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Developmental and molecular correlates of bovine preimplantation embryos

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Abstract

Expression of embryonic genes is altered in different culture conditions, which influence developmental potential both during preimplantation and fetal development. The objective of this study was to define the effects of culture conditions on: bovine embryonic development to blastocyst stage, blastocyst cell number, apoptosis and expression patterns of a panel of developmentally important genes. Bovine embryos were cultured *in vitro* in three culture media containing amino acids, namely potassium simplex optimization medium (KSOMaa), Charles Rosenkrans 1 (CR1aa) and synthetic oviductal fluid (SOFaa). Apoptosis in blastocysts was determined by TUNEL assay and expression profiles of developmentally important genes were assayed by real-time PCR. *In vivo*-produced bovine blastocysts were used as controls for experiments determining gene expression patterns. While the cleavage rates did not differ, embryos cultured in SOFaa had higher rates of development to blastocyst stage ($P < 0.05$). Mean cell numbers and percentages of apoptotic cells per blastocyst did not differ among the groups. Expression of the heat shock protein 70 (Hsp70) gene was significantly up-regulated in both CR1aa and KSOMaa when compared with SOFaa ($P < 0.001$). DNA methyltransferase 3a (Dnmt3a) expression was higher in embryos cultured in CR1aa than in those cultured in SOFaa ($P < 0.001$). Expression of interferon tau (IF-τ) and insulin-like growth factor II receptor (Igf-2r) genes was significantly up-regulated in KSOMaa when compared with CR1aa ($P < 0.001$). Gene expression did not differ between *in vivo*-derived blastocysts and their *in vitro*-derived counterparts. In conclusion, SOFaa supports higher development to blastocyst stage than KSOMaa and CR1aa, and the culture conditions influence gene expression.

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Introduction

Cattle are among the most economically important species for US agriculture. Both the dairy and beef industry rely heavily on a healthy reproductive system for more efficient product generation. *In vitro* maturation of bovine oocytes followed by *in vitro* fertilization (IVF) and culture for the production of cows around the world is rapidly increasing. Thousands of IVF-generated embryos are transferred to recipient cows annually to generate offspring of higher genetic merit. In addition to providing an inexpensive and readily available source of preimplantation bovine embryos, these methods have significant value in studying the basic biological events occurring during oocyte maturation, fertilization and early embryonic development.

Preimplantation-stage embryos can develop in different media whose compositions range from simple balanced salt solutions and carbohydrates (e.g. Charles Rosenkrans 1 (CR1), synthetic oviductal fluid (SOF) and potassium simplex optimization medium (KSOM)) to very complex constituents, such as tissue culture medium (TCM)-199, with further supplementation of serum and/or a feeder layer of somatic cells (Krisher *et al.* 1999, Niemann & Wrenzycki 2000, Summers & Biggers 2003). Major developmentally important events take place during development of embryos from post-fertilization to the blastocyst stage. These include zygote formation, first cleavage division, embryonic genome activation (EGA), compaction of the morulae (compacted embryos with more than 16 blastomeres) and blastocyst formation (Lonergan *et al.* 2003). Early embryonic development after fertilization depends heavily on stored maternal mRNAs and translated proteins in mature oocytes. EGA is an essential event initiating as early as the one-cell zygotic stage in the cow and

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increasing gradually as embryonic development advances. In the absence of proper EGA, the developing embryo dies because it can no longer support essential developmental functions (Memili & First 1998, 1999, Latham & Schultz 2001). It was shown that although the primary factor influencing the percentages of immature oocytes developing to the blastocyst stage was the quality of oocytes itself, the most important step of the embryo production system affecting the quality of blastocysts, determined in terms of the resistance against cryopreservation and relative abundance of gene transcripts, was the post-fertilization culture conditions (Lonergan *et al.* 2003, Rinaudo & Schultz 2004, Wrenzycki *et al.* 2004). Therefore, a suboptimal *in vitro* culture environment can seriously affect the developmental potential of *in vitro*-produced embryos. Furthermore, culture conditions have been previously shown to change the expression patterns of a number of genes during mammalian embryogenesis (Wrenzycki *et al.* 2001, Lazzari *et al.* 2002, Rizos *et al.* 2002, Rinaudo & Schultz 2004). The number of genes expressed at the blastocyst stage is limited as compared to $>25 \times 10^3$ genes expressed in the adult. However, the genes that are expressed during the blastocyst stage are expected to have vital roles in the development to the blastocyst stage and beyond. This is because a number of critical events take place during early embryogenesis. This stage consists of more than just dividing the cytoplasm, changing length of cell cycles and replicating DNA until the first cell differentiation occurs (Latham & Schultz 2001, Hamatani *et al.* 2004). In fact, early embryogenesis is as important as the other phases of developmental biology since the embryo dies if the EGA fails to occur properly. Available data demonstrate that the expression of various gene transcripts differs between embryos developed *in vivo* or *in vitro* (Knijn *et al.* 2002, Lonergan *et al.* 2003, Gutierrez-Adan *et al.* 2004). Altered expression of embryonic genes in different culture conditions may influence the developmental potential both during preimplantation and fetal development (Fernandez-Gonzalez *et al.* 2004). *In vivo*-produced embryos are the most reliable control group when comparing gene expression patterns in developing embryos since any *in vitro* embryo production system is not optimized enough to mimic *in vivo* conditions (Niemann & Wrenzycki 2000).

The effects of culture media gene expression in bovine blastocysts have been studied by Wrenzyki et al. (2004) and by Tesfaye *et al.* (2004). However, the genes studied in these reports were different, and the studies did not compare gene expression profiles in parallel culture media.

The experiments proposed here are aimed at comparing currently used embryo culture media containing amino acids (namely KSOMaa, CR1aa and SOFaa) on their ability to support embryonic development as determined by comparing the rates of embryonic development (number of embryos reaching blastocyst stage), mean cell number, percentages of apoptotic cells and the expression patterns of some developmentally important genes at the blastocyst stage. In the present study, we characterize expression of glucose transporter-1 (Glut-1), heat shock protein 70 (Hsp70), interferon tau (IF-τ), DNA methyltransferase 3a (Dnmt3a), desmosomal glycoprotein desmocollin III (DcIII), insulin-like growth factor II receptor (Igf-2r) relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These chosen genes involve metabolism (Glut-1), stress (Hsp70), maternal recognition of pregnancy (IF-τ), DNA methylation (Dnmt3a), compaction and cavitation (DcIII), and growth factor signaling (Igf-2r). In the present study, *in vivo* control blastocysts were only used for determining the expression patterns of a panel of developmentally important genes.

Materials and Methods

All the chemicals used in the present study were purchased from Sigma unless otherwise stated.

In vivo blastocysts

Bovine *in vivo* blastocysts frozen in 0.25 ml straws (one blastocyst/straw) were provided by North American Breeders, Inc. (Berryville, VA, USA). Straws were immersed in a water bath at 36 °C for 15 s. After thawing, the contents of the straws were poured into PBS solution containing 5% glycerol and 0.55 M sucrose and kept for 3 min. Blastocysts were then collected and transferred into PBS solution containing 0.55 M sucrose for 3 min. Blastocysts were transferred into TL-HEPES. Then, groups of ten blastocysts were rinsed three times in saline and transferred into 500 μl centrifuge tubes with a minimum amount of saline. Samples were quickly frozen and kept at − 80 °C for RNA isolation.

In vitro maturation (IVM)

Bovine oocytes were collected from 2–8 mm follicles of bovine ovaries obtained from a local abattoir; collection was carried out by aspiration using an 18-gauge needle attached to a vacuum system. Only oocytes with several layers of cumulus cells and homogenous cytoplasm were selected for use in the present study. Oocytes were washed three times in TL-HEPES and matured in tissue culture medium (TCM) -199, Gibco/Invitrogen) supplemented with 0.2 mM pyruvate, 0.5 μg/ml follicle-stimulating hormone (FSH; Sioux Biochemicals, Sioux City, IA, USA), 5 μg/ml luteinizing hormone (LH; Sioux Biochemicals), 10% fetal calf serum (FCS, Gibco/Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco/Invitrogen). Ten oocytes were matured in 50 μl maturation drops covered with mineral oil for 24 h at 39 °C in a humidified incubator with 5% CO_2. This culture condition was the same for IVF and *in vitro* culture (IVC).

IVF

Matured oocytes were washed twice in TL-HEPES and groups of ten oocytes were transferred to 44 μl fertilization drops under mineral oil. The fertilization medium was glucose-free TALP supplemented with 0.2 mM pyruvate, 6 mg/ml fatty acid-free BSA (BSA-FAF), 100 U/ml penicillin and 100 μg/ml streptomycin. Frozen sperm from a previously tested bull was used for the fertilization of *in vitro*-matured oocytes. A density gradient system using Percoll was used for the separation of live spermatozoa in frozen–thawed semen (Parrish *et al.* 1995). A straw of frozen sperm was thawed at 36 °C for 1 min, and then carefully layered on top of the Percoll gradient system. Sperm was diluted to 50×10^6 sperm cells/ml in TL-HEPES, which produced 2×10^6 spermatozoa/ml in fertilization drops. Fertilization of matured oocytes was completed by adding 2 μl diluted sperm, 2 μl of 5 μg/ml heparin and 2 μl PHE solution (20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine) into the 44 μl fertilization drops. Oocytes and sperm were co-cultured for 18 h in the incubator (Leibfried & Bavister 1982).

IVC

Eighteen hours after insemination, cumulus cells were removed by vortexing the embryos in a 1.5 ml Eppendorf tube at high speed for 3 min. Twenty-five cumulus-free presumptive zygotes were washed three times in TL-HEPES and transferred into 50 μl drops of three different embryo culture media: KSOM (Specialty Media, Phillipsburg, NJ, USA), CR1 (Rosenkrans *et al.* 1993, Sagirkaya *et al.* 2004) or SOF (Specialty Media) under mineral oil. The ingredients of each media are presented in Table 1. Ten per cent FCS was added to each drop on day 4. Developmental data were recorded for two-cell, eight-cell and morulae- and blastocyst-stage embryos at 48, 96, 120 and 192 h post-insemination respectively. Fertilization time in the present study was considered as hour 0. Rates of development to blastocyst stage were recorded on day 8, and the blastocysts were either fixed in 4% formaldehyde for apoptosis studies or frozen in 0.9% NaCl for gene expression studies.

Table 1 Compositions of KSOMaa, CRIaa and SOFaa media in bovine embryo culture.

Components	KSOMaa[a]	CRIaa[b]	SOFaa[a]
Inorganic salts (mM)			
NaCl	94.97	114.60	107.70
KCl	2.50	3.08	7.16
KH_2PO_4	0.35	—	1.19
$NaHCO_3$	25.00	26.18	25.07
$MgSO_4$	0.20	—	—
$CaCl_2 \cdot 2H_2O$	1.71	—	1.17
$MgCl_2 \cdot 6H_2O$	—	—	0.49
Other components			
Na lactate (60% syrup; mM)	21.9	2.52	3.3
L-Lactate (mg/ml)	—	0.55	—
Na pyruvate (mM)	0.6	0.4	0.4
BSA (fatty acid-free) (mg/ml)	3.0	3.0	8.0
D-Glucose (mM)	0.2	—	—
EDTA (mg/ml)	3.0	—	—
Penicillin (μg/ml)	100	100	100
Streptomycin (μg/ml)	50	100	100
Amino acids			
BME amino acid solution[c] (μl/ml)	10	20	10
MEM non-amino acid solution (μl/ml)	20	10	20
L-Glutamine (mg/ml)	1.46	1.5	—

[a]From Specialty Media; [b]Rosenkrans *et al.* (1993); [c]Without L-glutamine.

Detection of apoptosis by TUNEL assay

DNA fragmentation in the nuclei of cells was determined by the TUNEL assay using the DeadEnd Fluorometric Apoptosis Detection Kit (Promega). The TUNEL assay was performed according to a modified protocol of Fedorcsak and Storeng (2003) for *in vitro*-cultured embryos. All fixed embryos were stored at 4 °C until the TUNEL assay. The embryos were transferred from 4 °C to room temperature on the day of the TUNEL assay, washed three times in 100 μl drops of 1% poly vinyl pyrrolidone (PVP) in PBS, permeabilized in 50 μl of 0.5% Triton X-100 for 30 min at room temperature in a humidified chamber and washed twice in PBS. The embryos were then incubated with 100 μl DNase buffer for 5 min; 100 μl DNase buffer containing 20 U/ml DNase I were added to the embryos previously assigned as negative and positive controls, and 100 μl DNase buffer was added to the remaining embryos. All embryos were incubated at 37 °C for 30 min, and then washed four times in 100 μl double distilled water by transferring embryos from one drop to the next. Thereafter, the embryos were exposed to 100 μl equilibration buffer for 10 min. Subsequently, 51 μl of prepared rTdT reaction buffer (45 μl equilibration buffer, 5 μl nucleotide mix, 1 μl rTdT enzyme) were added to the ten embryos. For negative control slides, 1 μl autoclaved deionized water was used in place of rTdT enzyme. Embryos were incubated in the dark at 37 °C for 1 h. The reaction was completed by incubating the embryos in 2 × SSC solution for 15 min at room temperature and washing twice in PBS. The embryos were then incubated in 50 μg/ml of RNase A (Sigma) in 10 mM Tris–HCl (pH = 7.5) and 15 mM NaCl at 37 °C for 40 min. Finally, embryos were stained with 1 μg/ml propidium iodide for 15 min and washed with deionized water to eliminate extra stain. After the last wash, the embryos were mounted with a DABCO anti-fade solution and covered with a coverslip for evaluation. Assessment of total and TUNEL-positive cell numbers was determined by evaluating each embryo under an epifluorescent microscope (Nikon, Japan) equipped with a 450–490 nm excitation filter, a 520 nm dichroic mirror and a 520 nm barrier emission filter, using a × 40 objective. The apoptotic nuclei with fragmented DNA were stained yellowish green, while normal nuclei appeared orange-red in color.

Isolation of total RNA and cDNA synthesis

Similar to *in vivo* blastocysts, *in vitro*-produced blastocysts were first transferred into TL-HEPES from culture media and groups of ten blastocysts were rinsed three times in 0.9% NaCl, and finally transferred into 500 μl centrifuge tubes with a minimal amount of saline. After transferring into centrifuge tubes, samples were immediately frozen and kept at −80 °C until RNA isolation. Total RNA was isolated from a pool of ten *in vivo*- or *in vitro*-produced blastocysts using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of total RNA isolated from blastocysts were estimated using a Bioanalyzer 2100 RNA 6000 picochip kit (Agilent, Palo Alto, CA, USA). Total RNA (8 ng) was used for cDNA synthesis using the first-strand cDNA synthesis kit for RT-PCR (AMV, Roche Applied Sciences, IN, USA) according to the manufacturer's protocol. Samples were incubated for 10 min at 25 °C, 60 min at 42 °C and then at 99 °C for 5 min.

Primer and Taqman probe design

All PCR primers and probes were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). The primers and probes were carefully designed to avoid amplification of genomic DNA. The sequences and positions of the primers and TaqMan probes used, and the fragment size and the sequence references of the expected PCR products are shown in Table 2.

Table 2 Sequences of real-time PCR primers and TaqMan probes from bovine genome.

Genes	**Primer and TaqMan probe sequences and positions** (5′ → 3′)	**Fragment size** (bp)	**Sequence references** (accession no.)
Glucose transporter-l (Glut-l)	CCAAGGATCTCTCAGAGCACAG (1688–1709) TTCTTCTGGACATCACTGCTGG (1797–1776) FAM-GATAGATCTCAGCAGAGCCGGGCCT-TAMRA (1734–1758)	110	M60448
Heat shock protein 70 (Hsp70)	GACAAGTGCCAGGAGGTGATTT (1870–1891) CAGTCTGCTGATGATGGGGTTA (1986–1965) FAM-AGCAC AAGAGGAAGGAGCTGGAGCA-TAMRA (1934–1958)	117	U09861
Interferon tau (IF-τ)	TCCATGAGATGCTCCAGCAGT (433–453) TGTTGGAGCCCAGTGCAGA (535–517) FAM-AGCACTCGTCTGCTGCCTGGAACA-TAMRA (475–498)	103	X65539
DNA methltransferase 3a (Dnmt3a)	TGATCTCTCCATCGTCAACCCT (2062–2083) GAAGAAGGGGCGGTCATCTC (2185–2166) FAM-TGAGTTCTACCGCCTCCTGCATGATG-TAMRA (2125–2150)	124	AY271298
Desmocollin III (DeIII)	CCTCTGTGATTGTACTAACCCCG (2006–2028) GAAGTATGGCAAGGATCGCC (2078–2097) FAM-TGCTCGACGGAGTGCGGACGT-TAMRA (2042–2062)	92	L33774
Insulin-like growth factor II receptor (Igf-2r)	CAGGTCTTGCAACTGGTGTATGA (4935–4957) TTGTCCAGGGAGATCAGCATG (5071–5051) FAM-AAGAGCGTCATCAGCTTCGTGTGCA-TAMRA (4998–5022)	137	J03527
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AAGGCCATCACCATCTTCCA (178–197) CCACTACATACTCAGCACCAGGAT (253–230) FAM-AGCGAGATCCTGCCAACATCAAGTGG-TAMRA (200–225)	76	U85042

All of the primer sets for each gene were sequenced and confirmed.

Real-time quantitative PCR

Real-time quantitative PCR was performed to assess transcripts of Glut-1, Hsp70, IF-τ, Dnmt3a, DcIII and Igf-2r relative to the housekeeping gene GAPDH. Each cDNA sample was analyzed in duplicate using the LightCycler instrument (Roche). Quantitative assessment of RNA amplification was detected with the TaqMan probes, which are specific for the targeted genes. The TaqMan probe contains two dyes, a reporter and a quencher dye, the primers were fluorescence labeled at the 5′ end with 6-carboxyfluorescein (FAM) as a reporter dye and at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher (Tibmolbiol, Adelphia, NJ, USA). The real-time PCR reactions were carried out in a total volume of 10 μl according to the manufacturer's manuals for hybridization probes master mix (Roche). The primers and TaqMan probe concentrations were 0.3 and 0.2 μM respectively. The cycling parameters were 2 min at 95 °C for denaturation, 50 cycles of 5 s at 95 °C, 20 s at 60 °C for amplification and quantification. GAPDH RNA was quantified to adjust the amount of total RNA in each sample with a GAPDH probe and primer set. In real-time PCR reactions the same initial amounts of target molecules were used, and the cross point (C_p) values (22.90 ± 0.02) of GAPDH mRNA were constant in all *in vitro* and *in vivo* groups. A new software tool was used, named REST (relative expression software tool), and compared all samples of each group. The mathematical model used is based on the PCR efficiencies and the crossing point deviation between the samples (Pfaffl *et al.* 2002).

Statistical analysis

In vitro-embryo production experiments were repeated at least three times. Developmental rates to the different stages were determined from the number of fertilized oocytes. Mean developmental percentages of fertilized oocytes to various developmental stages, and mean cell numbers and mean percentages of apoptotic cells per blastocyst, were calculated as estimated marginal means using the SPSS statistical program (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL, USA). Significant differences were determined by one-way ANOVA using the SPSS program. In cases of more than two groups, ANOVA was followed by multiple pair-wise comparisons using Tukey's test.

Statistical analysis of the gene expression patterns of the developmentally important genes was performed using REST (384-beta version, May 2005), which runs in Microsoft Excel. The software combined gene quantification and normalization into a single calculation. REST is based on an efficiency corrected mathematical model for data analysis. It calculates the relative expression ratio on the basis of the PCR efficiency (E) and crossing point deviation (ΔCP) of the investigated transcripts and on a newly developed randomization test macro. REST uses the pair-wise fixed reallocation randomization test to calculate the significance of results (Pfaffl 2001, Pfaffl *et al.* 2002). Differences at $P < 0.001$ were considered significant. The software used for statistical analyses is an established method and analyzes real-time PCR results directly.

Results

Development of embryos in culture media

In the present study, a total of 538, 518 and 503 oocytes were used for KSOMaa, CR1aa and SOFaa groups respectively. Cleavage rates in KSOMaa, CR1aa and SOFaa groups were 79, 78 and 80% respectively; developmental rates to eight-cell, morulae and blastocyst stage are all summarized in Table 3. There was no statistical difference between groups until the blastocyst stage. The blastocyst development rate of the SOFaa group was significantly higher ($P < 0.05$) than those of KSOMaa and CR1aa groups (32.77 vs 22.67 and 23.08% respectively).

Cell numbers and apoptotic cell ratios

On day 8 of IVC, 19, 27 and 39 blastocysts were used to determine the mean cell numbers and the percentages of TUNEL-positive nuclei for the KSOMaa, CR1aa and SOFaa groups respectively. Mean cell numbers for KSOMaa, CR1aa and SOFaa groups were 109.3, 101.0 and 114.0, and the percentages of TUNEL-positive nuclei of these groups were 1.25, 1.91 and 1.87% respectively (Table 4). There was no significant difference among groups in terms of mean cell numbers and percentage of TUNEL-positive nuclei.

Table 3 Development of bovine embryos cultured in three different culture media.

Groups	Number of fertilized oocytes	Number of two cell embryos (%)	Number of eight cell embryos (%)	Number of morulae (%)	Number of blastocysts (%)
KSOMaa	538	426 (79.15)	249 (46.65)	215 (39.92)	119 (22.16)[a]
CRIaa	518	402 (77.53)	248 (48.31)	205 (39.88)	120 (23.41)[a]
SOFaa	503	403 (80.16)	248 (50.17)	222 (42.28)	164 (32.85)[b]

Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 4 Mean cell numbers of day-8 bovine blastocysts developed in three different culture media.

Groups	*n*	Mean cell number blastocyst (± S.D.)	%apoptotic cell number blastocyst (± S.D.)
KSOMaa	19	109.3 ± 29.5	1.25 ± 1.0
CR1aa	27	101.0 ± 26.0	1.91 ± 1.9
SOFaa	39	114.0 ± 28.7	1.87 ± 1.7

Expression patterns of some developmentally important genes

The Bioanalyzer gel image of the total RNAs for KSOMaa, CR1aa, SOFaa and *in vivo* control groups is shown in Fig. 1. The figure shows that the integrity of the total RNA was solid enough to proceed with further analysis of mRNAs. The dynamics of gene expression in different culture conditions is shown in Figs 2 and 3. The expression of the Hsp70 gene was significantly up-regulated in both CR1aa and KSOMaa when compared with SOFaa ($P < 0.001$) (Fig. 2A) while expression of the Dnmt3a gene was significantly up-regulated in CR1aa when compared with SOFaa ($P < 0.001$) (Fig. 2B). There was no significant difference between *in vivo* control and *in vitro*-cultured blastocysts for any of the gene transcripts (Fig. 2C–F) determined in the present study. The expression of IF-τ and Igf-2r genes was significantly up-regulated in KSOMaa when compared with CR1aa ($P < 0.001$) (Fig. 3). There was no significant difference between CR1aa and KSOMaa for Glut-1, Hsp70, Dnmt3a and DcIII gene transcripts (Fig. 3).

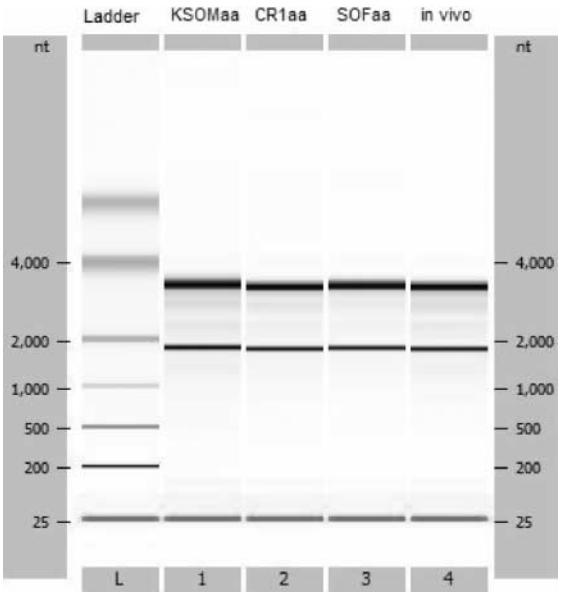

Figure 1 Total RNA was isolated from pools of ten blastocysts developed in KSOMaa, CR1aa, SOFaa and in vivo. RNA (1 μl) was loaded for each lane in the Bioanalyzer 2100 RNA 6000 picochip (Agilent). Lane L, size markers; RNA from control blastocysts in KSOMaa (lane 1), CR1aa (lane 2), SOFaa (lane 3) and in vivo (lane 4). The ratio of the sharp bands of 28S and 18S ribosomal RNA below the 4000 and 2000 molecular weight markers, respectively, demonstrate the integrity of the total RNA isolated.

Discussion

The preimplantation period of mammalian embryogenesis is notable for many critical events that affect the genome both before and after implantation. These include extensive changes in DNA methylation, changes in chromatin structure and histone acetylation–methylation, changes related to genomic imprinting and changes in the array of transcription factors, all of which, over the course of several cell divisions, lead to the establishment of a nuclear structure that is able to support embryogenesis and carry out the developmental program (Latham & Schultz 2001). It has been known that bovine early-stage embryos and blastocysts are transcriptionally active (Memili & First 1999), and embryo culture conditions influence gene expression (Wrenzycki *et al.* 2005). However, parallel studies focusing on the influence of the most commonly used culture media (CR1aa, KSOMaa and SOFaa) on gene expression in the same experiment have not been characterized.

In the present study, the development rate to blastocyst stage in SOF was significantly higher than that in CR1 and KSOM media ($P < 0.05$). However, the ingredients of these media differed slightly. While KSOM and CR1 have glutamine, SOF does not have it. There are recent reports investigating the inhibitory and teratological effects of glutamine on embryos (Devreker & Hardy 1997, Summers *et al.* 2005). Also, the concentration of BSA in SOF is higher than that in CR1 and KSOM. These differences could have an effect on the embryo development data in our study.

In an effort to achieve a higher development rate in bovine *in vitro* embryo production systems, researchers have used different *in vitro* culture strategies such as the use of somatic cell co-culture, cell-conditioned media and complex media containing serum (Bavister 1995). Recently, the use of somatic cell co-culture and cell-conditioned media has declined because of the extra work involved in cell culture production and the improvements in defined culture conditions. Culture media used in bovine *in vitro* embryo production have undergone dramatic improvement. Even though semi-defined or mostly defined culture media are used by some, they do not completely mimic *in vivo* conditions (Wrenzycki *et al.* 2005). The most commonly used culture media in bovine embryo production are SOFaa, KSOMaa and CR1aa. To our

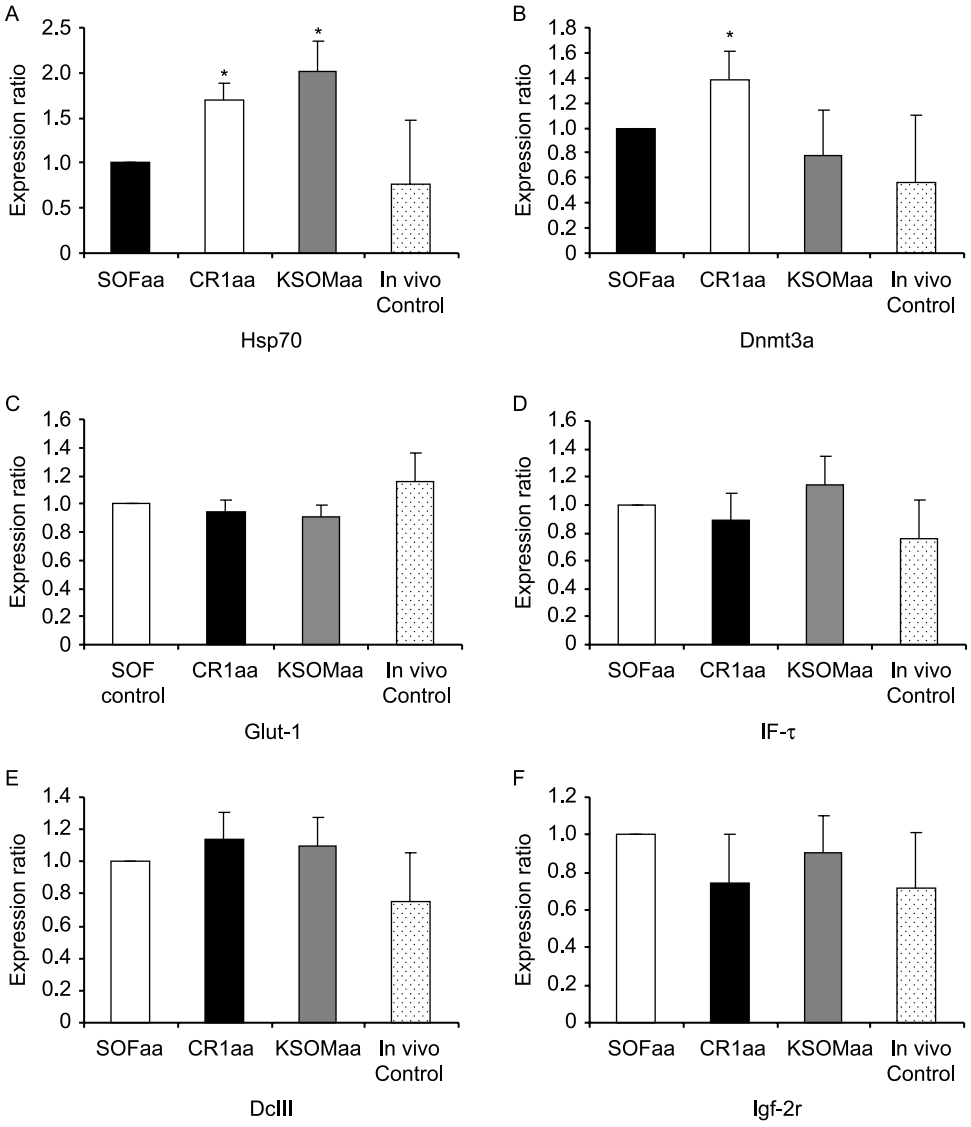

Figure 2 Relative abundance of various developmentally important gene transcripts in bovine blastocysts obtained from SOFaa (solid bars), CR1aa (open bars), KSOMaa (gray bars) culture media and *in vivo* controls (stippled bars). Expression ratios of Hsp70, Dnmt3a, Glut-1, IF-τ, DcIII and Igf-2r genes are demonstrated in panels A, B, C, D, E and F respectively. *Significant differences when compared with SOFaa ($P < 0.001$).

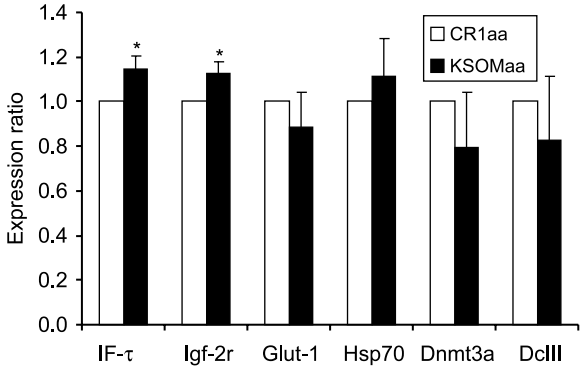

Figure 3 Relative abundance of IF-τ, Igf-2r, Glut-1, Hsp70, Dnmt3a and DcIII gene transcripts in bovine blastocysts obtained from CR1aa (solid bars) and KSOMaa (open bars) culture media. *Significant differences when compared with CR1aa ($P < 0.001$).

knowledge there are no studies comparing these three culture media side by side. Developmental data in the present study showed that SOFaa medium produced significantly higher numbers of blastocyst-stage embryos than the other two media ($P < 0.05$); even though there were no significant differences in developmental rates to two-cell, eight-cell and morulae stages among the three groups. TUNEL-positive nuclei of day-8 blastocysts derived from KSOMaa, CR1aa and SOFaa did not differ significantly (Table 4). However, other developmentally important genes could be affected.

Advances in the field of molecular biology have provided new ways to evaluate the quality of embryos (Wrenzycki *et al.* 2005). Determination of the expression patterns of genes relevant to early embryonic development provides an opportunity to assess the quality of the embryos produced *in vitro* and a chance to optimize *in vitro* embryo production systems. Real-time PCR is one of

the most reliable methods to compare some developmentally important gene transcripts (Gutierrez-Adan *et al.* 2004, Tesfaye *et al.* 2004, Miyazaki *et al.* 2005). The effects of *in vitro* culture conditions on gene expression have been mostly studied in later developmental stages such as morulae and blastocyst stages (Wrenzycki *et al.* 2005). Our study analyzed the expression of a panel of developmentally regulated genes in blastocysts cultured in KSOMaa, CR1aa and SOFaa, and supported the hypothesis that *in vitro* conditions affect the patterns of embryonic gene expression in bovine (Niemann & Wrenzycki 2000) and murine embryos (Ho *et al.* 1994, 1995). Others have reported that there are differences between the expression profiles of some specific genes in embryos derived *in vitro* and *in vivo* (Wrenzycki *et al.* 1996, Rizos *et al.* 2002, Lonergan *et al.* 2003). However, in the present study, there was no significant difference between the *in vivo* control group and *in vitro* groups (CR1aa, KSOMaa and SOFaa). In our study, frozen/thawed *in vivo* blastocysts were used. Freezing and thawing could potentially have an effect on the mRNA expression levels, and there is no comparative study examining alterations of mRNA amount in relation to freezing and thawing. However, we did not see any RNA degradation in our RNA samples. In addition, we checked transcript levels relative to the housekeeping gene GAPDH, and thus, we expect that the effects of freezing and thawing would not change our results. It has been reported that there was a significant difference between the metabolism pathways of *in vitro*-produced and *in vivo*-developed bovine embryos (Khurana & Niemann 2000). However, in the present study the expression of Glut-1 did not differ significantly among the four groups, which reflects the fact that this isoform might not play a crucial role in embryonic glucose uptake from the environment. It has been shown that environmental stressors, such as free oxygen radicals and increased temperature, induce gene expression prior to major activation of the gene expressions in bovine embryos (Edwards & Hansen 1996, 1997, Edwards *et al.* 1997). In the present study, the expression of the Hsp70 gene in CR1aa and KSOMaa culture media was significantly different from that in SOFaa ($P < 0.001$) (Fig. 2A). Our results indicated that the use of SOFaa culture media may create more sensitivity in response to heat shock.

It has been shown that while IF-τ mRNA was found in blastocysts, it was absent in morulae (Hernandez-Ledezma *et al.* 1993, Wrenzycki *et al.* 1999). IF-τ is exclusively secreted by the trophectodermal cells of blastocysts and primarily functions as a factor responsible for maternal recognition of pregnancy in cattle (Roberts *et al.* 1992). It also plays a crucial role in placentation. IF-τ mRNA was expressed at higher levels in hatched blastocysts cultured in the presence of poly vinyl alcohol (PVA) than those cultured when serum was present (Wrenzycki *et al.* 1999). Similarly, Rizos *et al.* (2003) reported a significantly higher level of IF-τ transcript in blastocysts produced in the absence of serum. It was hypothesized that deviation from normal placentation is one of the major causes of pregnancy loss after transferring *in vitro*-produced embryos (Hasler *et al.* 1995, Wells *et al.* 1999, Hill *et al.* 2000). It is commonly believed that a higher amount of IF-τ mRNA is an indicator of poor-quality blastocysts (Wrenzycki *et al.* 2001). In the present study, the expression of the IF-τ gene in KSOMaa culture media was significantly different from CR1aa ($P < 0.001$) (Fig. 3). Our results indicated that the bovine blastocysts cultured in KSOMaa might have higher implantation rates.

Methylation of DNA is an essential process in early embryonic mammalian development. It has important roles in regulation of transcription, X chromosome inactivation, cell differentiation and imprinting, and it usually causes gene silencing (Kaneda *et al.* 2004). The expression of the Dnmt3a gene in CR1aa was significantly different from that in SOFaa ($P < 0.001$) (Fig. 2B). The use of CR1aa culture media might affect global gene methylation, which may result in different transcription patterns in further development.

Desmosomal glycoprotein DcIII has a role in compaction and cavitation during blastocyst formation. In the present study, there was no significant difference in the expression of DcIII among the four groups. The use of different culture media had no effect on blastocyst formation. Expression of Igf-2r is detectable at all stages of embryonic development in bovine embryos (Watson *et al.* 1992, Yoshida *et al.* 1998, Yaseen *et al.* 2001). It has been postulated that insulin-like growth factors play a role as a survival factor in preimplantation bovine embryos (Yaseen *et al.* 2001). In the present study, the expression of the Igf-2r gene in KSOMaa culture media was significantly different from that in CR1aa ($P < 0.001$) (Fig. 3). This result indicates that the KSOMaa culture media has a positive effect on blastocyst development. In conclusion, SOFaa supports higher development to blastocyst stage than KSOMaa and CR1aa, and the *in vitro* culture conditions influence gene expression. The results of this study showed that the culture conditions alter the expression of a panel of genes that might affect their developmental potential. However, further studies are needed to examine genome-wide transcriptomes of embryos cultured in different media by DNA microarrays, and to study fetal development by embryo transfer into recipient cows.

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References

Bavister BD 1995 Culture of preimplantation embryos: facts and artifacts. *Human Reproduction Update* **1** 91–148.

Devreker F & Hardy K 1997 Effects of glutamine and taurine on preimplantation development and cleavage of mouse embryos in vitro. *Biology of Reproduction* **57** 921–928.

Edwards JL & Hansen PJ 1996 Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes. *Biology of Reproduction* **55** 341–346.

Edwards JL & Hansen PJ 1997 Differential responses of bovine oocytes and preimplantation embryos to heat shock. *Molecular Reproduction and Development* **46** 138–145.

Edwards JL, **Ealy AD**, **Monterroso VH & Hansen PJ** 1997 Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. *Molecular Reproduction and Development* **48** 25–33.

Fedorcsak P & Storeng R 2003 Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos in vitro. *Biology of Reproduction* **69** 1531–1538.

Fernandez-Gonzalez R, **Moreira P**, **Bilbao A**, **Jimenez A**, **Perez-Crespo M**, **Ramirez MA**, **Rodriguez De Fonseca F**, **Pintado B & Gutierrez-Adan A** 2004 Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *PNAS* **101** 5880–5885.

Gutierrez-Adan A, **Rizos D**, **Fair T**, **Moreira PN**, **Pintado B**, **de la Fuente J**, **Boland MP & Lonergan P** 2004 Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. *Molecular Reproduction and Development* **68** 441–448.

Hamatani T, **Carter MG**, **Sharov AA & Ko MS** 2004 Dynamics of global gene expression changes during mouse preimplantation development. *Developmental Cell* **6** 117–131.

Hasler JF, **Henderson WB**, **Hurtgen PJ**, **Jin ZQ**, **McCauley AD**, **Mower SA**, **Neely B**, **Shuey LS**, **Stokes JE & Trimmer SA** 1995 Production, freezing, and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43** 141–152.

Hernandez-Ledezma JJ, **Mathialagan N**, **Villanueva C**, **Sikes JD & Roberts RM** 1993 Expression of bovine trophoblast interferons by in vitro-derived blastocysts is correlated with their morphological quality and stage of development. *Molecular Reproduction and Development* **36** 1–6.

Hill JR, **Burghardt RC**, **Jones K**, **Long CR**, **Looney CR**, **Shin T**, **Spencer TE**, **Thompson JA**, **Winger QA & Westhusin ME** 2000 Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biology of Reproduction* **63** 1787–1794.

Ho Y, **Doherty AS & Schultz RM** 1994 Mouse preimplantation embryo development in vitro: effect of sodium concentration in culture media on RNA synthesis and accumulation and gene expression. *Molecular Reproduction and Development* **38** 131–141.

Ho Y, **Wigglesworth K**, **Eppig JJ & Schultz RM** 1995 Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Molecular Reproduction and Development* **41** 232–238.

Kaneda M, **Okano M**, **Hata K**, **Sado T**, **Tsujimoto N**, **Li E & Sasaki H** 2004 Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* **429** 900–903.

Khurana NK & Niemann H 2000 Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* **54** 741–756.

Knijn HM, **Wrenzycki C**, **Hendriksen PJ**, **Vos PL**, **Herrmann D**, **van der Weijden GC**, **Niemann H & Dieleman SJ** 2002 Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. *Reproduction* **124** 365–375.

Krisher RL, **Lane M & Bavister BD** 1999 Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. *Biology of Reproduction* **60** 1345–1352.

Latham KE & Schultz RM 2001 Embryonic genome activation. *Frontiers in Bioscience* **6** D748–D759.

Lazzari G, **Wrenzycki C**, **Herrmann D**, **Duchi R**, **Kruip 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.

Leibfried ML & Bavister BD 1982 Effects of epinephrine and hypotaurine on in-vitro fertilization in the golden hamster. *Journal of Reproduction and Fertility* **66** 87–93.

Lonergan P, **Rizos D**, **Gutierrez-Adan A**, **Moreira PM**, **Pintado B**, **de la Fuente J & Boland MP** 2003 Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. *Biology of Reproduction* **69** 1424–1431.

Memili E & First N 1998 Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development. *Molecular Reproduction and Development* **51** 381–389.

Memili E & First NL 1999 Control of gene expression at the onset of bovine embryonic development. *Biology of Reproduction* **61** 1198–1207.

Miyazaki K, **Tomii R**, **Kurome M**, **Ueda H**, **Hirakawa K**, **Ueno S**, **Hiruma K & Nagashima H** 2005 Evaluation of the quality of porcine somatic cell nuclear transfer embryo by gene transcription profiles. *Journal of Reproduction and Development* **51** 123–131.

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

Parrish JJ, **Krogenaes A & Susko-Parrish JL** 1995 Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. *Theriogenology* **44** 859–870.

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45.

Pfaffl MW, **Horgan GW & Dempfle L** 2002 Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30** e36.

Rinaudo P & Schultz RM 2004 Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction* **128** 301–311.

Rizos D, **Lonergan P**, **Boland MP**, **Arroyo-Garcia R**, **Pintado B**, **de la Fuente J & Gutierrez-Adan A** 2002 Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biology of Reproduction* **66** 589–595.

Rizos D, **Gutierrez-Adan A**, **Perez-Garnelo S**, **De La Fuente J**, **Boland MP & Lonergan P** 2003 Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction* **68** 236–243.

Roberts RM, **Cross JC & Leaman DW** 1992 Interferons as hormones of pregnancy. *Endocrine Reviews* **13** 432–452.

Rosenkrans CF Jr, **Zeng GQ**, **McNamara GT**, **Schoff PK & First NL** 1993 Development of bovine embryos in vitro as affected by energy substrates. *Biology of Reproduction* **49** 459–462.

Sagirkaya H, **Yagmur M**, **Nur Z & Soylu MK** 2004 Replacement of fetal calf serum with synthetic serum substitute in the in vitro maturation medium: effects on maturation, fertilization and subsequent development of cattle oocytes in vitro. *Turkish Journal of Veterinary and Animal Sciences* **28** 779–784.

Summers MC & Biggers JD 2003 Chemically defined media and the culture of mammalian preimplantation embryos: historical per-

spective and current issues. *Human Reproduction Update* **9** 557–582.

Summers MC, **McGinnis LK**, **Lawitts JA & Biggers JD** 2005 Mouse embryo development following IVF in media containing either L-glutamine or glycyl-L-glutamine. *Human Reproduction* **20** 1364–1371.

Tesfaye D, **Ponsuksili S**, **Wimmers K**, **Gilles M & Schellander K** 2004 A comparative expression analysis of gene transcripts in post-fertilization developmental stages of bovine embryos produced in vitro or in vivo. *Reproduction in Domestic Animals* **39** 396–404.

Watson AJ, **Hogan A**, **Hahnel A**, **Wiemer KE & Schultz GA** 1992 Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Molecular Reproduction and Development* **31** 87–95.

Wells DN, **Misica PM & Tervit HR** 1999 Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biology of Reproduction* **60** 996–1005.

Wrenzycki C, **Herrmann D**, **Garnwath JW & Neimann H** 1996 Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. *Journal of Reproduction and Fertility* **108**.

Wrenzycki C, **Herrmann D**, **Carnwath JW & Niemann H** 1999 Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Molecular Reproduction and Development* **53** 8–18.

Wrenzycki C, **Herrmann D**, **Keskintepe L**, **Martins A Jr**, **Sirisathien S**, **Brackett B & Niemann H** 2001 Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Human Reproduction* **16** 893–901.

Wrenzycki C, **Herrmann D**, **Lucas-Hahn A**, **Lemme E**, **Korsawe K & Niemann H** 2004 Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome? *Animal Reproduction Science* **82–83** 593–603.

Wrenzycki C, **Herrmann D**, **Lucas-Hahn A**, **Korsawe K**, **Lemme E & Niemann H** 2005 Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. *Reproduction, Fertility and Development* **17** 23–35.

Yaseen MA, **Wrenzycki C**, **Herrmann D**, **Carnwath JW & Niemann H** 2001 Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different in vitro systems. *Reproduction* **122** 601–610.

Yoshida Y, **Miyamura M**, **Hamano S & Yoshida M** 1998 Expression of growth factor ligand and their receptor mRNAs in bovine ova during in vitro maturation and after fertilization in vitro. *Journal of Veterinary Medical Science* **60** 549–554.

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