Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*

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Bovine embryos produced *in vitro* differ substantially from embryos produced *in vivo* in the mRNA expression patterns of genes important for development. Several factors in the *in vitro* production systems have profound effects on embryonic mRNA expression patterns. The effects of the type of maturation on the expression pattern of genes important for development in blastocysts produced *in vitro* have not yet been investigated. The aim of the present study was to investigate the effects of various maturational protocols on the relative abundance of a panel of six marker genes, indicative of compaction and cavitation, metabolism, stress susceptibility and RNA processing, in bovine blastocysts produced *in vitro*. Four groups of blastocysts were analysed by a sensitive semi-quantitative RT-PCR assay. Blastocysts were produced *in vitro* from oocytes of different origin from: (1) 3–8 mm follicles; (2) preovulatory follicles before the LH surge; and

(3) preovulatory follicles 24 h after the LH surge. The first two groups were matured *in vitro*, whereas the third group had undergone maturation *in vivo*. A fourth group comprised blastocysts developed entirely *in vivo*. Expression of glucose transporter 1 was significantly ($P < 0.05$) higher, and expression of desmocollin 2 and plakophilin tended to be higher ($P < 0.1$) for *in vivo* (group 4) compared with *in vitro* blastocysts (group 1), whereas no differences were found for heat shock protein 70.1, E-cadherin and poly(A) polymerase. Expression of the six transcripts did not differ among blastocysts produced *in vitro* from oocytes of groups 1, 2 and 3. Results indicate that alterations in the relative abundance of these transcripts in blastocysts produced *in vitro* cannot primarily be attributed to the origin of the oocyte, but are likely to have been induced by post-maturation or fertilization culture conditions.

Introduction

In cyclic cows, embryos develop after fertilization of oocytes that originate from an ovulatory follicle. These follicles have undergone the processes of selection, growth and dominance until oestrus, when the LH surge initiates the final maturation approximately 24 h before ovulation (Dieleman *et al.*, 1983a; Fortune, 1994). In contrast, for production of viable embryos *in vitro*, oocytes are used that differ from oocytes originating from ovulatory follicles *in vivo* in that they are: (1) derived from immature, subordinate follicles of 2–8 mm in diameter, and (2) matured *in vitro*, outside the follicle. Oocytes from small follicles (< 3 mm) are less likely to develop *in vitro* into blastocysts than are oocytes from larger (> 3 mm) follicles (Pavlok *et al.*, 1992; Blondin *et al.*, 1997; Van de Leemput *et al.*, 1999). A comparative analysis of the ultrastructure of oocytes derived from dominant, preovulatory follicles with

that of oocytes from subordinate, small follicles revealed first signs of maturation (referred to as ‘capacitation’ or ‘prematuration’) in the dominant follicle before the LH surge (Assey *et al.*, 1994; Hyttel *et al.*, 1997). Bovine oocytes contain large but changing populations of mRNAs and proteins. The oocyte supplies mRNAs that sustain embryonic development up to the stage of maternal–embryonic transition, and a few mRNAs persist throughout development until the blastocyst stage (Memili and First, 1999). In particular, the oocyte contains transcription factors involved in the minor and major activation of the embryonic genome at the two- to four-cell stage and eight-cell stage, respectively (Viuff *et al.*, 1996). So, within the mammalian oocyte, genes have to be properly imprinted; furthermore, oocyte-derived factors are important for changes in methylation and for recognition of imprinted genes that need to be methylated (Young and Fairburn, 2000). The mRNA contents of the oocyte are related to the developmental competence of the zygote after fertilization (Lequarre *et al.*, 1997). Furthermore, the length of the poly-A tail of RNA in bovine oocytes was to some extent correlated

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with the quality of the resulting blastocyst (Brevini-Gandolfi *et al.*, 1999). The amounts of mRNA encoding for Na-K-ATPase, Cu-Zn superoxide dismutase (SOD), basic fibroblast growth factor (bFGF), cyclin A and B in bovine oocytes are affected by the maturation medium (Watson *et al.*, 2000). The intrinsic quality of the oocyte is a key factor in determining blastocyst yields (Sirard and Blondin, 1996; Rizos *et al.*, 2002). These findings indicate that prematuration and maturation of the oocyte affect patterns of gene expression at the blastocyst stage.

Various differences have been described between embryos derived *in vitro* and *in vivo* (Holm and Callesen, 1998; Niemann and Wrenzycki, 2000), including morphology (Van Soom and de Kruif, 1992; Van Soom *et al.*, 1997a), number and allocation of cells (Van Soom *et al.*, 1996; Viuff *et al.*, 2001), frequency of apoptosis and mixoploidy (Viuff *et al.*, 1999, 2001; Gjørret *et al.*, 2001), tolerance to cryopreservation (Niemann, 1995; Enright *et al.*, 2000), embryonic metabolism (Khurana and Niemann, 2000) and expression profile of specific mRNAs (Wrenzycki *et al.*, 1996, 2001a; Eckert and Niemann, 1998; Lequarre *et al.*, 2001). The relative abundance of several gene transcripts thought to be critically involved in preimplantation development is affected by the choice of basic culture medium and the type of protein supplement (Wrenzycki *et al.*, 1999, 2001a). The effects of different maturational protocols on the relative abundance of genes important in development in blastocysts derived *in vitro* have not yet been investigated.

The aim of the present study was to determine the effects of prematuration-maturation (*in vitro* versus *in vivo*) of the oocyte on the relative abundance of a panel of six 'marker' genes important in development: glucose transporter 1, desmocollin 2, E-cadherin, plakophilin, heat shock protein 70.1 and poly(A) polymerase. These genes were selected because they are indicative of various mechanisms in preimplantation bovine development *in vitro*. Plakophilin and desmocollin 2 are of embryonic origin, whereas the other genes are of maternal and embryonic origin. All six transcripts are sensitive markers for compaction and cavitation, metabolism, RNA processing and stress susceptibility, and thereby indicate the quality of blastocysts (Wrenzycki *et al.*, 1999, 2001a). The effects of different oocyte origins on blastocyst gene expression were investigated by collecting and analysing four groups of blastocysts from: (i) oocytes lacking the preovulatory development (*in vitro* group); (ii) oocytes prematured *in vivo* and matured *in vitro* (pre-LH group); (iii) oocytes prematured and matured *in vivo* (post-LH group); and (iv) blastocysts developed entirely *in vivo* (*in vivo* group). Oocytes derived from these different sources were subjected to *in vitro* fertilization and cultured to blastocysts *in vitro* under identical conditions.

Materials and Methods

Experimental design

Four groups of blastocysts that differed with regard to either the origin of the oocyte or conditions of embryo

development were collected. In the first (*in vitro*) group, oocytes from ovaries obtained from an abattoir were matured, fertilized and cultured *in vitro* up to the blastocyst stage. In the second and third groups, two groups of cows were synchronized and treated with FSH to stimulate a large population of follicles to undergo the normal events of prematuration. The oocytes were collected either before the LH surge (pre-LH group) or 24 h after the induced LH surge (post-LH group). The oocytes from the pre-LH group were matured *in vitro*, and were then fertilized and cultured up to the blastocyst stage simultaneously with the oocytes from the post-LH group (Fig. 1). In the fourth (*in vivo*) group, blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. Oocytes and blastocysts were collected from Holstein Friesian cows. Oocytes were recovered from cows at a commercial abattoir for the *in vitro* group, whereas donor animals from the experimental herd of the Veterinary Faculty of Utrecht University were used for the other three groups.

Animals

For the pre-LH ($n = 8$) and post-LH ($n = 9$) groups, 17 clinically healthy, non-lactating Holstein Friesian cows were selected on the basis of progesterone concentrations in peripheral blood samples measured three times a week for at least 4 weeks before the experiment. The cows were fed 20 kg corn silage and 4 kg concentrate per day, and grass silage and water were supplied *ad libitum*. The cows were presynchronized using an ear implant for 9 days (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer), together with 3 mg norgestomet and 5 mg oestradiol valerate i.m. (Intervet International BV). Two days before the implant was removed, prostaglandin (15 mg Prostaglandin i.m.; Intervet International BV) was administered.

On day 8 of the synchronized cycle (oestrus = day 0), all follicles > 5 mm were removed by transvaginal ultrasound-guided aspiration to avoid the inhibitory effects of follicular dominance on the developmental capacity of oocytes from subordinate follicles (Bungartz and Niemann, 1994). On day 9 of the oestrous cycle, the cows received another ear implant (3 mg norgestomet, Crestar) for 5 days, but no further combined norgestomet and oestradiol valerate. From day 10 of the oestrous cycle onwards, the cows received o-FSH i.m. (Ovagen ICP, Auckland) twice a day in decreasing doses over 4 days (3.5 ml, 2.5 ml, 1.5 ml and 1.0 ml; in total 17 ml, equivalent to 299 iu NIH-FSH-S1). Prostaglandin (22.5 mg i.m.) was administered together with the fifth dose of FSH, and 55 h later the ear implants were removed (Fig. 1).

The day after the last FSH dose, transrectal ultrasound technology was used to collect oocytes from cows with more than eight follicles > 8 mm. For the pre-LH group, cows ($n = 8$) were ovariectomized 2 h after removal of the implant. In the post-LH group, cows ($n = 9$) received GnRH (1.0 mg Fertagyl in 10 ml saline i.m.; Intervet International BV) at the time of removal of the implant and were

Fig. 1. Schedule of treatments for superovulation with a fixed LH surge to collect prematured oocytes from FSH-treated cows for *in vitro* maturation (pre-LH) or *in vivo* maturation (post-LH), and subsequent simultaneous *in vitro* fertilization (IVF). FSH: administration of eight consecutive, decreasing doses of FSH; time: the time relative to start of *in vitro* maturation (start of IVM); PG: administration of prostaglandin; GnRH: administration of GnRH; LH surge: start of *in vivo* maturation: the time at which the maximum of the GnRH-induced LH surge occurs, starting *in vivo* maturation; OVX: ovariectomy.

ovariectomized 26 h after receiving GnRH. Ovariectomy was performed by laparotomy through a flank incision under local anaesthetic, using lidocaine cum adrenaline (Alfasan, Woerden). Ovaries were collected in 0.9% (w/v) NaCl at 37°C and were immediately transported to the laboratory. Heparinized blood samples were collected from the jugular vein every day during the experimental cycle and every hour after removal of the second implant for 6 h or until ovariectomy. After immediate centrifugation at 1800 *g* for 10 min at 4°C, plasma was stored at -25°C.

The seven cows in the *in vivo* group were treated with 3000 iu eCG (Intergonan; Intervet, Tönisvorst) between day 9 and day 13 of the oestrous cycle, and with prostaglandin (Estrumate; Schering-Plough, Munich) 48 h later. At oestrus, the donors were inseminated twice at an interval of 12 h with semen of a bull with proven fertility. At day 7 after insemination, blastocysts were recovered by non-surgical flushing of the uterine horns with 300 ml PBS (Sigma, St Louis, MO) supplemented with 1% newborn calf serum (NBCS, No. 295957; Boehringer, Mannheim) using established procedures.

Only blastocysts of morphological grades I and II (Robertson and Nelson, 1998) were included in this study. The blastocysts were stored in a minimum volume of PBS with 0.1% (w/v) polyvinyl alcohol (PVA; Sigma) and stored at -80°C.

Radioimmunoassays for progesterone and LH

Concentrations of progesterone in plasma were estimated by a solid-phase ¹²⁵I-radioimmunoassay (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA) according to the instructions of the manufac-

turer as validated by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol l⁻¹, and the intra- and interassay coefficients of variation were 8 and < 11%, respectively.

Concentrations of LH in plasma were estimated by a validated homologous radioimmunoassay (Dieleman *et al.*, 1983b). The sensitivity was 0.4 mg NIH-LH-B4 l⁻¹. The intra- and interassay coefficients of variation were < 9%.

Collection of oocytes and in vitro maturation

In vitro group. Bovine ovaries were collected in a thermos flask at a local abattoir from Holstein Friesian cows of unknown reproductive status and were then transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from 3–8 mm follicles, but only those with a multi-layered compact cumulus investment were used for the experiments. An average of ten usable COCs was collected per ovary. Selected COCs were rinsed once with HEPES-buffered M199 supplemented with 10% fetal calf serum (FCS; Gibco BRL, Paisley) and once with maturation medium. Groups of 35 oocytes were randomly allocated to wells of four-well culture plates (Nunc A/S, Roskilde) containing 500 µl maturation medium (M199; Gibco BRL) supplemented with 10% FCS (Gibco BRL), 0.01 iu porcine FSH ml⁻¹ (Sigma), and 0.01 iu equine LH ml⁻¹ (Sigma) per well and then cultured for 22 h (39°C, 5% CO₂ in humidified air).

Pre-LH group. Immediately after ovariectomy, COCs were aspirated from putative preovulatory follicles > 8 mm and were included in the experiment on the basis of a minimum of three layers of compact cumulus investment.

COCs of one cow were then processed in one well for *in vitro* maturation similar to the COCs of the *in vitro* group.

Post-LH group. Immediately after ovariectomy at 24 h after the LH surge, COCs matured *in vivo* were aspirated from preovulatory follicles > 8 mm and were selected for the experiment based on an expanded cumulus-cell mass. COCs with a compact cumulus investment and degenerated oocytes were discarded. Selected COCs from each cow were kept together in maturation medium in a single well until fertilization.

The interval between ovariectomy and transfer of COCs to maturation (pre-LH group) or fertilization (post-LH group) medium was 30–40 min. The duration of maturation was 24 h for the pre-LH group (*in vitro*) and 24 h *in vivo* (ovariectomy 24 h after the LH surge). Ovulation of fully matured oocytes occurs 24 h after the LH surge (Dieleman *et al.*, 1983a). Maturation was assessed by determining expansion of the cumulus cells. Cytological studies have revealed that in our system > 80% of the oocytes reach the metaphase II stage.

In vitro fertilization and embryo culture (IVF and IVC)

Procedures for *in vitro* fertilization (IVF) were performed as described by Izadyar *et al.* (1996). In brief, matured oocytes of the *in vitro*, pre-LH and post-LH groups were fertilized in wells of four-well culture plates. All oocytes were fertilized at the same time with the same batch of semen from one bull with proven fertility. Before fertilization, oocytes in each well were rinsed twice with 2 ml HEPES-buffered M199 medium.

Frozen-thawed semen used for IVF was centrifuged over a Percoll gradient for 30 min at 700 *g* at 25°C. The sperm sample was collected by removing the gradient except for the last 150 µl containing the sperm pellet. COCs were transferred to 0.43 ml fertilization medium (Fert-TALP) (Parrish *et al.*, 1988), but without glucose and with 10 µl penicillin–streptomycin ml⁻¹ (Gibco BRL) instead of gentamycin. Sperm suspension (20 µl; final concentration 0.5 × 10⁶ cells ml⁻¹), 20 µl heparin (final concentration 10 µl ml⁻¹; Sigma), and 20 µl PHE (consisting of 20 µmol D-penicillamine l⁻¹, 10 µmol hypotaurine l⁻¹, 1 µmol adrenaline l⁻¹; Sigma) were added to the fertilization medium. After 18–20 h of incubation at 39°C, with 5% CO₂ in humidified air, cumulus cells were completely removed from presumptive zygotes by vortexing for 3 min; a maximum of ten zygotes was placed in 20 µl droplets of synthetic oviductal fluid (SOF) medium under oil (Squibbs Oil, Princeton, NJ) (Van Wagtenonk-de-Leeuw *et al.*, 2000). Zygotes of the pre-LH and post-LH groups from each animal were cultured in individual droplets. *In vitro* culture (IVC) was performed at 39°C in a humidified atmosphere containing 5% CO₂, 7% O₂ and 88% N₂. On day 4 of culture, all cleavage stages were transferred to fresh culture droplets, and the proportion of each cleavage stage was assessed. On day 7 after insemination, blastocysts and expanded blastocysts

were collected, whereas morulae and early blastocysts were cultured for one additional day. On day 8, additional blastocysts and expanded blastocysts were collected. Day 1 of culture was defined as beginning immediately after completion of IVF. Embryos were washed four times in PBS with 0.1% (w/v) PVA (PBS-ET; Bio Whittaker Europe, Verviers) and transferred to 0.5 ml cups in a minimum volume (≤ 5 µl) of PBS with 0.1% (w/v) PVA and frozen at –80°C. Analysis of cultured embryos was confined to those of morphological grades I and II (Robertson and Nelson, 1998).

Selection of the embryos for RT-PCR

For each gene, embryos from as many different cows as possible were analysed. Furthermore, similar numbers of embryos collected on days 7 and 8 were assigned to each mRNA analysis. Depending on the type of gene, four to five transcripts were analysed per embryo and at least five embryos per gene were determined. The relative abundance of mRNAs from the six different genes important in the development of a preimplantation embryo was determined in single bovine blastocysts by semi-quantitative RT-PCR.

Isolation of RNA

Poly(A)⁺ RNA from a single embryo was isolated using a Dynabeads mRNA DIRECT kit (DynaL A.S., Oslo). RNA was isolated following the manufacturer's instructions with minor alterations (Wrenzycki *et al.*, 1999). In brief, frozen embryos were thawed by adding 30 µl lysis buffer (100 mmol Tris–HCl l⁻¹, pH 7.5; 500 mmol LiCl l⁻¹; 10 mmol EDTA l⁻¹, pH 8; 1% (w/v) LiDS (SDS); 5 mmol dithiothreitol l⁻¹). As an internal standard, 1.0 pg rabbit globin RNA (Life Technologies BV, Eggenstein) was added to each embryo. The samples were mixed for 10 s, centrifuged for 15 s at 12 000 *g*, and left for 10 min at room temperature. Dynabeads (5 µl; Dynal) were added and mixed for 5 min at room temperature. The samples were put in the magnetic separator to remove the lysis buffer and leave the Dynabeads. The Dynabeads were washed four times, once with 40 µl washing buffer A (10 mmol Tris–HCl l⁻¹, pH 7.5; 0.15 mol LiCl l⁻¹; 1.0 mmol EDTA l⁻¹; 0.1% (w/v) LiDS) and three times with 40 µl washing buffer B (10 mmol Tris–HCl l⁻¹, pH 7.5; 0.15 mol LiCl l⁻¹; 1.0 mmol EDTA l⁻¹). After removal of washing buffer B, 11 µl sterile H₂O was added and incubated at 65°C for 2 min to elute mRNA from the Dynabeads. The cups were put into the magnetic separator again; the supernatant was removed and immediately used for reverse transcription.

Reverse transcription

Poly(A)⁺ RNA isolated from a single embryo was reverse transcribed into cDNA in a total volume of 20 µl. The reaction mixture consisted of 1 × RT buffer (50 mmol KCl l⁻¹; 10 mmol Tris–HCl l⁻¹, pH 8.3; Perkin Elmer Biosystems, Vaterstatten), 5 mmol MgCl₂ l⁻¹, 1 mmol of each dNTP l⁻¹ (Amersham, Brunswick), 2.5 µmol random hexamers l⁻¹ (Perkin Elmer), 20 iu RNase inhibitor (Perkin Elmer) and

50 iu MuLV reverse transcriptase (Perkin Elmer). The samples were centrifuged for 15 s at 12 000 *g* and were overlaid with mineral oil to prevent evaporation. One sample was prepared with 1.0 pg rabbit globin RNA and 10 μ l H₂O. As negative controls, tubes without an RNA sample and tubes with an RNA sample, but without reverse transcriptase, were analysed. The reverse transcription reaction was carried out in a thermocycler (Biometra Triothermoblock; Biometra, Göttingen) for 10 min at 25°C, 60 min at 42°C, and 5 min at 99°C, and was kept on ice once the reaction was completed.

PCR

Immediately after reverse transcription the samples were subjected to PCR amplification. PCR was performed with a volume of the reverse transcriptase reaction corresponding to 0.1 (desmocollin 2 and Glut-1), 0.2 (heat shock protein, E-cadherin and poly(A) polymerase) and 0.4 (plakophilin) embryo equivalents in a final volume of 50 μ l, containing 1 $\times$ PCR buffer (20 mmol Tris-HCl l⁻¹; 50 mmol KCl l⁻¹, pH 8.4; Life Technologies), 1.5 mmol MgCl₂ l⁻¹, 200 μ mol of each dNTP l⁻¹ and 1 μ mol of each sequence-specific primer l⁻¹ (0.5 μ mol l⁻¹ for globin primers). A 'hot start' PCR was performed to obtain specific amplification. During the 'hot start' 1 iu Taq DNA polymerase (Life Technologies) was added at 72°C. The sequences and positions of the primers used, the annealing temperature, the fragment sizes of the expected products and the sequence references are shown (Table 1). The products of each primer pair were sequenced to confirm the identity of the RT-PCR fragments.

Running a linear cycle series established the optimal cycle number at which the transcript was amplified exponentially for the PCR reaction. The PCR programme started at 97°C for 2 min, decreased to 72°C for 2 min (hot start) and was followed by a number of cycles, which depended on the gene (Table 1), of 15 s at 95°C for denaturation, 15 s at the annealing temperature of the specific gene (Table 1) and 15 s at 72°C for primer extension. After finishing these cycles a final extension of 5 min at 72°C was performed, followed by cooling to 4°C. A PTC-200 thermocycler (MJ Research, Watertown, MA) was used.

Detection and semi-quantification of RT-PCR products

RT-PCR product (25 μ l) with 5 μ l of 10 $\times$ loading buffer (0.25% (w/v) xylene cyanol and 25 mmol EDTA l⁻¹ in 50% (w/v) glycerol) was loaded on to a 2% (w/v) agarose gel in TBE buffer (90 mmol Tris l⁻¹; 90 mmol borate l⁻¹; 2 mmol EDTA l⁻¹, pH 8.3) containing 0.2 μ g ethidium bromide ml⁻¹ and subjected to electrophoresis. The concentration of ethidium bromide in the running buffer and the gel was the same. After electrophoresis at 100 V for 5 min and at 80 V for 40 min, the fragments were visualised on a 312 nm UV transilluminator. A CCD camera (Quantix; Photometrics, Munich) and IP Lab spectrum (IP Lab Gel; Signal Analytics Corporation, Vienna, VA) were used to digitize the image of

Fig. 2. Concentration (mean $\pm$ SEM) of LH in peripheral blood of FSH-prostaglandin-norgestomet-treated cows without GnRH (pre-LH, *n* = 8; $\diamond$) and with GnRH administered (post-LH, *n* = 7; $\blacksquare$); GnRH was administered at the time of removal of the implant.

the gel. Densitometric scanning using a computer-assisted image analysis system (IP Lab Gel; Signal) was used to quantify the signal intensity of each band. The relative abundance of the mRNA of the different genes was estimated by dividing the intensity of the band of the gene of interest by the intensity of the globin band from the same sample.

The recovery rate of the RNA was estimated for all of the embryos. It was calculated as the ratio between the intensity of the globin bands with and without RNA extraction. On average, 42% of the poly(A)⁺ RNA of the blastocyst was extracted, which is similar to percentages obtained by Wrenzycki *et al.* (1999).

Statistical analysis

Rates of blastocyst formation were analysed with logistic regression and *P* $\leq$ 0.05 was considered significant. For the relative abundance of the gene transcripts, an analysis of variance was performed using the nlme library of S-PLUS 2000 with random cow effects (Pinheiro and Bates, 2000). Data are presented as mean $\pm$ SEM.

Results

Response to superovulation treatment (pre-LH and post-LH groups)

No LH surge was observed in peripheral blood of cows in the pre-LH group (Fig. 2). The cows that were treated with GnRH (post-LH group) showed a clear LH surge at 2 h after the administration of GnRH; in cows from which embryos were collected for RT-PCR, maximum LH concentration was 24.5 ± 2.5 μ g l⁻¹ (*n* = 7; Fig. 2).

Signs that an LH surge had occurred before the implant was removed were observed in four cows: cumulus cells were moderately expanded in two cows from the pre-LH group and two cows of the post-LH group had already ovulated. Oocytes from these animals were excluded from the experiment.

The superovulation response in the pre-LH and the

Table 1. Primers used for PCR

Genes	Primer sequence and positions	Number of cycles	Annealing temperature (°C)	Fragment size (bp)	Sequence references (EMBL accession number)
Globin	5' primer (241–260): GCAGCCACGGTGGCGAGTAT 3' primer (555–657): GTGGGACAGGAGCTTGAAT	27	60	257	Cheng <i>et al.</i> (1986) (X04751)
Desmocollin 2	5' primer (2085–2109): CTCCTGGCGATGACAAAGT GTATTC 3' primer (2503–2527): GCCGATCCTCTTCCTTCGTA GTTAT	31	57	443/397 (insertion of 46 bp, 2396–2441)	Koch <i>et al.</i> (1992) (M81190)
Plakophilin	5' primer (1337–1361): CCCGTGGACCCGAGGTCTT CTTCA 3' primer (1580–1604): CGGTGTAGGCGTTGCGGGCG TTGTAACCTCTGGCTGATAAAA	35	64	268	Heid <i>et al.</i> (1994) (Z37975)
Glucose transporter 1	5' primer (1609–1638): AGGAGCTGTTCCACCCCTG GGAGCTGACT 3' primer (1906–1935): TGTGGGTGAAGGAG	32	59	327	Boado and Pardridge (1991) (M60448)
Poly(A) polymerase	5' primer (886–915): GTTTCCTCGGTGGTGTTC TGGGCTATGC 3' primer (1108–1137): TGGAGTTCTGTTGTGGGTAT GCTGGTGTA	35	57	252	Raabe <i>et al.</i> (1991) (X63436)
Heat shock protein 70.1	5' primer (1861–1890): AAGGTGCTGGACAAGTGCC AGGAGGTGATT 3' primer (2319–2348): ACTTGAAGTAAACAGAAA CGGGTGAAAA	36	59	488	Gutierrez and Guerriero (1995) (U09861)
E-cadherin	5' primer (1486–1515): CTCAAGCTCGCGGATAACC AGAACAAAGAC 3' primer (1785–1814): AGGCCCCTGTGCAGCTGGC TAAATCAAAG	33	55	332	Ringwald <i>et al.</i> (1987) (X06339)

post-LH groups was 30.3 ± 10.5 (mean $\pm$ SD, $n = 6$) and 23.1 ± 10.0 (mean $\pm$ SD, $n = 7$) follicles > 8 mm on average per cow, respectively.

Oocyte recovery and in vitro culture

In the pre-LH group, 166 COCs were collected from 182 follicles of preovulatory size from six cows (recovery rate 91.2%). COCs that did not have an intact multi-layered

compact cumulus investment or that already showed cumulus expansion were excluded ($n = 26$) from analysis. In the post-LH group, 136 oocytes were collected from 162 follicles of preovulatory size from seven cows (recovery rate 84.0%). Denuded oocytes and COCs not showing cumulus expansion ($n = 20$) were excluded. In the *in vitro* group, 333 oocytes were collected and used for IVM, IVF and IVC.

Cleavage rates on day 4 of culture were similar in the three IVC groups (average 75%; Table 2). On day 7 after

Table 2. Rates of blastocyst formation after *in vitro* culture (IVC) and IVF of *in vivo*- or *in vitro*-matured oocytes obtained from preovulatory follicles from FSH-stimulated cows compared with the rates of *in vitro*-matured oocytes from 3–8 mm follicles

	Number of oocytes after IVF, day 1*	Number of cleaved embryos on day 5 (%)	Number of blastocysts		
			Day 7 [†] (%)	Day 8 (%)	Total
Preovulatory follicles					
Pre-LH (after <i>in vitro</i> maturation)	140	123 (87.9)	51 (36.4) ^a	19 (13.6) ^a	70 (50.0) ^a
Post-LH (after <i>in vivo</i> maturation)	116	85 (73.3)	29 (25.0) ^b	7 (6.0) ^a	36 (31.0) ^b
3–8 mm follicles					
After IVM	333	233 (70.0)	55 (16.5) ^c	33 (9.9) ^a	88 (26.4) ^b

*Oocytes were collected from preovulatory follicles before (pre-LH) or 24 h after (post-LH) an induced LH surge. Day 1 is start of IVC. Percentages of cleavage and blastocyst formation are calculated relative to the number of (non)-fertilized oocytes at day 1 of IVC.

[†]At day 7 of IVC, blastocysts were collected and stored for RT-PCR, and morulae and early blastocysts were cultured for an additional day and collected as blastocysts at day 8.

^{a–c} Values with different superscripts within a column are significantly different ($P < 0.05$).

Fig. 3. Expression of six genes important to development as detected by RT-PCR in a 0.1 to 0.4 proportion of a single bovine blastocyst; digitalized image after electrophoresis on an agarose gel containing ethidium bromide. PCR products are indicated at the top for heat shock protein 70.1 (HSP), poly(A) polymerase (polyA), glucose transporter 1 (Glut-1), desmocollin 2 (Dc-2), E-cadherin (E-cad) and plakophilin (plako), and for the RNA extraction control globin (– Ext: without extraction; + Ext: with extraction). Expected fragment sizes are indicated at the bottom; bpl: 100 bp ladder as marker for fragment size.

insemination, most (60–80%) of the blastocysts had already formed. Rates of blastocyst formation at day 7 were significantly ($P < 0.05$) different in the pre-LH, post-LH and *in vitro* groups. The overall rate of blastocyst formation was significantly ($P < 0.05$) higher in the pre-LH group (50%; Table 2) than in the post-LH (31.0%) and *in vitro* groups (26.4%). Most of the blastocysts in the three IVC groups were already expanded: 64.3, 52.7 and 54.5% for pre-LH, post-LH and *in vitro* group embryos, respectively.

Relative abundance of genes in single blastocysts derived from different oocyte origins

For RT-PCR products (Fig. 3), the bands represent gene transcripts derived from 0.1–0.4 parts of a bovine blastocyst. As negative controls, RNA or reverse transcriptase was

Fig. 4. Relative abundance of transcripts of six genes important to development as detected by semi-quantitative RT-PCR in single bovine blastocysts originating from oocytes subjected to different modes of maturation. Embryos were derived from: (i) immature oocytes from 3–8 mm follicles after IVM, IVF and IVC (*in vitro* group; ▨); (ii) prematured oocytes from preovulatory sized, FSH-stimulated follicles after IVM, IVF and IVC (pre-LH group; ▤); (iii) prematured oocytes from preovulatory sized, FSH-stimulated follicles after *in vivo* maturation, IVF and IVC (post-LH group; □); and (iv) similar oocytes to those in (iii) but also with fertilization and development entirely *in vivo* (*in vivo* group; ■). Bars represent the mean $\pm$ SEM relative abundance of a number of blastocysts as indicated in the bars; each value was the mean of at least five different cows. Asterisks indicate difference from all groups: * $P < 0.1$; ** $P < 0.05$.

omitted during the reverse transcription reaction. No amplified products were found at any time in the negative controls during these experiments.

The relative abundance of Glut-1 was significantly ($P < 0.05$) higher in the *in vivo* group compared with the pre-LH, post-LH and *in vitro* groups (Fig. 4). The relative abundance of desmocollin 2 and plakophilin tended to be higher in the fourth group (*in vivo*) compared with the other three groups, although this difference was not significant. No significant differences were detected in any of the gene transcripts between the three *in vitro* groups (*in vitro*, pre-LH and post-LH groups; Fig. 4).

Discussion

In the present study the effects of different maturation regimens of bovine oocytes on the relative abundance of transcripts from six genes important in development in blastocysts were investigated. *In vitro* conditions have profound effects on the patterns of expression of a range of gene transcripts in bovine and murine embryos (Ho *et al.*, 1994, 1995; Niemann and Wrenzycki, 2000). The present data show for the first time that prematuration–maturation does not necessarily affect the relative abundance of the genes and indicate that *in vitro* maturation may not be the critical factor contributing to variation in mRNA content between *in vivo*- and *in vitro*-derived embryos. The differences between *in vivo*- and *in vitro*-derived blastocysts were probably not due to variation in the rates of development. In parallel experiments, blastocysts derived *in vivo* and *in vitro* as well as those derived from pre-LH and post-LH groups had similar numbers of cells.

The semi-quantitative RT–PCR assay used in the present study provides sensitive and highly reproducible results from both pooled and single bovine embryos. It has constantly been updated and increased in sensitivity (Wrenzycki *et al.*, 1999, 2001a,b, 2002). The validity of the assay has been demonstrated previously and revealed efficient amplification of both the globin standard and the RNA of choice (Wrenzycki *et al.*, 2000). Its accuracy compares favourably with current real-time RT–PCR techniques (Stenman *et al.*, 1999). Similar to endpoint RT–PCR, real-time RT–PCR uses standards against which the amounts of mRNA of the genes of choice are compared (Freeman *et al.*, 1999; Bustin, 2000; Steuerwald *et al.*, 2000). The densitometric analysis of ethidium bromide-stained agarose gels described in the present study is a well established and sensitive enough approach to detect even subtle differences in amounts of mRNA in different biological materials (Grover *et al.*, 2001; Ringhoffer *et al.*, 2001). The degree of variation with this approach is rather small (Grover *et al.*, 2001), but can be further decreased by an optimized real-time RT–PCR protocol (Bustin, 2000). We have calculated the number of replicates necessary to obtain statistically significant differences to accommodate any inherent variability. This approach has recently proven valid for single bovine cloned and IVP embryos (Wrenzycki *et al.*, 2001b, 2002). The differences in patterns of expression described previously were related to the increased incidence of the large offspring syndrome in offspring

derived from IVP or cloned bovine embryos (Niemann and Wrenzycki, 2000; Niemann *et al.*, 2002). Even subtle changes in patterns of expression of specific genes were shown to have marked biological effects such as predisposition to tumourigenesis (Yan *et al.*, 2002) and may be causally involved in large offspring syndrome.

The mRNAs measured in blastocysts in the present study were probably transcribed during embryonic development and did not represent mRNAs that remained from the maternal mRNA pool. Plakophilin, desmocollin 2 and E-cadherin are predominantly expressed from the morula stage onwards (Wrenzycki *et al.*, 1999, 2001a). The mRNAs of glucose transporter 1 and poly(A) polymerase were found throughout early development up to the blastocyst stage, which is indicative of both maternal and embryonic origin, with a burst after maternal–embryonic transition of genomic activity (Wrenzycki *et al.*, 1999). The different amounts of mRNA observed in blastocysts derived from oocytes matured *in vitro* or *in vivo* in the present study are supported by findings from histological and biochemical studies. Embryos developed *in vitro* frequently form the blastocoel earlier than *in vivo*, without proper compaction, and have fewer cells than do *in vivo*-grown embryos (Van Soom *et al.*, 1997b). Desmocollin 2 and plakophilin molecules are involved in intercellular communication structures and compaction (Collins *et al.*, 1995). The lower expression of desmocollin 2 and plakophilin observed in blastocysts of the *in vitro* group might explain the impaired compaction in embryos produced *in vitro*. Differences in metabolism between bovine embryos produced *in vitro* and those developed *in vivo* have been detected, especially with regard to lactate production and glucose metabolism (Khurana and Niemann, 2000). The decreased amount of glucose transporter 1 in *in vitro* embryos found in the present study probably reflects differences in energy metabolism between blastocysts developed *in vitro* and *in vivo*.

A novel aspect of the present study was related to expression of the panel of genes in blastocysts derived from prematured oocytes (pre-LH group) and oocytes matured *in vivo* (post-LH group). The results of the present study indicate that blastocysts developed from pre-LH and post-LH oocytes are not different with respect to patterns of gene expression from those derived from oocytes that lacked a period of prematuration and *in vivo* maturation. Nevertheless, the mode of oocyte prematuration and maturation may affect the developmental competence after cleavage into morulae and blastocysts (Hytel *et al.*, 1997).

The rate of blastocyst development in the pre-LH group was higher than in the *in vitro* group, which is in accordance with earlier observations and confirms the stimulatory effects of prematuration on oocyte developmental competence. The rate of blastocyst development in the post-LH group differed from recent findings in which exceptionally high rates of blastocyst development were found (Van de Leemput *et al.*, 1999; Hendriksen *et al.*, 2000). When two cows with low rates of blastocyst development were excluded from analysis, the rate of

blastocyst development of the post-LH group increased to approximately 45% and the relative abundance of the genes did not change significantly. The high proportion of well-expanded blastocysts indicates the inherent high capacity of the blastocysts for development.

The results of the present study indicate that *in vitro* maturation – as the first step in IVP of embryos – has improved significantly (Bavister, 1995; Keskinetepe and Brackett, 1996). Blastocysts derived from oocytes that were matured differently, but cultured identically, did not show significant differences with respect to the panel of gene transcripts investigated in the present study. The differences may therefore be related to the *in vitro* conditions imposed on fertilized oocytes. This contention is supported by previous findings for sheep and cattle, in which zygotes were subjected to different culture conditions and developed abnormal phenotypes (Young *et al.*, 1998; Sinclair *et al.*, 2000). Nevertheless, it could be useful to analyse gene expression in oocytes immediately after completing different maturation protocols. However, this would require a different panel of gene transcripts to be informative, including genes involved in cell cycle regulation, meiotic competence and cumulus expansion. Little is known about transcriptional activity in bovine oocytes. Duration of oocyte maturation and quality of COCs affected transcription of cyclooxygenase-2 and various prostaglandin E receptors in *in vitro* culture (Calder *et al.*, 2001). Furthermore, it would be interesting to determine rates of transcription in the embryos in which development was arrested at an early stage and thus did not reach the blastocyst stage.

In conclusion, the difference in the relative abundance of the six gene transcripts found between blastocysts produced *in vitro* and *in vivo* can probably be attributed to embryo culture conditions after maturation and fertilization. Prematuration and maturation are thought to be critically involved in the ability of the oocyte to support early development (Sirard and Blondin, 1996; Hyttel *et al.*, 1997; Rizos *et al.*, 2002). Whether blastocysts derived from oocytes matured *in vivo* or *in vitro* differ with regard to quality has yet to be determined. It should be noted that this study was limited to a panel of six gene transcripts and it is possible that other gene transcripts would behave differently. cDNA array technology enables simultaneous determination of a potentially unlimited number of gene transcripts and it has already been applied to the diagnosis of various forms of human cancer, human autoimmune diseases and the characterization of murine embryonic stem cells (Alizadeh *et al.*, 2000; Kelly and Rizzino, 2000; Rogge *et al.*, 2000). A prototype of a suitable cDNA array for single bovine embryos has recently become available (Brambrink *et al.*, 2002) and its broader application will also improve determination of patterns of gene expression in embryos derived from IVP or cloning.

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