

Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived *in vitro* or *in vivo*

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In this study we have examined the presence of mRNA encoding connexin 43 (Cx43) in bovine embryos derived *in vivo* and *in vitro*. Cumulus–oocyte complexes, immature and matured oocytes liberated from cumulus cells, zygotes, 2–4-cell and 8–16-cell embryos, morulae, blastocysts and hatched blastocysts were produced *in vitro* from ovaries obtained from an abattoir using TCM 199 supplemented with hormones and 10% oestrous cow serum for maturation. Cumulus–oocyte complexes matured for 24 h were exposed to bull spermatozoa for 19 h and then cultured in TCM 199 supplemented with 10% oestrous cow serum to the desired developmental stage. Morulae and blastocysts derived *in vivo* were collected from superovulated donor cows. Total RNA was extracted from pools of 60–200 bovine oocytes or embryos using a modified phenol–chloroform extraction method and analysed by reverse transcriptase polymerase chain reaction. Before reverse transcription, aliquots of DNase-digested embryonic RNA were tested by polymerase chain reaction using bovine-specific primers to control for residual genomic DNA contamination. DNA-free, total RNA was reverse transcribed after preincubation with the Cx43 specific 3' primer. The resultant cDNA was amplified by polymerase chain reaction using Cx43 specific primers that define a 516 bp fragment of Cx43. The reverse transcriptase polymerase chain reaction product was verified by restriction enzyme analysis with Alu I and sequencing. Assays were repeated at least twice for each developmental stage and provided identical results between replicates. Cx43 transcripts were detected in bovine morulae and blastocysts grown *in vivo*. In contrast, whereas the early *in vitro* stages from cumulus–oocyte complexes to morulae expressed Cx43, blastocysts and hatched blastocysts did not have detectable concentrations of mRNA from this gene. Restriction enzyme cutting revealed three fragments of the predicted size (139, 177, 200 bp). The amplified product showed 100% identity with the published bovine genomic DNA sequence. Under our *in vitro* conditions the Cx43 gene either had never been activated, which would require that the maternal transcript was stable through early development, or embryonic gene expression that had been active was then terminated prematurely. The differences in transcription between bovine embryos derived *in vivo* or *in vitro* indicate that culture conditions affect gene expression. This affords a tool for the further optimization of *in vitro* production systems for bovine embryos and contributes towards physiological characterization by definition of transcription phenotype of bovine embryos produced *in vitro*.

Introduction

Gap junctions are aggregations of intercellular membrane channels that regulate the exchange of small metabolites and ions (up to 1 kDa) between adjacent cells and thereby coordinate metabolic and electrical activities (Loewenstein, 1981). Gap junctional communication is an important control mechanism of processes involved in growth, cellular differentiation, and embryonic development.

The hemichannels in a single cell are termed connexons and each connexon is composed of six connexin molecules. The

connexins comprise a multigene family (Dermitzel *et al.*, 1989; Bennett *et al.*, 1991; Willecke *et al.*, 1991; Beyer, 1993). In rodents, 12 different connexins have been identified (Willecke *et al.*, 1991; Haeflinger *et al.*, 1992). The theoretical molecular mass (kDa) deduced from the cDNA sequence is commonly used to distinguish the different members of this gene family (Beyer *et al.*, 1987).

Connexin43 (Cx43) and Connexin32 (Cx32) are the two most abundant connexins and are expressed in numerous tissues including ovaries, placenta and decidua in several species (Risek *et al.*, 1990; Wiesen and Midgley, 1993, 1994; Mayerhofer and Garfield, 1995; Pauken and Lo, 1995). In rodents, Cx43 and Cx32 expression changes during oocyte

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growth and maturation and preimplantation embryo development (Barron *et al.*, 1989; Nishi *et al.*, 1991; Valdimarsson *et al.*, 1991, 1993; De Sousa *et al.*, 1993). Cx43 is a single copy gene that consists of two exons and one intron (Sullivan *et al.*, 1993).

During murine preimplantation development functional gap junctions are first observed at compaction at the eight-cell stage (Lo and Gilula, 1979; McLachlin *et al.*, 1983; Goodall and Johnson, 1984) and are necessary for the maintenance of compaction, and thus for subsequent blastocyst formation (Lee *et al.*, 1987; Bevilacqua *et al.*, 1989). Injection of antibodies (Lee *et al.*, 1987) or antisense RNA (Bevilacqua *et al.*, 1989) into blastomeres of compacted murine morulae leads to uncoupling and decapsulation of the injected blastomeres. The onset of gap junctional coupling does not depend on microtubules or microfilaments and is unrelated to cell flattening and cytokinesis (Kidder *et al.*, 1987). The rate limiting step of coupling is regulated by the insertion of nascent subunits into plasma membranes downstream of transcription and translation (De Sousa *et al.*, 1993). Their assembly into functional gap junction channels follows quickly and presumably spontaneously (Kidder, 1992). In mouse embryos, Cx43 mRNA and protein are detected from the four-cell stage onwards, whereas it is not possible to detect Cx32 mRNA at any preimplantation stage (Nishi *et al.*, 1991; Valdimarsson *et al.*, 1991; De Sousa *et al.*, 1993). Interestingly, Cx32 protein is detectable in the cytoplasm of preimplantation mouse embryos up to the morula stage as a persistent oogenetic maternally derived product (Barron *et al.*, 1989).

Data on intercellular communication via gap junctions in preimplantation bovine eggs or embryos are limited. By using a dye transfer test, immunofluorescence and ultrastructural immunocytochemistry, it has been shown that during culture *in vitro*, the cumulus cells of bovine cumulus-oocyte complexes (COC) are connected to each other via Cx43 gap junctions throughout the entire culture period. However, the cumulus-to-oocyte gap junctions consisting of Cx43 disappear within 6–9 h of culture, whereas Cx32 is detectable in the cytoplasm of oocytes cultured for 6 h and increases progressively up to 24 h of culture (Sutovsky *et al.*, 1993). In 2–16-cell bovine embryos, gap junctions are not visible in electron micrographs (Brackett *et al.*, 1980). These structures have been observed in day 7 blastocysts and are seen in increased numbers in hatched blastocysts (Lineares and Ploen, 1981; Mohr and Trounson, 1982). Using a dye transfer method, Prather and First (1993) demonstrated cell-to-cell coupling in bovine morulae and expanded blastocysts grown *in vivo*, whereas they were not able to show this phenomenon in expanded blastocysts produced *in vitro*, indicating differences in an important morphological feature between *in vivo* and *in vitro* derived bovine embryos.

The development of the reverse transcriptase polymerase chain reaction (RT-PCR) has made it possible to study the transcription of known genes in preimplantation mammalian embryos (Rappolee *et al.*, 1988, 1989). The overwhelming proportion of the information on embryonic gene expression has been obtained from studies in mice. With the improvement of *in vitro* maturation/*in vitro* fertilization/*in vitro* culture (IVM/IVF/IVC) in cattle and sheep (for review see Trounson *et al.*, 1994), gene expression experiments with preimplantation bovine and ovine embryos have now become feasible. It has

been shown that bovine and ovine embryos produced *in vitro* express an interesting set of growth factor ligand and receptor genes (Watson *et al.*, 1992, 1994).

The purpose of our study was to investigate the temporal pattern of transcription of the gap junction gene Cx43 in bovine embryos produced *in vivo* and *in vitro*, using RT-PCR technology. The comparative approach should add to a physiological characterization of the 'normality' of *in vitro* derived bovine embryos and should reveal any differences in the regulation pattern or timing of transcription between these and control embryos developed in their natural maternal environment.

Materials and Methods

Production of bovine embryos in vitro

Collection of cumulus-oocyte complexes. Bovine ovaries obtained from a local abattoir were transported in PBS (Sigma Chemical Co., St Louis, MO) at 25–30°C to the laboratory, where they were washed twice in fresh PBS. Cumulus-oocyte complexes were isolated via slicing (Eckert and Niemann, 1995). Briefly, ovaries were cut with razor blades in PBS containing 2% heat-inactivated newborn calf serum (NBCS, Boehringer, Mannheim) and 2 iu heparin (Serva, Heidelberg). The resulting suspension was passed through a filter to isolate the COC. The COC were evaluated by the criteria of Pavlok *et al.* (1992). Category I (with a homogeneous evenly granulated cytoplasm possessing at least three layers of compact cumulus cells) and category II COC (with fewer than three layers of cumulus cells or partially denuded but also with a homogeneous evenly granulated cytoplasm) were pooled in TCM-air (TCM 199 containing L-glutamine and 25 mmol Hepes l⁻¹ (Sigma) supplemented with 22 µg pyruvate ml⁻¹, 350 µg NaHCO₃ ml⁻¹, 50 µg gentamicin ml⁻¹ and 1 mg BSA ml⁻¹ (fraction V, Sigma)). All other morphological types of COC were discarded.

Maturation in vitro. TCM 199 containing L-glutamine and 25 mmol Hepes l⁻¹ served as the basis for the culture media. One millilitre was supplemented with 22 µg pyruvate, 2.2 µg NaHCO₃ and 50 µg gentamicin. For oocyte maturation this medium was supplemented with 10% heat-inactivated (30 min at 56°C) oestrous cow serum (OCS) (collected on the first day of standing oestrus), 1 µg oestradiol ml⁻¹ (Serva), 0.5 µg FSH ml⁻¹ (Folltropin[®]; Vetrepharm, London, Ontario) and 0.06 iu hCG ml⁻¹ (Ekluton[®]; Vemie, Kempen).

Fresh COC were washed twice in TCM 199 supplemented with 10% OCS and then divided into groups of 20–25, transferred to 100 µl maturation drops under silicone oil and cultivated in a humidified atmosphere composed of 5% CO₂ in air at 39°C for 24 h.

To evaluate the success of the *in vitro* maturation period, a representative sample of oocytes was fixed in ethanol-acetic acid (3:1), stained with 2% (w/v) acetic orcein after removing the cumulus cells (by incubation for 5 min in trypsin/EDTA in TCM-air followed by pipetting) and examined under a phase-contrast microscope at × 400 magnification for the presence of metaphase II and the first polar body.

Fertilization in vitro. After *in vitro* maturation, COC were rinsed in fertilization medium (Fert-TALP supplemented with 6 mg BSA ml⁻¹) and fertilized in Fert-TALP containing 10 µmol hypotaurine l⁻¹ (Sigma), 1 µmol epinephrine l⁻¹ (Sigma), 0.1 iu heparin ml⁻¹ (Serva) and 6 mg BSA ml⁻¹. Frozen semen from one bull with proven fertility in *in vitro* fertilization (IVF) was used. For IVF, semen was prepared by a modified 'swim-up' procedure (Parrish *et al.*, 1986, 1988). Briefly, semen was thawed in a waterbath at 37°C for 1 min. After swim-up separation for 1 h in Sperm-TALP containing 6 mg BSA ml⁻¹, the semen was washed twice with Sperm-TALP by centrifugation at 350 g and 36°C for 10 min before being resuspended in Fert-TALP supplemented with hypotaurine, epinephrine, heparin, and BSA. The final sperm concentration in the fertilization drop was 1 × 10⁶ spermatozoa ml⁻¹.

Fertilization occurred during a 19 h coincubation under the same temperature and gas conditions as described for maturation. To evaluate the fertilization success, a representative group of fertilized oocytes was fixed and stained after 9 h coculture as described above. Oocytes containing one sperm tail and a female and male pronucleus were considered as being normally fertilized.

Culture in vitro. For *in vitro* culture, zygotes were transferred into 200 µl drops TCM 199 + 10% OCS. Culture was maintained for a maximum of 10 days under the same conditions as described above.

A total of 6708 COC from 787 ovaries was used to produce a complete developmental series from COC to hatched blastocysts. Immature and *in vitro* matured oocytes were treated with trypsin/EDTA to remove the cumulus cells. To ensure that the transcripts did not originate from residual cumulus cells, all ova and embryos produced *in vitro* were washed extensively and the absence of cumulus cells was verified at × 200 magnification.

Production of bovine embryos in vivo

Holstein Friesian donor cows were superovulated with a single intramuscular injection between day 9 and day 13 of the oestrous cycle of 3000 iu PMSG (Brumegon[®], Hydrochemie, München) followed 48 h later by cloprostenol (Estrumate[®], Pitman-Moore, Burgwedel). Forty eight hours later the donors were artificially inseminated twice at an interval of 12 h when superovulatory oestrus was detected. On day 7 after artificial insemination, morulae and blastocysts were recovered by nonsurgical flushing of the uterine horns with 300 ml PBS plus 1% NBCS.

Morphologically intact ova and embryos were used for the experiments, washed three times in PBS containing 0.4% PVA (polyvinyl alcohol, Sigma) and stored in pools of 200 (COC, immature oocytes, matured oocytes, and zygotes), 150 (2–4-cell embryos), 120 (8–16-cell embryos), 80 (morulae), 70 (blastocysts), or 60 (hatched blastocysts) at –80°C in a minimum volume (5 µl or less) of medium until use.

Isolation of RNA

Total RNA was extracted from ova and embryo pools using a modified phenol–chloroform extraction method (Braude and

Pelham, 1979; Arcellana-Panlilio and Schultz, 1993). Briefly, in a single tube the following mixture was prepared: 100 µl RNA extraction buffer (0.2 mol NaCl l⁻¹; 25 mmol Tris l⁻¹, pH 7.4; 1 mmol EDTA l⁻¹); 100 µl phenol (neutralized, Roti[®]-Phenol, Roth, Karlsruhe); and 100 µl chloroform–isoamylalcohol solution (24:1). Twenty micrograms of *Escherichia coli* ribosomal RNA (rRNA, Boehringer) was added to the ova and embryos to facilitate precipitation in subsequent steps.

This mixture was added to ova and embryos and vortexed twice vigorously for 10 s each time. The phases were separated by centrifugation at 13 000 g for 10 min. The supernatant aqueous phase was transferred to a new tube and re-extracted with 200 µl chloroform–isoamylalcohol solution. After centrifugation, the aqueous phase was transferred to a fresh tube, 2.5 volumes of cold ethanol were added, and the nucleic acids were allowed to precipitate at –20°C overnight. The next morning, RNA was recovered by centrifugation at 28 000 g for 30 min at 4°C. The RNA pellets were washed once with 70% ethanol, dried at 37°C, and redissolved in 6 µl sterile water. DNA was degraded by incubating the samples with 5 iu DNase (Boehringer) for 30 min at 37°C. To inactivate the DNase, samples were heated to 80°C for 10 min.

As a positive control, total RNA was also extracted from bovine heart by a modification of the method of Chomczynski and Sacchi (1987). The tissue was homogenized in a solution of 4 mol guanidine thiocyanate l⁻¹, 0.5% (w/v) sarkosyl, 100 mmol 2-mercaptoethanol l⁻¹ and 25 mmol sodium citrate l⁻¹, pH 7.0. Subsequently, 85 µl 2 mol sodium acetate l⁻¹, pH 4.0, 850 µl of phenol (acid, Aqua[®]-Phenol, Roth), and 170 µl chloroform–isoamylalcohol mixture (49:1) were added to an 850 µl aliquot of the homogenate, with thorough mixing after addition of each component. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10 000 g for 20 min at 4°C. After centrifugation, the aqueous phase was transferred to a fresh tube, mixed with 1 ml isopropanol, and then held at –80°C for 60 min to precipitate RNA. The sample was centrifuged again and the resulting RNA pellet was washed twice with 70% ethanol. After drying at 37°C, the pellet was dissolved in 100–200 µl sterile water and divided into 30 µl portions. DNA was digested as described above, except that 10 iu DNase was used. RNA concentration and purity were determined by spectrophotometry. The integrity of the RNA was analysed by ethidium bromide staining after electrophoresis on a 1% (w/v) agarose (Gibco, BRL, Eggenstein) gel.

Reverse transcription

To check for residual DNA, an aliquot (1 µl) of each DNase-digested embryonic RNA sample was submitted to a PCR reaction as described below with the following exceptions: 1.0 mmol MgCl₂ l⁻¹, 100 µmol l⁻¹ of each dNTP, 1.0 µmol l⁻¹ of each primer. Bovine specific primers (5' primer: 5'-AGGTCGCGAGATTGGTCGCTAGGTCATGCA-3', 3' primer: 5'-AAGACCTCGAGAGACCTCTTCAACACGT-3'; Ellis and Harpold, 1986; K. R. Bondioli, personal communication) produce a fragment of 300 bp when DNA is present. These primers are used routinely as controls in PCR based embryonic sex determination (Bondioli *et al.*, 1989).

The remaining 5 µl of total RNA from one pool of ova and embryos or 1 µg total RNA from bovine heart free of DNA contamination was reverse transcribed into cDNA in a total volume of 10 µl. The reaction mixture consisted of: 1 × RT-buffer (50 mmol KCl l⁻¹, 10 mmol Tris-HCl l⁻¹, pH 8.0, 0.1 mg gelatine ml⁻¹), 3 mmol MgCl₂ l⁻¹, 1 mmol l⁻¹ of each dNTP (Amersham, Brunswick), 10 iu RNase inhibitor (Perkin-Elmer, Vaterstetten, Germany) and 25 iu MuLV reverse transcriptase (Perkin-Elmer). Before the addition of the other reaction components, the RNA template was mixed with 1 mmol l⁻¹ of the downstream primer (3'primer), heated to 60°C for 5 min and then cooled on ice. Prior to heating, the mixture was overlaid with mineral oil to prevent evaporation. The RT-reaction was carried out at 42°C for 1 h, followed by a denaturation step at 99°C for 5 min and flash cooling on ice.

Polymerase chain reaction (PCR)

PCR was performed in a final volume of 20 µl (10 µl of the RT-reaction plus 10 µl PCR reaction mixture) using a Biometra thermocycler, Trio-Thermoblock. The PCR reaction mixture was: 1 × PCR-buffer (10 mmol Tris-HCl l⁻¹, pH 8.0, 50 mmol KCl l⁻¹, 0.1 mg gelatine ml⁻¹), 1.5 mmol MgCl₂ l⁻¹, 200 µmol l⁻¹ of each dNTP, and 0.5 µmol l⁻¹ of the upstream primer (5'primer). The mixture was overlaid with mineral oil to prevent evaporation during PCR.

The PCR programme used a holding step at 94.5°C for 5 min and 72°C for 3 min (hot start), then 35 cycles of 1 min at 94.5°C for DNA denaturation, 1 min at 60°C for annealing of primers, and 1 min at 72°C for primer extension, followed by cooling to 4°C. During hot start, 2 iu Taq DNA polymerase (Amersham, Braunschweig) were added. The 5' and 3'primers for Cx43 were obtained from MWG-Biotech, Ebersberg and the sequences were as follows: 5'primer (nucleotides 736–765): 5'-GGGAAAGAGCCATCCTTACCACACTACCAC-3'; 3'primer (nucleotides 1251–1222): 5'-CCACCTCCAATGAAACAAAATGAACACCTA-3' (Lash *et al.*, 1990). The primers define a fragment of 516 bp and were targeted to the sequence coding for the nonconserved carboxy terminus of the protein thereby ensuring that no crossreaction with Cx32 or other members of the multigene family could occur.

The tubes were then centrifuged to collect any condensation and after addition of 2 µl of 10 × loading buffer (0.25% (w/v) xylene cyanol and 25 mmol EDTA l⁻¹ in 50% (w/v) glycerol), the RT-PCR products were subjected to electrophoresis on a 2% (w/v) agarose (Gibco) gel containing 0.5 µg ethidium bromide ml⁻¹ along with a molecular weight marker (pBR322DNA-MspI Digest, New England Biolabs GmbH, Schwalbach) and photographed on a UV-transilluminator. All assays were repeated at least twice with different embryo pools and gave consistent results.

The authenticity of the RT-PCR products was initially verified by restriction enzyme analysis using Alu I (Pharmacia, Freiburg). Briefly, the samples were heated to 95°C for 10 min to inactivate the DNA polymerase. They were then digested with 1.5 iu Alu I (Pharmacia, Ebersberg) for 3 h at 37°C. The predicted fragments (139, 177, 200 bp) were distinguished by electrophoresis as described above on a 4% (w/v) agarose

Table 1. Developmental rates (means ± SD) of bovine embryos produced *in vitro*

Developmental rates	Percentage of embryos (relative to number of oocytes)	Percentage of embryos (relative to number of cleaved oocytes)
Cleavage (day 3)	50.0 ± 7.7 (2498/4987)	—
8–16-cell embryos (day 3–4)	33.3 ± 8.5 (436/1311)	74.3 ± 15.3 (436/587)
Morulae (day 6–7)	22.4 ± 11.4 (319/1424)	47.6 ± 19.5 (319/670)
Blastocysts (day 8)	15.6 ± 8.1 (233/1495)	28.7 ± 15.6 (233/813)

(Metaphor, FCM BioProducts, Rockland, ME) gel. To further ensure the identity of the RT-PCR fragment it was subsequently sequenced using Li-Cor 4000L DNA sequencer (Li-Cor, Inc., Lincoln, NE).

As a negative control, one sample was always prepared in which RNA was omitted from the RT reaction. In addition, the embryo culture and washing media and the carrier rRNA were also tested with the Cx43 primers. No product was detectable in these reactions (data not shown).

Results

Production of bovine embryos *in vitro*

On average 8.5 ± 2.2 COC (mean ± SD) were obtained per ovary. Maturation rate was 66.7 ± 9.8% and fertilization rate 71.6 ± 6.1%, based on the number of fixed oocytes. The rates of further development are summarized in Table 1.

Transcription of the Cx43 gene

All RNA preparations were screened for the presence of genomic DNA contamination using bovine specific primers (Ellis and Harpold, 1986) before the RT-PCR. mRNA encoding Cx43 was detected in bovine COC, immature, *in vitro* matured oocytes liberated from cumulus cells, *in vitro* produced zygotes, 2–4-cell embryos, 8–16-cell embryos and morulae (Fig. 1), but was not detectable in blastocysts and hatched blastocysts generated *in vitro* (Fig. 1). In contrast, bovine morulae and blastocysts produced *in vivo* continued to transcribe Cx43 (Fig. 1). Digestion of the RT-PCR product with Alu I endonuclease showed the three predicted fragments of correct size (Fig. 2). The identity of the amplified product was also confirmed by sequencing. The sequence of the fragment was compared with that of the published bovine genomic DNA sequence and showed 100% identity.

Discussion

In the present study, the temporal expression of the Cx43 gene, which plays an essential role in the maintenance of

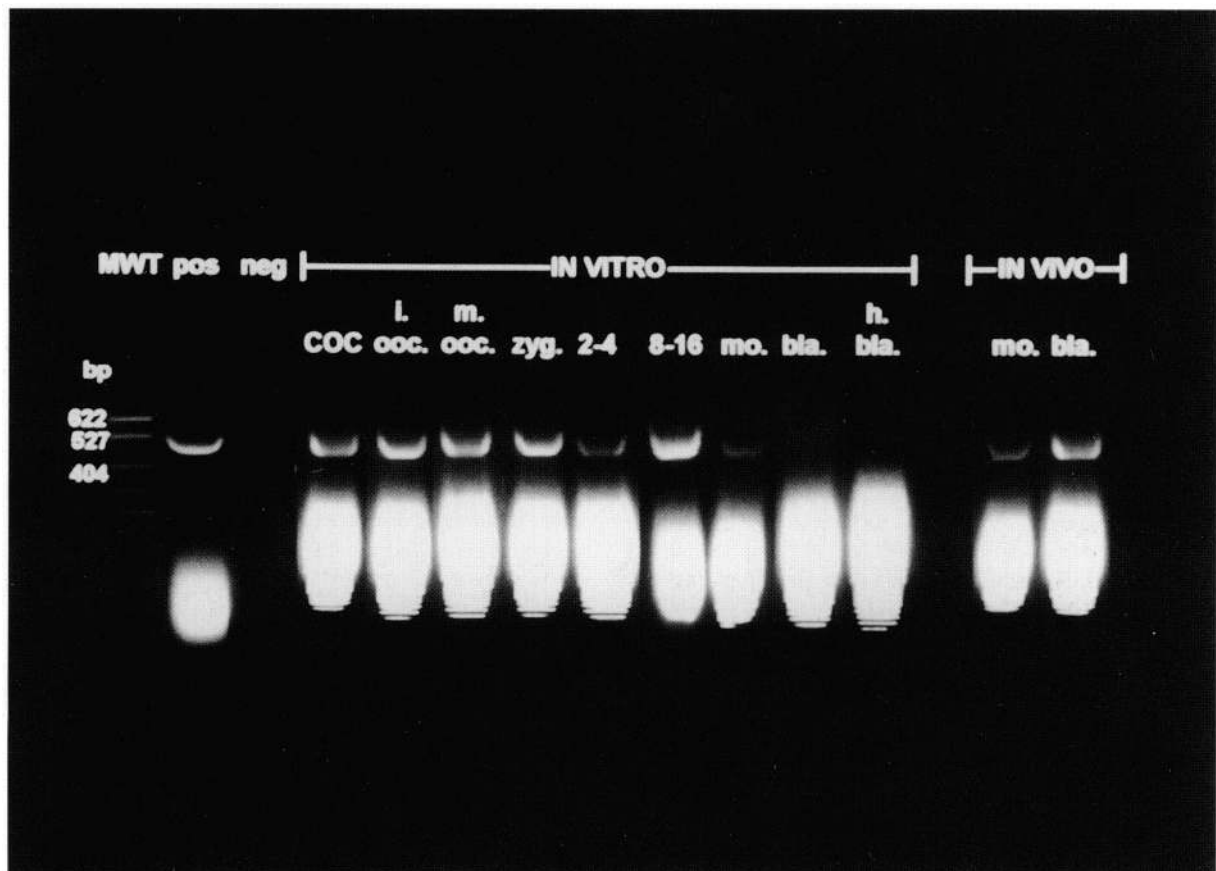


Fig. 1. mRNA encoding connexin43 (Cx43) in bovine oocytes and embryos. Total RNA from approximately 200 cumulus–oocyte complexes (COC), immature oocytes (i.ooc.), oocytes (matured *in vitro*) (m.ooc.) and zygotes (zyg.), 150 2–4-cell embryos, 120 8–16-cell embryos, 80 morulae (mo.), 70 blastocysts (bla.) and 60 hatched blastocysts (h.bla.) produced *in vitro* was analysed by the reverse transcriptase polymerase chain reaction (RT-PCR). To detect mRNA encoding Cx43 in morulae (mo.) and blastocysts (bla.) produced *in vivo*, the same number of embryos was used at each stage. A sample of bovine heart RNA was included as a positive control (pos). An aliquot of the RT-PCR reaction components without RNA was also included (neg). To verify the size of the amplified fragments a molecular weight marker (MWT, pBR322DNA-Msp I Digest) was included in each gel. This figure is a montage of individual gels run for each group of oocytes or embryos. Gels at each stage were repeated at least twice.

compaction in murine embryos grown *in vivo* (Lee *et al.*, 1987; Bevilacqua *et al.*, 1989), was analysed in bovine ova and embryos by RT-PCR. Ova and embryos produced *in vitro* and ranging in developmental stage from the oocyte to the hatched blastocyst were examined and compared with bovine morulae and blastocysts that had developed *in vivo*. The rates of development to the morula/blastocyst stage obtained in this study compare favourably with data from other laboratories (for review see Trounson *et al.*, 1994), and upon transfer of embryos produced *in vitro* under our laboratory conditions, pregnancy rates of approximately 50% were achieved (Pavlok *et al.*, 1992).

When using high sensitivity RT-PCR for the detection of rare transcripts in preimplantation ova and embryos, it is necessary to have similarly sensitive controls to ensure that the RNA is not contaminated with genomic DNA (Dirnhofer *et al.*, 1995). This is of critical importance when the PCR products do not include introns, as in the present case (Fishman *et al.*, 1990; Sullivan *et al.*, 1993), because the amplified fragment obtained from genomic DNA will have the same size as that from cDNA. The sensitivity of our RT-PCR procedure, the amount

of RNA extracted from 60 to 70 blastocysts per analysis as well as the consistency of the negative results over 4–5 replicates performed in these particular developmental stages should provide a solid scientific basis for our finding of the absence of expression of mRNA encoding Cx43 in bovine blastocysts produced *in vitro*. In addition, using exactly the same protocol, we were able to identify Cx43 expression in the bovine blastocysts derived *in vivo*. However, our data only provide qualitative evidence as to the presence or absence of Cx43 mRNA. Thus, further studies will aim at a characterization of the quantitative Cx43 expression patterns in bovine preimplantation embryos.

Our results indicate that Cx43 transcripts in bovine embryos produced *in vitro* could be of maternal and embryonic origin. In cattle, the maternal–zygotic transition normally occurs at the 8–16-cell stage in embryos of *in vivo* origin (Camous *et al.*, 1986; Telford *et al.*, 1990), whereas in embryos generated *in vitro* embryonic transcription is thought to begin earlier, at the 2–4-cell stage and is not dependent upon cell cycle number (Barnes and First, 1991; Iwasaki *et al.*, 1995). The Cx43 gene may not be activated when early embryonic development is

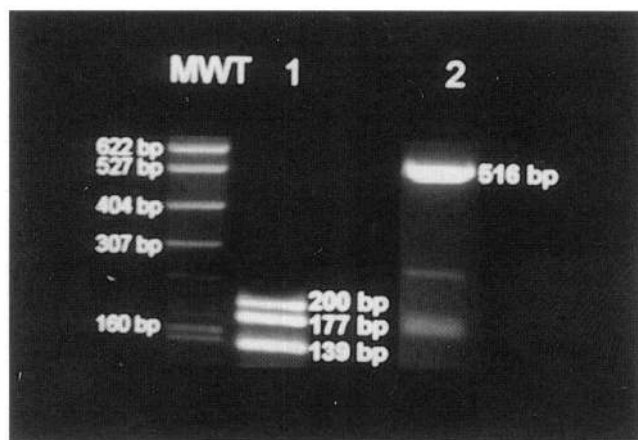


Fig. 2. Verification of the reverse transcriptase polymerase chain reaction (RT-PCR) product for connexin43 (Cx43) (516 bp) by digestion with Alu I using total RNA from bovine heart as starting material, showing the expected fragment sizes of 200, 177 and 139 bp. To estimate the size of the digested fragment (lane 1) and the amplified fragment (lane 2) a molecular weight marker (MWT, pBR322DNA-Msp I Digest) was also run.

performed *in vitro*; alternatively expression may be terminated prematurely by a breakdown of the regulatory machinery. In this regard, bovine embryos produced *in vitro* are abnormal. Speculatively, the abnormal compaction frequently observed in bovine embryos produced *in vitro* that leads to the absence or strong reduction of the perivitelline space in the morula (Greve *et al.*, 1994; Avery and Greve, 1995) could be the result of the lack of or insufficient expression of gap junction genes. Nevertheless, the gap junctions required for blastocyst formation could be constructed from precursor pools of connexin protein, which have been found in murine embryos from the four-cell stage onwards (McLachlin *et al.*, 1983; Valdimarsson *et al.*, 1991).

In contrast to blastocysts generated *in vitro*, bovine blastocysts produced *in vivo* contain mRNA encoding Cx43. This is also observed in murine morulae and blastocysts developed *in vivo* (Nishi *et al.*, 1991; C. Wrenzycki, D. Herrmann, J. W. Carnwath and H. Niemann, unpublished observations). In mice, Cx43 is transcribed by the embryonic genome from shortly after genome activation (Nishi *et al.*, 1991; Valdimarsson *et al.*, 1991; De Sousa *et al.*, 1993) which occurs at the two-cell stage (Howlett and Bolton, 1985). However, it is becoming clear that transcription of most genes in murine preimplantation development is regulated more by a cellular clock than by a specific morphogenetic transition (Kidder, 1992).

Using a dye transfer assay, bovine morulae and blastocysts derived *in vivo* and *in vitro* have been examined for the presence of functional gap junctions (Prather and First, 1993). Embryos derived *in vivo* show dye transfer, indicating the existence of open gap junctions, while in those produced *in vitro* the cell-to-cell channels are either not present or closed, as there is no dye transfer (Prather and First, 1993). Closed channels can result from *in vitro* culture conditions since the functional state of gap junctions is affected by many factors, including the concentration of Ca^{2+} , H^{+} , and organic acids, or the presence of free radicals in the culture medium (Turin and Warner, 1977; Loewenstein, 1981; Saez *et al.*, 1987; Giaume

et al., 1989). Another possible explanation could be the absence of gap junctions due to non-activation of the Cx43 gene. In the uterine smooth muscle cells of rats, the number and size of gap junction plaques is modulated by steroid and prostaglandin concentrations (MacKenzie and Garfield, 1985). During pregnancy in the rat, myometrial Cx43 transcript concentrations are upregulated by oestrogen and downregulated by progesterone (Lye *et al.*, 1993).

The difference in transcription of the Cx43 gene between bovine embryos developing *in vivo* and those produced *in vitro* supports the hypothesis that culture conditions alter the pattern of expression of genes during early embryogenesis. It validates the approach taken in this study, in which transcriptional activities of embryos produced *in vitro* are monitored and compared with embryos produced *in vivo* with the aim of creating culture conditions that promote the growth of embryos with normal gene expression patterns. Furthermore, these differences demonstrate that findings reported for bovine embryos generated *in vitro* (Watson *et al.*, 1992, 1994) are not necessarily valid for embryos developing *in vivo*.

Information about the effects of culture media on gene expression during preimplantation mouse development *in vitro* has been obtained using SOM (simplex-optimized medium) containing low concentrations of sodium chloride. Murine embryos grown in this medium show higher protein and poly(A)⁺-RNA synthesis (Anbari and Schultz, 1993; Ho *et al.*, 1994), mRNA stability, and increased abundance of specific transcripts (Ho *et al.*, 1994). Using a modified SOM (KSOM) supplemented with specific amino acids, Ho *et al.* (1995) found that the amount of expression of genes analysed is almost identical to that of murine embryos developing *in vivo*. Differences in the expression of genes are also observed between *in vitro* and *in vivo* murine embryos. Expression of the Glut-1 (Glucose transporter) gene and glucose uptake itself are reduced in murine blastocysts produced *in vitro* as compared with their *in vivo* counterparts (Morita *et al.*, 1994). Similarly, investigations to elucidate the activity of hexokinase in mouse blastocysts generated *in vivo* and *in vitro* demonstrated that enzyme activity is significantly reduced in embryos produced *in vitro* (Ayabe *et al.*, 1994).

Prolonged *in vitro* culture of early sheep embryos results in several abnormalities such as cytoplasmic fragmentation, premature blastocoel formation and a reduced number of nuclei per blastocyst (Walker *et al.*, 1992). Furthermore, upon transfer of ovine and bovine embryos produced *in vitro*, duration of gestation, lamb/calf birth mass and neonatal mortality are significantly increased over *in vivo* controls (Walker *et al.*, 1992; Holm *et al.*, 1994; Farin and Farin, 1995; Behboodi *et al.*, 1995). These abnormalities indicate destabilization of the complex and well orchestrated programme of gene expression caused by extended *in vitro* culture. The implications of this suggestion for current practice in embryo manipulation both in human medicine and in domestic animals demand further investigations.

In conclusion, we have demonstrated for the first time a difference in the expression of a developmentally important gene caused by extended *in vitro* culture of bovine embryos. The results contribute to the physiological characterization of bovine embryos produced *in vitro*. The RT-PCR analysis will facilitate the improvement of *in vitro* culture systems for

bovine embryos and will possibly elucidate the origin of the abnormalities observed in offspring obtained from embryos produced *in vitro*. Further studies are underway to investigate the expression of a diagnostic set of genes to obtain a more general insight into the transcriptional activities of preimplantation bovine embryos in different *in vitro* culture systems and using spermatozoa from different bulls for IVF. Further refinement in *in situ* hybridization, differential display, and RT-PCR technology itself with the possibility to detect multiple transcripts in single blastomeres (Zimmermann and Schultz, 1994; Collins *et al.*, 1995) promises detailed information on the temporal and spatial expression patterns of a number of genes in individual embryos in the future.

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References

- Anbari K and Schultz RM (1993) Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos *Molecular Reproduction and Development* **35** 24–28
- Arcellana-Panlilio MY and Schultz GA (1993) Analysis of messenger RNA in early mouse embryos. In *Guide to Techniques in Mouse Development – Methods in Enzymology* **225** pp 303–328 Eds PM Wassarman and ML DePamphilis. Academic Press, New York
- Avery B and Greve T (1995) Apparent (ab)normalities of *in vitro* produced bovine embryos. In *Reproduction and Animal Breeding: Advances and Strategy* pp 171–183 Eds G Enne, GF Greppi and A Lauria. Elsevier Biofutur, Paris
- Ayabe T, Tsutsumi O and Taketani Y (1994) Hexokinase activity in mouse embryos developed *in vivo* and *in vitro* *Human Reproduction* **9** 347–351
- Barnes F and First NL (1991) Embryonic transcription in *in vitro* cultured bovine embryos *Molecular Reproduction and Development* **29** 117–123
- Barron DJ, Valdimarsson G, Paul DL and Kidder GM (1989) Connexin32, a gap junction protein, is a persistent oogenic product through preimplantation development of the mouse *Developmental Genetics* **10** 318–323
- Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF and Murray JD (1995) Birth of large calves that developed from *in vitro*-derived bovine embryos *Theriogenology* **44** 227–232
- Bennett MVL, Barrio LC, Bargiello TA, Spray DC, Hertzberg E and Saez JC (1991) Gap junctions: new tools, new answers, new questions *Neuron* **6** 305–320
- Bevilacqua A, Loch-Carusio R and Erickson RP (1989) Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos *Proceedings of the National Academy of Sciences USA* **86** 5444–5448
- Beyer EC (1993) Gap junctions *International Review of Cytology* **137C** 1–37
- Beyer EC, Paul DL and Goodenough DA (1987) Connexin43, a protein from rat heart homologous to gap junction protein from liver *Journal of Cell Biology* **105** 2621–2629
- Bondioli KR, Ellis SB, Pryor JH, Williams MW and Harpold (1989) The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos *Theriogenology* **31** 95–104
- Brackett BG, Oh YK, Evans JF and Donawick WJ (1980) Fertilization and development of early cow ova *Biology of Reproduction* **23** 189–205
- Braude PR and Pelham HRB (1979) A microsystem for the extraction and *in vitro* translation of mouse embryo RNA *Journal of Reproduction and Fertility* **56** 153–158
- Camous S, Kopecky V and Flechon JE (1986) Autoradiographic detection of the earliest stage 3H-uridine incorporation in the cow embryo *Biology of the Cell* **58** 195–200
- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction *Analytical Biochemistry* **162** 156–159
- Collins ME, Stevens DA, Jeuner LJ and Brownlie J (1995) A rapid method for mRNA detection in single-cell biopsies from preimplantation bovine embryos *Theriogenology* **43** 1227–1238
- Dermitzel R, Traub O, Hwang TK, Beyer E, Bennett MVL, Spray DC and Willecke (1989) Differential expression of three gap junction proteins in developing and mature brain tissues *Proceedings of the National Academy of Sciences USA* **86** 10 148–10 152
- De Sousa PA, Valdimarsson G, Nicholson BJ and Kidder GM (1993) Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos *Development* **117** 1355–1367
- Dirnhofer S, Berger C, Untergasser G, Geley S and Berger P (1995) Human β -actin retrovirus genes interfere with RT-PCR *Trends in Genetics* **11** 380–381
- Eckert J and Niemann H (1995) *In vitro* maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media *Theriogenology* **43** 1211–1225
- Ellis SB and Harpold MM (1986) Nucleic acid probes for prenatal sexing *PCT WO* **86/07095**
- Farin PW and Farin CE (1995) Transfer of bovine embryos produced *in vivo* or *in vitro*: survival and fetal development *Biology of Reproduction* **52** 676–682
- Fishman GI, Spray DC and Levinwand LA (1990) Molecular characterization and functional expression of the human cardiac gap junction channel *Journal of Cell Biology* **111** 589–598
- Giaume C, Randriamampita C and Trautman A (1989) Arachidonic acid closes gap junctional channels in rat lacrimal glands *Pflügers Archiv* **413** 273–279
- Goodall H and Johnson MH (1984) The nature of intercellular coupling within the preimplantation mouse embryo *Journal of Embryology and Experimental Morphology* **79** 53–76
- Greve T, Callesen H, Hyttel P and Avery B (1994) From oocyte to calf: *in vivo* and *in vitro*. In *Animal Production and Biotechnology* pp 71–97 Eds GF Greppi and G Enne. Elsevier Biofutur, Paris
- Haeflinger JA, Bruzzone R, Jenkins NA, Gilbert DJ, Copeland NG and Paul DL (1992) Four novel members of the connexin family of gap junction protein *Journal of Biological Chemistry* **267** 2057–2064
- Ho Y, Doherty AS and 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 and 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
- Holm P, Walker SK, Petersen BA, Ashman RJ and Seamark RF (1994) *In vitro* vs *in vivo* culture of ovine IVF-IVF ova: effect on lambing *Theriogenology* **41** 217 (Abstract)
- Howlett SK and Bolton VN (1985) Sequence and regulation of morphological and molecular events during first cell cycle of mouse embryogenesis *Journal of Embryology and Experimental Morphology* **87** 175–206
- Iwasaki S, Wilmut I and Campbell KHS (1995) Time-dependent RNA synthesis in early bovine embryos derived from *in vitro* fertilization *Journal of Reproduction and Fertility Abstract Series* **15** 59–60
- Kidder GM (1992) The genetic program for preimplantation development *Developmental Genetics* **13** 319–325
- Kidder GM, Rains J and McKeon J (1987) Gap junction assembly in the preimplantation mouse conceptus is independent of microtubules, microfilaments, cell flattening, and cytokinesis *Proceedings of the National Academy of Sciences USA* **84** 3718–3722
- Lash JA, Critser ES and Pressler ML (1990) Cloning of a gap junctional protein from vascular smooth muscle and expression in two-cell mouse embryos *Journal of Biological Chemistry* **265** 13 113–13 117
- Lee S, Gilula NB and Warner AE (1987) Gap junctional communication and compaction during preimplantation stages of mouse development *Cell* **51** 851–860
- Lineares T and Ploen L (1981) On the ultrastructure of seven-day-old normal (blastocysts) and abnormal bovine embryos *Zentralblatt der Veterinärmedizin, Reihe C Anatomie, Histologie, Embryologie* **12** 212–226
- Lo CW and Gilula NB (1979) Gap junctional communication in the preimplantation mouse embryo *Cell* **18** 399–409
- Loewenstein WR (1981) Junctional intercellular communication *Physiological Reviews* **61** 29–915

- Lye SJ, Nicholson BJ, Mascarenhas M, MacKenzie L and Petrocelli T (1993) Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in plasma estrogen:progesterone ratio *Endocrinology* **132** 2380–2386
- MacKenzie LJ and Garfield RE (1985) Hormonal control of gap junctions in the myometrium *American Journal of Physiology* **248** 297–300
- McLachlin JR, Caneney S and Kidder GM (1983) Control of gap junction formation in early mouse embryos *Developmental Biology* **98** 155–164
- Mayerhofer A and Garfield RE (1995) Immunocytochemical analysis of the expression of gap junction protein Connexin43 in the rat ovary *Molecular Reproduction and Development* **41** 331–338
- Mohr RL and Trounson AO (1982) Comparative ultrastructure of hatched human, mouse and bovine blastocysts *Theriogenology* **66** 499–504
- Morita Y, Tsutsumi O, Oka Y and Taketani Y (1994) Glucose transporter GLUT 1 mRNA expression in the ontogeny of glucose incorporation in mouse preimplantation embryos *Biochemistry and Biophysical Research Communications* **199** 1525–1531
- Nishi M, Kumar N and Gilula N (1991) Developmental regulation of gap junction gene expression during mouse embryonic development *Developmental Biology* **146** 117–130
- Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH and First NL (1986) Bovine *in vitro* fertilization with frozen-thawed semen *Theriogenology* **25** 591–600
- Parrish JJ, Susko-Parrish JL, Winer MA and First NL (1988) Capacitation of bovine sperm by heparin *Biology of Reproduction* **38** 1171–1180
- Pauken CM and Lo CW (1995) Nonoverlapping expression of Cx43 and Cx26 in the mouse placenta and decidua: a pattern of gap junction gene expression differing from that in rat *Molecular Reproduction and Development* **41** 195–203
- Pavlok A, Lucas-Hahn A and Niemann H (1992) Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles *Molecular Reproduction and Development* **31** 63–67
- Prather RS and First NL (1993) Cell-to-cell coupling in early-stage bovine embryos: a preliminary report *Theriogenology* **39** 561–567
- Rappolee DA, Brenner CA, Schultz R, Mark D and Werb Z (1988) Developmental expression of PDGF, TGF- α , and TGF- β genes in preimplantation mouse embryos *Science* **241** 1823–1825
- Rappolee DA, Wang A, Mark D and Werb Z (1989) Novel method for studying mRNA phenotypes in single or small numbers of cells *Journal of Cell Biochemistry* **39** 1–11
- Risek BS, Guthrie S, Kumar NM and Gilula NB (1990) Modulation of gap junction transcript and protein expression during pregnancy in rat *Journal of Cell Biology* **110** 269–282
- Saez JC, Bennett MVL and Spray DC (1987) Oxidant stress blocks hepatocyte gap junctions independently of changes in intercellular pH, Ca^{2+} or MP27 phosphorylation state *Biophysical Journal* **51** 39a
- Sullivan R, Ruangvoravat C, Joo D, Morgan J, Wang BL, Wang XK and Lo CW (1993) Structure, sequence and expression of the mouse Cx43 gene encoding connexin 43 *Gene* **130** 191–199
- Sutovsky R, Flechon JE, Flechon B, Motlik J, Peynot N, Chesné P and Heyman Y (1993) Dynamic changes of gap junctions and cytoskeleton during *in vitro* culture of cattle oocyte cumulus complexes *Biology of Reproduction* **49** 1277–1287
- Telford NA, Watson AJ and Schultz GA (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species *Molecular Reproduction and Development* **26** 90–100
- Trounson A, Pushett D, MacLellan LJ, Lewis I and Gardner DK (1994) Current status of IVF/IVF and embryo culture in humans and farm animals *Theriogenology* **41** 57–66
- Turin L and Warner AE (1977) Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo *Nature* **270** 56–57
- Valdimarsson G, De Sousa PA, Beyer EC, Paul DL and Kidder GM (1991) Zygotic expression of the connexin 43 gene supplies subunits for gap junction assembly during mouse preimplantation development *Molecular and Reproduction Development* **30** 18–26
- Valdimarsson G, De Sousa PA and Kidder GM (1993) Coexpression of gap junction proteins in the cumulus–oocyte complex *Molecular and Reproduction Development* **36** 7–15
- Walker SK, Heard TM and Seamark RF (1992) *In vitro* culture of sheep embryos without co-culture: successes and perspectives *Theriogenology* **37** 111–126
- Watson AJ, Hogan A, Hahnel A, Wiemer KE and Schultz GA (1992) Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo *Molecular and Reproduction Development* **31** 87–95
- Watson AJ, Watson PH, Arcellana-Panlilio M, Warnes D, Walker SK, Schultz GA, Armstrong DT and Seamark RF (1994) A growth factor phenotype map for ovine preimplantation development *Biology of Reproduction* **50** 725–731
- Willecke K, Hennemann H, Dahl E, Jungbluth S and Heynkes R (1991) The diversity of connexin genes encoding gap junctional proteins *European Journal of Cell Biology* **56** 1–7
- Wiesen JF and Midgley AR (1993) Changes in expression of connexin43 gap junction messenger ribonucleic acid and protein during ovarian follicular growth *Endocrinology* **133** 741–746
- Wiesen JF and Midgley AR (1994) Expression of connexin43 gap junction messenger ribonucleic acid and protein during follicular atresia *Biology of Reproduction* **50** 336–348
- Zimmermann JW and Schultz RM (1994) Analysis of gene expression in the preimplantation mouse embryo: use of mRNA differential display *Proceedings of the National Academy of Sciences USA* **91** 5456–5460