Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different *in vitro* systems

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The aim of this study was to determine the relative abundance of mRNAs for the insulin-like growth factor I (IGF-I) and IGF-II ligands, and for the IGF receptors (IGF-IR and IGF-IIR) in *in vitro* preimplantation bovine embryos from the oocyte to the hatched blastocyst stage using two different culture systems: TCM-199 supplemented with oestrous cow serum, or synthetic oviduct fluid supplemented with polyvinyl alcohol. Development to the two- to four-cell stage and blastocyst stage was significantly higher ($P \leq 0.05$) in embryos cultured in TCM supplemented with oestrous cow serum than in those cultured in synthetic oviduct fluid supplemented with polyvinyl alcohol (61 and 25% versus 55 and 17%, respectively). A semi-quantitative RT-PCR assay did not detect IGF-I transcripts at any stage of preimplantation bovine development, including the hatched blastocyst stage. In both culture systems, IGF-IR, IGF-II and IGF-IIR were expressed throughout preimplantation development up to the hatched blastocyst stage in a varying

pattern. The expression patterns of IGF-IR, IGF-II and IGF-IIR in embryos generated in the two culture systems were not significantly different, except at the expanded blastocyst stage, at which significantly higher amounts of IGF-IIR were observed in the TCM system than in the synthetic oviduct fluid system. These results indicate that transcripts of IGF-IR and IGF-IIR follow the standard pattern in which maternal stores of mRNA in the oocyte are slowly depleted up to the 16-cell stage and then re-established at the onset of embryonic expression of these genes. The lack of detectable IGF-I transcripts in the bovine embryo indicates a predominantly paracrine mode of action. The bovine embryo is capable of producing IGF-II, IGF-IIR and IGF-IR in large amounts, particularly after hatching, which may be important for the formation of the filamentous conceptus. Results indicate an autocrine mechanism for IGF-II and modulation of IGF family expression by culture conditions.

Introduction

Insulin and insulin-like growth factors (IGFs) are a group of structurally related polypeptides that regulate the growth of many types of mammalian cells. The growth-promoting activity of these polypeptides is mediated by binding to receptors on the cell membrane. Receptors with primary binding specificity for these polypeptides have been identified (Baxter, 1988). IGF transport and function are modulated by interactions with at least six insulin-like growth factor binding proteins (IGFBPs), which are present in many extracellular fluids and in early embryos (Heyner *et al.*, 1993; Kaye and Harvey 1995; Winger *et al.*, 1997; Luciano *et al.*, 2000). IGFs enhance cell proliferation, mitogenesis and steroid hormone activity (Adashi *et al.*, 1985; Kamada *et al.*, 1992).

Several lines of evidence indicate that the polypeptide

growth factors of the IGF family have an important role in early development. Growth factors have been identified in follicular fluid (Hammond *et al.*, 1988) and in the female reproductive tract (Buhi *et al.*, 1997). The addition of exogenous IGF-I or -II to the culture medium results in improved bovine oocyte maturation and embryonic development (Herrler *et al.*, 1992; Kaye *et al.*, 1992; Matsui *et al.*, 1995; Palma *et al.*, 1997; Pawshe *et al.*, 1998; Mihalik *et al.*, 2000). Expression of mRNA transcripts for the IGF family has been detected in embryos of a variety of species. Although IGF-IR, IGF-II and IGF-IIR are expressed throughout preimplantation development up to the blastocyst stage, expression of IGF-I ligand by preimplantation embryos has also been reported. For example, IGF-I expression was detected in bovine and ovine embryos produced *in vitro* (Schultz *et al.*, 1992; Watson *et al.*, 1992, 1994; Yoshida *et al.*, 1998), but was not observed in embryos from water buffalo (Daliri *et al.*, 1999), mice (Rappolee *et al.*, 1992), rats (Zhang *et al.*, 1994) and humans (Lighten *et al.*, 1997a).

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Niemann and Wrenzycki (2000) reported that transcription of several developmentally important genes in preimplantation embryos is affected by the *in vitro* environment, possibly leading to perturbation of differentiation and organogenesis, and 'large offspring syndrome'. The amounts of various transcripts were correlated with the presence or absence of the respective protein in the culture medium. Bovine embryos responded to changes in their environment very early on in development by modifying the synthesis of specific mRNAs (Wrenzycki *et al.*, 1999). mRNA expression of IGFs, their receptors and IGF-BPs during bovine preimplantation development varies qualitatively (Schultz *et al.*, 1992; Watson *et al.*, 1992; Winger *et al.*, 1997; Yoshida *et al.*, 1998; Lonergan *et al.*, 2000) and the changes in the abundance of IGF-I mRNA and IGF-IR in relation to culture medium composition have not yet been investigated.

The aim of this study was to determine the relative abundance of mRNAs for the IGF-I and IGF-II ligands and their receptors (IGF-IR and IGF-IIR) in preimplantation bovine development up to the hatched blastocyst stage using two different *in vitro* culture systems: TCM-199 with oestrous cow serum, or synthetic oviduct fluid with polyvinyl alcohol. These two contrasting culture systems (complex and undefined versus simple and defined) were used to determine effects on mRNA expression originating from the *in vitro* production process. The study also aimed to clarify whether *in vitro* produced bovine embryos express IGF-I ligand during preimplantation development.

Materials and Methods

In vitro production of embryos

Bovine oocytes and embryos were produced using standard protocols for *in vitro* maturation, fertilization and culture (Eckert and Niemann, 1995; Wrenzycki *et al.*, 1996, 1999). Briefly, bovine ovaries obtained from a local abattoir were transported in PBS (No. D5773; Sigma, St Louis, MO) at 25–30°C to the laboratory, where they were washed twice in fresh PBS. Ovaries were sliced with razor blades in PBS containing 2 iu heparin l⁻¹ (Serva, Heidelberg) and 2.0% NBCS (newborn calf serum; Boehringer Mannheim), or 0.1% (w/v) polyvinyl alcohol (No. P8136, Sigma), to release cumulus-oocyte complexes (COCs) (Eckert and Niemann, 1995). The resulting suspension was passed through a filter to isolate COCs. Category I COCs (with a homogeneous evenly granulated cytoplasm with at least three layers of compact cumulus cells) and category II COCs (with fewer than three layers of cumulus cells or partially denuded, but also with a homogeneous evenly granulated cytoplasm; Pavlok *et al.*, 1992) 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) or polyvinyl alcohol). TCM-199

containing L-glutamine and 25 mmol Hepes l⁻¹ served as the basic medium for *in vitro* maturation. TCM-199 (1 ml) was supplemented with 22.0 µg pyruvate l⁻¹, 2.2 mg NaHCO₃ l⁻¹ and 50.0 µg gentamicin l⁻¹. For oocyte maturation, the same medium was supplemented with 10 iu eCG ml (Intervet Tönisvorst), 5 iu hCG ml⁻¹ (Intervet Tönisvorst) and either 10.0% heat-inactivated (30 min at 56°C) oestrous cow serum (collected on day 1 of standing oestrus) or 0.1% polyvinyl alcohol. Fresh COCs were washed three times in TCM-199 supplemented with 10.0% oestrous cow serum or 0.1% polyvinyl alcohol, and then divided into groups of 20–25, transferred into 100 µl maturation drops under silicone oil and cultured in a humidified atmosphere of 5% CO₂ in air at 39°C for 24 h. After *in vitro* maturation, COCs were rinsed in fertilization medium (fert-Tyrodé's albumin lactate pyruvate (TALP) supplemented with 6 mg BSA ml⁻¹) and fertilized in fert-TALP containing 10.0 µmol hypotaurin l⁻¹ (Sigma), 1.0 µmol adrenaline l⁻¹ (Sigma), 0.1 iu heparin l⁻¹ (Serva) and 6.0 mg BSA ml⁻¹. Frozen semen from one bull of proven fertility in 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 by centrifugation (at 350 g at 36°C for 10 min) before being resuspended in fert-TALP supplemented with 10.0 µmol hypotaurin l⁻¹ (Sigma), 1.0 µmol adrenaline l⁻¹ (Sigma) and 0.1 iu heparin l⁻¹ (Serva), and BSA. The final concentration of spermatozoa added to each fertilization drop was 1 × 10⁶ spermatozoa ml⁻¹. Fertilization occurred during a 19 h coincubation period using the same temperature and gas conditions as described for maturation. For *in vitro* culture in TCM-199, presumptive zygotes were transferred into 200 µl drops of TCM-199 supplemented with 10% oestrous cow serum and were then cultured for up to 9 days under the same conditions as described above, to produce a complete developmental series from zygotes to hatched blastocysts. For culture in the synthetic oviduct serum system, fertilized oocytes (18–19 h after insemination) were denuded from the cumulus cells by vortexing for 3 min in TCM-air followed by gentle pipetting, and were then transferred in groups of six to eight oocytes (after washing three times in culture medium) into 30 µl drops of synthetic oviduct fluid (Tervit *et al.*, 1972) containing 0.33 mmol pyruvate l⁻¹, 50.0 µg gentamicin ml⁻¹, 10.0 µg phenol red ml⁻¹, 1 ml per 100 ml essential amino acids of basal Eagle medium (Sigma), 2 ml per 100 ml non-essential amino acids of minimum essential medium (Sigma) and 6 mg polyvinyl alcohol ml⁻¹ (Keskinetepe and Brackett 1996; Eckert *et al.*, 1998). Embryos were cultured under silicone oil in 5% CO₂, 7% O₂ and 88% N₂ (Air Product, Hattingen) in a humidified atmosphere in modular incubator chambers (ICN Biomedicals, Inc., Aurora, No. 615300, OH) at 39°C.

The cumulus cells were removed from immature and *in vitro* matured oocytes as described above. All oocytes or embryos were washed extensively and the absence of

Table 1. Primers used for PCR

Genes and sequence references (EMBL accession no.)	Primer sequences and positions	Annealing (°C)	Fragment size (base pair)
Globin Cheng <i>et al.</i> (1986) (X04751)	GCAGCCACGGTGGCGAGTAT (241–260) GTGGGACAGGAGCTTGAAAT (555–657)	60	257
IGF-I Fotsis <i>et al.</i> (1990) (X15726)	GATGGGCATTTCCCCCAATGAA-ATAAGTAA (580–609) CTGTAAAACAAACAGCCTGTGT-TGCGTAGA (884–913)	55	334
IGF-IR Sneyers <i>et al.</i> (1991) (X54980)	CATCTCCAACCTCCGGCCTTTT-ACTCT (186–213) CCCAGCCTGCTGCTATTTCTTTT-TCTAT (695–722)	59	538
IGF-II Brown <i>et al.</i> (1990) (X53553)	CTTCAGCCGACCATCCAGCCGC-ATAAAC (67–96) TCAGCGGACGGTGACTCTTGCC-CTCTCT (362–389)	64	323
IGF-IIR Lobel <i>et al.</i> (1987, 1988) (J03527 and M15869)	CGCCTACAGCGAGAAGGGGTGA-GTC (4799–4823) AGAAAAGCGTGCACGTGCGCTT-GTC (5067–5091)	62	393

IGF: insulin-like growth factors; IGF-R: insulin-like growth factor receptors.

cumulus cells was verified at $\times 200$ magnification to ensure that the transcripts did not originate from residual cumulus cells. Pools of immature and matured oocytes, zygotes, two- to four-cell embryos, 8–16-cell embryos, morulae, expanded blastocysts and hatched blastocysts were collected at the appropriate stages of development. Oocytes or embryos that had not reached the expected developmental stage at the respective time points in both treatment groups were discarded from this study. Presumptive matured oocytes or zygotes were examined under a phase-contrast microscope at $\times 400$ for the presence of the first polar body or two pronuclei. All embryos at all stages of development were examined carefully for uniform size and quality. Representative cell counts in subgroups of embryos produced in these systems revealed an average of 50–60 cells in morulae and 110–120 cells in blastocysts (Wrenzycki *et al.*, 2001). After washing three times in PBS containing 0.1% polyvinyl alcohol, embryos were stored in pools of 20–25 at -80°C in a minimum volume (5 μl or less) of medium until use.

Isolation of RNA

Poly(A)⁺ RNA was isolated using a Dynabeads mRNA DIRECT Kit (Dyna, Oslo) according to the manufacturer's instructions, with minor modifications (Wrenzycki *et al.*, 1999). Briefly, oocytes or embryos were lysed by the addition of 150 μl lysis/binding buffer (100 mmol Tris-HCl l^{-1} , pH 8.0, 500 mmol LiCl l^{-1} , 10 mmol EDTA l^{-1} , 1% LiDS (SDS) and 5 mmol DDT l^{-1}). Subsequently, 0.1 pg rabbit globin mRNA (BRL, Gaithersburg, MD) per oocyte or

embryo was added to each tube as an internal standard. After vortexing for 10 s, brief centrifugation (12 000 *g*, 15 s) and incubation at room temperature for 10 min, 10 μl prewashed Dynabeads oligo (dT)₂₅ was pipetted into the fluid. After incubation of poly(A)⁺ RNAs with oligo(dT) Dynabead at room temperature for 5 min, the beads were separated using a Dynal MPC-E-1 magnetic separator, washed once using 100 μl washing buffer 1 (10 mmol Tris-HCl l^{-1} , pH 8.0, 0.15 mol LiCl l^{-1} , 1 mmol EDTA l^{-1} , 0.1% LiDS) and then washed three times with 100 μl washing buffer 2 (10.0 mmol Tris-HCl l^{-1} , pH 8.0, 0.15 mol LiCl l^{-1} , 1.0 mmol EDTA l^{-1}). Poly(A)⁺ RNAs were then eluted from the beads by incubation in 11 μl sterile water at 65°C for 2 min, and aliquots were used immediately for reverse transcription (RT). As a positive control, total RNA was also extracted from bovine oviduct tissue (Chomczynski and Sacchi, 1987) with minor modification as described by Wrenzycki *et al.* (1998).

Reverse transcription

Poly(A)⁺ RNA isolated from different numbers of oocytes or embryos (20–25), or total RNA from oviductal tissue, was reverse transcribed into cDNA in a total volume of 20 μl using 2.5 μmol random hexamers (Perkin-Elmer, Vaterstetten) to obtain the widest range of cDNA. The reaction mixture consisted of 1 $\times$ RT buffer (50 mmol KCl l^{-1} , 10 mmol Tris-HCl l^{-1} , pH 8.3, Perkin-Elmer), 5 mmol MgCl_2 l^{-1} , 1 mmol of each dNTP l^{-1} (Amersham, Brunswick), 20 iu Rnase inhibitor (Perkin-Elmer) and 50 iu MuLV reverse

transcriptase (Perkin-Elmer). The RT reaction was carried out at 25°C for 10 min and at 42°C for 1 h followed by a denaturation step at 99°C for 5 min, and flash cooling on ice. The reaction mixture was diluted to get a final concentration of 0.5 oocyte or embryo equivalent per μL , and 50 fg globin RNA μL^{-1} .

Polymerase chain reaction

PCR was performed with cDNA equivalents corresponding to four oocytes or embryos (IGF-II: eight embryo equivalents) from different pools of oocytes or embryos generated in different *in vitro* produced runs, and 100 fg of globin RNA in a final volume of 50 μL of $1 \times$ PCR buffer (20 mmol Tris-HCl L^{-1} , pH 8.4, 50 mmol KCl L^{-1} , Gibco BRL, Eggenstein), 1.5 mmol MgCl_2 L^{-1} , 200 μmol of each dNTP L^{-1} , 0.5 μmol of each sequence-specific primer L^{-1} (except for IGF-II primer which was used at a concentration of 1.0 μmol L^{-1}) using a PTC-200 thermocycler (MJ Research, Watertown, MA). A 'hot start' was used by adding 1 iu Taq DNA polymerase (Gibco) at 72°C to ensure specific amplification. PCR primers were designed from the coding regions of each gene sequence using the OLIGO program. The sequence and positions of the primers used, the annealing temperatures, the fragment sizes and the sequence reference of the expected PCR products are summarized (Table 1).

The PCR program used an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by 35 cycles (globin: 27 cycles and IGF-II: 37 cycles) of 15 s each at 95°C for DNA denaturation, 15 s at different temperatures for annealing of the primers, and 15 s at 72°C for primer extension. The last cycle was followed by an additional 5 min at 72°C and then a cooling period to 4°C. The products from each primer pair were sequenced to confirm the identity of the RT-PCR fragments. As negative controls, tubes were always prepared in which RNA or reverse transcriptase was omitted during the RT-reaction. Poly(A)-polymerase proved to be a good positive control for the presence of embryonic RNA as these transcripts were detectable in all preimplantation stages. Generation of the diagnostic fragments was strictly dependent on the presence of RNA in the RT reaction, as omission of reverse transcriptase from the RT did not generate any amplified fragments (data not shown).

After the addition of 5 μL of $10 \times$ loading buffer (0.25% (w/v) xylene cyanol and 25 mmol EDTA L^{-1} in 50% glycerol), the RT-PCR products were subjected to electrophoresis on a 1.5% agarose gel in $1 \times$ TBE buffer (90 mmol Tris L^{-1} , 90 mmol borate L^{-1} , 2 mmol EDTA L^{-1} , pH 8.3) containing 0.2 μg EtBr mL^{-1} with further addition of EtBr in the same concentration as the running buffer. After they were run at 80 V for 45 min, the fragments were visualized on a 312 nm UV-transilluminator. The image of each gel was recorded using a CCD camera (Quantix, Photometrics, München) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band was determined by densitometry using an image analysis program (IPLab Gel). The relative amount

of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage. Experiments were repeated for each mRNA, with a minimum of four or five separate oocyte or embryo batches each derived from different *in vitro* produced runs.

For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of numbers of cycles over which linear amplification occurred, and the number of PCR cycles was kept within this range. For the growth factor genes and their receptors, a linear range was observed from 31 to 39 amplification cycles (data not shown). These preliminary amplification experiments were performed with a fixed amount of RNA. The fixed number of cycles was used to demonstrate that the amount of RNA added gave a proportional output of RT-PCR product (data not shown). Because the total efficiency of amplification for each set of primers during each cycle is not known, these assays can be used only to compare relative abundances of a specific mRNA among different samples (Temeles *et al.*, 1994). The recovery rate of RNA was estimated as the ratio between the intensity of the globin band with and without the RNA preparation procedure, starting with an equivalent of 50 fg in the PCR reaction. On average, 66% of poly(A)-tailed RNA was recovered using our Dynabead oligo d(T) mRNA isolation method, which is in agreement with published yields (Shim *et al.*, 1996). After optimization, the RT-PCR assay was sensitive enough to detect specific bovine mRNA from bovine oviduct at concentrations from 0.5 to 5.0 ng of total RNA (a blastocyst contains approximately 10 ng of total RNA) (data not shown). Pools of embryos were used to minimize the effect of individual variation of oocytes or embryos (DeSousa *et al.*, 1998). Krüssel *et al.* (1998) have shown that mRNA patterns differ markedly even among single blastomeres from the same embryo.

Statistical analysis

Data were analysed using the SigmaStat 2.3 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kolmogorov-Smirnov test with Lilliefors correction) and equal variance (Levene Median test), a one-way ANOVA (or a one-way ANOVA on ranks) followed by multiple pairwise comparisons using either Tukey's test or Duncan's method was used to determine differences between both treatments at the same developmental stage and in the temporal expression patterns within one treatment group. Differences were considered to be significant at $P \leq 0.05$.

Results

Development in vitro

The developmental rates of bovine embryos generated in the TCM system or synthetic oviduct fluid system are summarized (Table 2). A significantly higher percentage of

Table 2. Culture efficiency (mean $\pm$ SEM) of bovine embryos produced *in vitro*

Stage of development	Percentage of embryos (relative to absolute number of oocytes)	
	TCM-199 + ECS	SOF + PVA
2–4-cell embryo (day 2)	60.7 $\pm$ 1.0 ^a (281/468)	54.8 $\pm$ 1.8 ^b (447/828)
8–16-cell embryo (days 3–4)	47.0 $\pm$ 1.7 (167/371)	46.8 $\pm$ 2.3 (236/514)
Morulae (day 6)	33.1 $\pm$ 0.7 (135/410)	28.3 $\pm$ 2.8 (198/523)
Expanded blastocysts (days 8–9)	25.0 $\pm$ 1.2 ^a (220/819)	16.5 $\pm$ 0.6 ^b (174/1061)

^{ab}Values with different superscripts within one stage of development are significantly different ($P \leq 0.05$).

ECS: oestrous cow serum; SOF: synthetic oviduct fluid; PVA: polyvinyl alcohol.

two- to four-cell embryos and blastocysts was obtained in the TCM system than in the synthetic oviduct fluid system. Embryos cultured in the presence of serum appeared darker and had more 'granules' than those cultured in the serum-free system.

Ontogenetic expression pattern of IGFs

Representative gel photos of mRNA expression in bovine hatched blastocysts cultured in the TCM system or synthetic oviduct fluid system are shown (Fig. 1). IGF-I transcripts were not detected at any stage of preimplantation bovine development even when PCR was performed with cDNA equivalents corresponding to > 16 oocytes or embryos (Fig. 2). Matured oocytes generally had more mRNA for IGF-IR, IGF-II and IGF-IIR than did immature oocytes (except for IGF-IR oocytes in the TCM system). At maturation, mRNA expression of IGFs and their receptors decreased significantly during early development until activation of the embryonic genome at the 8–16-cell stage, when an increase was observed. There was a significant increase in mRNA expression of IGFs and their receptors at the hatched blastocyst stage.

Effects of medium conditions

The relative abundances of IGF-IR, IGF-II and IGF-IIR gene transcripts found in the two culture systems are summarized (Fig. 3). In the TCM system, IGF-IR was expressed in the immature oocyte up to the two- to four-cell embryo stage. Expression of IGF-IR decreased up to the morula stage and then increased up to the hatched blastocyst stage. In the synthetic oviduct fluid system, a similar pattern of expression for IGF-IR was observed. There was a decrease in IGF-IR mRNA up to the maternal–embryonic transition at the 8–16-cell stage followed by an increase during further embryonic development up to the blastocyst stage. The amount of IGF-IR mRNA was consistently lower in embryos cultured in the synthetic

oviduct fluid system than in those cultured in the TCM system. The individual differences in IGF-IR, IGF-II and IGF-IIR gene expression of embryos generated in the two media were not significant except at the expanded blastocyst stage when IGF-IIR transcripts were significantly higher in the TCM system than in the synthetic oviduct fluid system.

Discussion

This study is the first to report on changes in the relative abundance of mRNAs for IGF-I, IGF-II and their receptors throughout bovine preimplantation development *in vitro*. Bovine embryos were cultured in two different culture systems and a semi-quantitative RT–PCR assay was used with the addition of exogenous globin as internal standard (Wrenzycki *et al.*, 1999). Changes in mRNA expression in the two different culture systems (a TCM system and a synthetic oviduct fluid system) were detected, providing additional information to the findings reported by Wrenzycki *et al.* (1999, 2001) on the effect of culture conditions on mRNA expression. However, it is not possible to use the semi-quantitative RT–PCR assay to differentiate between an increase in transcription, an increase in RNA stability or a change in the poly(A) tail length (Wrenzycki *et al.*, 1999).

The concentrations of mRNA for IGF-II, IGF-IIR and IGF-IR seem to follow a pattern similar to most genes expressed during oocyte maturation in that they increased, indicating that maturation may depend on increased mRNA from these genes. After maturation, IGF concentrations decreased gradually, as expected, up to maternal–embryonic transition at the 8–16-cell stage. After the maternal–embryonic transition, concentrations of IGF-II, IGF-IIR and IGF-IR mRNA increased up to the hatched blastocyst stage. In human embryos, the relative abundance of mRNAs for IGFs and their receptors increases from the three- to four-cell stage (for example, after maternal–embryonic transition) up to the blastocyst stage (Liu *et al.*, 1997). In the present study

Fig. 1. Semi-quantitative RT-PCR analysis of mRNA insulin-like growth factor I receptor (IGF-IR), IGF-II and IGF-IIR in bovine hatched blastocysts produced *in vitro*. Each lane represents the RT-PCR product derived from poly(A)⁺ RNA from the equivalent of four embryos (IGF-II: eight embryos). The RNA recovery rate was estimated as the ratio between the intensity of the fragment with (globin +prep) and without preparation (–prep). SOF: synthetic oviduct fluid; PVA: polyvinyl alcohol; ECS: oestrous cow serum; bp: base pairs.

Fig. 2. Detection of insulin-like growth factor I (IGF-I) transcripts in mRNA from bovine expanded blastocysts and RNA from bovine oviduct and uterus. Lane 1: eight expanded blastocysts; lane 2: 16 expanded blastocysts; lane 3: 20 ng oviduct-RNA; lane 4: 40 ng oviduct-RNA; lane 5: 20 ng uterus-RNA; lane 6: 40 ng uterus-RNA; lane 7: negative control without RT; and lane 8: negative control without RNA. Arrow indicates expected size of the fragment in base pairs.

on bovine embryos, the greatest increase in IGF mRNA occurred after hatching. The increase in IGF-IR, IGF-II and IGF-IIR mRNA observed as the bovine embryo reached the hatched blastocyst stage may indicate an important role for growth factors of the IGF family as the bovine embryo becomes filamentous in shape. Blondin *et al.* (2000) reported that the extended *in vitro* culture period used to generate viable bovine blastocysts from immature oocytes led to significant alterations in IGF-II mRNA steady state concentrations in the liver and skeletal muscle of fetuses at day 70 of gestation. This finding indicates that further studies into the role of IGFs in 'large offspring syndrome', which is frequently observed in calves derived from certain *in vitro* production systems (Niemann and Wrenzycki, 2000), might be valuable.

In contrast to other reports in which IGF-I mRNA was

found in preimplantation bovine and mouse embryos (Schultz *et al.*, 1992; Watson *et al.*, 1992; Doherty *et al.*, 1994; Yoshida *et al.*, 1998; Lonergan *et al.*, 2000), in the present study, mRNA for IGF-I was not detected in embryos derived from the two culture systems. This finding is consistent with observations in preimplantation embryos of mice (Rappolee *et al.*, 1992), rats (Zhang *et al.*, 1994), water buffalo (Daliri *et al.*, 1999) and humans (Lighten *et al.*, 1997a), in which IGF-I mRNA was not detected. Lonergan *et al.* (2000) used nested PCR to identify IGF-I transcripts in early bovine embryos. Furthermore, bovine embryos produced *in vitro* did not release IGF-I protein, although large amounts of IGF-II were observed (Winger *et al.*, 1997). The conflicting results with regard to IGF-I expression in bovine embryos may be due to differences in culture systems or interaction of RT-PCR with specific components used in the experiments,

Fig. 3. Relative abundance of (a) insulin-like growth factor I receptor (IGF-IR), (b) IGF-II and (c) IGF-IIR gene transcripts (mean $\pm$ SEM) throughout bovine preimplantation development (i.ooc: immature oocyte; m.ooc: matured oocyte; Zyg.: zygote; 2-4-cell: 2-4-cell embryo; 8-16-cell: 8-16-cell embryo; mo.: morula; bla.: blastocyst; h.bla.: hatched blastocyst) using TCM-oestrous cow serum (ECS) (□) or synthetic oviduct fluid (SOF)-polyvinyl alcohol (PVA) (■) as culture systems. Significant differences throughout preimplantation development are indicated by different letters (TCM-ECS: A,B,C,D = $P < 0.05$; SOF-PVA a,b,c,d = $P < 0.05$), whereas values with the same symbol (*) indicate significant differences ($P < 0.05$) between embryos at the same development stage in the two culture systems.

particularly the design of primer pairs. Results from the present study indicate that the paracrine rather than the autocrine mechanism is favoured as the mode of action for IGF-I. Embryonic expression of IGF-I most likely does not begin until after implantation. In contrast to mRNA for IGF-I, mRNA for IGF-IR, IGF-II and IGF-IIR was detected at all stages

of preimplantation bovine development, which is consistent with previous observations (Rappolee *et al.*, 1992; Watson *et al.*, 1992; Yoshida *et al.*, 1998, Lonergan *et al.*, 2000). Autocrine and paracrine IGF-II activity appear to be important as IGF-II mRNA is present in preimplantation embryos and in the reproductive tract (Buhi *et al.*, 1997). In

other studies, autocrine and paracrine mechanisms for IGF-II and TGF α action have been suggested (Eicher *et al.*, 1993; Zhang *et al.*, 1994).

Alterations in mRNA expression can be associated with differences in the culture environment (Wrenzycki *et al.*, 1999, 2001). The timing and magnitude of these alterations vary among the different transcripts and are significantly affected by the presence of exogenous protein in a stage-specific manner, predominantly at critical developmental time points. In the present study, the amounts of IGF-IR mRNA were slightly higher at all stages of development in embryos derived and cultured in the TCM system than in embryos that had been cultured in the synthetic oviduct fluid system. Although individual stage-specific concentrations were not significantly different, concentrations of IGF-IR were consistently lower in the synthetic oviduct fluid system. The similarity of the patterns (an increase during oocyte maturation, a decrease up to maternal-embryonic transition and an increase after maternal-embryonic transition) of growth factor gene expression in the two media indicates an important role of growth factors in preimplantation development.

The presence of transcripts for IGF-IR, IGF-II and IGF-IIR in all preimplantation bovine embryos from the immature oocyte to the hatched blastocyst stage indicates that maternal transcripts occur in the oocyte and that these transcripts persist at cleavage stages before activation of the embryonic genome. IGF-II mediates growth in early mouse embryos and forms a pathway in which imprinted genes affect development during preimplantation stages (Rappolee *et al.*, 1992). The IGF-II ligand is imprinted when inherited maternally and IGF-IIR is imprinted when inherited paternally (De-Chiara *et al.*, 1990; Barlow *et al.*, 1991; Latham *et al.*, 1994), consistent with the hypothesis of Haig and Graham (1991) which predicts that imprinting of growth factors such as IGF-II, IGF-IIR and IGF-IR regulates embryonic growth in the mammalian uterus. Lighten *et al.* (1997b) suggested that IGF-II is parentally imprinted in human preimplantation embryos. However, there is little information available with regard to imprinting of these genes in cattle.

Wrenzycki *et al.* (2001) showed that embryos generated in the synthetic oviduct fluid system appear to be more similar to their *in vivo* counterparts with regard to gene expression patterns than those generated in the TCM system. This similarity may also be attributed to the reduced oxygen tension of the synthetic oviduct fluid system via the reduction of deleterious effects of reactive oxygen species on early development (Johnson and Nasr-Esfahani, 1994). In the present study, the abundance of IGF-IR, IGF-II and IGF-IIR transcripts in preimplantation bovine embryos generated in the TCM system was greater than it was in the synthetic oviduct fluid system. From these results, it is possible to speculate that IGFs play a role as survival factors in preimplantation bovine embryos. IGF-I has been shown to prevent apoptosis in early rabbit embryos and to act as a survival factor (Herrler *et al.*, 1998). These findings indicate

a potentially more versatile role of IGFs in preimplantation development than previously suggested.

Determination of growth factor gene expression in preimplantation bovine development can be used for optimization of *in vitro* culture systems, with the goal of improving the quality of embryos generated *in vitro* and ensuring normal offspring. Further studies are underway to investigate growth factor gene expression in embryos produced *in vivo* in an effort to clarify the effects of culture conditions.

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