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Temporal Divergence in the Pattern of Messenger RNA Expression in Bovine Embryos Cultured from the Zygote to Blastocyst Stage In Vitro or In Vivo[1]

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ABSTRACT

The objective of this study was to examine the time during the postfertilization period that gene expression patterns in in vitro-cultured bovine embryos diverge from those of their in vivo-cultured counterparts. Presumptive bovine zygotes were produced by in vitro maturation and fertilization of immature oocytes collected from the ovaries of slaughtered animals. Approximately 20 h post insemination (hpi), zygotes were denuded and randomly divided into two groups for culture either in vitro, in synthetic oviduct fluid medium, or in vivo, in the ewe oviduct. Embryos were recovered from both systems at approximately 30 hpi (2-cell), 2 (4-cell), 3 (8-cell), 4 (16-cell), 5 (early morula), 6 (compact morula), or 7 (blastocyst) days post insemination. On recovery, they were examined for stage of development and snap frozen in liquid nitrogen for the analysis of transcript abundance using real-time polymerase chain reaction. The transcripts studied were glucose transporter 5, sarcosine oxidase, mitochondrial Mn-superoxide dismutase, connexin 43, interferon tau, insulin-like growth factor II, apoptosis regulator box-α and insulin-like growth factor-I receptor, most of which are known from our previous work to differ in terms of transcript abundance in blastocysts derived from culture in vitro or in vivo. The results demonstrate that the relative abundance of the transcripts studied varies throughout the preimplantation period and is strongly influenced by the culture environment. In addition, the data demonstrate that changes in transcript abundance in blastocyst stage embryos are in many cases a consequence of perturbed transcription earlier in development. Depending on the transcript, these differences may be evident by as little as 10 h of initiation of culture. Such information has implications not only for basic biology but also for human assisted reproduction in which there is a move toward culturing embryos to the blastocyst stage, necessitating prolonged culture in vitro under potentially deleterious conditions.

developmental biology, embryo, female reproductive tract, gene regulation, in vitro fertilization

INTRODUCTION

After fertilization, the early embryo is dependent on mRNA that was transcribed during oocyte growth (i.e., maternal), and it is only after a few cleavage divisions (8- to 16-cell stage in cattle) that the embryonic genome is activated and the embryo takes control of its own destiny. During the 6-day window in the bovine embryo between zygote formation and attainment of the blastocyst stage, several major developmental events take place. These include the first cleavage division, the timing of which is known to be of critical importance in determining the subsequent development of the embryo [1, 2]; the switching on of the embryonic genome [3]; compaction of the morula, which involves the establishment of the first intimate cell-to-cell contacts in the embryo [4]; and blastocyst formation, involving the differentiation of two cell types, the trophectoderm, and the inner cell mass [5]. Clearly any modifications of the culture environment in the postfertilization period, which could affect any, or all, of these processes, could have a major influence on the quality of the resulting blastocyst.

We have recently shown that although the quality of the oocyte is the main factor affecting the proportion of immature oocytes developing to the blastocyst stage, the postfertilization culture environment is the critical part of the embryo production process influencing the quality of the blastocyst, measured in terms of cryotolerance and relative transcript abundance [6–8]. Blastocysts produced following culture in vitro in synthetic oviduct fluid (SOF) had significantly altered patterns of mRNA expression for genes involved in gap junction formation, apoptosis, oxidative stress, and differentiation, compared with those cultured in vivo [6]. In addition, simply omitting serum from the culture medium during the postfertilization period had a positive effect on cryotolerance, which was mirrored in changes in mRNA relative abundance for several developmentally important genes [8].

Although it is clear from our work and that of others [9–13] that the postfertilization culture environment can have a dramatic effect on the pattern of mRNA abundance of many developmentally important genes in the embryo, this effect has generally been measured in blastocysts at the end of the culture period. It is not clear whether a temporal association between culture environment and gene expression exists. Wrenzycki et al. [9] used a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay to analyze the effects of two media supplements, serum or polyvinyl alcohol, on the relative abundance of gene transcripts in bovine embryos cultured in vitro. However, to date, a comparison between in vitro- and in vivo-cultured embryos throughout the early preimplantation period has not been made. Such information is of relevance not only to the study of basic biology but also to human assisted

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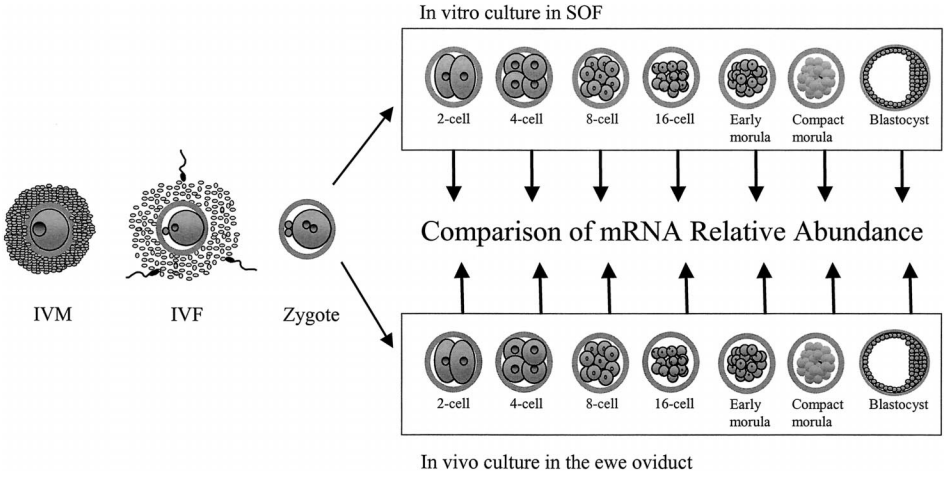

FIG. 1. Experimental design. Bovine zygotes produced by in vitro maturation and fertilization were cultured in vitro, in synthetic oviduct fluid (SOF), or in vivo in the ewe oviduct. Embryos were recovered from both systems at Day 1 (2-cell), Day 2 (4-cell), Day 3 (8-cell), Day 4 (16-cell), Day 5 (early morula), Day 6 (compact/late morula), or Day 7 (blastocyst) post insemination for analysis of transcript abundance.

reproductive technology; until recently it was customary to transfer in vitro-fertilized human embryos back to the mother relatively soon after fertilization thus avoiding prolonged exposure to potentially detrimental in vitro conditions [14]. However, nowadays there is a move toward blastocyst transfer in an effort to reduce the incidence of high-order multiple pregnancies [15, 16]. This necessitates prolonged culture in vitro under potentially deleterious conditions.

The objective of this study was to extend our previous work [6, 8] by examining the time during the postfertilization period that gene expression patterns in the in vitro cultured embryo diverge from those of their in vivo cultured counterparts. This was done using the now well-established ewe oviduct model [6, 7, 17, 18].

MATERIALS AND METHODS

Zygote Production

Bovine presumptive zygotes were produced following in vitro maturation (IVM) and fertilization (IVF). Briefly, cumulus oocyte complexes (COCs) were obtained by aspirating follicles from bovine ovaries collected after slaughter. After four washes in PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamicin, and 0.5 mg/ml BSA (Sigma, St. Louis, MO; catalog no. A-9647), groups of up to 50 COCs were placed in 500 μl maturation medium in four-well dishes (Nunc, Roskilde, Denmark) and cultured for 24 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor. For IVF, COCs were washed four times in PBS and then in fertilization medium before being transferred in groups of up to 50 into four-well dishes containing 250 μl of fertilization medium per well (Tyrode medium with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml fatty acid–free BSA, and 10 μg/ml heparin-sodium salt (184 U/mg heparin; Calbiochem, San Diego, CA). Motile spermatozoa were obtained by centrifugation of frozen-thawed semen (Dairygold A.I. Station, Mallow, Ireland) on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 ml 45% Percoll over 2.5 ml 90% Percoll) for 8 min at 700 ×g at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction, were washed in Hepes-buffered Tyrode and pelleted by centrifugation at 100 × *g* for 5 min. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of fertilization medium to give a concentration of 2×10^6 spermatozoa/ml. A 250-μl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml. Plates were incubated for approximately 20 h at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Semen from the same bull was used for all experiments.

Culture of Zygotes to the Blastocyst Stage

At approximately 20 h post insemination (hpi), presumptive zygotes produced following IVM/IVF were denuded by gentle vortexing. They were then randomly divided into two groups and cultured either in vitro in SOF medium (25 zygotes in 25 μl of medium under mineral oil) or in vivo in the ewe oviduct (Fig. 1). In vitro culture took place in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at maximum humidity. FCS (10%, v/v) was added at 48 hpi. For in vivo culture, zygotes were surgically transferred by midventral laparotomy to the ligated ewe oviduct (approximately 100 per oviduct) at 20 hpi [7, 18]. Embryos were recovered from both systems at approximately 30 hpi (i.e., approximately 10 h after initiation of culture) or 2, 3, 4, 5, 6, or 7 days post insemination. On recovery, embryos were examined for stage of development and snap frozen in liquid nitrogen for the analysis of transcript abundance.

For the comparison of transcript abundance between in vivo- and in vitro-cultured embryos, only embryos at the correct stage for age were compared (Day 1: 2-cell; Day 2: 4-cell; Day 3: 8-cell; Day 4: 16-cell; Day 5: early morula; Day 6, compact/late morula; Day 7: blastocyst).

RNA Extraction and Reverse Transcription

Poly(A) RNA was prepared from 4–5 groups of pools of 10 embryos, following the manufacturer's instructions using the Dynabeads mRNA direct kit (DYNAL, Oslo, Norway). Briefly, samples were lysed in 50 μl lysis/binding buffer (DYNAL). After vortexing and a brief centrifugation, the samples were incubated at room temperature for 10 min. Ten microliters of prewashed Dynabeads oligo $(dT)_{25}$ were added to each sample. After 5 min of hybridization, the beads were separated from the binding buffer using the DYNAL magnetic separator. Then the beads were washed in buffer A and B (DYNAL) and the poly(A) RNA was eluted from the beads by adding 11 μl of diethylpyrocarbonate-treated water. The resultant poly(A) mRNA was used in the RT-PCR in a total volume of 20 μl using 2.5 μM random hexamer primer, 1 × RT buffer, 20 IU RNase inhibitor, 50 IU AMV reverse transcriptase enzyme (Promega, Madrid, Spain), 5 mM $MgCl_2$, and 1 mM of each deoxynucleotide triphosphate (dNTP). Tubes were heated to 70°C for 5 min to denature the secondary RNA structure before the addition of RT enzyme. The RT reaction was then incubated at room temperature for 10 min and then at 42°C for 60 min to allow the RT of RNA, followed by 93°C for 1 min to denature the RT enzyme.

Quantitative RT-PCR

The quantification of all gene transcripts was carried out by real-time quantitative RT-PCR. Three replicate PCR experiments were conducted for all genes of interest using embryos collected from the experimental pools. Experiments were conducted to contrast relative levels of each transcript and bovine Histone H2a in every sample. PCR was performed by adding a 4-μl aliquot of each sample to the PCR mix containing the specific primers to amplify Histone H2a, glucose transporter 5 (GLUT-5), sarcosine oxidase (SOX), mitochondrial Mn-superoxide dismutase (Mn-SOD), connexin 43 (Cx43), interferon tau (IFN-τ), insulin-like growth factor II (IGF-II), apoptosis regulator box-α (Bax), or insulin-like growth factor-I receptor (IGF-IR). Primer sequences, annealing temperature, the approximate sizes of the amplified fragments, and the Gene Bank accession number are shown in Table 1.

Bovine Histone H2a amplification was used as a standard control of the RT-PCR. For quantification, PCR was performed using a Rotorgene 2000 real time cycler (Corbett Research, Sydney, Australia) and SYBR

TABLE 1. Details of primers used for RT-PCR.

Gene	Primer sequence (5′-3′)	Fragment size (bp)	Annealing temperature (°C)	Gene Bank accession no.
Histone H2a	5′ AGGACGACTAGCCATGGACGTGTG	208	59	NM_174809
	3′ CCACCACCAGCAATTGTAGCCTTG			
Glut-5	5′ CATCTCCATCATCGTCCTCA	531	56	AF308830
	3′ GTAGATGGTGGTGAGGAGAC			
SOX	5′ CCTGAGGCCAGGAGTTCAAGACC	200	59	G1857444
	3′ GCAATAGTGTGATCTCGGCTCACTG			
MnSOD	5′ CCCATGAAGCCTTTCTAATCCTG	307	56	L22092.1
	3′ TTCAGAGGCGCTACTATTTCCTTC			
Cx43	5′ TGGAATGCAAGAGAGGTTGAAAGAGG	293	56	NM_17046 8.1
	3′ AACACTCTCCAGAACACATGATCG			
IF-τ	5′ GCCCTGGTGCTGGTCAGCTA	564	46	AF238612
	3′ CATCTTAGTCAGCGAGAGTC			
IGF-IR	5′ TTGAACTGATGCGCATGTGCTGG	210	56	BTIGF1B
	3′ TGCTTCTTGCGGCCCCCGTTCAT			
Bax	5′ TGCAGAGGATGATCGCAGCTGTG	198	59	BTU92569
	3′ CCAATGTCCAGCCCATCATGGTC			
IGF-II	5′ GTGCTGCATTGCTGCTTACC	161	59	E01192
	3′ TTCGGAAGCAACACTCTTCCA			
G6PDH	5′ CGCTGGGACGGGGTGCCCTTCATC	347	56	XM_0493
	3′ CGCCAGGCCTCCCGCAGTTCATCA			

Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. The PCR reaction mixture (25 μl) contained 2.5 μl 10× buffer, 3 mM $MgCl_2$, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 μM of each dNTP, and 0.2 μM of each primer. In addition, the double-stranded DNA dye, SYBR Green I (1:3000 of 10 000× stock solution) was included in each reaction. The PCR protocol included an initial step of 94°C (2 min), followed by 40 cycles of 94°C (15 sec), 56–59°C (30 sec), and 72°C (30 sec). Fluorescent data were acquired at 85°C (a temperature associated with the beginning of the peak for the specific products). The melting protocol consisted of a hold temperature at 40°C for 60 sec and then heating from 50°C to 94°C, holding at each temperature for 5 sec while monitoring fluorescence. Product identity was confirmed by ethidium-bromide–stained 2% agarose gel electrophoresis. As negative controls, tubes were always prepared in the RNA or reverse transcriptase that was omitted during the RT reaction. In addition, amplicon identities were confirmed by appropriate restriction digests of PCR products (data not shown).

The method used for quantification of expression was the relative standard curve method. The quantification was normalized to an endogenous control (Histone H2a), and standard curves were prepared for each target and the endogenous reference. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence rose above background for each sample. The Rotor Gene software (Corbett Research) generated a best-fit line and extrapolated the unknown concentration from the threshold cycle of titered known quantities. For each experimental sample, the amount of mRNA of each transcript and Histone H2a was determined from the appropriate standard curve. Subsequently, the quantity of each transcript was divided by Histone H2a to obtain a normalized value for each transcript. The sample with the higher value was assigned a value of 1. The normalized target values were divided by the calibrator normalized target values to generate the relative expression levels.

Statistical Analysis

Data on mRNA expression were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student-Newman-Kleus method) was used for the analysis of differences in mRNA expression assayed by quantitative RT-PCR. Differences of $P < 0.05$ were considered significant.

RESULTS

The mean recovery rate from the ewe oviduct was 73.6% (1876/2550; range: 38.8%–91.5%). The relative abundance of the gene transcripts studied is shown in Figure 2. Transcripts for IGF-IR, IGF-II, glucose-6-phosphate dehydrogenase (G6PD), SOX, MnSOD, and Cx43 were detected throughout preimplantation development from zygote to blastocyst stage, and the remaining transcripts (IFN-τ, GLUT-5, and Bax) were present only at specific stages of development.

Transcripts for IFN-τ were detected from the 8-cell stage onward in in vitro cultured embryos but not until the early morula stage in those cultured in vivo. Levels of this transcript increased significantly ($P < 0.01$) at the compact morula and blastocyst stages in both groups but were significantly higher in in vitro cultured embryos at both stages.

The kinetics of appearance of mRNA for Bax differed between embryos cultured in vivo or in vitro. The mRNA for Bax was not detected before the 8-cell stage in in vitro cultured embryos and not until the 16-cell stage in in vivo cultured embryos. The abundance of this transcript increased significantly thereafter up to the blastocyst stage in both groups ($P < 0.01$). The level of expression was significantly higher at all stages of development in in vitro cultured embryos than those cultured in vivo ($P < 0.05$ for D4 and D5; $P < 0.01$ for D6 and D7).

The relative abundance of Cx43 transcripts decreased in both in vitro and in vivo cultured embryos at the 8- to 16-cell stage. Levels remained low thereafter in the in vitro cultured embryos but significantly increased ($P < 0.01$) in those cultured in vivo. Transcript abundance was significantly higher ($P < 0.01$) in in vivo cultured embryos from Day 4 onward with a 10-fold difference presence at the blastocyst stage.

GLUT-5 mRNA was not detected before the 8-cell stage in either in vitro- or in vivo-cultured embryos. From the 8-cell stage onward, the relative abundance of this transcript increased significantly irrespective of whether culture took place in vitro or in vivo ($P < 0.05$). From the 16-cell stage onward, the relative transcript abundance was significantly higher in embryos cultured in vivo ($P < 0.05$).

The abundance of transcripts for MnSOD decreased significantly ($P < 0.01$) from the zygote to the 8-cell stage after which it increased up to the blastocyst stage. Transcript abundance was significantly higher in in vitro cultured embryos at the 8-cell stage, but at the blastocyst stage, levels were significantly higher in the in vivo group ($P < 0.05$).

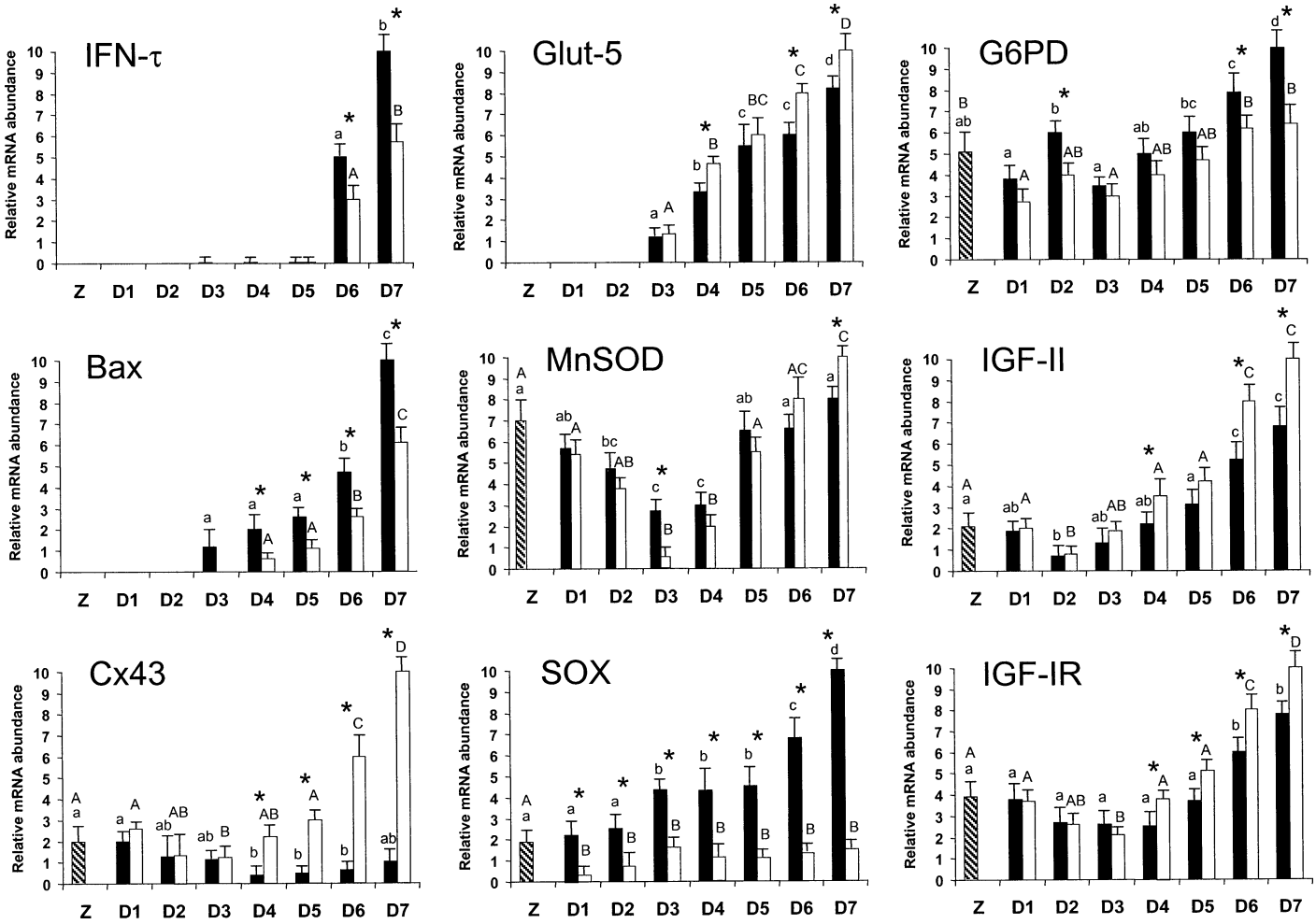

FIG. 2. Relative abundance of specific transcripts in bovine embryos cultured in vitro, in synthetic oviduct fluid (SOF, black bars) or in vivo in the ewe oviduct (white bars). abcd refers to significant differences in relative transcript abundance among in vitro-cultured embryos throughout the early preimplantation period; ABCD refers to significant differences in relative transcript abundance among in vivo-cultured embryos throughout the early preimplantation period. An asterisk (*) indicates a significant difference in relative transcript abundance between in vitro- and in vivo-cultured embryos at a given stage of development. Z, Zygote before transfer to treatment group; D1, 2-cell; D2, 4-cell; D3, 8-cell; D4, 16-cell; D5, early morula; D6, compact/late morula; D7, blastocyst.

The relative abundance of SOX transcripts increased continuously throughout the culture period from zygote to blastocyst stage in in vitro-cultured embryos. In contrast, levels of this transcript decreased from the zygote to the 2-cell stage and remained low thereafter throughout the preimplantation period in in vivo cultured embryos. The transcript abundance was significantly higher in in vitro-cultured embryos at all stages of development with more than a 5-fold difference present at the blastocyst stage ($P < 0.05$ for D2; $P < 0.01$ for D1, D3, D4, D5, D6, and D7).

Levels of G6PD increased from the 8-cell stage onward in embryos irrespective of whether culture took place in vivo or in vitro. Levels were significantly higher in in vitro-cultured embryos at the 4-cell, compact morula, and blastocyst stages ($P < 0.05$ for D2 and D6; $P < 0.01$ for D7).

The pattern of expression of IGF-IR mRNA was similar for both groups, remaining relatively constant from the zygote to the 16-cell stage and increasing thereafter. Transcript abundance was significantly higher in in vivo-cultured embryos from the 16-cell stage onward ($P < 0.05$ for D4, D5, and D6; $P < 0.01$ for D7).

The relative abundance of IGF-II mRNA decreased from the zygote stage to the 4-cell stage in both groups but increased subsequently up to the blastocyst stage irrespective of whether culture took place in vivo or in vitro ($P < 0.05$). The relative abundance was higher at all stages in in vivo-cultured embryos, the difference being significant at the 16-cell, compact morula and blastocyst stages ($P < 0.05$ for D4; $P < 0.01$ for D6 and D7).

DISCUSSION

The present study is the first to use real-time PCR to analyze the changes in the relative abundance of mRNAs throughout bovine preimplantation development in embryos cultured in vitro or in vivo. The set of genes analyzed in the present study characterizes several important physiological processes including transport and metabolism of fructose (GLUT-5), stress (SOX), mitochondrial activity and detoxification of reactive oxygen species (MnSOD), cell communication (Cx43), maternal recognition of pregnancy (IFN-τ), imprinting (IGF-II), apoptosis (Bax), growth factor binding (IGF-IR), and metabolism and oxidative stress (G6PD).

In this study we used bovine histone H2a mRNA as an endogenous standard for the quantification of the expression of other mRNAs. This gene was selected because in a recent comparison of the pattern of expression of different

housekeeping genes, histone H2a was the only one that had constant mRNA levels across the entire preimplantation period [19]. In agreement with Robert et al. [19], we found that the mRNA levels of bovine histone H2a are more constant across the preimplantation period than bovine β-actin (data not shown), but we found higher levels of expression of H2a than in their report. These differences could be due to the fact that in this article we used primers specific for the amplification of bovine histone H2a instead of the primers for murine H2a as used for Robert et al. [19].

The results demonstrate clearly that the relative abundance of various transcripts varies throughout the early preimplantation period and is strongly influenced by the culture environment. These observations are entirely consistent with our previous work [6, 8] demonstrating differences in transcript abundance between blastocysts cultured in vitro or in vivo. More importantly, however, the present data demonstrate clearly that changes in transcript abundance in blastocyst stage embryos are in many cases a consequence of perturbed transcription earlier in development.

IFN-τ is the primary agent responsible for maternal recognition of pregnancy in cattle [20]. Bovine embryos begin to express IFN-τ as the blastocyst forms [21], although there is considerable variability between individual embryos in the amount they produce [22], which may be related to the source of the embryo [23], the age at which blastocyst formation occurs [24, 25], the group size in which culture takes place [26], the medium composition [8, 9, 25, 27], or to the sex of the embryo [28]. In a previous study from our group [8], we observed a significantly higher level of expression of IFN-τ mRNA in blastocysts produced in the absence of serum, which was correlated with significantly higher cryotolerance. This would be consistent with the notion that mRNA levels for this transcript are higher in good-quality embryos. In agreement, Wrenzycki et al. [9] reported increased levels of IFN-τ mRNA in hatched blastocysts produced in the presence of polyvinyl alcohol, compared with those in which serum was present. However, such a hypothesis would seem to apply only to embryos cultured in vitro; in contrast to these observations, in the present study, levels of transcripts for IFN-τ were significantly higher in in vitro–cultured embryos at the compact morula and blastocyst stages, compared with those cultured in vivo. The reasons for this discrepancy are unclear but are entirely consistent with the findings of Wrenzycki et al. [10], who observed a significantly increased relative abundance for IFN-τ transcripts in in vitro-generated blastocysts, compared with those derived in vivo. It may be that the temporal expression of IFN-τ transcripts is a better indicator of embryo quality than the absolute expression at a particular stage because the latter is known to vary widely [22]. Kubisch et al. [24] observed a negative relationship between early IFN-τ production and developmental competence. The results of the present study support this notion because IFN-τ transcripts were detected from Day 3 in in vitro-cultured embryos but not until Day 5 in those cultured in vivo.

The mRNA for Bax was detected from the 8-cell stage onward in in vitro-cultured embryos but not until the 16-cell stage in in vivo-cultured embryos. The level of mRNA abundance was significantly higher for this transcript at all stages thereafter in in vitro-cultured embryos than those cultured in vivo. These observations are consistent with our previous studies comparing blastocysts produced in vitro or in vivo [6] or those produced in vitro in the presence or absence of serum [8]. In agreement, Gjorret et al. [29] reported that apoptosis is more frequent in in vitro- than in vivo-produced blastocysts. Taken together, these data would indicate that this gene might represent a useful marker of blastocyst quality.

In previous work from our group, the level of expression of Cx43 was significantly higher in blastocysts produced following in vivo culture than those produced in vitro [6]; in addition culture from the zygote to blastocyst stage in the absence of serum significantly increased the relative transcript abundance for Cx43 transcripts, compared with those in which serum was present [8]. This pattern of expression reflects the quality of these blastocysts measured in terms of cryotolerance.

Wrenzycki et al. [30] reported that Cx43 mRNA was detectable in in vitro-produced bovine embryos from the oocyte to the morula stage but was not detectable in blastocysts or hatched blastocysts, in contrast to its detection in in vivo-derived blastocysts. The same authors subsequently observed that the expression pattern for Cx43 in vitro was altered in the presence of serum, disappearing at the 8- to 16-cell stage and reappearing at the hatched blastocyst stage [9]. These observations are supported by the findings of the present study; the relative abundance of Cx43 transcripts decreased in both in vitro and in vivo-cultured embryos at the 8- to 16-cell stage. Levels remained low thereafter in the in vitro-cultured embryos but significantly increased in those cultured in vivo. Transcript abundance was significantly higher in in vivo-cultured embryos from Day 4 onward with a 10-fold difference present at the blastocyst stage.

GLUT-5 mRNA was not detected before the 8-cell stage in either in vitro- or in vivo-cultured embryos. From the 8-cell stage onward, the relative abundance of this transcript increased irrespective of whether culture took place in vitro or in vivo. These results are consistent with those of Augustin et al. [31], who noted that transcription of GLUT-5 started at the 8- to 16-cell stage in in vitro-produced bovine embryos. In the present study, the relative abundance of GLUT-5 transcripts was significantly higher in embryos cultured in vivo from the 16-cell stage onward, indicating a culture environment-induced differential expression pattern. As pointed out by Augustin et al. [31], because GLUT-5 transcripts were detectable from the 8-cell stage onward and given the high affinity of this transporter for fructose, it would appear that the early bovine embryo is capable of transporting this energy substrate, which is found in the uterine fluid [32].

In the present study, MnSOD transcripts were detected throughout development. Irrespective of whether culture took place in vitro or in vivo, the abundance of transcripts for MnSOD tended to decrease from the zygote stage to the 8- to 16-cell stage after which it increased up to the blastocyst stage. Transcript abundance was significantly higher in in vitro-cultured embryos at the 8-cell stage, but at the blastocyst stage, levels were significantly higher in those cultured in vivo. This supports our previous observations of a higher level of expression of MnSOD mRNA in in vivo-produced blastocysts and those cultured in the ewe oviduct (i.e., high-quality blastocysts), compared with those produced by culture in vitro in serum-supplemented SOF [6]. The lower level of expression in in vitro-produced embryos at blastocyst stage may be indicative of lower mitochondrial activity [33].

Lequarre et al. [34] demonstrated a culture environment-dependent expression of transcripts for MnSOD in bovine embryos. In that study, no expression of MnSOD was de-

tected at the 5- to 8-cell, 9- to 16-cell, and morula stages when culture took place in the absence of serum, but it was detected in almost 80% of blastocysts. In contrast, in the presence of 5% serum or in in vivo-produced embryos, mRNA expression was detectable in 58% of morulae and 74% of blastocysts. We previously reported that the expression of MnSOD was higher in blastocysts produced in SOF in the presence of serum than when it was absent [8].

The relative abundance of SOX transcripts increased continuously throughout the culture period from zygote to blastocyst stage in in vitro-cultured embryos. In contrast, levels of this transcript decreased from the zygote to the 2-cell stage and remained low thereafter throughout the preimplantation period in in vivo-cultured embryos. We previously observed a higher level of expression of the SOX enzyme in blastocysts produced in vitro in SOF than in those produced in vivo [6]. In addition, SOX expression was highest in blastocysts generated in serum-supplemented SOF, compared with those cultured in the absence of serum [8]. This would suggest that SOX expression could be used as a marker of blastocyst quality. The results of the present study are entirely consistent with that notion; transcript abundance for SOX was significantly higher in in vitro-cultured embryos at all stages of development with a more than a 5-fold difference present at the blastocyst stage.

Levels of G6PD were significantly higher in in vitro-cultured embryos at the 4-cell, compact morula, and blastocyst stages. Glucose-6-phosphate-dehydrogenase catalyzes the first and irreversible step of the pentose phosphate pathway (PPP), and it is a potential indicator of the PPP activity. Moreover, the G6PD gene is described as a sentinel for oxidative stress, leading rapidly to the generation of NADPH for maintenance of the cellular redox state. The higher expression of the in vitro-produced embryos after Day 5 of development may be an adaptive response to the oxidative stress induced by the in vitro conditions. However, the higher expression observed in vitro could be due to the differences in X-chromosome-linked gene transcript levels caused by perturbed dosage compensation in female IVP bovine embryos [35]. Because the process of X-chromosome inactivation is not completed until near the time of implantation, there is a preimplantation developmental window during which there may be basic differences in cellular chemistry between female and male embryos. We reported that female IVP bovine blastocysts have twice the expression of G6PD than their male counterparts [35]. This effect has not been reported for in vivo-produced embryos [36]; however, these authors also reported that female IVP blastocysts have more expression than males, and together, both male and female IVP embryos have more expression than those produced in vivo. Such differential expression between male and female IVP bovine blastocysts has been reported also for IFN-τ [28].

Aberrant expression patterns of imprinted genes have been implicated in embryonic and fetal abnormalities [37, 38]. It has been reported that in vitro culture alters the expression of imprinted genes in murine [39–41] and bovine embryos [42–44]. In the present study, morulae and blastocysts produced following in vivo culture had significantly more mRNA for IGF-II and IGF-IR than those cultured in vitro. IGF-II mediates growth in early embryos, is imprinted when inherited maternally, and has been related with the large offspring syndrome [43, 44]. Semiquantitative analysis of the expression of IGF-IR and IGF-II showed that the intensity of gene expression was positively correlated with the morphology of embryo [45]. In mice, optimization of culture media produces embryos with an mRNA expression of IGF-IR and IGF-II similar to that in in vivo embryos [46].

With regard to testing the functionality of the genes, to date there have been no knock-out experiments in cattle. An alternative, transgenic interference RNA has only very recently been reported in mammals (mice) [47] and to our knowledge has not yet been applied to bovine embryos. Deletion of some of the genes analyzed in this study (e.g., Cx43, IGF-IR) is apparently not deleterious to murine preimplantation development. Consistent with this, we previously carried out fresh transfers (i.e., without cryopreservation) of bovine embryos produced by culture in vitro in SOF or in vivo in the ewe oviduct as in this study to recipients, and the pregnancy rates were similar [18], suggesting that implantation rates of fresh embryos are unaffected by altered levels of these transcripts. However, such blastocysts differ significantly in terms of cryotolerance [7, 17, 18]. These differences are also evident at the ultrastructural level [48], particularly in relation to cell-to-cell communication, in which the connexins, among other genes, are implicated. Thus, there clearly is a relationship between altered transcript abundance patterns and some aspects of embryo quality (i.e., ultrastructural morphology and cryotolerance), which render the embryo capable of establishing a pregnancy, if transferred fresh, but incapable of withstanding cryopreservation.

It should also be pointed out that null mutant mice for Cx43 are far from normal. Juneja et al. [49] found that the gonads of both sexes of null mutant mice for Cx43 were unusually small because of, at least in part, a deficiency in germ cells that was traced back to Day 11.5 of gestation. Null mutants die soon after birth because of heart abnormalities. They concluded that Cx43 is required for the earliest stages of folliculogenesis, which can proceed to the primary follicle stage but beyond which it is impaired. It is possible that such embryos with altered transcript abundance survive to implantation because of the contribution of one or more additional connexin genes that are normally expressed along with connexin43 in preimplantation development. It has been reported that Cx31 serves as a compensatory channel during preimplantation development in rat [50], and we also previously showed that the abundance of transcripts for this gene can be modified by the culture environment [6].

Similarly, we know that the expression patterns of growth factors in general, and in particular IGF-IR, are different between mice and bovine embryos [51], and even between buffalo and bovine embryos [52]. Moreover, mice null for IGF-IR are by no means normal; they die invariably at birth of respiratory failure and exhibit a severe growth deficiency (45% normal size). In addition to generalized organ hypoplasia, including the muscles, and developmental delays in ossification, deviations from normalcy are observed in the central nervous system and epidermis [53].

In conclusion, the data presented here indicate that despite a similar rate of development to the blastocyst stage in in vitro-produced zygotes cultured in vitro or in vivo [7, 18] profound differences exist at the transcriptional level. Depending on the transcript, these differences may be evident within as little as 10 h of initiation of culture, and in all cases are still evident at the blastocyst stage. It is evident that the postfertilization culture environment is the key determinant of blastocyst quality [7]. Therefore, any efforts aimed at improving blastocyst quality will have to focus on the postfertilization period. It is clear from this and other

studies [14, 54–56] that bovine embryos exhibit temporal sensitivities to their culture conditions. This is in part reflected in the recognition that the developing embryo has vastly different nutrient requirements because it develops from the zygote to the blastocyst stage [57, 58] and in the development of sequential media [59, 60]. What is required is a culture system in vitro that induces a pattern of mRNA expression in the embryo similar to that observed in embryos cultured in vivo at all stages during the early preimplantation window.

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