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IGF paracrine and autocrine interactions between conceptus and oviduct

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Development *in vitro* is influenced by embryo density, serum, somatic cell co-culture and the production of 'embryotrophic' paracrine and autocrine factors. Research in our laboratory has focussed principally on the insulin-like growth factor (IGF) family. We have demonstrated that pre-attachment bovine and ovine embryos express mRNAs encoding a number of growth factor ligand and receptor genes including all members of the IGF ligand and receptor family throughout this developmental interval. In addition, early embryos express mRNAs encoding IGF-binding proteins (IGFBPs) 2–5 from the one-cell to the blastocyst stage and IGFBP5 mRNA at the blastocyst stage. Cultured bovine blastocysts release up to 35 pg per embryo in 24 h, whereas release of IGF-I was below detectable values. Analysis extended to bovine oviductal cultures has also demonstrated that mRNAs encoding these IGF family members are present throughout an 8 day culture period. Transcripts encoding IGFBPs 2–6 were also present. Release of both IGFs was recorded over an 8 day culture period. IGF-II release was significantly greater than that observed for IGF-I. Therefore, the IGFs are present throughout the maternal environment during early embryo development. The oocyte, within the follicle, is held in an environment high in IGFs and IGFBPs. The zygote, after fertilization, is maintained in an IGF-rich environment while free-living in the oviduct and the uterus. This review is focused on the IGF family and IGFBPs and their roles in enhancing development up to the blastocyst stage.

Overview

The production of mammalian zygotes *in vitro* is an important approach for studying oocyte maturation, fertilization, and early development. We are still, however, unable to mimic the environment of the female reproductive system for any mammalian species, and zygotes produced *in vitro* generally result in lower pregnancy rates after transfer to the uterus than do zygotes produced *in vivo*. *In vitro* derived embryos often display a developmental lag, contain fewer cells, and are morphologically distinct (Farin and Farin, 1995; Thompson *et al.*, 1995; Walker *et al.*, 1996). Successful progression through the first week (preimplantation phase) of development is essential for implantation and establishment of pregnancy. Studies are required to define and optimize culture environments and technologies for treating various forms of human infertility, to increase developmental frequencies of livestock embryos *in vitro* for commercial applications, and to understand better the genetic programme regulating early development.

Preimplantation development is somewhat autonomous as development to the blastocyst stage can be supported *in vitro* in simple defined conditions for many mammalian species (Chatot *et al.*, 1990; Gardner *et al.*, 1994; Keskintepe *et al.*, 1995; Summers *et al.*, 1995; Walker *et al.*, 1996). Despite this autonomous nature it is clear that development *in vitro* is enhanced by factors such as embryo density (O'Neill, 1997), serum and somatic cell co-culture (Gandolfi and Moor, 1987; Sirard *et al.*, 1988; Xia *et al.*, 1996). The desire to support development of preimplantation mammalian embryos *in*

vitro has driven the characterization of 'embryotrophic' factors and unleashed a period of analysing growth factor and cytokine expression patterns during early development (for review see Schultz *et al.*, 1993; Schultz and Heyner, 1993; Kaye and Harvey, 1995; Kane *et al.*, 1997; Stewart and Cullinan, 1997). These types of study have largely outpaced functional studies and to date a convincing foundation illuminating specific roles for these factors in supporting early development has been put forward only for the mouse preimplantation embryo (Kaye and Harvey, 1995; Stewart and Cullinan, 1997). There are, however, compelling data that support similar roles for these factors in influencing early development in other species too.

Research in our laboratories has focussed principally on the insulin-like growth factor (IGF) family. The IGFs are present throughout the maternal environment during early embryo development (for review see Clemmons, 1993; Murphy and Barron, 1993; Jones and Clemmons, 1995). The oocyte, within the follicle, is held in an environment high in IGFs and insulin-like growth factor binding proteins (IGFBPs) (Ling *et al.*, 1993; de la Sota *et al.*, 1996). The zygote, following fertilization, is maintained in an IGF-rich environment while free-living in the oviduct and the uterus (Giudice *et al.*, 1992; Wiseman *et al.*, 1992; Carlsson *et al.*, 1993; Xia *et al.*, 1996; Winger *et al.*, 1997). For these reasons, the current review will focus on the IGF family and IGFBPs and their roles in enhancing development up to the blastocyst stage. Emphasis will be placed on the early mouse embryo (because the majority of information has been generated from this species). We will, however, summarize data supporting roles for these modulators in regulating early development of livestock embryos as well.

Mammalian Embryo Culture

Early embryos from different mammalian species exhibit broad variations in their capacity to complete the first week of development *in vitro*. Simple media capable of supporting mouse development from the two-cell to blastocyst stage *in vitro* have been in use for over two decades. Initially, the persistence of a 'two-cell' culture block impeded development of one-cell zygotes to the blastocyst stage but, recently, media (most notably CZB and KSOMaa) have been designed specifically to circumvent this problem in this species (Chatot *et al.*, 1990; Summers *et al.*, 1995). In this regard, mouse embryos are somewhat atypical as their apparent ability to adapt to culture environments is not equally shared by early embryos of other species.

The development of serum-supplemented media and embryo co-culture methods for supporting bovine development *in vitro* occurred during the mid- to late 1980s (Gandolfi and Moor, 1987; Sirard *et al.*, 1988). These first systems were critical for advancing research investigating early mammalian development and also for applying assisted reproductive technologies to domestic species. Ovine embryo culture systems originated from synthetic oviduct fluid medium (SOFM) which was designed from concentrations of salts and energy metabolites found in sheep oviductal fluid (Tervit *et al.*, 1972; Walker *et al.*, 1996).

The complete removal of serum from culture protocols has been more difficult, but is clearly an important goal as evidenced by increasing reports of the negative effects of serum on early development (Farin and Farin, 1995; Thompson *et al.*, 1995; Walker *et al.*, 1996). Transitional stages from serum supplementation have included replacement with bovine serum albumin (BSA), and most recently the addition of polyvinyl alcohol (PVA) and amino acids (Gardner *et al.*, 1994; Keskintepe *et al.*, 1995; Walker *et al.*, 1996). With these advances it is now possible to record similar developmental frequencies to the blastocyst stage for bovine zygotes placed into culture under complex or simple media systems, as is displayed in Table 1.

The use of simple defined conditions is imperative for studies directed at examining the physiological roles of 'embryotrophic' growth factors on early development. It is very difficult to formulate clear conclusions about effects on development when suboptimal culture conditions are used. We believe this limitation in culture conditions has especially impeded progress in understanding the regulation of development in early livestock embryos.

Table 1. Development of bovine *in vitro* matured and inseminated oocytes in complex versus simple culture media

Treatment	Zygotes[a]	Cleavage[b]	Morulae[c]	Blastocysts[d]
TCM-199+ serum + embryo co-culture[e]	285	73.8 ± 8.3%	43.0 ± 6.8%	31.2 ± 4.6%
cSOFM[f]	108	71.2 ± 7.4%	46.9 ± 8.7%	39.1 ± 5.1%[g]

[a]number of replicates = 3; [b]frequency (mean + SD) of zygotes at two-cell stage or further at 72 h post insemination (p.i.); [c]frequency (mean + SD) of cleaved zygotes reaching the morula stage (> 32 cell zygotes) at day 6 p.i.; [d]frequency (mean + SD) of cleaved zygotes reaching the blastocyst stage at day 8 p.i; [e]culture medium composed of TCM-199 medium + 10% steer serum; zygotes cultured in 50 µl culture drops with primary bovine oviduct vesicle cultures and under a 5% CO_2 in air culture atmosphere; [f]serum-free culture medium composed of modified synthetic oviduct fluid medium (cSOFM; Keskintepe *et al.*, 1995); zygotes cultured in 50 µl culture drops under a 5% CO_2:5%O_2:90%N_2 culture atmosphere; [g]$P < 0.05$, blastocyst formation for cSOFM versus TCM-199 + serum + coculture.

Insulin-like Growth Factors, Receptors and Binding Proteins

IGF ligands

Bovine IGF-I is a 70 amino acid, basic, single chain polypeptide, with a molecular mass of 7649 daltons. The bovine cDNA is 93% identical to the human sequence, and the amino acid sequence is 96% conserved (Fotsis *et al.*, 1990). Three disulfide bridges maintain tertiary structure of the molecule. The IGF-II protein is highly conserved between species (the 180 amino acids that encode the mature bovine and ovine IGF-II clones are identical) and rat, human, bovine and ovine forms differ at only one amino acid (Brown *et al.*, 1990). The precursor molecule contains a 24-residue amino-terminal signal peptide, a 67 amino acid, mature IGF-II polypeptide and an 89 amino acid carboxyl terminal. Bovine IGF-II has over 60% identity with IGF-I (Brown *et al.*, 1990; Fotsis *et al.*, 1990).

IGF-I/type-1 receptor

The actions of IGF-I and IGF-II are mediated largely through the IGF-I receptor (LeRoith *et al.*, 1995). The IGF-I receptor is synthesized as a single chain polypeptide. Post-translational modifications include cleavage of a signal polypeptide and further cleavage into a 707 amino acid, extracellular α-subunit and a 626 amino acid, transmembrane β-subunit. The α- and β- subunits are linked by disulfide bonds. Two αβ complexes are joined by additional disulfide bonds creating the mature $\alpha_2\beta_2$ receptor. Binding of IGF ligands is mediated by the extracellular α-subunit within a cysteine-rich region. Tyrosine kinase activity occurs in the cytoplasmic β-domain. Binding of IGF ligand to the α-subunit stimulates phosphorylation of both tyrosine and serine residues (LeRoith *et al.*, 1995). Autophosphorylation of the IGF-I receptor results in multiple signalling pathway cascades leading to the stimulation of cell growth (LeRoith *et al.*, 1995; Jones and Clemmons, 1995).

IGF-II–mannose-6-phosphate receptor

The IGF-II/M6P receptor is a monomeric 215 kDa glycoprotein with high IGF-II binding affinity, binding IGF-I at 500-fold lower level than IGF-II, with no affinity for insulin (for review see Schultz and Heyner, 1993; Jones and Clemmons, 1995). Sequence comparisons of the IGF-II receptor and the cation-independent mannose-6-phosphate receptor revealed identical molecules. The binding sites

for IGF-II and M6P are distinct and both ligands can bind simultaneously to the receptor (Morgan *et al.*, 1987). The IGF-II receptor protein contains a large extracellular domain, comprising 93% of the total receptor, a single transmembrane domain and a small cytoplasmic tail. Fifteen repeat sequences of eight conserved cysteine residues, a single fibronectin type II repeat and 19 *N*-linked glycosylation sites are located on the extracellular domain (Morgan *et al.*, 1987). The binding of IGF-II to the receptor results in internalization and degradation of IGF-II (Morgan *et al.*, 1987). It is still unclear whether the IGF-II receptor has a biological role beyond regulating free concentrations of IGF-II. A soluble form of the IGF-II/M6P receptor generated by proteolytic cleavage of the membrane bound receptor has been identified in rats (for review, see Jones and Clemmons, 1995).

Insulin-like growth factor binding proteins (IGFBPs)

The IGFs are almost entirely bound *in vivo* to high-affinity IGF-binding proteins of which there are at least six members (for review see Clemmons, 1993; Murphy and Barron, 1993; Jones and Clemmons, 1995). All IGFBPs display structural homology, bind IGF-I and IGF-II specifically and have a negligible affinity for insulin. Sequence alignments of IGFBPs reveal regions of homology within the amino- and carboxyl-terminal regions. The positions of 18 cysteines, which participate in the formation of disulfide bridges and contribute to three-dimensional structure, are conserved in IGFBPs 1–5. The rat IGFBP-6 sequence lacks two and the human IGFBP-6 sequence lacks four of the 18 conserved cysteines found in the other IGFBPs. In serum, approximately 75% of the circulating IGF is complexed with IGFBP-3, and an 88 kDa glycoprotein, the acid labile subunit (ALS), forming a 150 kDa protein complex. This 150 kDa complex prolongs the half-life of IGFs in serum to 12–15 h, which is considerably longer than the 10 min half-life of free IGFs. The half-life of free binding proteins is between 30 and 90 min. IGFBPs can inhibit or potentiate IGF action under various conditions (for review see Clemmons, 1993; Murphy and Barron, 1993; Jones and Clemmons, 1995). The characterization of specific proteases for the IGFBPs has further complicated the situation as these proteases cleave binding proteins into forms with altered affinity for the IGFs. IGFBPs are subject to post-translational modifications, and direct cellular effects, in which binding to IGF ligand is not necessary, have been described (Jones and Clemmons, 1995).

Oviductal Growth Factors and Binding Proteins

Oviductal fluid provides an environment in which fertilization and early embryonic growth take place (Leese, 1988). The oviduct may provide an environment rich in 'embryotrophic factors' capable of enhancing development (Wiseman *et al.*, 1992). The precise role these molecules play in supporting early mammalian development is still under investigation but growth factors certainly perform roles expected for 'embryotrophic' factors. We characterized the expression of transcripts encoding basic fibroblast growth factor (bFGF), transforming growth factor α (TGF-α), TGF-β1, TGF-β2; platelet-derived growth factor (PDGF-A), IGF-I, and IGF-II in bovine and ovine oviduct primary cultures by applying reverse transcription–polymerase chain reaction methods (RT–PCR; Watson *et al.*, 1992, 1994).

The production of IGF-I and IGF-II by the oviduct has been established for several species (Giudice *et al.*, 1992; Wiseman *et al.*, 1992; Carlsson *et al.*, 1993; Winger *et al.*, 1997). Insulin, IGF-I and IGF-II of maternal origin have been detected in the murine reproductive tract (Murphy and Barron, 1993). Likewise transcripts and polypeptides encoding IGF-I have been reported in the rat fallopian tube (Carlsson *et al.*, 1993). Our more recent analysis of oviduct growth factors mapped out the distribution of both mRNAs and polypeptides encoding IGF-I and IGF-II in bovine oviduct and in primary cultures (Xia *et al.*, 1996). Bovine primary oviduct cultures released ten times more IGF-II than IGF-I into the culture medium (Winger *et al.*, 1997). Schmidt *et al.* (1994) reported the localization of IGF-I mRNAs in the bovine oviduct during the entire oestrous cycle by RT–PCR and northern blot analysis. IGF-I mRNA expression increased after ovulation, indicating a possible role for IGF-I following ovulation by either influencing oviduct function or early embryo development.

Table 2. Growth factor ligand mRNAs during preimplantation development

Factor	Mouse	Cow	Sheep
Insulin	No	No	No
IGF-I	2C-blast	1C-blast	1C-blast
IGF-II	2C-blast	1C-blast	1C-blast
TGF-α	1C-blast	1C-blast	1C-blast
EGF	No	No	No
NGF	No	No	No
bFGF	No	1C-8/16C	1C-8/16C
TGFβ1	1C-blast	1C-blast	1C-blast
PDGF-α	2C-blast	1C-blast	not investigated

IGF: insulin-like growth factor; TGF-α: transforming growth factor α; EGF: epidermal growth factor; NGF: nerve growth factor; bFGF: basic fibroblast growth factor; PDGF: platelet-derived growth factor; blast: blastocyst; C: cell.
Data from Schultz and Heyner (1993); Kaye and Harvey (1995).

IGF-I protein has also been localized in human oviductal epithelial cells (Giudice *et al.*, 1992) and in porcine oviductal fluid (Wiseman *et al.*, 1992). Furthermore, IGF-I and IGF-II polypeptides may be transported to the oviduct via the circulation, as reported in the mouse (Murphy and Barron, 1993). This source of IGFs could augment the low expression observed within intact oviduct and increase the overall amounts of IGF polypeptides associated with these epithelial cells.

IGFBPs 1–4 have been detected in the human oviduct (Giudice *et al.*, 1992). Experiments conducted in our laboratory detected transcripts encoding IGFBP-2, -3, -4, and -5 in bovine oviduct and primary oviduct cultures by RT–PCR analysis (Winger *et al.*, 1997). The mRNAs encoding IGFBP-1 were not detected and IGFBP-6 mRNAs were not consistently detected in all oviduct sample replicates. Western ligand blot analysis revealed four IGFBPs of approximate molecular masses 24 kDa, 31 kDa, 36 kDa and a broad band extending from 46 to 53 kDa in bovine oviduct conditioned media. We confirmed the identity of the 24, 31 and 36 kDa proteins by western immunoblot as IGFBP -4, -5, and -2, respectively. The 46–53 kDa broad band represented IGFBP-3.

Embryonic Growth Factors and Binding Proteins

Data collected from studies applying RT–PCR and immunolocalization to characterize the expression of a number of growth factor genes during murine preimplantation development are summarized in Table 2 (for review see Schultz *et al.*, 1993; Schultz and Heyner, 1993; Kaye and Harvey, 1995). These include mRNAs encoding TGF-α , TGF-β1, TGF-β2, TGF-β3, PDGF-A, Kaposi's sarcoma-type growth factor (kFGF) , IGF-I and IGF-II. Transcripts encoding cytokines such as interleukin 3 (IL-3), interleukin 6 (IL-6) and leukaemia inhibitory factor (LIF) also are expressed by the blastocyst during early mouse development. The activation of growth factor ligand and receptor genes is selective, as transcripts encoding several factors including EGF, nerve growth factor (NGF) and insulin have not been detected during the first week of development for any mammalian species (Watson *et al.*, 1992, 1994). Until recently it was thought that IGF-I was not produced by the preimplantation mouse embryo until the eight-cell stage. However, the use of RNA extracts from larger embryo pools has led to the detection of IGF-I mRNAs in all murine preimplantation stages (Doherty *et al.*, 1994). Our failure to detect IGF-I mRNAs in rat embryos (Zhang *et al.*, 1994) may reflect this problem of transcript abundance, as we were able to detect IGF-I mRNAs during both bovine and ovine early development (Watson *et al.*, 1992; 1994).

RT–PCR amplicons for TGF-β 2, TGF-α , PDGF-A and IGF-II were detected throughout bovine early development (Watson *et al.*, 1992) and transcripts encoding IGF-II and TGF-α throughout ovine development (Watson *et al.*, 1994). Similar to mouse and rat early embryos, products encoding EGF, NGF, or

Table 3. Growth factor receptor mRNAs during preimplantation development

Factor	Mouse	Bovine	Ovine
Insulin-r	8C-blast	1C-blast	1C-blast
IGF-Ir	8C-blast	1C-blast	1C-blast
IGF-IIr	2C-blast	1C-blast	1C-blast
EGF-r	1C-blast	blast	no reports
PDGF-a	1C-blast	1C-blast	not investigated

IGF: insulin-like growth factor; EGF: epidermal growth factor; PDGF: platelet-derived growth factor; blast: blastocyst; C: cell;
Data from Schultz and Heyner (1993); Kaye and Harvey (1995).

insulin were not detected in bovine or ovine early embryos. However, transcripts encoding bFGF were detected only up to the eight- to16-cell stage in bovine embryos, and then declined markedly following the eight- to16-cell stage during early ovine development (Watson *et al.*, 1992, 1994).

A number of receptor genes are expressed in the early mouse embryo (summarized in Table 3) including the insulin-r, IGF-Ir, IGF-IIr, EGFr, PDGF-α r, and colony-stimulating factor 1r (CSF-I) (for review see Schultz and Heyner, 1993; Kaye and Harvey, 1995). In the mouse, the IGF type 1 receptor was detected by cell surface binding of IGF-I and IGF-II at the morula and blastocyst stages of development (Mattson *et al.*, 1988) and by gold-labelled IGF-I binding as early as the eight-cell stage (Smith *et al.*, 1993). The IGF-II/M6P receptor was first detected in two-cell mouse embryos (for review see Schultz and Heyner, 1993; Kaye and Harvey, 1995). Bovine and ovine early embryos express transcripts encoding PDGF α-r, insulin-r, and IGF-I-r and IGF-II-r throughout the first week of development (Watson *et al.*, 1992,1994).

Transcripts encoding IGFBPs 2–4 were detected throughout early bovine development while IGFBP-5 mRNAs were detected only weakly in bovine blastocysts (Winger *et al.*, 1997). mRNAs encoding IGFBPs 1 and 6 were not detected during this developmental interval in bovine embryos. In the mouse embryo, mRNAs encoding IGFBP-6 were detected only in blastocysts, while transcripts encoding IGFBP-2, -3 and -4 were detected throughout murine preimplantation development (Hahnel and Schultz, 1994). Transcripts encoding IGFBP-5 were not detected in any preimplantation stage in mice (Hahnel and Schultz, 1994).

IGFs in Bovine Parthenogenotes

Parthenogenetic embryos contain only maternal genetic material representing a set of genes derived completely from oogenesis. Imprinting implies that through epigenetic modification a particular genetic allele will become silenced, with expression resulting from the second (non-silenced) allele (DeChiara *et al.*, 1991; Latham *et al.*, 1994). IGF-II is an imprinted gene in which expression stems from only the paternal allele (DeChiara *et al.*, 1991). Imprinting of IGF-II may regulate IGF-II expression, since overexpression of IGF-II can be detrimental (see below). In contrast, expression of IGF-II receptor is the result of the maternal allele only (Baker *et al.*, 1993). Although imprinting of the IGF-II gene is well established by birth, a debate continues regarding the state of the imprint during preimplantation development (Rappolee *et al.*, 1992; Latham *et al.*, 1994). We have measured amounts of IGF-II released into medium by bovine parthenogenotes and contrasted these values with those observed for inseminated embryos. Blastocysts produced following fertilization released significantly greater amounts (mean ± SEM) of IGF-II (36.2 ± 3.9 pg per embryo) compared with parthenogenetic embryos (9.6 ± 2.8 pg per embryo, $P < 0.05$; Fig. 1). Bovine parthenogenetic blastocysts expressed transcripts encoding IGFBP-2, -3, –4 and -5 as was observed for *in vitro* fertilized controls (Fig. 2). The release of IGF-II by bovine parthenogenetic embryos is of interest. If imprinting influences gene expression (Rappolee *et al.*, 1992), it is possible that the IGF-II released is the result of an incomplete imprint, aberrant gene expression due to chromosomal ploidy or perhaps

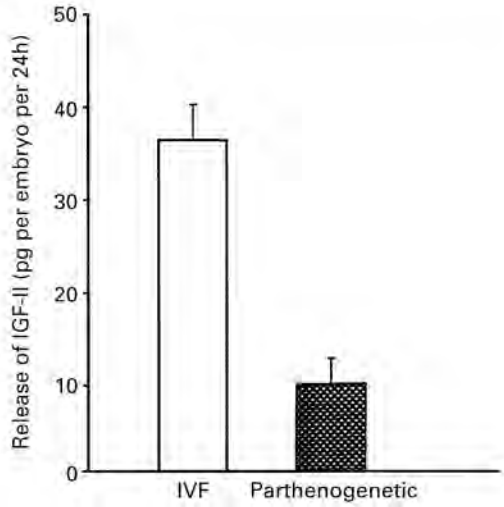

Fig. 1. Release of insulin-like growth factor II (IGF-II) from bovine parthenogenetic and *in vitro* fertilized (IVF) bovine blastocysts. For collection of conditioned media, inseminated and parthenogenetic blastocysts were removed from culture on day 7. They were washed three times in serum-free medium and groups of ten blastocysts (from both groups) were placed in 200 μl of serum-free TCM-199 incubation medium for 24 h (experimental, $n = 6$). Conditioned media were collected, lyophilized and resuspended in 50 μl for radioimmunoassay using recombinant human IGF-I and IGF-II iodinated to specific activities of 150–250 μCi μg^{-1} protein as outlined by Winger *et al.* (1997). Insulin-like growth factor binding proteins (IGFBPs) were extracted to release IGF–IGFBP complexes and precipitate the binding proteins. IVF blastocysts ($n = 6$) released significantly greater ($P < 0.05$) amounts of IGF-II than did parthenogenetic blastocysts. Release of IGF-I was below the limits of detection of the assay for both groups of embryos.

due to release of membrane bound IGF-II from embryos accumulated during culture. Alternatively, the IGF-II gene may not become imprinted until later stages of embryo development (Latham *et al.*, 1994), or the lower IGF-II release may be the result of lower overall transcription in less healthy parthenogenetic embryos. However, it would certainly appear that even bovine parthenogenotes are exposed to autocrine IGF-II during the first week of development.

The presence of gene products encoding the IGFs, their receptors and binding proteins in early embryos from several species indicates that IGFs expressed by the embryo or maternal tissues could exert receptor-mediated actions on the embryo and therefore influence growth and development by supporting the progression of embryos through the first week of development.

Biological Actions of IGFs

The actions of IGF *in vitro* include effects on protein and carbohydrate metabolism, and effects on cell replication and differentiation (Murphy and Barron, 1993; Schultz and Heyner, 1993; Jones and

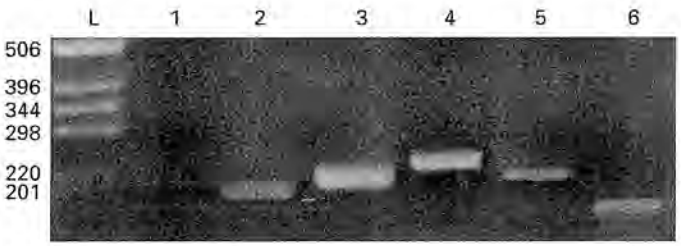

Fig. 2. Detection of mRNAs encoding insulin-like growth factor binding proteins (IGFBPs) in bovine parthenogenetic blastocysts by reverse transcription–polymerase chain reaction (RT–PCR). Bovine oocytes were activated by treatment with 7% ethanol for 5 min after *in vitro* maturation for 24 h. Activated oocytes were further treated with cytochalasin D (5 μg ml^{-1} for 6 h) to produce diploid chromosomal complements. Parthenogenetic zygotes were co-cultured with oviductal vesicles in TCM-199 medium supplemented with 10% steer serum to support development to the blastocyst stage. For RT–PCR analysis, total RNA was isolated from pools of 10–20 parthenogenetic blastocysts, and the RNA was reverse transcribed into cDNA as described by Watson *et al.* (1992, 1994). IGFBP primer sequences were derived from published human and bovine cDNA sequences. Expected sizes of products are: 239 bp (IGFBP-1); 186 bp (IGFBP-2); 210 bp (IGFBP-3); 222 bp (IGFBP-4); 215 bp (IGFBP-5) and 345 bp (IGFBP-6). The identity was confirmed by cloning each amplified product into a pCRII vector by the use of the TA cloning kit (Invitrogen) followed by base-specific termination of enzyme catalysed primer extension reactions using a T7 sequencing kit. Lanes are (L) ladder (bands from top to bottom: 516/506 bp, 396 bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp), while lanes 1–6 represent IGFBPs 1–6. Transcripts encoding IGFBP-2, -3, -4, and -5 were detected in bovine parthenogenetic blastocysts. Transcripts encoding IGFBP-1 and -6 were not detected; however, a smaller than expected product was detected with the IGFBP-6 primers. After sequence analysis, this product was found not to represent IGFBP-6.

Clemmons, 1995). IGF-I acts as a progression factor in the cell cycle. Quiescent cells in G0 when treated with a competence factor (PDGF, bFGF) progress to G1 and arrest. Treatment with IGF-I induces progression through the cell cycle leading to DNA synthesis and cell proliferation.

The functions of IGF-I and IGF-II on fetal development have been studied using gene targeting and transgenic approaches and the outcomes of these experiments are summarized in Table 4. Mice carrying copies of the human IGF-I gene fused to the metallothionein-I promoter have high IGF-I (for review see Baker *et al.*, 1993). These mice have increased body weight largely due to increased muscle, brain, spleen, kidney and pancreas mass. Most mice with a disrupted IGF-I gene died at birth and those that survived displayed growth retardation, reaching only 60% of normal birth weight (Liu *et al.*, 1993). IGF-II homozygous null mutants produce live pups with birth weights 60% of normal size with prenatal growth defects starting at about day 13.5 (DeChiara *et al.*, 1991). IGF-I and IGF-II double 'knock-outs' displayed complete neonatal lethality with birth weights 30% of normal. Type-1 receptor deficient mice displayed fetal growth deficits of 45% of normal birth weight and complete neonatal lethality (Liu *et al.*, 1993). The IGF-I–IGF-Ir mutants displayed the same phenotype as the IGF-Ir (−/−) mice, thus indicating that the essential functions of IGF-I are mediated through the IGF-Ir. IGF-II–IGF-Ir knockouts resulted in lower fetal birth weight than IGF-Ir knockouts alone, indicating that IGF-II must be acting by a route in addition to IGF-Ir pathways. Mice deficient in the IGF-II/M6P receptor resulted in larger birth weights and lethality in nearly all

Table 4. Summary of principal IGF family null mutant phenotypes in mice

Gene	Phenotype
IGF-I	60% of normal weight; most die at birth
IGF-II	60% of normal weight; prenatal defects start at day 13.5
IGF-I and IGF-II	30% of normal weight; neonatal lethality
IGF-Ir	45% of normal weight; neonatal lethality
IGF-I and IGF-Ir	45% of normal weight; neonatal lethality
IGF-II and IGF-Ir	30% of normal weight; neonatal lethality
IGF-IIr	Increased birth weight; lethality
IGF-II and IGF-IIr	Normal birth weights

IGF: insulin-like growth factor.
Data from Baker *et al.* (1993).

mutants. If the IGF-II gene of these mice was knocked-out in combination with the IGF-II/M6P receptor, the phenotype was rescued and normal birth weights were observed (for review see Baker *et al.*, 1993; Kaye and Harvey, 1995; Jones and Clemmons, 1995). This result indicated that in the embryo the IGF-II/M6P receptor regulates amounts of IGF-II that may become lethal if increased. Imprinting of the IGF-II gene may represent an additional control important for regulating IGF-II (Rappolee *et al.*, 1992).

IGF Regulation of Preimplantation Development

Several growth factors including IGFs, PDGF, bFGF, TGF-α , and TGF-β , when added exogenously to culture environments stimulate embryo development (for review see Schultz and Heyner, 1993; Schultz *et al.*, 1993; Kaye and Harvey, 1995; Stewart and Cullinan, 1997). These effects on murine preimplantation embryos include increased amino acid uptake, DNA, RNA and protein synthesis, increased embryo cell number and increased frequencies of development to blastocyst stage. The majority of experiments have investigated influences on mouse preimplantation development. Our knowledge of other species is very limited. As our ability to support preimplantation development of non-murine species in simple media improves, it is expected that progress in understanding the physiology of growth factor action on early development in these species will increase sharply.

The anabolic and mitogenic influences of IGF-I and IGF-II on murine preimplantation development are well characterized (see Schultz and Heyner, 1993; Kaye and Harvey, 1995). Treatment of murine blastocysts with IGF-I stimulates increased protein synthesis, inner cell mass proliferation, and increased endocytosis and glucose transport by trophectoderm (Kaye and Harvey, 1995). Effects on early development can be grouped into short and long term and may be mediated by interactions with either the insulin or IGF-Ir. For example, increased protein synthesis can be observed after treatment for 4 h and this response is probably mediated by the insulin-r (Kaye and Harvey, 1995). In contrast, proliferative and morphological responses are probably mediated by the type I-r (Kaye and Harvey, 1995). Recent studies have indicated that IGF-I exerts a beneficial effect on development of early porcine embryos *in vitro* (Xia *et al.*, 1994). The results from bovine studies are not as clear, some authors suggest that IGF-I has little influence on developmental frequencies (Larson *et al.*, 1992), while others report dose responsive influences on development of bovine zygotes to the blastocyst stage (Herrler *et al.*, 1992). It is too early to make definitive conclusions regarding the impact of IGF-I on early development of species such as the cow.

Addition of IGF-II to the culture medium of mouse embryos stimulates increased protein synthesis, cell number and frequencies of development to the blastocyst stage (Rappolee *et al.*, 1992; Schultz and Heyner, 1993; Kaye and Harvey, 1995). These stimulatory effects of IGF-II can be negated by embryo culture in the presence of IGF-II antisense oligodeoxynucleotides (Rappolee *et al.*, 1992). It is unlikely that these IGF-II effects on early development are mediated by interactions with the type 2/M6P receptor. Experiments with mutant IGF-II (modified to interact only with the

type 2 and not type 1 receptor) did not result in any stimulatory influences on early development (Rappolee *et al.*, 1992). Furthermore, IGF-II-mediated influences are propagated at the same EC_{50} value as are IGF-I effects implying that both are mediated via the type I IGF-I-r (Kaye and Harvey, 1995). Although the IGF-II mRNA and protein expression patterns have been characterized in early embryos from several other mammalian species (as summarized above), very few additional studies have investigated the physiological effects of IGF-II on early development. We have documented that small pools of bovine blastocysts release greater amounts of IGF-II than IGF-I into culture medium (Winger *et al.*, 1997) and human blastocysts in culture also release IGF-II (Hemmings *et al.*, 1992).

IGFBPs are certain mediators of IGF-I and IGF-II influences on early development. Although mRNAs encoding these genes have been detected during mouse and bovine early development as well as their polypeptides in oviductal fluids from several species (Hahnel and Schultz, 1994; Winger *et al.*, 1997), no studies to date have defined their specific interactions during the first week of development. Studies must be conducted to provide insight into their roles and to elucidate fully the impact of IGF paracrine and autocrine circuits in regulating this early developmental interval.

Significance of IGF Regulation of Early Development

It is clear that growth factors collectively influence a number of events during early development. The preimplantation mammalian embryo develops in an environment that includes all of the necessary gene products (ligands, receptors and binding proteins) required to support the development of 'embryotrophic' maternal and embryonic IGF circuits (Fig. 3). However, phenotypes arising from IGF null mutants indicate that expression of IGF ligand and receptor genes are not essential for preimplantation development. These outcomes may be explained by the autonomous nature of early development. Alternatively, the expression of other growth factor ligand and receptor gene families may regulate their expression in the absence of IGF gene products to provide necessary regulation of early developmental events. It is of concern that the results obtained from rodent species may be simply extended to include all mammalian species. In livestock species it has been possible to influence fetal growth and development (without compromising development to the blastocyst stage) by exposing preimplantation embryos to serum supplemented culture environments (Farin and Farin, 1995; Thompson *et al.*, 1995; Walker *et al.*, 1996). These observations demonstrate that it is possible to affect the preimplantation developmental programme and induce longer term consequences that are not revealed until fetal or postpartum stages. The impact of creating a null mutant may therefore not reveal itself simply by influencing developmental frequencies. Although blastocyst development occurs in all mouse IGF ligand and receptor null mutants, the blastocysts may not be 'normal' and the deleterious influences contributing to abnormal fetal phenotypes may be initiated during the first week of development. The analysis of null mutants should therefore include contrasting gene expression patterns of mutant and wild type preimplantation embryos.

Clearly further experimentation directed at elucidating the functional significance of IGF expression during preimplantation development of other species is required. With the development of effective simple media for supporting early development of bovine, ovine and pig embryos, it is now possible to apply antisense oligodeoxynucleotide approaches to examine consequences of downregulating IGF expression in these species. Since the 'primary goal' of all culture regimens is to support the production of large numbers of healthy blastocysts, it is imperative that the effects of culture on blastocyst quality be defined. Moreover, blastocyst quality can no longer be evaluated solely on the basis of morphology, since there is a clear disparity between morphological appearance of transferred blastocysts and pregnancy outcome. We would propose that evaluation procedures be expanded to include an assessment of the biological/biochemical events occurring within the zygote. Monitoring variations in IGF expression patterns may represent a means for attaining this goal. Continuation of such experimental approaches should ultimately assist in the production of defined conditions for the production of viable bovine preimplantation embryos and also will certainly elucidate the contributions of these growth factor genes during early development.

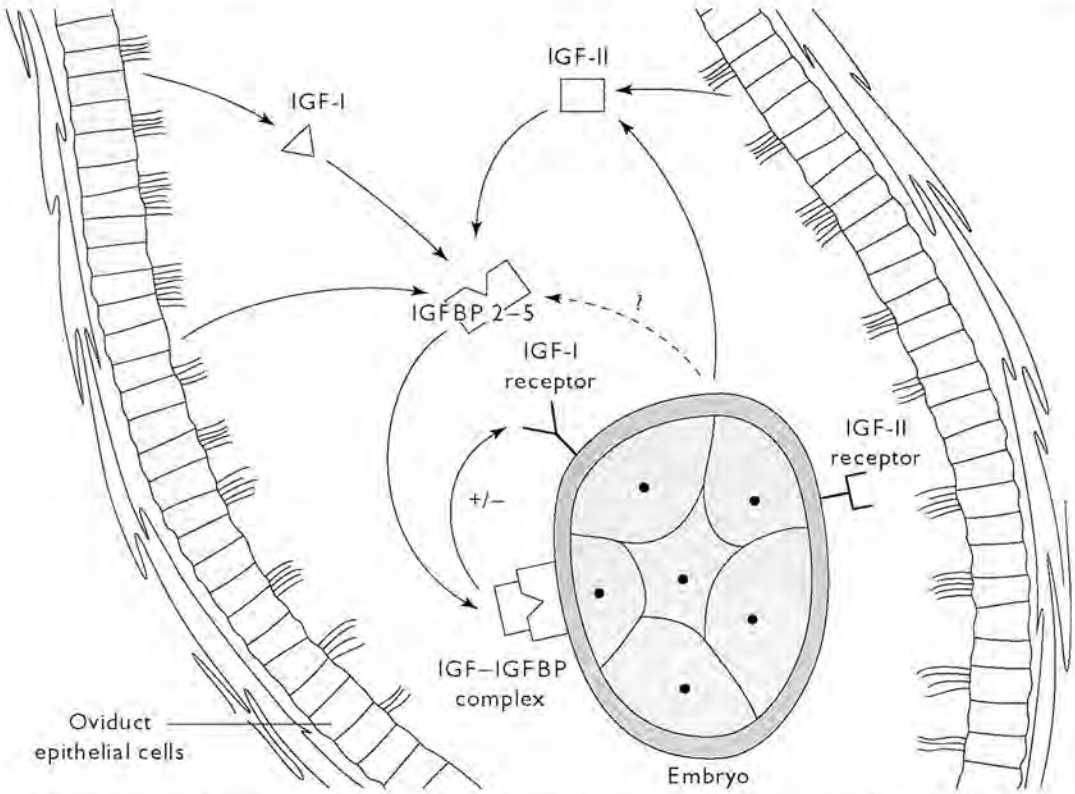

Fig. 3. Maternal and embryonic IGF circuits: detectable amounts of immunoreactive IGF-I and IGF-II are released from primary bovine oviduct cell cultures, and detectable amounts of IGF-II are released from bovine blastocysts. Bovine oviduct primary cultures express transcripts encoding IGFBPs 2–5 and release IGFBPs 2–5 into conditioned media. Bovine zygotes express mRNAs encoding IGFBPs 2–4 through to the blastocyst stage. mRNAs encoding IGFBP-5 were detected in bovine blastocyts. The detection of IGF-I, IGF-II and IGFBPs 2–5 in the culture environment indicates that IGF paracrine and autocrine regulatory circuits are present and may contribute to the events that regulate bovine early development. Future efforts must be directed at measuring the direct influences of these ligands on early development and determining the IGF–IGFBP dynamics that oversee their actions.

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References

Baker J, Lie JP, Robertson EJ and Efstratiadis A (1993) Role of insulin-like growth factors in embryonic and postnatal growth *Cell* **75** 73–82

Brown WM, Dziegielewska KM, Foreman RC and Saunders NR (1990) The nucleotide and deduced amino acid sequences of insulin-like growth factor II cDNAs from adult bovine and fetal sheep liver *Nucleic Acids Research* **18** 4614

Carlsson B, Hillensjo T, Nilsson A, Tornell J and Billig H (1993) Expression of insulin-like growth factor I (IGF-I) in the rat fallopian tube: possible autocrine and paracrine action of fallopian tube-derived IGF-I on the fallopian tube and on the preimplantation embryo. *Endocrinology* **133** 2031–2039

Chatot CL, Lewis JL, Torres I and Ziomek CA (1990) Development of 1-cell embryos from different strains of mice in CZB medium *Biology of Reproduction* **42** 432–440

Clemmons DR (1993) IGF binding proteins and their functions *Molecular Reproduction and Development* **35** 368–375

DeChiara TM, Efstratiadis A and Robertson EJ (1991) Paternal

imprinting of the mouse insulin-like growth factor II gene *Cell* **64** 849–859

de la Sota RL, Simmen FA, Diaz T and Thatcher WW (1996) Insulin-like growth factor system in bovine first-wave dominant and subordinant follicles *Biology of Reproduction* **55** 803–812

Doherty AS, Temeles GJ and Schultz R (1994) Temporal pattern of IGF-I expression during mouse preimplantation embryogenesis *Molecular Reproduction and Development* **37** 21–26

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

Fotsis T, Murphy C and Gannon F (1990) Nucleotide sequence of the bovine insulin-like growth factor I (IGF-I) and its IGF-IA precursor *Nucleic Acids Research* **18** 676

Gandolfi F and Moor RM (1987) Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells *Journal of Reproduction and Fertility* **81** 23–28

Gardner DK, Lane M, Spitzer A and Batt PA (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development *Biology of Reproduction* **50** 390–400

Giudice LC, Dsupin BA, Irwin JC and Eckert RL (1992) Identification of insulin-like growth factor binding proteins in human oviduct *Fertility and Sterility* **57** 294–301

Hahnel A and Schultz GA (1994) Insulin-like growth factor binding proteins are transcribed by preimplantation mouse embryos *Endocrinology* **134** 1956–1959

Hemmings R, Langlais J, Falcone T, Granger L, Miron P and Guyda H (1992) Human embryos produce transforming growth factor activity and insulin like growth factor II *Fertility and Sterility* **58** 101–104

Herrler A, Lucas-Hahn A and Niemann H (1992) Effects of insulin-like growth factor-I in *in vitro* production of bovine embryos *Theriogenology* **37** 1213–1224

Jones JI and Clemmons DR (1995) Insulin-like growth factors and their binding proteins: biological actions *Endocrine Reviews* **16** 3–34

Kane MT, Morgan PM and Coonan C (1997) Peptide growth factors and preimplantation development *Human Reproduction Update* **3** 137–157

Kaye PL and Harvey MB (1995) The roles of growth factors in preimplantation development *Progress in Growth Factor Research* **6** 1–26

Keskintepe L, Burnley CA and Brackett BG (1995) Production of viable bovine blastocyst in defined *in vitro* conditions *Biology of Reproduction* **52** 1410–1417

Larson RC, Ignotz GG and Currie WB (1992) Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle *Development* **115** 821–826

Latham KE, Doherty AS, Scott CD and Schultz RM (1994) Igf2r and Igf2 gene expression in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos: absence of regulation by genomic imprinting *Genes and Development* **8** 290–299

LeRoith D, Werner H, Beitner-Johnson D and Roberts CT, Jr (1995) Molecular and cellular aspects of the insulin-like growth factor I receptor *Endocrine Reviews* **16** 143–163

Lesse HJ (1988) The formation and function of oviduct fluid *Journal of Reproduction and Fertility* **82** 843–856

Ling NC, Liu XJ, Malkowski M, Guo YL, Erickson GF and Shimasaki S (1993) Structural and functional studies of insulin-like growth factor binding proteins in the ovary *Growth Regulation* **3** 70–74

Liu JP, Baker J, Perkins AS, Robertson EJ and Efstratiadis A (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igfr) *Cell* **75** 59–72

Mattson BA, Rosenblum IY, Smith RM and Heyer S (1988) Autoradiographic evidence for stage specific insulin binding to mouse embryo development *Diabetes* **37** 585–590

Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA and Rutter WJ (1987) Insulin-like growth factor II receptor as a multifunctional binding protein *Nature* **326** 300–307

Murphy LJ and Barron DJ (1993) The IGFs and their binding proteins in murine development *Molecular Reproduction and Development* **35** 376–381

O'Neill C (1997) Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos *in vitro*. *Biology of Reproduction* **56** 229–237

Rappolee DA, Sturm KS, Behrendsten O, Schultz GA, Pedersen RA and Werb Z (1992) Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos *Genes and Development* **6** 939–952

Schmidt A, Einspanier R, Amselgruber W, Sinowatz F and Schams D (1994) Expression of insulin-like growth factor I (IGF-I) in the bovine oviduct during the oestrus cycle *Experimental and Clinical Endocrinology* **102** 364–369

Schultz GA and Heyner S (1993) Growth factors in preimplantation embryos *Oxford Reviews of Reproductive Biology* **15** 43–81

Schultz GA, Hahnel A, Arcellana-Panlilio M, Wang L, Goubau S, Watson AJ and Harvey M (1993) Expression of IGF ligand and receptor genes during preimplantation mammalian development *Molecular Reproduction and Development* **35** 414–420

Sirard MA, Parrish JJ, Ware MI, Leibfried-Rutledge MI and First NL (1988) The culture of bovine oocytes to obtain developmentally competent embryos *Biology of Reproduction* **39** 546–552

Smith RM, Garside WT, Aghayan M, Shi C-Z, Shah N, Jarret L and Heyner S (1993) Mouse preimplantation embryos exhibit receptor-mediated binding and transcytosis of maternal insulin-like growth factor I *Biology of Reproduction* **49** 1–12

Stewart CL and Cullinan EB (1997) Preimplantation development of the mammalian embryo and its regulation by growth factors *Developmental Genetics* **21** 91–101

Summers MC, Bhatnagar PR, Lawitts JA and Biggers JD (1995) Fertilization *in vitro* of mouse ova from inbred and outbred strains: complete preimplantation embryo development in glucose-supplemented KSOM *Biology of Reproduction* **53** 431–437

Tervit HR, Whittingham DG and Rowson LEA (1972) Successful culture *in vitro* of sheep and cattle ova *Journal of Reproduction and Fertility* **30** 493–497

Thompson JG, Gardner DK , Pugh PA, McMillan WH and Tervit HR (1995) Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos *Biology of Reproduction* **53** 1385–1391

Walker SK, Hill JL, Kleemann DO and Nancarrow CD (1996) Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations *Biology of Reproduction* **55** 703–708

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 Reproduction and 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** 725–733

Winger QA, de los Rios P, Han VKM, Armstrong DT, Hill DJ and Watson AJ (1997) Bovine oviductal and embryonic insulin-like growth factor binding proteins: possible regulators of embryotrophic insulin-like growth factor circuits *Biology of Reproduction* **56** 1415–1423

Wiseman DL, Henricks DM, Eberhardt DM and Bridges WC (1992) Identification and content of insulin-like growth factors in porcine oviductal fluid *Biology of Reproduction* **47** 126–132

Xia P, Tekpetey FR and Armstrong DT (1994) Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development *in vitro*, and on granulosa and cumulus cell biosynthetic activity *Molecular Reproduction and Development* **38** 373–379

Xia P, Han VKM, Viuff D, Armstrong DT and Watson AJ (1996) Expression of insulin-like growth factors in two bovine oviductal cultures employed for embryo co-culture *Journal of Endocrinology* **148** 41–53

Zhang X, Kidder GM, Watson AJ, Schultz GA and Armstrong DA (1994) Possible roles of insulin and insulin-like growth factors in rat preimplantation development. Investigation of gene expression by reverse transcription–polymerase chain reaction *Journal of Reproduction and Fertility* **100** 375–380