Expression of growth factor ligand and receptor genes in preimplantation stage water buffalo (*Bubalus bubalis*) embryos and oviduct epithelial cells

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The temporal pattern of expression of genes for several growth factor ligands and receptors was examined in preimplantation water buffalo embryos and oviduct epithelial cells using RT-PCR. The identity of the resulting PCR products was confirmed by their expected size, restriction analysis, Southern blot hybridization and nucleotide sequence analysis. Preimplantation stage embryos from the one-cell to the blastocyst stage were derived after maturation, fertilization and culture of oocytes *in vitro*. Expression of members of the insulin-like growth factor (IGF) family was observed predominantly in preimplantation stage embryos and oviduct epithelial cells. Similarly, transcripts encoding insulin and IGF-I receptors were detected at each stage of embryonic development. The mRNA transcript of the IGF-I receptor was not detected in oviduct epithelial cells, but a prominent band corresponding to the insulin receptor was observed. Insulin and IGF-II mRNA were expressed as maternal transcripts that were not detected at the two- to four-cell stage but were present as zygotic transcripts at the eight-cell stage. Transcripts encoding IGF-I were detected in oviduct epithelial cells, but were not observed in any of the preimplantation stage embryos. Transforming growth factor (TGF) α and β and epidermal growth factor mRNA transcripts were not detected in any of the preimplantation stage embryos. These results indicate that IGF-I acts via a paracrine mechanism to promote growth and development of preimplantation water buffalo embryos. Similarly, IGF-II appears to act through a heterologous autocrine mechanism via the IGF-I or the insulin receptor. Furthermore, the presence of TGF- α in oviduct epithelial cells indicates that it may have a critical role during development.

Introduction

Successful methods for producing large numbers of water buffalo embryos from abattoir-derived ovaries are well documented (Totey *et al.*, 1992, 1993a,b, 1996a; Madan *et al.*, 1994). However, development of specific culture regimens that are capable of supporting *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and subsequent development of water buffalo zygotes to the blastocyst stage is required to produce good quality embryos.

The small number of inner cell mass (ICM) cells in IVF-derived blastocysts is indicative of reduced viability, and may result in lower pregnancy rates in water buffalo (Totey *et al.*, 1996a). However, addition of IGF-I during *in vitro* culture of embryos significantly increased the number of trophoblast and ICM cells (Narula *et al.*, 1996). IGF-I also improved bovine embryonic development *in vitro* (Kaye *et al.*, 1992; Palma *et al.*, 1997).

Insulin improved the development of preimplantation embryos in mice (Gardner and Kaye, 1991), rats (Hertogh *et al.*, 1991), pigs (Lewis *et al.*, 1992), cattle (Matsui *et al.*, 1995) and water buffalo (Totey *et al.*, 1996b). Larson *et al.* (1992) reported that addition of basic fibroblast growth factor and transforming growth factor β (TGF- β) at physiological concentrations to the culture medium improved the development of bovine *in vitro* matured and fertilized zygotes beyond the 16-cell stage and up to the blastocyst stage.

Growth factors have a regulatory role in ovarian function (Adashi *et al.*, 1985). Pawshe *et al.* (1998) reported that IGF-I induced proliferation of granulosa cells and maturation of oocytes in water buffalo. There was a significant increase in cleavage rate and blastocyst development when the oocytes were matured in the presence of IGF-I (Pawshe *et al.*, 1998). This implies that IGFs have a key role in preimplantation development in mammals (Larson *et al.*, 1992).

A number of growth factors and their cognate receptors are expressed by embryos, for example IGF-I, IGF-II,

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epidermal growth factor (EGF), TGF- β , TGF- α and insulin (Schultz and Heyner, 1993a). The growth factor ligand and receptor genes show differential expression before and after the onset of activation of the embryonic genome. However, the types of growth factor expressed vary in the different stages of development of the preimplantation embryo. Expression of a number of growth factor ligand and receptor genes has been demonstrated in preimplantation stage embryos in mice (Rappolee *et al.*, 1989; Telford *et al.*, 1990a), cattle (Schultz *et al.*, 1992; Watson *et al.*, 1992), rats (Zhang *et al.*, 1994) and humans (Lighten *et al.*, 1997). Various growth factors such as EGF, TGF- α , TGF- β and IGF-I have been identified in the oviducts of some mammalian species (Lei and Rao, 1992).

Since the precise role of growth factors in supporting early embryonic development in water buffalo is unclear, the aim of this study was to investigate their expression at various stages of embryonic development. Expression of mRNA transcripts encoding members of the IGF family of ligands and receptors, TGF- α , TGF- β and EGF has been detected in theca and cumulus and mural granulosa cells in water buffalo using RT-PCR and immunohistochemistry (S. M. Totey, unpublished). Therefore, the profile of expression of IGFs and other growth factors during embryogenesis was investigated. The prevalence of species-specific patterns of expression of these growth factors in mice (Schultz *et al.*, 1993b), rats (Zhang *et al.*, 1994) and cows (Watson *et al.*, 1992) confirms their differential mode of action, which is critical for development. Therefore, in the present study, the mRNA phenotype of growth factor ligand and receptor gene expression in preimplantation buffalo embryos and oviduct epithelial cells was examined.

Materials and Methods

Collection of oocytes and oviduct epithelial cells

Collection of preimplantation water buffalo embryos at various stages and IVM and IVF of immature oocytes were carried out as described by Totey *et al.* (1993a). Briefly, ovaries were collected from a local abattoir and intact cumulus-oocyte complexes (COCs) that had homogeneous ooplasm and were surrounded by four to five layers of unexpanded cumulus cells were isolated by aspirating small visible follicles of 2–6 mm in diameter within 1 h after the animals were killed.

Oviducts from cyclic water buffalo were transferred from the abattoir to the laboratory on ice. Oviducts from freshly ovulated water buffalo (identified by the presence of haemorrhagic corpora lutea on the ovaries) were collected. After removal of extraneous tissue, the oviducts were rinsed thoroughly with normal saline containing 50 μ g gentamycin ml^{-1} and blotted dry with sterile tissue paper. Epithelial cells were collected by scraping the outside of the oviduct from the infundibulum to the isthmus with a sterile glass slide. The cells were washed five to six times with TCM-199 supplemented with 10% fetal calf serum (FCS) and 50 μ g gentamycin ml^{-1} by gravity sedimentation. The pellet was resuspended and the cells were seeded in 24-well plates with

200 μ l culture medium. The cells were maintained at 39°C under 5% CO_2 in air and 95% relative humidity. After 2 days of culture, the cells were removed and washed with TCM-199 supplemented with 10% FCS and used for co-culture and RNA isolation.

In vitro maturation

COCs were washed three times in Tyrode lactate-Hepes (TL-Hepes) medium containing 0.3% (w/v) BSA (Sigma Chemical Co., St Louis, MO). The final two washings were carried out in maturation medium consisting of Ham's F-10 medium containing 10% FCS (Gibco BRL Life Technologies, Grand Island, NY) and supplemented with 1.25 mmol sodium pyruvate l^{-1} , 40 μ g gentamycin ml^{-1} , 5 μ g ovine LH ml^{-1} , 0.5 μ g ovine FSH ml^{-1} (National Institute of Diabetes and Digestive and Kidney Disease, National Hormone and Pituitary Program, MD), and 1 μ g oestradiol ml^{-1} (Sigma). Groups of 20 COCs were randomly allocated to 100 μ l microdrops of maturation medium under mineral oil (Sigma) and incubated for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization

Motile water buffalo spermatozoa were isolated from frozen stocks using the Percoll gradient method described by Totey *et al.* (1993b). Matured oocytes were washed with TL-Hepes and distributed in 50 μ l drops of fertilization medium containing 6 mg fatty acid free BSA ml^{-1} , 0.25 mmol sodium pyruvate l^{-1} and 10 μ g heparin ml^{-1} (Sigma) under mineral oil (ten oocytes per drop) in a 60 mm Petri dish. A 2 ml aliquot of sperm suspension (2×10^6 spermatozoa ml^{-1}) was added to each drop. Oocytes and spermatozoa were co-incubated for 22 h at 39°C in a humidified atmosphere of 5% CO_2 in air to enable *in vitro* fertilization.

In vitro culture

After 22 h of co-incubation of spermatozoa and oocytes, the cumulus cells of the fertilized embryos were removed by repeated pipetting and the embryos were washed several times in TL-Hepes medium. The final two washings were carried out in development medium consisting of TCM-199 supplemented with 10% FCS. Twenty presumptive zygotes were randomly distributed to 100 μ l drops of TCM-199 under mineral oil and co-cultured with oviductal cells in 5% CO_2 in air and 95% relative humidity at 39°C. The morphology and development of the embryos were examined. Matured oocytes and embryos that were developing normally at the two-cell, four-cell, eight-cell, compact morula and mid-blastocyst stages from day 2 to day 6 of culture were selected for this study (Narula *et al.*, 1996).

Isolation of RNA

Cumulus cells of matured unfertilized oocytes were removed by repeated pipetting. The zona pellucida was

Table 1. Sequence of growth factor primers from conserved regions of murine and human sequences and major features of the expected PCR fragments and restriction sites

Gene	Fragment size (bp)	Restriction site	Primer sequence (5'-3')	Reference
IGF-I ligand	210	Msp I	5' GGACCAGAGACCCCTTTCGCGGG 3' GGCTGCTTTTGTAGGCTTCAGTGG	Bell <i>et al.</i> , 1986
IGF-II ligand	256	Sac II	5' CGCCCCAGCGAGACTCTGTGC 3' GCCCACGGGGTATCTGGGGAA	Dull <i>et al.</i> , 1984
Insulin ligand	300	Sma I	5' GATGCGCTTCCTGCCCTGGTGGC 3' GTTGGTAGAGGGAGCAGATGTGG	Bell <i>et al.</i> , 1980
Insulin receptor	324	Hinc II	5' GTCAC TGACCTCATGCGCATGTGCTGG 3' CCGCCCGTTTTCTTGCCTCCGTTTCAT	Ullrich <i>et al.</i> , 1985
IGF-I receptor	354	Ban II	5' ATGCTGTTTGAAGTATGCGCATGTGCTGG 3' CCGCTCGTTCCTGCGGCCCCCGTTCAT	Ullrich <i>et al.</i> , 1986
IGF-II receptor	186	Taq I	5' TGTACACTCTTCTTCTCTGGCA 3' AGAGATGTTGATGTAGAAGACAGG	Morgan <i>et al.</i> , 1987; Telford <i>et al.</i> , 1990b
TGF- β 2	273	Sph I	5' AGAAATGTGCAGGATAATTGCTGC 3' TTCGATCTGGGCGTATTTCGAAT	Miller <i>et al.</i> , 1989
TGF- α	239	Sph I	5' ACCTGCAGGTTTTTGGTGCAG 3' GCAGACGAGGGCAGGCACCA	Derynck <i>et al.</i> , 1986
EGF	247	Rsa I	5' CCAGTTCAGTAGAACTGGG 3' TGGTTTCTAATGATTTTCTCC	Gray <i>et al.</i> , 1983
β actin	243	Bgl II	5' CGTGGGCCGCCCTAGGCACCA 3' TTGGCCTTAGGGTTCAGGGGGG	Tokunaga <i>et al.</i> , 1986

IGF, insulin-like growth factor; TGF, transforming growth factor; EGF, epidermal growth factor.

β actin was included as an internal control.

removed by treating the oocytes and embryos with acid-Tyrode's medium. Zona free oocytes and embryos were collected according to their stage of development. Oviduct epithelial cells were collected after 2 days of culture. A buffalo lung-derived cell line (Bu-IMR-31) and a rat liver-derived cell line (BRL-3A) were cultured simultaneously in McCoy's medium and minimum essential medium (Gibco), respectively, supplemented with 10% FCS and antibiotics and used as controls. Before extraction of RNA, all the cells, including the oocytes and embryos, were washed with collection medium consisting of bicarbonate-free minimal essential medium (Earle's salt) containing 100 μ g sodium pyruvate ml^{-1} , 10 μ g gentamycin ml^{-1} , 3 mg polyvinyl pyrrolidone (PVP) ml^{-1} , and 25 mmol Hepes l^{-1} , pH 7.2, and transferred in a minimal volume of medium to a chilled 0.6 ml microfuge tube on ice. Total RNA was isolated from 300 matured unfertilized oocytes, 200 embryos at each of the two-cell, four-cell, eight-cell and morula stages, 150 blastocysts, approximately 10000 oviduct epithelial cells, 1×10^6 BRL-3A cells, and 1×10^6 Bu-IMR-31 cells as described by Doherty *et al.* (1994). Each experiment was repeated three times using the same number of oocytes, embryos and cells to enable comparative analysis.

RT-PCR procedures

Total RNA from BRL-3A and Bu-IMR-31 cells (3 μ g) and from embryos at the different stages of development (1 μ g) was used for reverse transcription. The reaction was performed in a total volume of 30 μ l consisting of 10 μ l total RNA, 6 μ l of 5 $\times$ buffer (250 mmol Tris-HCl l^{-1} , pH 8.3, 375 mmol KCl l^{-1} , 15 mmol MgCl_2 l^{-1}), 1.5 ml 2'-deoxyribonucleoside triphosphates

(dNTPs) (1 mmol l^{-1} each of dATP, dGTP, dCTP, dTTP), 3 μ l of 0.1 mol dithiothreitol (DTT) l^{-1} , 1 μ l (0.5 μ g) oligo (2'-deoxyribosylthymidine) 12-18 (Gibco) and 0.5 μ l (14 U) RNasin (Promega). The volume was made up to 30 μ l with distilled water. The reaction was carried out in 0.6 ml Gen Amp thin walled tubes (Perkin Elmer Cetus, Norwalk, CT). The tubes were incubated at 37°C for 2 min in a thermocycler (Perkin Elmer Cetus) before addition of 1 μ l (200 U) Superscript II reverse transcriptase enzyme (Gibco). The tubes were returned to the thermocycler and reverse transcription was carried out at 42°C for 1 h. The tubes were heated to 99°C for 5 min to inactivate the enzyme, and were then placed immediately on ice.

Oligonucleotide primer pairs were chemically synthesized at Bio-synthesis, Inc., Louisville, TX, and Rama Biotechnologies Pvt Ltd, Hyderabad, India. The sequences, expected size of PCR fragments and restriction sites are shown (Table 1). A primer pair for mouse β actin cDNA was included as an internal control. All the growth factor primers included an intron, so that product derived from genomic DNA contamination would be larger than the product of the reverse transcribed mRNA.

PCR was carried out in a total volume of 25 μ l in a Gen Amp thin walled tube. Each reaction mixture contained 2-3 μ l cDNA solution, 2.5 μ l of 10 $\times$ buffer (200 mmol Tris-HCl l^{-1} , pH 8.4, 500 mmol KCl l^{-1}), 0.9 μ l of 50 mmol MgCl_2 l^{-1} , 0.5 μ l dNTPs (10 mmol l^{-1} each of dATP, dGTP, dCTP, dTTP), 1 ml each of appropriate 3' and 5' primers (20 pmol) and 2.5 U Taq DNA polymerase (Gibco). The volume was made up to 25 μ l with distilled water and the mixture was overlaid with mineral oil. After an initial denaturation at 94°C for 1 min, the thermocycler was programmed for 40 cycles. Each cycle consisted of 94°C for 30 s, 60°C for 45 s (annealing), and 72°C

for 45 s (extension). The final cycle included an additional thermal delay for 5 min at 72°C for complete extension. The tubes were chilled on ice and centrifuged at 400 g for 30 s. After PCR amplification, 3.3 U RNase H (Gibco) was added to the sample and the tubes were incubated at 37°C for 20 min. After addition of 3 µl of 6 × loading buffer (0.25% (v/v) bromophenol blue in 30% (v/v) glycerol), 15 µl of each sample was subjected to electrophoresis on a 1.8% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light.

Verification of RT-PCR products

The authenticity of the PCR products was analysed by digestion with a specific restriction endonuclease or by Southern blot analysis after the PCR products were transferred to a genescreen plus membrane (NEN Research Product, Boston, MA) as described by Telford *et al.* (1990a), and subsequently by cloning and sequencing of the PCR product. PCR amplified products were resolved on 1.8% (w/v) agarose gels. The distinct fragments were eluted from the gel and cloned directly into the pMos Blue T-vector according to the manufacturer's instructions (Amersham International, Amersham). The plasmid DNA was isolated from the positive clones using the alkaline lysis method and purified by precipitation with polyethylene glycol (Sambrook *et al.*, 1989). The double-stranded DNA was sequenced by Sanger's dideoxynucleotide chain termination procedure using a sequenase version 2.0 DNA sequencing kit (USB Corp., Cleveland, OH), universal primers and growth factor specific oligonucleotide primers. The sequences obtained were compared with known sequences from other species and were analysed using GCG and DNASIS software packages.

Results

Expression of various growth factor ligand (insulin, IGF-I, IGF-II, TGF- α , TGF- β and EGF) and receptor mRNA transcripts (insulin, IGF-I and IGF-II) was studied in preimplantation water buffalo embryos at various stages of development and in oviduct epithelial cells. The oligonucleotide primers used were derived from conserved regions of murine and human sequences (Table 1). Two transformed cell lines were used to test whether these primers would primarily amplify similar cDNA sequences in water buffalo: BRL-3A (derived from rat liver) and Bu-IMR-31 (derived from water buffalo lung). The cell lines were included as controls to validate the performance of the PCR. The primers worked well in the buffalo-derived cell line, and amplified similar cDNA sequences as observed in the rat-derived cell line, unless otherwise stated (Fig. 1 and Table 2). β actin was included as a positive control to evaluate the quality of the isolated RNA, the extent of DNA contamination, and the PCR procedure. The primers were designed to amplify 330 bp fragments in the event of genomic DNA contamination and 243 bp fragments from cDNA. A single 243 bp band was obtained in all the RNA

samples used in the study, indicating that there was no contamination by genomic DNA during RNA extraction. Similarly, all the growth factor primers selected from the literature (Table 1) included an intron, so that product derived from genomic DNA contamination would be larger than the product of the reverse transcribed mRNA. Final confirmation of each PCR product was carried out by sequencing.

Expression of IGF-I and IGF-I receptor

An expected 210 bp fragment encoding IGF-I was observed in the rat-derived (Fig. 1a) and buffalo-derived cell lines (Fig. 2, lanes 1–3) and oviduct epithelial cells (Fig. 1i; Fig. 2, lanes 4–6). However, IGF-I ligand was not detected at any stage in development of the preimplantation embryos (Fig. 1c–h). In the Bu-IMR-31 cell line (Fig. 1b) and oviduct epithelial cells, IGF-I primers occasionally amplified a major 432 bp fragment instead of the expected 210 bp fragment, or a 610 bp fragment was observed in addition to the expected 210 bp IGF-I fragment (Fig. 2). Restriction analysis showed that Msp I digestion of the 210 bp product yielded 149 and 61 bp subfragments in both cell lines and in oviduct epithelial cells, whereas the 432 bp fragment yielded 244 and 188 bp subfragments (Fig. 3a) in Bu-IMR-31 and oviduct epithelial cells. The identity of the two amplified products was verified by Southern blot hybridization with a ³²P-labelled murine IGF-I cDNA probe. The probe hybridized strongly to both 210 and 432 bp sequences (Fig. 3b). cDNA was freshly transcribed from poly(A)+ mRNA instead of total RNA from the BU-IMR-31 cell line and oviduct epithelial cells to characterize both fragments further. The 432 bp fragment was not observed when poly(A)+ mRNA was used, whereas the 210 and 610 bp fragments were amplified. All the PCR fragments from the Bu-IMR-31 cell line and oviduct epithelial cells were subjected to sequence analysis. The 210 bp fragment obtained from the Bu-IMR-31 cell line consisted of 207 nucleotides (Genbank accession no. Y 10691) and from the oviduct epithelial cells consisted of 210 nucleotides (Genbank accession no. Y 16248); both were IGF-IA. Deletion of three bases in the fragment from the Bu-IMR-31 cell line at positions 40, 41 and 47 (each a thymidine) was not detected in the oviduct epithelial cells (Fig. 4). Both nucleotide sequences encode 70 amino acids and contain insulin family signatures at positions 46–60. The sequences showed up to 98% homology to mouse, 86% homology to human, 83.3% homology to ovine and 84.2% homology to marmoset IGF-I ligand sequences. Partial sequence analysis of the 432 bp PCR fragment revealed it to be 99% homologous to rat 18S rRNA (Fig. 5). The sequence has been deposited with Genbank (accession no. Y 12516). The 610 bp fragment did not show homology to any of the databases in Genbank.

Transcripts encoding the IGF-I receptor were detected in all developmental stages of the preimplantation embryos (Fig. 1c–h). The 357 bp RT-PCR product for the IGF-I receptor was not detected in oviduct epithelial cells, but a prominent amplified product of 327 bp corresponding to the size of the insulin receptor was detected (Fig. 1i). Restriction digestion with Ban II enzyme resulted in cleavage of the 357

Fig. 1. Detection of mRNA transcripts by RT-PCR. (a) A rat liver-derived cell line (BRL-3A): lane 1, 210 bp insulin-like growth factor I (IGF-I) ligand; lane 2, 243 bp β actin fragment; lane 3, IGF-I receptor; lane 4, IGF-II ligand; lane 5, IGF-II receptor; lane 6, insulin; lane 7, insulin receptor; lane 8, transforming growth factor α (TGF- α); lane 9, TGF- β 2; lane 10, epidermal growth factor (EGF). (b) A water buffalo lung-derived cell line (Bu-IMR-31). (c-h) Preimplantation water buffalo embryos: (c) oocyte, (d) two-cell, (e) four-cell, (f) eight-cell, (g) morula, (h) blastocyst. (i) Water buffalo oviduct epithelial cells. (b-i) Lane 1, single 243 bp fragment for β actin expression, indicating that there is no contamination by genomic DNA; lane 2, IGF-I ligand; lane 3, IGF-I receptor; lane 4, IGF-II ligand; lane 5, IGF-II receptor; lane 6, insulin ligand; lane 7, insulin receptor; lane 8, TGF- α ; lane 9, TGF- β 2; lane 10, EGF. The PCR products were resolved on 1.8% (w/v) agarose gels. m, molecular size marker (OX 174 RF DNA digested with Hae III restriction enzyme).

bp product into three fragments of 176, 101 and 80 bp (Fig. 3d), but did not cleave the 327 bp band, which was identified as a crossreactive product of the insulin receptor. Similarly, in the rat-derived cell line and in four-cell embryos, in addition to the expected 357 bp fragment of the IGF-I receptor, a crossreactive 327 bp fragment of the insulin receptor was

observed (Fig. 1a,c). Sequence analysis revealed the authenticity of both PCR fragments. The 357 bp fragment amplified by IGF-I receptor primers was identified as the IGF-I receptor (Genbank accession no. Y 12700), whereas the crossreactive product of the 327 bp fragment was identified as the insulin receptor (Genbank accession no. Y 14017).

Table 2. Growth factor ligand and receptor mRNA transcripts expressed in preimplantation stage water buffalo embryos and oviduct epithelial cells

Growth factor	Stage of embryo development						Oviductal cells	Controls	
	Oocyte	Two-cell	Four-cell	Eight-cell	Morula	Blastocyst		Bu-IMR-31	BRL-3A
β actin	+	+	+	+	+	+	+	+	+
IGF-I	-	-	-	-	-	-	+	+	+
IGF-I receptor	+	+	+	+	-	+	-	+	+
IGF-II	+	-	-	+	-	+	-	+	+
IGF-II receptor	-	-	-	-	-	-	-	+	+
Insulin	+	-	-	+	-	+	-	+	+
Insulin receptor	+	+	+	+	+	+	+	+	+
TGF- α	-	-	-	-	-	-	+	+	+
TGF- β	-	-	-	-	-	-	-	+	+
EGF	-	-	-	-	-	-	-	-	+

IGF, insulin-like growth factor; TGF, transforming growth factor; EGF, epidermal growth factor.

Bu-IMR-31, buffalo lung-derived cell line; BRL-3A, rat liver-derived cell line.

Fig. 2. Detection of 210 bp fragment of insulin-like growth factor I (IGF-I) mRNA transcripts in water buffalo lung-derived cell line (Bu-IMR-3) (lanes 1–3) and water buffalo oviduct epithelial cells (lanes 4–6). m, molecular size marker (ϕ X 174 RF DNA digested with Hae III restriction enzyme).

Fig. 3. Verification of RT-PCR fragment identity by restriction enzyme analysis. (a) Uncut (u) and MspI cut (c) 210 and 432 bp PCR products of insulin-like growth factor I (IGF-I) were resolved on 1.8% (w/v) agarose gel. m, molecular size marker (ϕ X 174 RF DNA digested with Hae III restriction enzyme). (b) Southern hybridization of IGF-I ligand PCR product cut with Msp I. Hybridization was carried out with 32 P-labelled rat IGF-I ligand cDNA probe.

Expression of insulin and insulin receptors

A single 300 bp RT-PCR product for the insulin ligand was detected in oocytes (Fig. 1c), but was not detected in embryos at the two-cell (Fig. 1d) and the four-cell (Fig. 1e) stages. The transcript was apparent in embryos at the eight-cell (Fig. 1f) and the blastocyst stages (Fig. 1h), but was not detected in morulae (Fig. 1g) and oviduct epithelial cells (Fig. 1i).

Analysis of the pattern of transcription of the insulin receptor by RT-PCR showed the presence of a single expected 327 bp amplified product in oocytes (Fig. 1c), all embryonic stages (Fig. 1d–h) and oviduct epithelial cells (Fig. 1i). In addition, a 280 bp PCR fragment was present in these samples. Restriction digestion using the Hinc II enzyme confirmed the presence of a single restriction site in the 327 bp insulin receptor in the rat-derived cell line, but not in the water buffalo-derived cell line. Further restriction analysis of the 327 and 280 bp fragments of the water buffalo-derived insulin receptor using a panel of restriction enzymes revealed the presence of Msp I sites. The 327 bp buffalo cDNA sequence (Genbank accession no. Y 14017) shares 95% homology with the human insulin receptor gene sequence, whereas the co-amplified product of 280 bp (Genbank accession no. Y 14610) did not show significant homology to any of the databases in Genbank.

Expression of IGF-II and IGF-II receptor

A single IGF-II-derived fragment of 256 bp was detected in oocytes (Fig. 1c) and embryos at the eight-cell and blastocyst stages (Fig. 1f,h), but not in morulae (Fig. 1g) and oviduct epithelial cells (Fig. 1i). Similar to insulin, the transcript for the IGF-II ligand was not detected in embryos at the two- or four-cell stages (Fig. 1d,e). Restriction digestion with the appropriate enzyme, Sac II, yielded fragments of expected size (189 and 67 bp).

The transcripts for the IGF-II receptor were not observed in the oviduct epithelial cells, oocytes and or any of the embryonic stages (Fig. 1c–i), although an expected 186 bp IGF-II receptor product was identified in both the control cell lines (Fig. 1a,b). The authenticity of the 186 bp fragment was verified by Taq I digestion which resulted in fragments of 105 and 81 bp.

Fig. 4. (a) Nucleotide and (b) predicted amino acid sequences of insulin-like growth factor I (IGF-I) ligand amplicon of 207 bp (Genbank accession no. Y 10691). The percentage homology with other species is shown. M, murine; H, human; O, ovine; Mo, marmoset.

Expression of TGF and EGF

Although expected 239, 273 and 247 bp TGF- α , TGF- β 2 and EGF products were detected in the positive control cell line (BRL-3A) (Fig. 1a), only the TGF- α and TGF- β 2 transcripts were present in the Bu-IMR-31 cell line (Fig. 1b). The RT-PCR analysis showed an absence of these growth factors in oocytes (Fig. 1c) and embryos (Fig. 1d-h). However, the 239 bp amplified product, corresponding to the transcript for TGF- α , was detected in oviduct epithelial cells (Fig. 1i). The authenticity of the 239 bp fragment of TGF- α was verified with restriction analysis using Sph I.

Discussion

The expression of various growth factors and their receptors in preimplantation stage water buffalo embryos and oviduct epithelial cells in primary culture was characterized using RT-PCR. The oligonucleotide primers used were designed from known mouse or human cDNA sequences. The sequence of the exons encoding the mature insulin protein and IGF peptides are highly conserved among vertebrate species (Le Roith *et al.*, 1993). Initially, the heterologous primers were investigated using a cell line derived from water buffalo lung (Bu-IMR-31) and also a rat liver cell line (BRL-3A) to determine whether they would amplify growth

factor ligand and receptor sequences in water buffalo. It was confirmed that the human and mouse primer sequences of various growth factors recognized similar heterologous growth factor gene sequences in water buffalo, except possibly EGF. Although a fixed amount of RNA was used for the experimental samples, which was a smaller amount than in the positive control, it was enough to confirm the presence or absence of the transcript in the specific types of cell tested. Thus, these data provide qualitative evidence on the presence or absence of growth factor mRNA transcripts.

The mRNA transcripts encoding the IGF-I ligand were not detected in any of the preimplantation stage embryos, although the ligand was detected in oviduct epithelial cells. This indicates that IGF-I is unlikely to act via an autocrine mechanism during preimplantation embryo development in water buffalo. IGF-I mRNA is translated in oviductal cells and its action appears to be regulated by four IGF binding proteins (S. M. Totey, unpublished). Although *in vitro* development of embryos from many mammalian species can be maintained independently of the maternal environment, co-culturing with oviductal cells significantly increases the rate of development of blastocysts (Totey *et al.*, 1992). The expression of IGF-I in oviduct epithelial cells and the presence of the IGF-I receptor on preimplantation stage embryos indicates a paracrine mode of action in water buffalo. These results are in contrast to other reports in cattle,

	1	ggtggtgcat	ggccgttctt	agttggtgga	gcgatttgtc	tggttaattc
R						
M				...t.g...		
H				...t.g...		
	51	cgataacgaa	cgagactctc	ggcatgctaa	ctagttacgc	gacccccgag
R						
M						
H						
	101	cggtcggcgt	cccccaactt	cttagaggga	caagtggcgt	tcagaccccc
R						C.a...
M						C.a...
H						C.a...
	151	ccgagattga	gcaataaagg	ctgtgNNNNN	NNNNNNNNNN	NNNNNNNNNN
R			ca.	g.c..		
M			ca.	g.c..		
H			ca.	g.c..		
	201	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
R						
M						
H						
	251	Ngcgcgggta	acccgttgaa	ccccattcgt	gatggggatc	ggggattgca
R						
M						
H						
	301	attattcccc	atgaacgagg	aattcccagt	aagtgcgggt	cataagcttg
R						
M						
H						
	351	cgttgattaa	gtccctgccc	ttgtacaca	ccgccc	
R						
M						
H						

Fig. 5. Nucleotide sequence of water buffalo 18S rRNA (390 bp) co-amplified using insulin-like growth factor I (IGF-I) primers (Genbank accession no. Y 12516). The percentage homology with other species is shown. R, rat; M, mouse; H, human.

in which transcripts for IGF-I were not detected in oviductal cells (Watson *et al.*, 1992; Viuff *et al.*, 1995). The IGF-I transcript was present in cattle and mouse embryos at all stages (Watson *et al.*, 1992; Doherty *et al.*, 1994). However, in rats and humans, the IGF-I transcript was not detected in preimplantation embryos, but was found in the oviduct and uterus (Zhang *et al.*, 1994; Lighten *et al.*, 1997).

The oligonucleotide primers of IGF-I resulted in two distinct amplified products of 210 and 432 bp. The verification of these amplified products revealed that the 432 bp product was detected only when a preparation of total RNA was used as the template for the RT-PCR and was not apparent when poly(A)⁺ mRNA was used. The 432 bp

fragment was shown to be 99% homologous to 18S ribosomal RNA. This is the first report to indicate that oligonucleotide primers of IGF-I can crossreact with 18S ribosomal RNA in water buffalo during PCR. Viuff *et al.* (1995) demonstrated that an IGF-I cDNA probe cross-hybridizes with the 28/18S ribosomal band obtained from total RNA. There is evidence that base pairings between some eukaryotic mRNAs, such as human IGF-I and the 3' end of 18S rRNA, might have a role in the initiation of eukaryotic translation (Jansen *et al.*, 1983). In the present study, the abundance of the transcript and the appearance of the expected 210 bp amplified product often varied between different batches of RNA. This may be due to the short half-

life of the IGF-I transcript. Hepler *et al.* (1990) reported that the functional significance of different IGF-I mRNA size classes is related to differences in their stability.

IGF-II and insulin were detected as a maternal transcript in unfertilized eggs, but they were not apparent at the two- to four-cell stage when most maternal mRNA in the mammalian embryo is destroyed (Telford *et al.*, 1990a). However, IGF-II and insulin were resynthesized in the zygote at the eight-cell stage as zygotic transcripts. In contrast, transcripts for insulin were not detected in preimplantation stage embryos of murine, bovine or human origin (Schultz *et al.*, 1992; Lighten *et al.*, 1997). The expression of insulin in mouse and human embryos commences after implantation (Rappolee *et al.*, 1992; Lighten *et al.*, 1997). Although there were strong signals of transcripts encoding the IGF-I receptor, insulin and IGF-II at the eight-cell and the blastocyst stages, they were not visualized by ethidium bromide staining in morula stage embryos. An increase in the number of PCR cycles did not increase the ability to detect the transcript in morula stage embryos. However, mRNA transcripts for the insulin receptor were detected in water buffalo embryos at all stages of development, including morulae, as well as in oviduct epithelial cells. The presence of insulin receptors within the embryo and oviductal cells indicates that embryonic development is supported by both autocrine and paracrine modes of action of insulin. The degradation of the transcript at the two- to four-cell stage in buffalo may indicate that embryogenesis is affected by the expression of zygotic growth factor genes and that the maternal-zygotic transition takes place at the two- to four-cell stage. However, this hypothesis requires further confirmation, since IGF-I receptor, insulin and IGF-II transcripts were not visualized by ethidium bromide staining in morula stage embryos, it is also possible that the relevant zygotic genes are not expressed until the blastocyst stage and, if so, the transcripts at the eight-cell stage may not be from the zygotic genome. There is evidence that IGF-II exerts its growth promoting effect through IGF-I receptors or insulin receptors rather than through the IGF-II receptor (Czech and Ullrich, 1988; MacDonald *et al.*, 1988; Rappolee *et al.*, 1992). In the present study, IGF-II receptors were not detected in either preimplantation stage embryos or in oviductal cells, thus it is postulated that in water buffalo IGF-II may also act through IGF-I or insulin receptors.

There is considerable homology between IGF-I and insulin receptors (Ebina *et al.*, 1985; Ullrich *et al.*, 1985, 1986; Telford *et al.*, 1990b) and this may increase the tendency for primer pairs to crossreact with each other. Indeed, the primers used in the PCR of the IGF-I receptor amplified a 327 bp cross-reactive sequence of the insulin receptor and an additional 280 bp fragment. Differential restriction digestion using Ban II, which is appropriate for IGF-I receptor, and Hinc II, which is specific for the insulin receptor, did not cleave any of the crossreactive PCR products of the insulin receptor. This indicates that the Hinc II site may be absent from the water buffalo insulin receptor fragment. The 280 bp fragment amplified in addition to the insulin receptor and the 610 bp fragment amplified in addition to IGF-I did not show significant homology to any of the database sequences in Genbank and are worthy of further investigation.

Although the TGF- α transcript was detected in oviduct epithelial cells, it was not observed at any stage of development of the preimplantation embryo. The role of TGF- α in the water buffalo embryo remains to be elucidated. Addition of exogenous TGF- α to *in vitro* culture medium did not show any beneficial effect on embryonic development (S. M. Totey, unpublished). Other growth factor transcripts such as TGF- β 2 and EGF were also not detected in preimplantation buffalo embryos and oviduct epithelial cells.

Although many of the growth factors and their receptors are expressed in murine, bovine and water buffalo embryos, the embryonic stages at which transcripts can be detected differ among these species. This indicates that the pattern of growth factor expression and mode of action at different stages of embryonic development is species specific. The addition of insulin and IGF enhances embryonic development in water buffalo *in vitro* (Totey *et al.*, 1996b). Similarly, the number of trophoblast and ICM cells is significantly increased in blastocysts cultured in the presence of IGF-I (Narula *et al.*, 1996). Gardner and Kaye (1991) reported similar results in mice.

In conclusion, the results of the present study indicate that growth factors such as insulin, IGF-I and IGF-II and their receptors are selectively expressed at various stages of embryonic development. This indicates that they are important in the development of preimplantation stage water buffalo embryos, but further confirmation is required. In addition, IGF-I is produced by oviduct epithelial cells and may act via a paracrine mechanism within the water buffalo oviduct to provide an optimal environment for embryonic development.

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