* **74** 972–977. (doi:10.1002/mrd.20677)
- Di Pasquale E, Beck-Peccoz P & Persani L 2004 Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *American Journal of Human Genetics* **75** 106–111. (doi:10.1086/422103)
- Diskin MG & Sreenan JM 1980 Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *Journal of Reproduction and Fertility* **59** 463–468. (doi:10.1530/jrf.0.0590463)
- Dobson AT, Raja R, Abeyta MJ, Taylor T, Shen S, Haqq C & Pera RA 2004 The unique transcriptome through day 3 of human preimplantation development. *Human Molecular Genetics* **13** 1461–1470. (doi:10.1093/hmg/ddh157)
- Ebner T, Yaman C, Moser M, Sommergruber M, Polz W & Tews G 2001 Embryo fragmentation *in vitro* and its impact on treatment and pregnancy outcome. *Fertility and Sterility* **76** 281–285. (doi:10.1016/S0015-0282(01)01904-5)
- Edwards JL, King WA, Kawarsky SJ & Ealy AD 2001 Responsiveness of early embryos to environmental insults: potential protective roles of HSP70 and glutathione. *Theriogenology* **55** 209–223. (doi:10.1016/S0093-691X(00)00455-6)
- El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K & Tesfaye D 2006 Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological Genomics* **28** 84–96. (doi:10.1152/physiolgenomics.00111.2006)
- Farin PW, Piedraita JA & Farin CE 2006 Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. *Theriogenology* **65** 178–191. (doi:10.1016/j.theriogenology.2005.09.022)
- Galaviz-Hernandez C, Stagg C, de Ridder G, Tanaka TS, Ko MS, Schlessinger D & Nagaraja R 2003 Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis. *Gene* **309** 81–89. (doi:10.1016/S0378-1119(03)00508-0)
- Hesse M, Franz T, Tamai Y, Taketo MM & Magin TM 2000 Targeted deletion of keratins 18 and 19 leads to trophoblastic fragility and early embryonic lethality. *EMBO Journal* **19** 5060–5070. (doi:10.1093/emboj/19.19.5060)
- Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K & Yodoi J 1997 AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *PNAS* **94** 3633–3638. (doi:10.1073/pnas.94.8.3633)
- Hoelker M, Rings F, Lund Q, Ghanem N, Phatsara C, Griesse J, Schellander K & Tesfaye D 2009 Effect of the microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured *in vitro*. *Reproduction* **137** 415–425. (doi:10.1530/REP-08-0370)
- Huang ZP, Ni H, Yang ZM, Wang J, Tso JK & Shen QX 2003 Expression of regulator of G-protein signalling protein 2 (RGS2) in the mouse uterus at implantation sites. *Reproduction* **126** 309–316. (doi:10.1530/rep.0.1260309)
- Imakawa K, Chang K & Christenson RK 2004 Pre-implantation conceptus and maternal uterine communications: molecular events leading to successful implantation. *Journal of Reproduction and Development* **50** 155–169. (doi:10.1262/jrd.50.155)
- Jackson BW, Grund C, Schmid E, Burki K, Franke WW & Illmensee K 1980 Formation of cytoskeletal elements during mouse embryogenesis. Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. *Differentiation* **17** 161–179. (doi:10.1111/j.1432-0436.1980.tb01093.x)
- Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP *et al.* 2002 Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biology of Reproduction* **67** 1777–1789. (doi:10.1095/biolreprod.102.007146)
- Khosla S, Dean W, Brown D, Reik W & Feil R 2001 Culture of preimplantation mouse embryos affect fetal development and the expression of imprinted genes. *Biology of Reproduction* **64** 918–926. (doi:10.1095/biolreprod64.3.918)
- Khurana NK & Niemann H 2000 Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. *Biology of Reproduction* **62** 847–856. (doi:10.1095/biolreprod62.4.847)
- Klein C, Bauersachs S, Ulbrich SE, Einspanier R, Meyer HHD, Schmidt SEM, Reichenbach HD, Vermehren M, Sinowatz F, Blum H *et al.* 2006 Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the pre-attachment period. *Biology of Reproduction* **74** 253–264. (doi:10.1095/biolreprod.105.046748)
- Knijin HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Zeinstra EC, van der Weijden GC, Niemann H & Dieleman SJ 2005 *In vitro* and *in vivo* culture effects on mRNA expression of genes involved in metabolism and apoptosis in bovine embryos. *Reproduction, Fertility, and Development* **17** 775–784. (doi:10.1071/RD05038)
- Kobayashi-Miura M, Nakamura H, Yodoi J & Shiota K 2002 Thioredoxin, an anti-oxidant protein, protects mouse embryos from oxidative stress-induced developmental anomalies. *Free Radical Research* **36** 949–956. (doi:10.1080/1071576021000006626)
- Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruij T, Niemann H & Galli C 2002 Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biology of Reproduction* **67** 767–775. (doi:10.1095/biolreprod.102.004481)
- Liu CH, Yang CC, Lin DP, Wu MH & Tsai KJ 2004 Stored of Hsp72/Hsp73 in germinal vesicle-stage mouse oocytes. *Reproduction in Domestic Animals* **39** 19–24. (doi:10.1046/j.1439-0531.2003.00470.x)
- Lopata A, Sibson MC, Enders AC, Bloomfield KL, Gregory MS, Trapani GD, Perkins AV, Tonissen KF & Clarke FM 2001 Expression and localization of thioredoxin during early implantation in the marmoset monkey. *Molecular Human Reproduction* **7** 1159–1165. (doi:10.1093/molehr/7.12.1159)
- Loureiro B, Brad AM & Hansen PJ 2007 Heat shock and tumor necrosis factor- α induce apoptosis in bovine preimplantation embryos through a caspase-9 dependent mechanism. *Reproduction* **133** 1129–1137. (doi:10.1530/REP-06-0307)
- Mamo E, Sargent CA, Affara NA, Tesfaye D, El-Halawany N, Wimmers K, Gilles M, Schellander K & Ponsuksili S 2006 Transcripts profiles of some developmentally important genes detected in bovine oocytes and *in vitro*-produced blastocysts using RNA amplification and cDNA microarrays. *Reproduction in Domestic Animals* **41** 527–534. (doi:10.1111/j.1439-0531.2006.00708.x)
- Nonogaki T, Noda Y, Narimoto K, Umaoka Y & Mori T 1991 Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized *in vitro*. *Human Reproduction* **6** 1305–1310.
- Nowak-Imialek M, Wrenzycki C, Herrmann D, Lucas-Hahn A, Lagutina I, Lemme E, Lazzari G, Galli C & Niemann H 2008 Messenger RNA expression patterns of histone-associated genes in bovine preimplantation embryos derived from different origins. *Molecular Reproduction and Development* **75** 731–743. (doi:10.1002/mrd.20816)

- Oshima RG, Howe WE, Klier FG, Adamson ED & Shevinsky LH 1983 Intermediate filament protein synthesis in preimplantation murine embryos. *Developmental Biology* **99** 447–455. (doi:10.1016/0012-1606(83)90294-4)
- Ozawa M, Nagai T, Fahrudin M, Karja NW, Kaneko H, Noguchi J, Ohnuma K & Kikuchi K 2006 Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage. *Molecular Reproduction and Development* **73** 998–1007. (doi:10.1002/mrd.20533)
- Powis G, Gasdaska JR & Baker A 1997 Redox signaling and the control of cell growth and death and the effects of inhibitors. *Chemico-Biological Interactions* **111** 23–34. (doi:10.1016/S0009-2797(97)00148-8)
- Rizos D, Lonergan P, Ward F, Duffy P & Boland MP 2002 Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61** 234–248. (doi:10.1002/mrd.1153)
- Salilew-Wondim D, Rings F, Holker M, Gilles M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Sukhorukov VL, Zimmermann U *et al.* 2007 Dielectrophoretic behavior of *in vitro*-derived bovine metaphase II oocytes and zygotes and its relation to *in vitro* embryonic developmental competence and mRNA expression pattern. *Reproduction* **133** 931–946. (doi:10.1530/REP-06-0277)
- Salilew-Wondim D, Hölker M, Rings F, Ghanem N, Ulas-Cinar M, Peippo J, Tholen E, Looft C, Schellander K & Tesfaye D 2010 Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer. *Physiological Genomics* **42** 201–218. (doi:10.1152/physiolgenomics.00047.2010)
- Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W & Dean W 2003 Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Current Biology* **13** 1116–1121. (doi:10.1016/S0960-9822(03)00419-6)
- Shao GB, Ding HM, Gong AH & Xiao DS 2008 Inheritance of histone H3 methylation in reprogramming of somatic nuclei following nuclear transfer. *Journal of Reproduction and Development* **54** 233–238. (doi:10.1262/jrd.19173)
- Smith SL, Everts RE, Tian XC, Du F, Sung LY, Rodriguez-Zas SL, Jeong BS, Renard JP, Lewin HA & Yang X 2005 Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. *PNAS* **102** 17582–17587. (doi:10.1073/pnas.0508952102)
- Smith SL, Everts RE, Sung LY, Du F, Page RL, Henderson B, Rodriguez-Zas SL, Nedambale TL, Renard JP, Lewin HA *et al.* 2009 Gene expression profiling of single bovine embryos uncovers significant effects of *in vitro* maturation, fertilization and culture. *Molecular Reproduction and Development* **76** 38–47. (doi:10.1002/mrd.20927)
- Snabes MC & Harper MJK 1984 Site of action of indomethacin on implantation in the rabbit. *Journal of Reproduction and Fertility* **71** 89–98. (doi:10.1530/jrf.0.0710089)
- Soto P, Natzke RP & Hansen PJ 2003 Actions of tumor necrosis factor- α on oocyte maturation and embryonic development in cattle. *American Journal of Reproductive Immunology* **50** 380–388. (doi:10.1034/j.1600-0897.2003.00101.x)
- Watson AJ, Westhusin ME, De Sousa PA, Betts DH & Barcroft LC 1999 Gene expression regulating blastocyst formation. *Theriogenology* **51** 117–133. (doi:10.1016/S0093-691X(98)00236-2)
- Wu YT, Tang L, Cai J, Lu XE, Xu J, Zhu XM, Luo Q & Huang HF 2007 High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Human Reproduction* **22** 1526–1531. (doi:10.1093/humrep/dem029)
- Yamagishi N, Saito Y, Ishihara K & Hatayama T 2002 Enhancement of oxidative stress-induced apoptosis by Hsp105 α in mouse embryonal F9 cells. *European Journal of Biochemistry* **269** 4143–4151. (doi:10.1046/j.1432-1033.2002.03109.x)
- Zmijewski JW, Song L, Harkins L, Cobbs CS & Jope RS 2001 Oxidative stress and heat shock stimulate RGS2 expression in 1321N1 astrocytoma cells. *Archives of Biochemistry and Biophysics* **392** 192–196. (doi:10.1006/abbi.2001.2430)

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