Expression of Insulin-like Growth Factors (IGF) and Their Binding Proteins (BP) in Fertilized and Parthenogenetic Bovine Embryos: Regulators of Early Development

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ABSTRACT

IGFBP -2, -3, -4 and -5 transcripts were observed in oviduct primary cultures and bovine blastocysts. IGFBP-1 transcripts were not detected in any samples and IGFBP-6 transcripts were detected inconsistently in oviduct cells. Ligand blot analysis with [^{125}I]-IGF-II revealed four polypeptide bands in oviductal conditioned media samples. Western immunoblot analysis confirmed the identity of the 24 kDa, 31 kDa and 36 kDa species as IGFBP -4, -5, and -2 respectively. The IGF-II release from oviductal vesicles was greater than from monolayer cultures ($p<0.005$). No difference in IGF-I release between monolayer and vesicle cultures was observed. Pools of ten fertilized blastocysts released 36.2 $\pm$ 3.9 pg of IGF-II/embryo/24h, significantly greater levels than released from parthenogenetic embryos. Parthenogenetic blastocysts expressed IGFBP 2-5 in an identical pattern to fertilized blastocysts. The results suggest that the biological availability of maternally derived IGF may be regulated by IGFBPs in support of bovine preattachment development.

Keywords: bovine culture, embryo development, oviduct, insulin-like growth factor binding protein, parthenogenesis

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LIST OF ABBREVIATIONS

aa	Amino acid
AcAcid	Acetic acid
AIB	Aminoisobutyric acid
ALS	Acid labile subunit
bFGF	Basic fibroblast growth factor
bp	Base pair
BRL	Buffalo rat liver
°C	Degrees Celsius
Ca^{++}	Calcium
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
COC	Cumulus oocyte complex
CPM	Counts per minute
DAB	3-3' diaminobenzidine
DMAP	Dimethylaminopurine
DNA	Deoxyribonucleic acid
DSS	Disuccinimidyl suberate
ECM	Extracellular matrix

EGF	Epidermal growth factor
EtOH	Ethanol
g	Grams
GH	Growth hormone
h	Hour
HBSS	Hank's balanced salt solution
ICM	Inner cell mass
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-Ir	Insulin-like growth factor-I receptor
IGF/M6P	Insulin-like growth factor-II/manose 6 phosphate receptor
IGFBP	Insulin-like growth factor binding protein
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVMF	*In vitro* matured and *in vitro* fertilized
IVM	*In vitro* maturation
K^+	Potassium
Kb	Kilo base
kDa	Kilo daltons
L	Litres
LH	Luteinizing hormone

M	Molar
M6P	Mannose-6-phosphate receptor
MeOH	Methanol
Mg	Milligrams
Mg^{++}	magnesium
mM	Millimolar
mono	Monolayer
mRNA	Messenger ribonucleic acid
Na^{+}	Sodium
NaOH	Sodium hydroxide
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet-derived growth factor
PHEM	Pipes, hepes, EGTA, $MgCl_2$ buffer
RGD	Arg-Gly-Asp (amino acids)
RIA	Radioimmunoassay
RT-PCR	reverse transcription-polymerase chain reaction
SDS	Sodium-dodecyl-sulfate
SEM	Standard error of the mean
SS	steer serum
TCA	Trichloroacetic acid

TCM 199	Tissue culture media 199
TGF	Transforming growth factor
TPA	teradecanoylphorbol acetate
TBS	Tris buffered saline
TTBS	Tris buffered saline + Tween-20
µg	Microgram
µl	Microliter
µm	Micrometer

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 *The Preattachment Period of Mammalian Embryo Development*

Fertilization, beginning with the fusion of a single sperm cell with an oocyte, represents the beginning of new life. This union results in the joining of two haploid sets of chromosomes, thereby restoring the diploid complement of chromosomes. The ovary is the site of oocyte production and initiation of maturation. Each oocyte develops within a fluid filled structure called the follicle. Follicular oocytes are arrested at diplotene of the first meiotic division. Ovulation, the release of usually one oocyte in the cow, occurs upon the rupture of the follicle wall and results in the completion of the first meiotic division. Following the extrusion of the first polar body the oocyte again arrests awaiting sperm penetration (Xu & Greve, 1988). The oocyte migrates into the oviduct during this period. The oviduct provides passage for the sperm and becomes the site of fertilization. The oviduct then provides passage for the transport of the developing zygote to the uterus, where attachment to the uterine wall, placentation and fetal development occurs (Bazer & First, 1983).

The oviductal fluid provides an environment in which fertilization and early embryonic growth take place (Leese, 1988; Gandolfi *et al.*, 1989a,b; Heyner *et*

al., 1993). The embryo begins rapid mitotic divisions immediately following fertilization. This developmental interval is known as the early cleavage stages, and is characterized by rapid DNA replication (period S of cell cycle) and mitosis (period M) and almost no G_1 and G_2 periods (Xu & Greve, 1988). Little mRNA transcription occurs during this period and the mRNA present is the result of maternal transcripts stored in the oocyte (Plante *et al.*, 1994; Barnes & First, 1991; Barnes & Eyestone, 1990). The number of cells, called blastomeres, in the zygote continues to increase with the progression of these early divisions. Early cleavage occurs by partitioning of the cytoplasmic material, and little increase in zygote size occurs.

Compaction and Cavitation

The first morphogenic event of early development is called compaction. Compaction is a process that includes an increase in intrablastomeric cell to cell contact that obscures the individual cell outlines forming an uniform embryonic structure called a morula (Pratt *et al.*, 1982). This stage arises at the 32-64 cell stage of bovine development (McLaren, 1982). The morula begins to produce gene products necessary for the establishment of cell polarity (Kidder & McLachlin, 1985; Wiley *et al.*, 1990; Ziomek & Johnson, 1980). The differentiation of outer blastomeres into a polarized epithelium is a critical component for the next morphogenic event, cavitation. Cavitation results in the

formation of a fluid-filled structure called a blastocyst. A blastocyst is composed of two cell types, an outer epithelial trophectoderm (first epithelium) and the inner cell mass (progenitor cells of the embryo proper, Watson, 1992). Several gene products play a role in the onset and establishment of the blastocyst cavity; tight junction associated polypeptides (ZO-1), Na/K-ATPase, uvomorulin, gap junction, and growth factors (such as transforming growth factor-α, insulin-like growth factor (IGF) and epidermal growth factor) are all believed to participate in the events that coordinate trophectoderm cell polarity (Kidder & Watson, 1990; Watson, 1992). It is hypothesized that the blastocoel cavity may arise as a consequence of water movement into the cavity, driven, in part from the establishment of a trans-trophectoderm Na^+ gradient, that arises from a basolaterally localized trophectoderm Na^+/K^+ATPase (Watson & Kidder, 1988). The final morphogenetic event of early development involves the release of the zygote from the zona pellucida (zona hatching). Preattachment development, initiated in the oviduct at fertilization and leading to the formation of a hatched blastocyst, is essential for further embryonic development.

In vitro Bovine Culture

Understanding the events necessary for successful fertilization and development leading to a healthy pregnancy and birth will increase our ability to treat infertility. It is likely that many spontaneous abortions occur undetected

during early stages of pregnancy resulting in implantation failure steming from errors in early development. Bovine embryo production *in vitro* is an important vehicle for the study of the biological processes of oocyte maturation, fertilization and early embryo development. It is clear that the female reproductive tract is the optimal environment for embryo production, and it is unlikely that culture conditions can ever fully mimic these conditions. This is highlighted by the fact that *in vitro* produced embryos contain fewer cells, display morphological differences and lag in developmental rate compared to their *in vivo* counterparts (Bavister, 1988; Bowman & McLaren, 1970; Walker *et al.*, 1992; Iwasaki *et al.*, 1990). Culture methods employed for the mouse embryo have had limited application to the development of sucessful bovine embryo culture environments; however our ability to culture embryos to the blastocyst stage is steadily improving. Embryo co-culture employing primary bovine oviductal epithelial cells represents one such culture system that is effective for supporting cow (Xu *et al.*, 1992; Wiemer *et al.*, 1991) sheep (Watson *et al.*, 1994; Gandolfi and Moor, 1987) and pig early development (White *et al.*, 1989). The role of the oviduct in regulating development *in vivo* is not clearly defined. The production of IGF-I and IGF-II by the oviduct has been established for several species (Carlsson *et al.*, 1993; Wiseman *et al.*, 1992; Pfeifer & Chegini, 1994), including the cow (Kirby *et al.*, 1996). The expression of IGFs in the oviduct supports the hypothesis that the oviduct exerts some capacity to regulate early embryo development. Oviduct

specific glycoproteins, that are capable of binding to cow embryos, have been detected in oviductal fluid (Bavister, 1988; Boice *et al.*, 1990; Gandolfi *et al.*, 1989b). Furthermore, a recent study investigating the expression of Hoxa-10 indicates that this gene product is necessary for sucessful embryo implantation. Animals with a disrupted Hoxa-10 gene are sterile due to implantation failure (Satokata *et al.*, 1995) and Hoxa-10 mRNAs are preferentially expressed in the distal oviduct and uterus. Gene products such as the ones mentioned above may account for improved rates of embryo development that occur with co-culture systems.

One of the possible roles of the oviductal cells in culture is to provide "embryotrophic factors" capable of enhancing development. *In vivo*, the presence of growth factors in the oviduct may support the rapid cleavage period following fertilization (Wiseman *et al.*, 1992). Several growth factors when added exogenously to *in vitro* culture stimulated embryo development including; IGF (Harvey & Kaye, 1991b, 1992), platelet derived growth factor (PDGF) (Larson *et al.*, 1992a) epidermal growth factor (EGF) and transforming growth factor (TGF-α, TGF-β) (Dardik & Schultz, 1991; Larson *et al.*, 1992b). All are possible candidates for further study into their effects on bovine embryo development. The present study is focused on characterizing the insulin-like growth factor family to demonstrate a possible role of maternal paracrine growth factor circuits in regulating early embryonic development. 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 insulin-like growth factor binding proteins (IGFBPs) (de la Sota *et al.*, 1996). Seminal fluid contains both IGF-I, IGF-II and IGFBPs (Rosenfeld *et al.*, 1990; Baxter *et al.*, 1984; Rasasharma *et al.*, 1986). The zygote, following fertilization, is maintained in an IGF containing environment while free-living in the oviduct and the uterus (Geisert *et al.*, 1991; Wiseman *et al.*, 1992; Kirby *et al.*, 1996). For these reasons, my study was directed at characterizing the expression and presence of oviductal IGFs and IGFBPs.

Growth Factors in Cultured Early Bovine Embryos and Bovine Oviduct Cell Cultures

Bovine oviductal primary cell cultures express transcripts encoding bFGF, TGF-α, TGF-β1, TGF-β2, PDGF, IGF-I and IGF-II as detected by applying reverse transcription-polymerase chain reaction (RT-PCR) methods (Watson et al., 1992). Bovine preattachment embryos express mRNAs encoding these growth factors and the receptors for IGF-I (type-1 receptor), IGF-II (mannose-6-phosphate receptor) and the insulin receptor (Watson *et al.*, 1992). In the mouse, the presence of the 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). An intact type-1 receptor was detected by chemical cross-linking of ^{125}I-IGF-I using disuccinimidyl suberate (DSS) applied to freshly isolated and cultured pig trophoderm cells at day 15-19 of pregnancy (Corps *et al.*, 1990). The type-1 receptor was however, undetected in sections of day 4-10 intact pig embryos by immunocytohistochemistry but type-1 receptor was detected at day 20 of pregnancy. The IGF-II/M6P receptor was detected in 2-cell stage mouse (Harvey & Kaye, 1991a) and day 4 to 10 pig embryos (Chastant *et al.*, 1994). The presence of mRNA encoding both the IGFs and their receptors in bovine embryos and the presence of type-1 receptor in other mammalian preimplantation embryos, indicates that IGFs expressed by the embryo or maternal tissues could exert receptor mediated actions on the embryo and therefore influence growth and development. The distribution of polypeptides and transcripts encoding IGF-I and IGF-II has been recently mapped out in bovine oviductal monolayer and vesicle primary cultures (Xia *et al.*, 1996).

1.2 INSULIN-LIKE GROWTH FACTORS

1.2.1 *IGF-I*

Structure

Bovine IGF-I is a 70 amino acid, basic, single chain polypeptide, with a molecular weight 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 (Raschdorf *et al.*, 1988; Smith *et al.*, 1988).

The rat IGF-I gene produces two distinct mRNAs by differential splicing of six exons, differential polyadenylation and the use of multiple promoters. IGF-Ia is produced from exons 1 or 2 and 3, 4, and 6, while IGF-Ib is the product of exons 1 or 2 and 3, 4, 5 and 6. Exons 1 and 2 contain the 5' untranslated region including multiple translation initiation sites (Roberts *et al.*, 1987a,b). Transcription is regulated by two different promoters; the major promoter produces mRNA containing exon 1, the second produces mRNA containing exon 2 (Adamo *et al.*, 1993). Expression from the major promoter is found in all tissues, while mRNA derived from the second promoter is restricted to the liver (Lowe *et al.*, 1987; Hall *et al.*, 1992; Adamo *et al.*, 1989) The major promoter lacks TATA, CAAT and GC-rich regions, but contains a protein binding site isolated to nucleotides -18 to +78 of exon 1 (Ra An & Lowe, 1995; Adamo *et al.*, 1989, 1991). Promoter and repressor elements have been identified 500 kb upstream of exon 1 of the rat IGF-I gene (Huang *et al.*, 1995).

Regulation

Levels of circulating IGF-I are regulated by growth hormone (GH) (Mathews *et al.*, 1986; Roberts *et al.*, 1987b; Hynes *et al.*, 1987; Chin *et al.*, 1992). Hypophysectomy reduces serum IGF-I, and IGF-I in the liver, skeletal

muscle, heart, white adipose tissue, kidney, spleen, and testes, but not in the brain (Gosteli-Peter *et al.*, 1994). Infusion of exogenous GH restores the normal level of expression of IGF-I in these tissues. Dietary influences such as fasting and protein restriction, and disease such as diabetes reduce IGF-I expression (Clemmons & Underwood, 1991; Goldstein *et al.*, 1988; Bornfelt *et al.*, 1989). In these situations, supply of IGF-I in a maternal environment prior to implantation may be inadequate to support proper growth and development. Regulation of IGF-I in the fetus is independent of GH, and IGF-I is expressed by most tissues during early development (Han *et al.*, 1987, 1988a). In the uterus estrogen regulates IGF-I and is independent of GH levels (Murphy *et al.*, 1987; Norstedt *et al.*, 1989).

1.2.2 *IGF-II*

Structure

The IGF-II protein is highly conserved between species (within the 180 amino acids that produce 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% homology with IGF-I (Brown *et al.*, 1990; Fotsis *et al.*, 1990). The rat IGF-II gene contains 6 exons, 1,

2, and 3 are non-coding, 4, 5 and 6 encode pre-pro IGF-II (Frunzio *et al.*, 1986; Yamamoto *et al.*, 1990). Rat IGF-II contains 3 promoter regions P1, P2 and P3 initiating transcription of 3.8, 4.6 and 3.6 mRNAs respectively (Soares *et al.*, 1986). P2 and P3 contain TATA and GC-rich sequences recognizing transcription factor Sp1, and produce the more abundant transcripts (Evans *et al.*, 1988; Matsuguchi *et al.*, 1990). P1 lacks both TATA and GC-rich regions (Ueno *et al.*, 1989).

Regulation

IGF-II levels are reduced in the rat by fasting (Phillips *et al.*, 1989) and in the liver by treatment with glucocorticoids (Beck *et al.*, 1988). Protein and mRNA levels are high in the rat fetus and decline after birth (Moses *et al.*, 1980; Soares *et al.*, 1985, 1986; Brown *et al.*, 1986). In contrast human and bovine IGF-II serum levels are higher in the adult than in the fetus (Boulle *et al.*, 1993). In the mouse IGF-II is maternally imprinted and produces transcripts from only the paternal allele, except in the choroid plexus and leptomeninges where genomic imprinting does not arise (DeChiara *et al.*, 1990, 1991).

1.2.3 *IGF-I/Type-1 receptor*

The actions of IGF-I and IGF-II are largely mediated through the IGF-I receptor (Jones & Clemmons, 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 disufide 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 cysteine-rich region of the α-subunit stimulates phosphorylation of both tyrosine and serine residues (Steele-Perkins *et al.*, 1988; Kato *et al.*, 1993). Intracellular tyrosines 1131, 1135 and 1136 within the kinase domain of the β-subunit are the primary phosphorylation sites on the IGF-I receptor (Kato *et al.*, 1993; Li *et al.*, 1994). Autophosphorylation then occurs where the tyrosine kinase of one β-subunit phosphorylates residues on the other β-subunit (Frattali *et al.*, 1993). Autophosphorylation of the IGF-I receptor results in multiple signalling pathway cascades leading to the stimulation of cell growth (Izumi *et al.*, 1987; Chuang *et al.*, 1993; Sun *et al.*, 1993; Blenis, 1993; White & Kahn, 1994; LeRoith *et al.*, 1995).

1.2.4 *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 then IGF-II, with no affinity for insulin. Sequence comparisons of the IGF-II receptor and the cation-independent mannose-6-phosphate receptor revealed identical molecules (Lobel *et al.*,1987; Kiess *et al.*, 1988). The binding sites for IGF-II and M6P are distinct and both ligands can bind simultaneously (Braulke *et al.*, 1988). The IGF-II receptor protein contains a large extracellular domain, which comprises 93% of the total receptor, a single transmembrane domain and a small cytoplasmic tail. Fifteen repeat sequences of 8 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 the IGF-II (Oka *et al.*, 1985). It is still unclear if the IGF-II receptor has a biological role in addition to regulating levels of IGF-II by a scavenger like action. Experiments using blocking antibodies against the M6P receptor applied to various cell types show that IGF-II responses were not blocked (Mottola & Czech, 1984; Kiess *et al.*, 1987a). Similarly experiments blocking the IGF-I receptor with αIR3 antibody blocked IGF-II biological responses, indicating that IGF-II signals through the IGF-I receptor and not the IGF-II receptor (Adashi *et al.*, 1990; Conover *et al.*, 1986). A soluble form of the IGF-II/M6P receptor generated by proteolytic cleavage of the membrane bound form has been identified in rat (Kiess *et al.*, 1987b) and human serum (Causin *et al.*, 1988). In response to treatment with IGF-I, IGF-II, insulin or EGF the number

of IGF-II/M6P receptors found on cell membranes increase, as a result of redistribution from internal membranes to the cell surface (Braulke *et al.*, 1989).

1.3 BIOLOGICAL ACTIONS OF IGFs

The actions of IGF *in vitro* include effects on protein and carbohydrate metabolism, and effects on cell replication and differentiation (Giudice, 1992a; Cohick & Clemmons, 1993). IGF-I acts as a progression factor in the cell cycle. Quiescent cells in G_0 when treated with a competence factor (PDGF, bFGF) will progress to G_1 and will arrest. Treatment with IGF-I will induce the progression through the cell cycle leading to DNA synthesis and cell proliferation (Porcu *et al.*, 1992). This G_1 progression is believed to be Ras related (Lu & Campisi, 1992). IGF-I and IGF-II are able to promote cell differentiation in myoblasts (Florini *et al.*, 1991), osteoclasts (Mochizuki *et al.*, 1992), chondrocytes, (Geduspan & Solursh, 1993), and neural cells (Pahlman *et al.*, 1991).

IGF-I when added exogenously to culture stimulates a number of effects on preimplantation embryos including; an increased cell number in the inner cell mass (ICM) (without increasing the number of trophectoderm cells in cow (Herrler *et al.*, 1992) and pig (Xia *et al.*, 1994)) and also increased developmental frequencies with greater numbers of embryos reaching the blastocyst stage in the mouse (Harvey & Kaye, 1991b, 1992). IGF-II has similar effects when added to culture media, as higher rates of development to the

blastocyst stage occur with an increase in the number of ICM cells (Rappolee *et al.*, 1992). Rao *et al.* (1990) reported that insulin stimulated significant increases in DNA, RNA and protein synthesis.

The functions of IGF-I and IGF-II on fetal development have been studied using gene targeting and transgenic approaches. Mice carrying copies of the human IGF-I gene fused to the metallothionein-I promoter have elevated IGF-I levels (Mathews *et al.*, 1988). These mice displayed increases in body weight largely due to increased muscle, brain, spleen, kidney and pancreas mass. Most mice with a disrupted IGF-I gene die at birth and those that survive have growth retardation, reaching only 60% of normal birth weight (Baker *et al.*, 1993; Liu *et al.*, 1993). A gene-targeted mouse line for IGF-II produced live pups with birth weights 60% of normal size with prenatal growth defects starting around day 13.5 (DeChiara *et al.*, 1990; 1991). However, the effect of the IGFs on normal development is best observed in murine IGF-I and IGF-II double "knocked-outs". These mutants have complete neonatal lethality and birth weights 30% of normal. The phenotype of mice deficient for the type-1 receptor displays greater fetal growth deficits at 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. The phenotype of IGF-II/IGF-Ir (-/-) mutants is similar producing a lethal phenotype with slightly lower birth weights. These results

suggest that IGF-II is acting only in part through the IGF-I receptor. IGF-II/IGF-Ir knockouts result in lower fetal birth weight then IGF-Ir knockouts alone, suggesting that IGF-II must be acting by a route in addition to IGF-Ir pathways. The more severe phenotype observed for the IGF-I/IGF-II and the IGF-II/IGF-Ir knockout further supports IGF-II acting through a mechanism other than the IGF-Ir. Mice deficient in the IGF-II/M6P receptor result in larger birth weights and lethality in nearly all mutants. If in these mice the IGF-II gene is knocked-out in combination with the IGF-II/M6P receptor the phenotype is rescued and normal birth weights are observed (Filson *et al.*, 1993; Lau *et al.*, 1994; Wang *et al.*, 1994). This indicates that in the embryo the function of the IGF-II/M6P receptor is to control IGF-II levels which can be lethal if elevated. Imprinting of the IGF-II gene may represent an addition control measure important for regulating levels of IGF-II (Rappolee *et al.*, 1992; Vu & Hoffman, 1994). The phenotypes resulting from the gene targeting method provide a functional map of IGF ligand and receptor regulation and demonstrates their importance during mammalian development.

1.4 INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBP)

The IGFs are almost entirely bound *in vivo* to one of six IGFBPs. All IGFBPs contain structural homology, bind IGF-I and IGF-II specifically and have a negligible affinity for insulin (Jones & Clemmons, 1995). Sequence alignments

of the six IGFBPs reveal regions of homology within the amino- and -carboxyl terminal regions. The position of 18 cysteines, which participate in the formation of disulphide bridges and contribute to three-dimensional structure, are conserved in IGFBPs 1-5 (Shimaski *et al.,* 1991). The rat IGFBP-6 sequence is missing 2 and the human IGFBP-6 sequence lack 4 of the 18 conserved cysteines found in the other IGFBPs. This omission results in the absence of the invariant Gly-Cys-Gly-Cys-Cys sequence found in the amino terminal region (Shimaski *et al.*, 1991). The maintenance of the disulfide bridges is important for IGF-I binding as reduction of IGFBPs or mutation of the conserved cysteines results in loss of IGF binding (Brinkman *et al.*, 1991). 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 (Baxter & Martin, 1989; Leong *et al.*, 1992). The ALS contains several leucine-rich domains that facilitate protein-protein binding of IGFBP-3. IGF-I or IGF-II binds to the IGFBP-3-ALS complex and stabilizes the ternary complex (Baxter & Martin, 1989). 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 (Guler *et al.*, 1989; Hodgkinson *et al.*, 1989). The half-life of free binding proteins is between 30-90 min (Zapf *et al.*, 1986). IGF binding to IGFBP-3 is dependent on amino acids 3, 4, 15 and 16 of the β-chain of the ligand. Mutant IGF-I in which these amino-acids have been substituted for the insulin amino acids result in a

100-fold reduction in binding affinity (Bayne *et al.*, 1988). The ability of IGFBPs to regulate IGF action is not easily discerned from the current literature. IGFBPs can inhibit (Zapf *et al.*, 1979), and potentiate IGF action (Elgin *et al.*, 1987; Blum *et al.*, 1989) under various conditions. The characterization of specific proteases for the IGFBPs has further complicated the story as these proteases cleave binding proteins into forms with altered affinity for the IGFs. Thus experimental responses of systems to the effects of exogenous IGFBP may be modulated by protease levels in the system. Proteolysis occurs in IGFBPs 2-5, by calcium-dependent serine proteases, specific for each IGFBP. IGFBPs may also be affected by postranslational modifications and localization of IGFBPs in different systems resulting in different levels of interaction with IGFs. IGFBPs may themselves elicit direct cellular effects in which binding to IGF ligand is not necessary (Oh *et al.*, 1993; Bar *et al.*, 1989). These actions may in turn be regulated by the levels of IGFs present in the system (Jones & Clemmons, 1995). Serine phosphorylation of IGFBP-1, -3, and -5 has been detected and is proven to lower IGF affinity of IGFBP-1 (Jones *et al.*, 1991, 1992; Mukku & Chu, 1990). N-linked glycosylation occurs in IGFBP-3 and IGFBP-4, and O-linked glycosylation is present in IGFBP-5 and IGFBP-6. IGFBP-1 and IGFBP-2 contain an Arg-Gly-Asp (RGD) sequence at the C-terminal end which may be involved in binding to cell surface receptors (Ruoslathi & Pierschlaber, 1987). IGFBP-1 binds specifically to the $\alpha 5\beta 1$-intregrin receptor molecule and stimulates

migration in Chinese hamster ovary cells through an RGD-dependent mechanism. IGFBP-3 and IGFBP-5 bind to different unidentified molecules on the cell surface (Jones *et al.*, 1993a). The IGFBPs contain many similarities as a family, individual differences prevent the formulation of a common mechanism of action.

1.5 BIOLOGICAL ACTIONS OF IGFBPs

In the mouse pre-implantation blastocyst transcripts encoding insulin-like growth factor binding proteins (IGFBP) -2, -3, -4, and 6 have been detected by RT-PCR (Schultz *et al.*, 1993a; Hahnel & Schultz, 1994). Six IGFBPs bind with high affinity to IGFs and regulate IGF biological action by transporting IGFs, influencing ligand half-life, contributing to tissue and cell specific distribution and modulating and potentiating IGF action with receptors (Clemmons, 1993; Jones & Clemmons, 1995). IGFBPs may have the additional capacity to directly influence metabolic events as exemplified by IGFBP-3 action on human breast cancer cell growth (Oh *et al.*, 1993).

IGFBPs have been detected in the ovary of several species, and their expression varies between species (de la Sota *et al.*, 1996; Mason *et al.*, 1996; Kirby *et al.*, 1996; Grimes *et al.*, 1994; Mondschein *et al.*,1990). The developing oocyte grows within an IGFBP rich follicular fluid during follicular development.

IGFBPs 2-5 have been identified in bovine follicular fluid, and an increased level of IGFBP -2, -4 and -5 have been detected within atretic follicles (de la Sota *et al.*, 1996). Human follicular fluid contains IGFBPs 1-4, with IGFBP -2 and -4 being the most abundant, and the level of expression of each is follicle stage dependent (Giudice *et al.*, 1990; Mason *et al.*, 1996). The dominant follicles contain decreased amounts of IGFBP -2 and -4 when compared to non-dominant and atretic follicles. In the pig, transcripts encoding IGFBPs 2-5 were detected in the corpora lutea (Gadsby *et al.*, 1996). Analysis of follicular fluid collected from pig ovaries demonstrates that IGFBP -2 and -3 were the two most abundant IGFBPs in pig follicular fluid (Mondschein *et al.*, 1990). Quantification of IGFBP levels showed that IGFBP-2 levels were greater in atretic follicles and IGFBP-3 levels were greater in large healthy follicles (Grimes *et al.*, 1994). This increase in IGFBP-2 production in atretic follicles was also observed in sheep (Monget *et al.*, 1993), cow (de la Sota *et al.*, 1996) and human ovaries (San Roman & Magoffin, 1993; Cataldo & Giudice, 1992).

Murphy and Ghahary (1990) characterized IGFBPs in murine uterine luminal fluid and detected the same IGFBPs present in serum samples. The production of IGFBPs in the rat uterus is under regulation by growth hormone and oestradiol (Yallampalli *et al.*, 1993). In addition a dramatic increase in IGFBP-4 levels were observed in the mouse uterus at the time of implantation (Markoff *et al.*, 1995). In the cow, IGFBP-2 mRNA levels increased between

days 10 and 18 of the estrous cycle and during early pregnancy indicating that progesterone may be involved in the upregulation of IGFBP-2 (Geisert *et al.*, 1991). Human endometrial stroma cells produce increased amounts of IGFBP 1-4 when cultured *in vitro* in the presence of mouse embryos (Liu *et al.*, 1995). This suggests that the endometrial cells are responsive to stimuli produced from the embryo.

In the bovine oviduct IGFBP-2 and IGFBP-3 mRNAs are detected at equal levels in both pregnant and non-pregnant animals (Kirby *et al.*, 1996). IGFBPs 1-4 have been detected in the human oviduct (Giudice *et al.*, 1992b; Pfeifer & Chegini, 1994). These studies suggest a possible presence of IGFBPs in the reproductive tracts of other mammalian species.

A pig trophoderm cell line released an IGF specific binding protein of molecular weight 32-42 kDa detected by Western ligand blot technique (Corps *et al.*, 1990). This result suggests the possible release of IGFBPs from developing preimplantation pig embryos.

Recent gene "knock-out" studies and transgenic overexpression studies have started to elucidate the functional roles of IGFBPs (Dai *et al.*, 1994; D'Ercole *et al.*, 1994; Murphy & Barron, 1993, Wood *et al.*, 1993). Overexpression of IGFBP-1 resulted in no significant differences in growth and fertility. Those mice that expressed the IGFBP-1 construct in the brain however, displayed brain growth retardation (Dai *et al.*, 1994). IGFBP-1 is not normally

found in the brain and growth may be restricted by the inhibition of IGF action by the IGFBP-1 expression (Dai *et al.*, 1994). The lack of other phenotypic abnormalities suggests that the IGFBP system is able to compensate by altering levels of expression of other IGFBPs. Levels of IGFBP-3 may be reduced in these mice in order to compensate for the increase in IGFBP-1 (D'Ercole *et al.*, 1994). Mice with a null mutation of the IGFBP-2 gene display increased expression of other IGFBPs resulting in mice with normal phenotypes (Wood *et al.*, 1993). In order to demonstrate essential functions of IGFBPs by gene "knock-out" studies, double "knock-outs", similar to the IGF ligand and receptor studies may be required.

1.6 IGFBP-1

Structure

The first structural information regarding any of the IGFBPs arose from the amino acid sequencing of IGFBP-1 purified from human amniotic fluid (Pavoa *et al.*, 1984). The gene was characterized following the isolation of a cDNA product (Brinkman *et al.*, 1988). In the rat, cDNA sequencing revealed coding of a 247 amino acid peptide, with molecular weight of 32 kDa and an Arg-Gly-Asp (RGD) recognition sequence in the carboxyl-terminal domain (Murphy *et al.*, 1990). Rat IGFBP-1 is a 5.2 kb gene spanning four exons. The promoter region includes TATA and CAAT boxes and a putative homeodomain, AP-1, and

at least one insulin and glucocorticoid response element (Goswami *et al.*, 1994). Human, rat and bovine IGFBP-1 proteins are identical for 152 of 234 amino-acids (Lee *et al.*, 1993; Sneyers *et al.*, 1991). The human IGFBP-1 gene spans 5.2 Kb of genomic DNA including 4 exons of 514 bp, 170 bp, 129 bp and 701 bp respectively. Analysis of human IGFBP-1 transcripts suggests that there is only one cDNA present for this molecule and that differential splicing does not occur (Cubbage *et al.*, 1989). Human IGFBP-1 mRNA contains 1.55 kb containing a 777 bp coding sequence. The 612 bp 3'-untranslated region contains 5 ATTTA motifs which are characteristic of transcripts with short half-lives (Shaw & Kamen, 1986). The human IGFBP-1 protein consists of 234 amino-acids and a molecular mass of 25.3 kDa. The 25-residue N-terminal signal peptide and the cysteine rich N-terminal of the molecule are extremely hydrophobic, while the remaining areas are hydrophillic (Lee *et al.*, 1988). Dividing the protein based on structural characteristics produces three regions. The first is 79 amino-acids containing the N-terminal cysteine cluster, which is conserved at 82% sequence identity between human, cow and rat. Residues 30-53 form a hydrophobic domain, that is involved in IGF binding, and are conserved in human, rat and cow (Lee *et al.*, 1988). The second region from residue 80 to 144 displays a reduced identity. Only 40% of residues are conserved between human, rat and bovine proteins. In addition homology between IGFBP-1 and other IGFBPs is also very low in this region. Within this region Pro-Glu-Ser-Thr (PEST) domains

are found in all three species (Julkunen *et al.*, 1988). PEST sites are characteristic of proteins with rapid turnover rates (Rogers *et al.*, 1986), and along with the presence of ATTTA motifs in the mRNA allows IGFBP-1 levels to fluctuate rapidly. The final region spans residues 145-234 including the C-terminal cysteine cluster, and is 68% identical among rat, human and bovine proteins. The RGD sequence is located in region 3 at residues 221-223 in all three species (Lee *et al.*, 1993).

Regulation

In adult serum circulating levels of IGFBP-1 are low but are increased in individuals with diabetes, low GH or glucocorticoid levels and while fasting (Ooi *et al.*, 1990; Senevirante *et al.*, 1990; Murphy *et al.*, 1991; Luo *et al.*, 1990). IGFBP-1 transcription is inhibited by increased insulin levels in rat hepatoma cells (Orlowski *et al.*, 1991).

Post-translational modifications

IGFBP-1 is modified by serine phosphorylation, resulting in four different phosphorylated forms with a greater affinity for IGF-I. Phosphorylation principally occurs at Ser-101 (70%), Ser-169 (25%) and Ser-119 (5%) in the human. Rat and cow have Ser-101 and Ser-119 conserved but do not have Ser-169 (Frost & Tseng, 1991; Jones *et al.*, 1991). The phosphorylated forms have been shown to

inhibit IGF action while the unphosphorylated form stimulates IGF actions (Jones *et al.*, 1991).

Biological actions

IGFBP-1 potentiation was demonstrated by an increase in DNA synthesis in porcine aortic smooth muscle cells when purified human IGFBP-1 was added with a low concentration of platelet-poor plasma (Elgin *et al.*, 1987). IGFBP-1 added alone had no effect. In addition MDA-231 breast carcinoma cells grew better in the presence of IGFBP-1 alone, and it was found that IGF-I binding to IGFBP-1 was essential for potentiation (Camacho-Hubner *et al.*, 1991). It may be important in studies involving potentiation to regulate closely the concentrations of the constituents added. In human fibroblast cells incubation of 20 ng/ml IGF-I with 20-50 ng/mg IGFBP-1 resulted in increased DNA synthesis (Koistinen *et al.*, 1990), while addition of 2 and 200 ng/ml IGFBP-1 enhanced the proliferative response to IGF-I by 3-fold (Kratz *et al.*, 1992). Interestingly the same responses were not elicited by IGF-II, indicating that the effects are specific for IGF-I.

The presence of an RGD recognition sequence within IGFBP-1 suggests that this binding protein may bind to cell surfaces, specifically to the $\alpha5\beta1$-integrin receptor. Binding of IGFBP-1 to the $\alpha5\beta1$-integrin receptors on cultured CHO fibroblastic cells results in the stimulation of cell migration independent of IGFs (Jones *et al.*, 1993b).

The addition of molar excess of IGFBP-1 in serum-free culture clearly results in the inhibition of IGF action. IGFBP-1 when added in a 4:1 molar excess to IGF-I, inhibited DNA synthesis in cultured human granulosa and luteal cells (Angervo *et al.*, 1991). It is possible that the inhibition is due to the formation of IGF/IGFBP-1 complexes preventing binding of IGF to the cell surface receptors. Rutanen *et al.*, (1988) showed that direct inhibition of receptor binding by the addition of IGFBP-1 led to the inhibition of IGF-I effects in human endometrial membranes. This influence of IGFBP on IGF action was observed in other cell types including: thyroid cells (Frauman *et al.*, 1989), human osteosarcoma cells (Campbell & Novack, 1991), and porcine aortic smooth muscle cells (Busby *et al.*, 1988). *In vivo* injection of supraphysiological amounts of IGFBP-1 into rats resulted in a small increase in blood glucose suggesting excess exogenous IGFBP-1 inhibits the glucose-lowering actions of IGF-I (Lewit *et al.*, 1991).

1.7 IGFBP-2

Structure

IGFBP-2 was first purified from Buffalo rat liver (BRL)-3A cells (Lyons & Smith, 1986; Mottola *et al.*, 1986). The mature IGFBP-2 contains 284 amino-acids with molecular mass 31.5-33 kDa (Bourner *et al.*, 1992). IGFBP-2, like IGFBP-1, has an RGD recognition motif and contain no glycosylation sites. Bovine IGFBP-2 has a high degree of DNA sequence identity with rat (81%) and

human (87%) sequences (Bourner *et al.*, 1992; Upton *et al.*, 1990). The rat IGFBP-2 gene spans 36 kb containing 4 exons (Brown & Rechler, 1990). The promoter region lacks TATA and CAAT sequences but contains three GC boxes, as well as AP-1 and AP-2 consensus sequences. Transcription factor Sp1 binds to the GC boxes and initiates transcription of the gene (Kutoh *et al* 1993; Boisclair *et al.*, 1993). The bovine cDNA contains 388 nucleotides of 5'-untranslated region, 942 base open reading frame and a 381 base 3'untranslated region. A signal peptide of 33 amino-acids is cleaved to produce the mature IGFBP-2 protein (Bourner *et al.*, 1992).

Regulation

IGFBP-2 is present in fetal serum and expressed in many fetal tissues but declines postnatally, except in the brain where expression remains high (Donovan *et al.*, 1989). Expression of IGFBP-2 increases as a result of fasting, hypophysectomy and diabetes and is decreased by glucocorticoid treatment (Orlowski *et al.*, 1990; Ooi *et al.*, 1990, 1992). Pituitary expression of IGFBP-2 is increased by estrogen injection (Michels *et al.*, 1993). *In vitro* experiments show IGFBP-2 levels increased in response to treatment with tetradecanoyl phorbol acetate (TPA) in sheep thyroid cells (Eggo *et al.*, 1991), and decreased under treatment with insulin in hepatocytes (Boni-Schnetzler *et al.*, 1990), and forskolin in bovine kidney epithelial cells (Cohick & Clemmons, 1991).

IGFBP-2 levels are influenced by the day of estrus in cycling cows. IGFBP-2 mRNA levels are elevated on days 15 and 18 compared to days 5 and 10 of estrus in samples isolated from bovine endometrium (Geisert *et al.*, 1991).

Post translational modifications

IGFBP-2 is not glycosylated (Bourner *et al.*, 1992) or phosphorylated but can be cleaved in plasma by proteases (Jones & Clemmons 1995).

Biological action

IGFBP-2 has the ability to elicit both inhibition and potentiation of IGF actions *in vitro*. IGFBP-2 enhanced the IGF induced increase in glucose transport and amino-acid uptake in microvascular endothelial cells (Bar *et al.*, 1989). In this system, IGFBP-2 independent of additional IGF was able to produce similar responses. Porcine aortic smooth muscle cells when treated with a combination of IGFBP-2 and platelet-poor plasma displayed an 80% increase in IGF-I induced DNA synthesis. When added to serum-free treatment, IGFBP-2 was a potent inhibitor of the IGF-I stimulated response (Bourner *et al.*, 1992).

IGFBP-2 inhibits ^{3}H-thymidine incorporation in a number of *in vitro* systems including, human lung carcinoma (Reeve *et al.*, 1993) and rat astroglial cells (Han *et al.*, 1988b). IGFBP-2 may inhibit the actions of IGF-I and IGF-II by

preventing binding to the cell surface thereby limiting accessibility and binding of IGFs to their receptors (Ross *et al.*, 1989).

1.8 IGFBP-3

Structure

IGFBP-3 is the predominant binding protein isolated from adult serum and is commonly found complexed with the ALS and IGF-I, forming the 150 kDa complex. Bovine IGFBP-3 cDNA displays 80% homology with human IGFBP-3 and like the human form produces a 264 amino-acid mature protein. The porcine sequence encodes a 266 amino-acid protein while the rat form encodes a 265 amino-acid protein (Spratt *et al.*, 1991). Characterization of the bovine sequence reveals three conserved N-linked glycosylation sites at amino-acids 90, 109 and 172. Rat IGFBP-3 has one additional glycosylation site (Albiston *et al.*, 1990). The bovine IGFBP-3 mRNA is 1.65 kb, containing a 992 base open reading frame, a 119 nucleotide 5' untranslated region and a 591 nucleotide 3' untranslated region (Spratt *et al.*, 1991). Rat genomic IGFBP-3 is 10 kb, including a promoter region containing a TATA box and a GC rich box. Also within the promoter region are consensus sequences for AP-2, ATF, nuclear factor-1, estrogen response elements, glucocorticoid response elements, thyroid specific transcription factor-1 and -2 and growth hormone response elements (Albiston *et al.*, 1995).

Regulation

Uterine IGFBP-3 may act as a regulator of early development. Uterine levels of IGFBP-3 increase in response to estradiol (Huynh & Pollack *et al.*, 1994). IGFBP-3 levels are decreased in diabetes, and by fasting and protein restriction (Donovan *et al.*, 1991; Clemmons & Underwood, 1991). IGFBP-3 levels are largely regulated by circulating levels of growth hormone and IGF-I (Baxter & Martin, 1989). IGF-I when added to cultured cells increases IGFBP-3 levels in several cell types including; bovine and human fibroblasts (Conover, 1990), mammary epithelial (Romagnolo *et al.*, 1994) and sertoli cells (Smith *et al.*, 1990). In addition to IGF-I, the growth factors TGF-β and EGF also increase IGFBP-3 levels (Yateman *et al.*, 1993; Corps & Brown, 1991; Mondschein *et al.*, 1990).

Post-translational modifications

IGFBP-3 can undergo extensive post-translation modification involving glycosylation, phosphorylation and proteolytic cleavage (Jones & Clemmons, 1995). Glycosylation is confirmed by the reduction in molecular mass of IGFBP-3 upon glycanase treatment (Zapf *et al.*, 1988). IGFBP-3 does not require glycosylation to bind to cell surfaces or to potentiate IGF-I action (Conover, 1991).

Proteolysis of IGFBP-3 by a calcium dependent serine protease produces a 30 kDa fragment with a lower affinity for IGF-I (Davenport *et al.*, 1992; Gargosky *et al.*, 1992). Fowlkes *et al.* (1994) suggested that the cleavage of IGFBP-3 is also influenced by matrix metalloproteinases. The result of this cleavage may be that the 30 kDa fragment potentiates IGF-I action because it can release IGF more readily than intact IGFBP-3 (Schmid *et al.*, 1991). The addition of the truncated IGFBP-3 alone resulted in a greater increase in cell growth than when added with IGF-I indicating that the 30 kDa form may act independently.

Phosphorylation occurs at two major serine residues within the IGFBP-3 protein (Hoeck & Mukku, 1994). It remains unclear if phosphorylation alters the affinity of the IGFBP-3 for the ligand.

Biological actions

The addition of IGF-I and 180 ng/ml IGFBP-3 to baby hamster kidney cells resulted in a 90% increase in DNA synthesis compared to IGF-I alone. Concentrations of IGFBP-3 higher than 180 ng/ml inhibited the IGF-I mediated increase in DNA synthesis (Blum *et al.*, 1989). Bovine IGFBP-3 when coincubated with IGF-I resulted in inhibition of aminoisobutyric acid (AIB) uptake by fibroblasts. Fibroblasts that were preincubated with IGFBP-3, then treated with IGF-I, had a dose-dependent AIB uptake response to IGF-I (Conover *et al.*,

1990). In a similar study preincubation of human fibroblasts with IGFBP-3 resulted in a potentiation of IGF-I induced DNA synthesis and inhibition of DNA synthesis if incubated together without preincubation with IGFBP-3 (DeMellow & Baxter, 1988). IGFBP-3 binds to a yet unidentified protein on the cell surface and bound IGFBP-3 has a 10-fold lower affinity for IGF-I than free IGFBP-3 (Conover, 1991). This lower affinity of bound IGFBP-3 and the finding that preincubation with IGFBP-3 prevents an IGF-I induced down regulation of IGF-I receptors (Conover & Powell, 1991) may result in an overall potentiation of IGF action.

Free IGFBP-3 has the ability to inhibit IGF action by preventing IGF binding to receptors. The IGF/IGFBP-3 complex can bind to the cell surface without the involvement of the IGF receptor (Clemmons *et al.*, 1986; 1987). Expression of a recombinant IGFBP-3 cDNA in BALB/c3T3 fibroblasts resulted in growth inhibition. The cells had a longer doubling time and grew to lower cell density (Cohen *et al.*, 1993). The inhibition of IGF action was demonstrated to be concentration dependent. IGFBP-3 inhibited the IGF-I stimulated glucose oxidation in pig fat cells, but the inhibition was overcome by increased concentrations of IGF-I (Walton *et al.*, 1989). IGFBP-3 inhibits IGF-I stimulated glucose incorporation in BALB/c3T3 cells (Okajima *et al.*, 1993). Inhibition of IGF-I stimulated DNA synthesis by IGFBP-3 was observed in rat granulosa (Bicsak *et al.*, 1990), rat and mouse osteoblasts (Schmid *et al.*, 1991; Andress &

Birnbaum, 1992) and chick embryo fibroblasts (Blat *et al.*, 1989).

In vivo infusion of IGFBP-3/IGF-I complex lowers blood glucose and stimulates glycogen synthesis in hypophysectomized but not in normal rats (Zapf *et al.*, 1995). Combined IGF-I and IGFBP-3 application in a wound healing model demonstrated accelerated wound healing and increased amounts of wound tissue (Sommer *et al.*, 1991). Rats deficient in GH when given combined subcutaneous injections of IGFBP-3 and IGF-I had a 2-fold increased weight gain and greater epiphyseal width (Clark *et al.*, 1993).

Some evidence indicates that IGFBP-3 may inhibit the growth stimulating effects of other growth factors like TGF-β (Imbenotte *et al.*, 1992), and EGF (Villaudy *et al.*, 1991).

1.9 IGFBP-4

Structure

Bovine IGFBP-4 cDNA analysis predicts a protein product of 258 amino acids (Moser *et al.*, 1992). IGFBP-4 contains one N-linked glycosylation site (Shimasaki *et al.*, 1990). Amino-acid sequence comparisons show near identity between bovine IGFBP-4 and human (97%) and rat (92%). The full length cDNA product determined by Northern blot analysis is 2.3 kb in length (Moser *et al.*, 1992). The molecular weight of bovine IGFBP-4 under non-reducing conditions is 24-25 kDa for the unglycosylated form and 30 kDa for the glycosylated form.

The promoter region of the rat IGFBP-4 gene contains TATA and CAAT sequences, many cis-elements, three cAMP response elements, three AP-1 sites and a progesterone response element (Gao *et al.*, 1993).

Regulation

The expression of IGFBP-4 in various tissues is decreased in diabetes (Chen *et al.*, 1994). Mice overexpressing IGF-II or lacking IGF-II receptor produce higher than normal serum levels of IGFBP-4 (Wolf *et al.*, 1994; Lau *et al.*, 1994), and hypophysectomy reduces liver IGFBP-4 mRNA levels while treatment with GH or IGF-I partially restores IGFBP-4 mRNA levels (Gosteli-Peter *et al.*, 1994). IGFBP-4 gene expression is stimulated by forskolin, parathyroid hormone and parathyroid hormone-related peptide in both rat and human cell lines (Torring *et al.*, 1991; Mohan *et al.*, 1989). The effect of IGF treatment on the expression of IGFBP-4 by cultured cell lines has produced conflicting results. Expression of IGFBP-4 mRNA was decreased in human fibroblasts in response to IGF-I and IGF-II, unlike an epidermal squamous carcinoma cell line in which IGFBP-4 was stimulated by IGF treatment (Neely & Rosenfeld, 1992).

Post-translational modifications

Treatment with N-glycanase results in the loss of the 30 kDa IGFBP-4 product under ligand blot analysis indicating that this form represents the glycosylated form (Moser *et al.*, 1992). The possible alterations of action due to glycosylation are not yet proven.

IGFBP-4 protease activity has been detected in several cell types, and is enhanced by IGF treatment and inhibited by metallo-serine proteases and phorbol ester tumour promoters (Myers *et al.*, 1993; Cheung *et al.*, 1994; Conover *et al.*, 1993; Chernausek *et al.*, 1995). The proteolytic fragments have a much lower affinity for the IGF ligands and the ability to inhibit IGF stimulated AIB uptake is lost (Jones & Clemmons, 1995).

Biological actions

IGFBP-4 inhibits IGF actions under most experimental conditions. IGFBP-4 does not bind to cell surfaces and inhibits IGF action by binding to free IGFs preventing their interaction with the IGF-I receptor. IGFBP-4 inhibition is partially overcome by the addition of large excess of IGF-I, presumably due to saturation of IGFBP-4 binding capacity (Mohan *et al.*, 1989). IGFBP-4 inhibits cell growth in neuroblastoma cells (Cheung *et al.*, 1991), granulosa cells (Ui *et al.*, 1989; Lin *et al.*, 1993) and colon carcinoma cells (Culouscou & Shoyab, 1991).

1.10 IGFBP-5

Structure

Rat IGFBP-5 is a 252 amino acid protein, translated from a 6.0 kb cDNA (Zhu *et al.*, 1993a). The promoter region contains TATA and CAAT sites and binding sites for AP-1, AP-2 and progesterone receptor. IGFBP-5 is the most conserved of the six binding proteins across species (James *et al.*, 1993). A partial amino acid sequence of the bovine IGFBP-5 protein demonstrates 99% identity to a human clone (Moser *et al.*, 1992).

Regulation

IGFBP-5 production is increased in response to IGF-I, IGF-II and insulin in a rat thyroid cell line (Backeljauw *et al.*, 1993).

Post-translational modification

IGFBP-5 has the potential for both serine phosphorylation and O-linked glycosylation (Jones & Clemmons, 1995). A specific protease has been characterized and found to cleave IGFBP-5 into three smaller fragments. The fragmented IGFBP-5 has greatly reduced affinity for IGF compared to the intact IGFBP-5. A 23 kDa fragment of IGFBP-5 potentiates the mitogenic effect of IGF-I and IGF-II, and may do so by associating with the cell surface (Andress & Birnbaum, 1992; Andress *et al.*, 1993). Proteolysis of IGFBP-5 is regulated by

IGF-I, the addition of IGF-I preserves intact IGFBP-5 and inhibits proteolytic cleavage in conditioned medium (Conover & Kiefer, 1993). Proteases, capable of degrading IGFBP-5 have been isolated at 97 kDa, and proteases of 52-72 kDa in size have been characterized as matrix metalloproteinase-1 and -2 (Thrailkill *et al.*, 1995).

Biological actions

IGFBP-5 has the ability to bind to the extracellular matrix (ECM), and when associated with the surface reduces IGF-I affinity 7-fold. This suggests a mechanism for increasing the availability of IGF-I to surface IGF-I receptors. IGFBP-5 potentiates fibroblast cell response to IGF-I by 100% (Jones *et al.*, 1993a). In addition IGFBP-5 potentiates IGF-I and IGF-II actions in osteoblasts (Bautista *et al.*, 1991; Andress & Birnbaum, 1991).

Inhibition of IGF-I stimulated DNA synthesis has been demonstrated by the addition of a molar excess of IGFBP-5 (Kiefer *et al.*, 1992). IGFBP-5 also inhibited IGF-stimulated steroidogenesis in granulosa cells (Ling *et al.*, 1993).

1.11 IGFBP-6

Structure

Isolation and characterization of cDNAs from human and rat predict proteins of 216 a.a. and 206 a.a., respectively. Both sequences lack cysteines

two and four of the 18 conserved cysteines in IGFBPs 1-5 (Shimasaki *et al.*, 1991). Bovine IGFBP-6 when aligned with human amino acid sequence contains 84% identity and 73% to rat (Moser *et al.*, 1992). The rat promoter region does not contain TATA or CAAT sequences and is not GC-rich but contains Sp1, estrogen receptor and retinoic acid recognition sites (Zhu *et al.*, 1993b).

Regulation

IGF-I and IGF-II show stimulation of IGFBP-6 in L6E9 myoblasts (Silverman *et al.*, 1995) and NIH 3T3 cells (Claussen *et al.*, 1995).

Post-translational modifications

IGFBP-6 is O-linked glycosylated as demonstrated by a reduction in size upon treatment with O-glycanase (Bach *et al.*, 1993).

Biological actions

IGFBP-6 has a 10-fold greater affinity for IGF-II than for IGF-I. This may be partly a result of the two deleted cysteine residues (Roghani *et al.*, 1989). A possible role of IGFBP-6 is one of selectively regulating the action of IGF-II. In L6A1 myoblasts IGFBP-6 inhibited IGF-II induced cell differentiation (Bach *et al.*, 1994).

1.12 IMPRINTING AND PARTHENOGENESIS

Parthenogenesis (development of an unfertilized female zygote) occurs naturally in a select group of birds, fish and amphibians resulting in the production of viable offspring. Successful full term parthenogenetic development has not been recorded in a mammalian species (Whittingham, 1980). The "typical" mammalian oocyte maturation pattern (as exemplified by the murine or bovine oocyte) involves the vast majority of oocytes arresting at metaphase II following ovulation and awaiting fertilization before progressing onto further developmental events. Although spontaneous oocyte activation is observed *in vitro* for aged bovine oocytes (King *et al.*, 1988; Plante and King, 1996), in mouse oocytes carrying a disrupted c-mos gene (Vande Woude, 1994; Colledge *et al.*, 1994; Hashimoto *et al.*, 1994) and in oocytes from the murine LT/SV strain (Whittingham, 1980), these occurrences are otherwise not frequently observed. Artificial parthenogenetic activation of mammalian oocytes has been demonstrated by a variety of treatments, such as ethanol (Kaufman, 1982; Nagai, 1987; O'Neil and Kaufman, 1989; Kubiak *et al.*, 1991; Fukui *et al.*, 1992; Minamihashi *et al.*, 1993; Presicce and Yang, 1994a,b) electric shock (Collas *et al.*, 1993a,b) strontium chloride treatment (Fraser, 1987; O'Neil *et al.* 1991) and ionomycin treatment (Navara *et al.*, 1994; Susko-Parrish *et al.*, 1994). These methods activate matured oocytes by mimicking the transient calcium increases in the oocyte at the time of sperm penetration (Swann and Ozil, 1994), and they

result in the production of parthenogenetic embryos at a much higher rate than spontaneous oocyte activation levels (Kaufman, 1982; O'Neil and Kaufman, 1989; Plante and King, 1996).

In addition to assessing the relative contributions of the maternal and paternal genomes to the developmental program, parthenogenetic embryos are invaluable for the investigation of genomic imprinting of specific genes. IGF-II, Snrpn, and Xist are among a growing list of imprinted genes in which expression is determined by epigenetic modifications (Surani, 1994; Latham *et al.*, 1994; Leighton *et al.*, 1995). Parthenogenetic embryos may also be of use for the establishment of female embryonic stem cell lines and will also certainly be of use in determining the comparative developmental potential of parthenogenotes among mammalian species. In my study parthenogenetic bovine blastocysts were produced to determine the levels of IGF-II released by these embryos. IGF-II expression may be altered in parthenogenetic blastocysts due to genomic imprinting. The IGFs are a family of genes regulated by genomic imprinting in adult tissues. The study of parthenogenetic blastocysts will provide information regarding maternal contribution to regulation of the IGF genes prior to fetal development.

1.13 HYPOTHESIS

The principal hypothesis of my study is that early bovine development *in vitro* is subjected to regulation by growth factors of maternal origin. The focus of my study was to investigate the interactions of these putative bovine maternal paracrine circuits by characterizing the expression of mRNAs and polypeptides encoding IGFBPs in bovine primary oviductal cultures and preattachment embryos.

1.14 RATIONALE

1. Detection of IGF-I and/or IGF-II in the culture system is imperative for the establishment of autocrine and paracrine IGF circuits influencing embryo development.
2. IGFBPs bind IGFs with high affinity and modulate the actions of IGFs. Determining the specific IGFBPs released from the oviductal cells will aid in understanding potential IGFBP regulation of IGFs.
3. In parthenogenotes gene expression is solely derived from the maternal genome, and thus they allow the maternal contribution to early embryonic gene expression to be studied.

1.15 SPECIFIC AIMS

1. To verify the role and requirement of oviductal cell co-culture to support the development of bovine embryos *in vitro*.

2. To determine the levels of IGF-I and IGF-II ligand released by bovine blastocysts and cultured oviductal cells.

3. To determine the presence of IGFBP mRNA in early bovine embryos and primary bovine oviductal cell cultures, and the release of IGFBP polypeptides into conditioned media.

4. To produce bovine parthenogenetic embryos to contrast the release of IGF-II and the presence of IGFBP mRNAs with fertilized bovine embryos.

CHAPTER 2 EFFECT OF CULTURE ENVIRONMENT

2.1 INTRODUCTION

The ability to sustain bovine development through to the blastocyst stage *in vitro* required the characterization of conditions capable of overcoming a culture block at the 8-16 cell stage. Development beyond this culture block is largely dependent upon the composition of the culture system (Larson *et al.*, 1992a,b; Bavister *et al.*, 1992). The *in vitro* culture (IVC) system employed in this study involved co-culture on bovine oviductal cells in a 10% serum supplemented medium (Ellington *et al.*, 1990; Eyestone & First, 1989; Xu *et al.*, 1992). Bavister *et al.* (1992) suggested that the greatest influence the co-culture environment provides is in the removal of inhibitory compounds, but acknowledged that oviductal cultures also produce factors that likely stimulate embryo growth. Efforts to culture bovine embryos in a chemically defined system are advancing rapidly and are now reporting comparable blastocyst rates to those observed with co-culture systems (Bavister *et al.*, 1992; Keskintepe *et al.*, 1995; Pinopummintr & Bavister, 1991; Kim *et al.*, 1990). EGF, bFGF, PDGF, TGFα and TGFβ1 represent several growth factors, possibly released by co-culture cells, that may be involved in the regulation of embryo development (Paria & Dey, 1990; Rappollee *et al.*, 1988; Larson *et al.*, 1992 a,b).

Both IGFs and IGFBPs have been detected in murine (Markoff *et al.*,

1995), rat (Yallampalli *et al.*, 1993), porcine (Ko *et al.*, 1991), and bovine (Geisert *et al.*, 1991) uterine fluids. IGFs have also been detected in rat (Carlsson *et al.*, 1993) and pig oviductal fluids (Wiseman *et al.*, 1992) and both IGF and IGFBPs were detected in the human oviduct (Giudice *et al.*, 1992b; Pfeifer & Chegini, 1994). The presence of growth factors in the reproductive tracts of these mammals indicates the likelihood of a maternal regulatory influence on the developing zygote. Remy *et al.* (1995), using 15 antibodies specific for oviductal proteins were able to detect 11 of the 15 proteins released into conditioned media from bovine oviductal cell monolayers, suggesting that many observed oviductal products expressed *in vivo* are retained by oviductal primary cell cultures.

The objective of the following culture experiments was to establish oviductal cell co-culture to support development of bovine embryos *in vitro*. Several studies have reported the ability of oviductal co-culture to improve rates of development (Wiemer *et al.*, 1991; Watson *et al.*, 1994; Sirard *et al.*, 1988; Xu *et al.*, 1992). The present study focused on the capacity of our oviductal cell co-culture to improve developmental rates to the blastocyst stage.

2.2 MATERIALS AND METHODS

Primary Oviductal Cultures

Bovine oviductal cultures were established as outlined by Xu *et al.* (1992),

Xia *et al.* (1996) and Harvey *et al.* (1995). For establishment of monolayer cultures the epithelial cells were isolated by filling the oviduct lumen with 0.05% trypsin in Ca^{++}, Mg^{++} free Hank's Balanced Salt Solution (HBSS; GIBCO BRL) and incubating at 38.6 °C for 20 min. The oviduct contents were squeezed into a 35 mm petri dish. The cells were dispersed by forcing them through a 18 gauge needle attached to a 5 ml syringe three times before transferring the samples into 15 ml conical tubes containing 10 ml of HBSS for washing by centrifugation. The cells were re-suspended in fresh HBSS and washed 3 more times before final re-suspension in the appropriate volume of TCM-199 medium + 10% steer serum (SS). The cultures were established by addition of 1 x 10^6 cells per well to 24 well plates containing 1 ml of TCM-199 +10%SS medium per well. By 48 h, approximately 50% of the surface of each well was covered by attached cells. By 72 h the monolayers were confluent. The cultures were maintained for up to 8 days by removing the old medium and adding 1 ml of fresh culture medium every 48 h.

For establishment of epithelial vesicle cultures, cell sheets were collected from trimmed oviducts and were washed four times with HBSS (GIBCO). Up to 70 µl of cell suspension was placed into individual 35 mm petri dishes containing 3 ml of TCM-199 medium supplemented with 10% SS. The cell sheets were cultured for 24 h under an atmosphere of 5% CO_2 in air at 38.6°C to allow for the formation of vesicles. From this point on, the vesicle cultures were maintained for

up to 8-days by moving vesicles to new culture dishes containing fresh medium every 48 h.

Bovine Embryo Culture

Bovine preattachment embryos were produced by standard *in vitro* oocyte maturation, fertilization and embryo culture methods (Wiemer *et al.*, 1991; Watson *et al.*, 1994; Sirard *et al.*, 1988; Xu *et al.*, 1992) applied to cumulus-oocyte-complexes (COCs) collected from slaughterhouse ovaries. COCs were harvested within 4 h of removal of ovaries from the animal, by a razor blade slashing technique. The contents were pooled and the COCs collected and washed 4 times with oocyte collection medium (Hepes buffered-TCM-199 medium + 2% SS). COCs were placed into maturation medium consisting of TCM-199 medium (GIBCO, BRL) + 10% (v/v) (SS) supplemented with 35 µg/ml sodium pyruvate (Sigma), 5 µg/ml FSH (Follitropin; Vetrapharm, London, ON, Canada), 5 µg/ml LH (Vetrapharm) and 1 µg/ml estradiol-17b (Sigma) for 22 h at 38.6°C in a humidified atmosphere containing 5% CO_2 in air. Matured oocytes were fertilized *in vitro* with frozen-thawed bovine semen (Semex Canada Inc., Guelph, ON, Canada) prepared by standard "swim-up" procedures (Parrish *et al.*, 1986). COCs were removed from the maturation medium and washed 4 times in Hepes-buffered modified Tyrode's solution (Parrish *et al.*, 1986) just prior to their placement into pre-equilibrated fertilization drops (50 COCs/300 µl drop) consisting of bicarbonate buffered modified Tyrode's solution under light

paraffin oil (BDH Inc., Toronto, ON, Canada). The sperm/COC droplets (2.25 x 10^5 motile spermatozoa/drop) were incubated for 18 h at 38.6°C in a humidified 5% CO_2 in air atmosphere before removal of the remaining cumulus cell investment. Fertilized oocytes were placed into 50 µl culture drops specified in experiments 1 and 2. To sustain development through to the blastocyst stage, 50 µl of fresh media was added to each culture drop following 48 h of culture. No oocyte selection strategy was employed in this study. These conditions routinely support an overall cleavage rate of 70% of inseminated oocytes with up to 30% of the inseminated oocytes (40% of cleaved zygotes) progressing to the blastocyst stage. Pools of 50-100 bovine embryos including 1-cell zygotes; 2-5 cell embryos; 6-8 cell embryos; morulae, and blastocysts were collected by removing the embryos from culture at the appropriate developmental times.

Experiment 1.

The purpose of experiment 1 is to determine the ability of oviductal cell co-culture to support bovine early development *in vitro* in a 10 % serum supplemented media. Cumulus-oocyte complexes were collected, matured and fertilized *in vitro* as described. A total of 834 presumptive zygotes representing up to 6 replicates were allocated into one of 4 groups: 1) oviductal cell monolayers established 48h prior to embryo culture in 1 ml of TCM-199 + 10% SS medium (158); 2) oviductal cell monolayer established 72h prior to embryo

culture in 1 ml of TCM-199 + SS medium (153); 3) 50 µl culture drops under oil, containing up to 40 oviduct epithelial cell vesicles (270); and 4) 50 µl culture drops containing no co-culture cells (253). Embryos were cultured for 8 days at 38.6 °C in a humidified 5% CO_2 in air atmosphere.

Experiment 2.

In experiment 2 the ability of bovine oviductal cell co-culture to sustain development of bovine embryos *in vitro* in a serum free environment is determined. 292 zygotes consisting of 3 replicates were allocated into one of two culture groups: 1) 50 µl culture drops containing 40 oviductal vesicles (145); or 2) 50 µl culture drops without cells (147). Embryos were cultured for 8 days at 38.6 °C in a humidified 5% CO_2 in air atmosphere.

Statistical Analysis

Cleavage frequency and development to the blastocyst stage was analyzed by Chi-square test. Values were considered significantly different at $P<0.05$.

2.3 RESULTS

The purpose of experiment 1 was to determine if the presence of bovine oviductal cell co-culture positively influenced bovine development *in vitro*. The rates of cleavage were consistently high in the 4 groups, averaging 64.6%. The

proportions (mean ± SEM) of inseminated oocytes which developed to blastocysts were not significantly different ($p>0.05$) in the 48 h monolayer (29.7 ● 5.3%, n=3), vesicle (25.2 ± 1.2%, n=6) and no cell (20.2 ± 1.9%, n=6) groups (Figure 2-1). Developmental rates to the blastocyst stage in the 72 h monolayer groups were significantly lower (5.8 ± 2.2%, n=3) than the other treatments, suggesting that monolayer cultures are not supportive of development once confluent.

In experiment 2, serum was removed from the co-culture environment to investigate the ability of oviductal co-culture to support development to the blastocyst stage independent of serum factors. Frequency of cleavage (mean ± SEM) did not vary significantly ($p>0.05$) in the 2 treatments (Fig 2-2). Development to the blastocyst stage was significantly greater in the vesicle group (21.5 ± 1.2%, n=3) compared to the group cultured without cells (3.3 ± 1.4%, n=3).

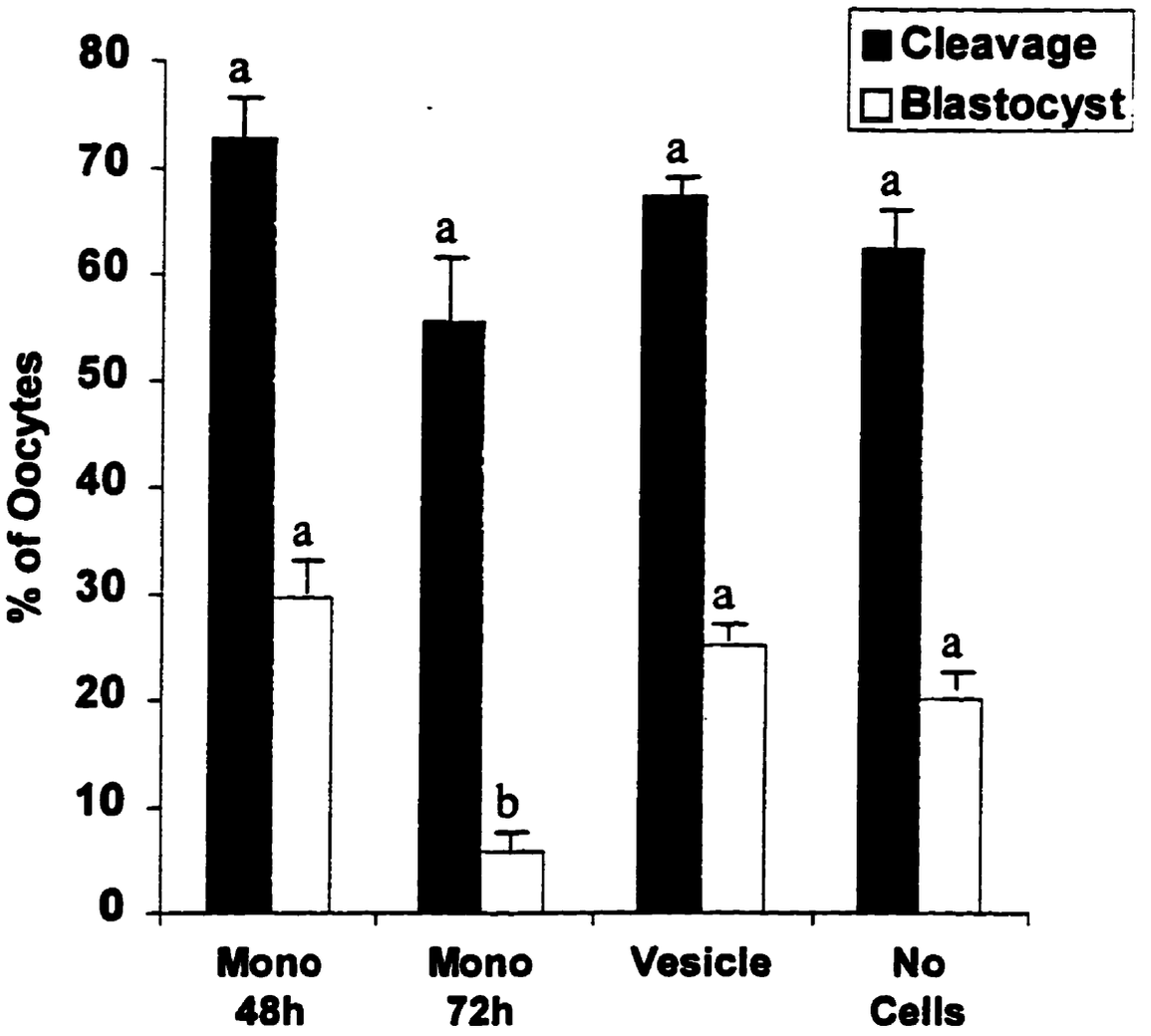
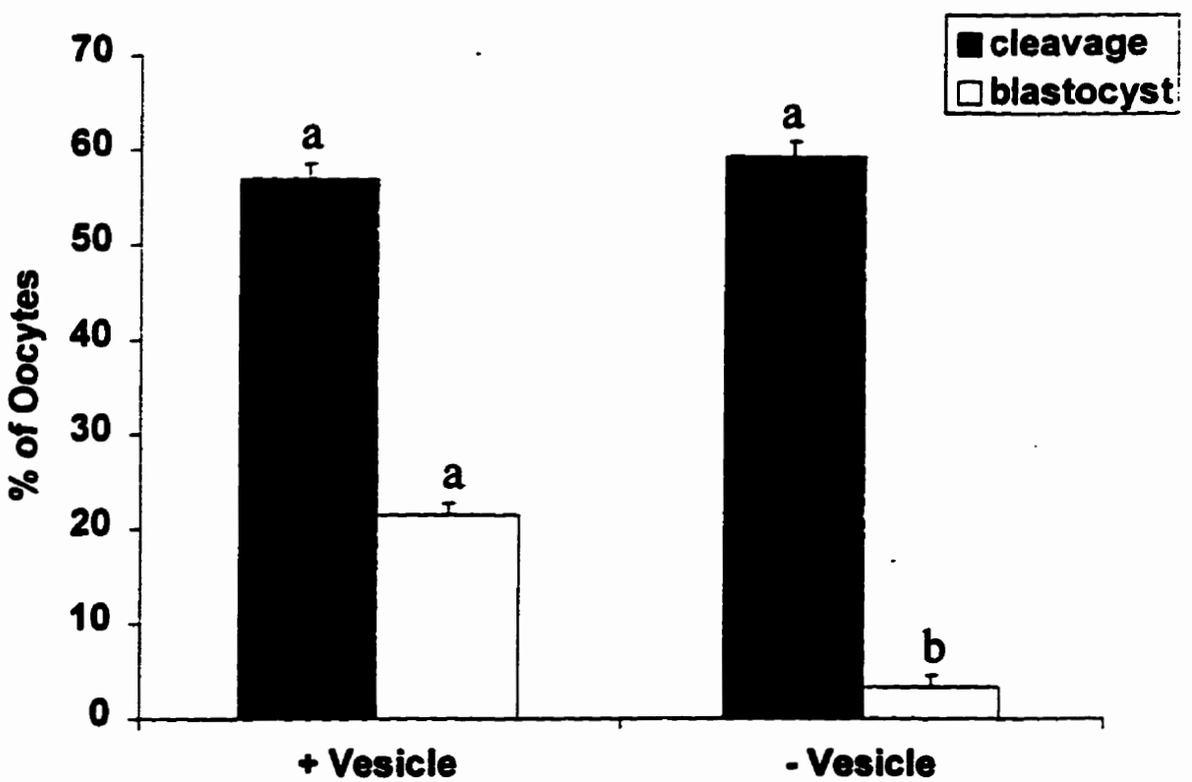

Cleavage
Blastocyst
% of Oocytes
80
70
60
50
40
30
20
10
0
a
a
a
b
a
a
a
a
Mono 48h
Mono 72h
Vesicle
No Cells

FIGURE 2-1. Comparison of bovine embryo culture in the presence and absence of oviductal cell co-culture. 834 presumptive zygotes were allocated into 4 culture treatments as follows: 48h monolayers (mono 48h, 158 zygotes, n=3), 72h monolayers (mono 72h, 153 zygotes, n=3), vesicles (270 zygotes, n=6), and no cells (253 zygotes, n=6). The % of oocytes refers to the number of zygotes progressing to that developmental stage as a percentage of the total number of oocytes in the culture group. Co-culture employing bovine oviductal cells positively influenced development ($p<0.05$). In this and in subsequent figures bars with different letters are different at $p<0.05$.

FIGURE 2-2. Comparison of bovine embryo development *in vitro* employing oviductal cell vesicle co-culture in the absence of serum. 292 presumptive zygotes were allocated into 2 treatments, + vesicles (145, n=3) and - vesicles (147, n=3). Both treatments employed culture in 50 μl culture micro drops. Vesicles significantly increased ($p<0.05$) the rate of blastocyst development (mean ± SEM). The embryos (21.5 ± 1.2%) produced in the vesicle treatment represent embryos that developed in an environment conditioned by the oviduct cells alone.


cleavage
blastocyst
% of Oocytes
70
60
50
40
30
20
10
0
a
a
a
b
+ Vesicle
- Vesicle

2.4 DISCUSSION

The development of bovine embryos to the blastocyst stage can be greatly influenced by the culture environment (Eyestone & First, 1989; Xu *et al.*, 1992; Bavister *et al.*, 1992; Keskintepe *et al.*, 1995). It is clear from experiment 1 that co-culture employing serum supports early development. In this experiment embryo development to the blastocyst stage was supported by culture without cells in the serum only group. Experiment 2 demonstrated that in the absence of serum significantly greater numbers (21.5 ± 1.2%) of zygotes progress to the blastocyst stage in the group employing vesicle co-culture vs culture without cells (3.3 ± 1.4%). This group represents a pool of embryos that survived in an environment conditioned only by the oviduct cells. The results support the proposition that the oviduct cells were able to condition the medium with "embryotrophic factors" that in turn facilitate early development. We hypothesize that the insulin-like growth factor (IGF) family represents one of the possible growth factor families provided by the oviductal co-culture cells. In the human both IGFs and IGFBPs were detected in the oviduct (Giudice *et al.*, 1992b; Pfeifer & Chegini, 1994).

The culture treatment that supported the greatest number of embryos through to the blastocyst stage is considered optimal for embryo production. For these reasons, I employed a serum supplemented culture environment to

maximize embryo production rates and produce all of the zygotes employed in the following experiments.

CHAPTER 3 IGF-I AND IGF-II RELEASE FROM BOVINE PREATTACHMENT EMBRYOS AND PRIMARY OVIDUCTAL CELL CULTURES

3.1 INTRODUCTION

The precise role(s) of growth factors in supporting early ovine and bovine development remains unclear, but evidence demonstrating that a significant number of bovine IVMF zygotes can progress beyond the 16 cell stage, reaching the blastocyst stage (Larson *et al.*, 1992a,b) in media supplemented with basic fibroblast growth factor (bFGF) and transforming growth factor (TGF-β) suggests that growth factors could certainly perform roles expected of "embryotrophic" factors. These molecules are, therefore, good candidates for further experimentation directed at understanding the molecular nature of the beneficial co-culture influence on early mammalian development. Bovine oviductal primary cultures express transcripts encoding, bFGF, TGF-α, TGF-β, platelet derived growth factor (PDGF-A), and insulin-like growth factors I and II (IGF-I, IGF-II) as determined by RT-PCR analysis (Watson *et al.*, 1992). Furthermore, bovine preattachment embryos express the same growth factor transcripts including mRNAs encoding IGF-I receptor, IGF-II receptor, and insulin-receptors (Watson *et al.*, 1992). We have confined our recent analysis to the IGF family since this family is one of the best characterized growth factor families in early development. The distribution of both mRNAs and polypeptides encoding IGF-I

and IGF-II has recently been mapped out in bovine oviductal monolayer and vesicle cultures (Xia *et al.*, 1996) by *in situ* hybridization and immunocytochemistry.

In order for the oviduct cells to act on the embryo through an IGF paracrine circuit, the cells must synthesize IGFs and release them into the culture media. The purpose of the following experiments was to measure the release of IGF-I and IGF-II in primary oviductal cell conditioned media. The level of release of IGF-II in blastocyst conditioned media is also determined for two purposes: first, to determine if the embryo has the capacity to release IGF-II locally as a possible autocrine route of control and secondly, to quantify the release of IGF-II in early mammalian embryos for the first time.

3.2 MATERIALS AND METHODS

Primary Oviductal Cell Cultures

Oviductal cells were isolated from fresh oviducts collected from the animals at slaughter and cultures were established as described in chapter 2.

Collection of Conditioned Media

Oviductal Monolayers

Conditioned medium was collected from monolayer cultures on days 2, 5 and 8 by first removing the serum supplemented medium and washing the cells

3 times (1 ml/well) with HBSS followed by 2 washes in serum-free TCM-199. The final wash medium was collected as a control to ensure that the possible transfer of serum proteins into the conditioned medium was avoided. Monolayer conditioned medium was prepared by adding 200 μl of TCM-199 to each culture well and incubating the cultures at 38.6°C under a 5% CO_2 in air atmosphere for 24 h. Conditioned medium from 8 wells was pooled, filtered through a 0.2 μm filter to remove any cells, and stored for up to 3 weeks at -20°C.

Oviductal Vesicles

Oviductal vesicle conditioned medium was collected from day 2, 5 and 8 cultures by first pooling the oviductal vesicles from 10 ml cultures into 15 ml plastic conical tubes and allowing the vesicles to settle forming a lose 250 μl pellet. The pellet was washed 4 times with serum-free TCM-199. The final wash was collected and analyzed as a control. Approximately half of the vesicles in each sample (ie 125 μl of vesicles) was cultured in 1 ml of serum-free TCM-199 medium. The vesicles were incubated at 38.6°C under a 5% CO_2 in air atmosphere for 24 h. Conditioned medium was collected, filtered and stored for up to 3 weeks at -20°C.

Blastocysts

Blastocyst-stage embryos were removed from culture on day 7 and washed 3 times in serum-free medium. Groups of 10 blastocysts were placed in 200 μl of serum-free TCM-199 medium for a 24 h incubation period. Conditioned medium was collected, lyophilized and re-suspended in 50 μl volumes for radioimmunoassay (RIA).

Radioimmunoassay (RIA) of IGF-I and IGF-II

A standard radioimmunoassay method applied routinely to plasma samples was modified to determine the concentration of the IGF ligands released into oviduct primary culture and blastocyst conditioned culture media (Hill *et al.*, 1984; Swenne *et al.*, 1987). Recombinant human IGF-I and IGF-II (Gro-Pep Pty Ltd, Adelaide, Australia) were iodinated to specific activities of 150-250 μCi/μg of protein using a chloramine-T method (Hill, 1990). Oviduct samples were concentrated by lyophilizing and re-suspending in a volume of 100 μl dH_20. IGFBPs were removed from all samples using an acidic environment (pH <3.4, formic acid) to release IGF/IGFBP complexes and precipitate the BPs. 100 μl of media was combined, in a 5 ml polypropylene tube, with 50 μl of 8M formic acid + 0.5% Tween-20. This solution was mixed and then 350 μl of acetone was added (Bowsher *et al.*, 1991). The tube was covered to prevent evaporation and

was centrifuged at 4°C for 30 min at 3000 x g. The supernatant (200 μl) was removed and neutralized in 1M Tris-base (200 μl). The loss of IGF during extraction was determined by the addition of labelled [^{125}I]IGF-I and -II to extraction reactions. The rate of recovery of labelled IGF-I and IGF-II was 52.2% ± 1.5 cpm (mean ± SEM, n=8) per sample. Conditioned media, standard or control samples (100 μl) + primary antibody (100 μl, anti-human IGF-I or IGF-II, GroPep) and RIA buffer (100 μl of 0.01 mol phosphate buffer/l containing 0.1% (w/v) sodium azide, 0.01M EDTA and 0.05% (v/v) Tween-20, pH 7.5) were combined in a 5 ml polystyrene test tube. The tubes were mixed briefly and incubated at 4°C overnight. 100 μl of ^{125}I-IGF was added to each tube at 20,000 cpm/tube. Tubes were mixed and incubated for a further 3 days before 250ml of PEG-2000 (Sigma) mix containing bovine gamma globulin (1.5 g/L, Sigma) was added to each tube. After overnight incubation, the tubes were centrifuged for 30 min at 6500 x g, the supernatant decanted, and the radioactivity of the pellet determined by γ spectroscopy. A standard curve was generated using 0.15-20 ng/ml recombinant IGF-I or IGF-II (GroPep human recombinant). Minimal detectable amounts and half maximal displacement of the radio-ligand occurred at 0.35 ng/ml and 2.2-2.8 ng/ml for IGF-I and at 0.32 ng/ml and 3.5-7 ng/ml IGF-II. For IGF-I, the intra- and interassay coefficients of variation were 10% and 12% respectively. For IGF-II, the intra- and interassay coefficients of variation were

8% and 13% respectively. Unknown samples were assayed in duplicate, with results corrected for loss during the extraction. Results are expressed as ng IGF-I or II released per μg cell DNA. For DNA quantification oviductal cells were lysed in 10% trichloroacetic acid (TCA) at 4°C for 20 min, then solubilized in 0.1 M NaOH overnight at 38.6°C. The solubilized cells were then assayed for DNA content by fluorometric spectroscopy as described in Kissane and Robins (1958).

Statistical Analysis

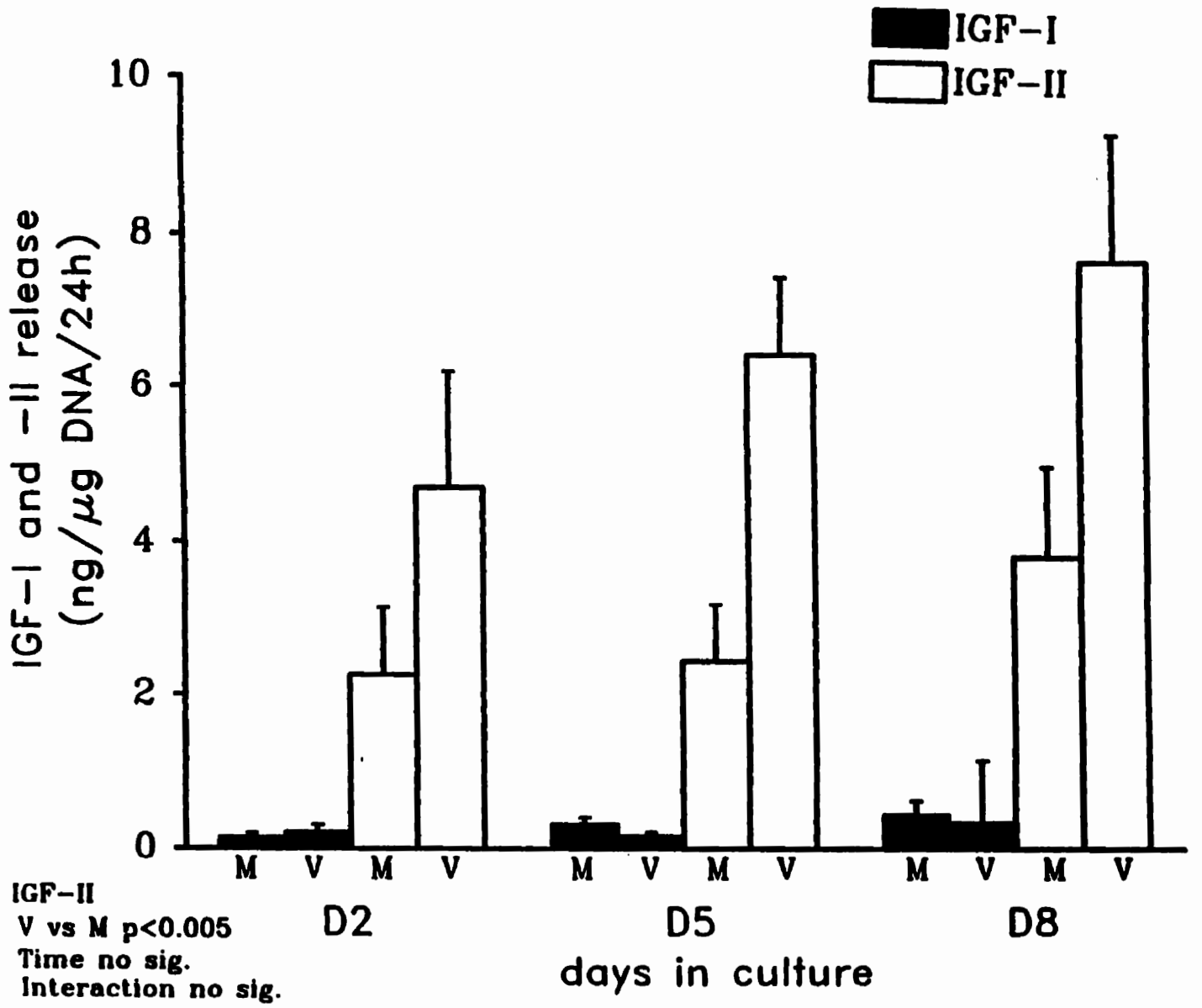
The effects of primary culture (monolayer or vesicle) and culture interval (days 2, 5, and 8) on IGF-I and IGF-II release from conditioned media was determined by a 2X3 factorial analysis of variance. Results were considered significant at $p < 0.05$. Oviductal cell conditioned media samples were collected in six replicate samples, and blastocyst conditioned media in four replicates.

3.4 RESULTS

RIAs were conducted to measure IGF-I and IGF-II accumulation in oviductal cell (Figure 3-1) and bovine blastocyst conditioned media. IGFBP extraction in these samples was confirmed by the absence of an IGFBP signal following Western ligand blot analysis prior to RIA (Figure 3-2). No significant variation in either IGF-I or IGF-II release was detected in either monolayer or

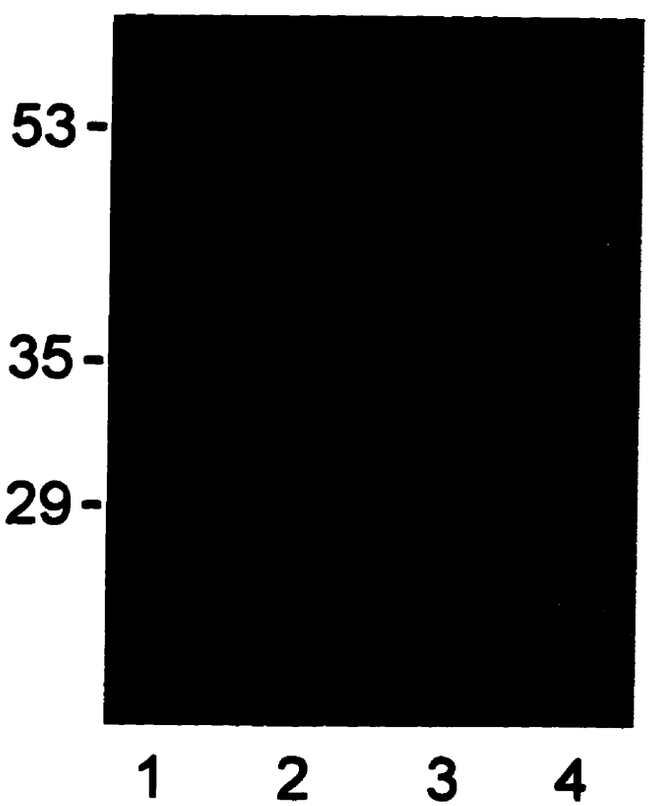
vesicle cultures over the 8 day culture interval. However, the release of IGF-II was 25 times that of IGF-I in vesicle cultures (6.25 ± 0.88 ng/μg DNA, IGF-II versus 0.25 ± 0.05 ng/μg of DNA, IGF-I), with a 9 fold difference in the accumulation of IGF-II over IGF-I in monolayer cultures (2.8 ± 0.56 ng/μg DNA, IGF-II versus 0.31 ± 0.08 ng/μg DNA, IGF-I; Figure 3-1). The release of IGF-II by vesicle cultures was significantly greater ($p<0.005$) than that observed for monolayer cultures. No significant difference in IGF-I release was observed. Pools of ten blastocysts released on average 36.2 ± 3.9 pg/embryo of IGF-II. Release of IGF-I from blastocysts was below the detectable levels of the assay.

FIGURE 3-1. Release of IGF-I and IGF-II from oviductal cultures over 24 h into serum-free medium. Following the extraction of binding proteins IGF levels were measured by RIA in 24h conditioned media samples collected at days 2, 5 and 8 of culture. No significant variation in the levels of IGF-I or IGF-II release was detected for vesicle (V) or monolayer (M) cultures over an 8-day culture interval. IGF-II is released at 25 times higher levels in vesicle cultures and 9 times higher in monolayer cultures than IGF-I release levels. The release of IGF-II from vesicle cultures was significantly greater than released from monolayer cultures ($p<0.005$). No significant difference in the levels of IGF-I release between monolayer and vesicle cultures was observed.



IGF–II
V vs M p<0.005
Time no sig.
Interaction no sig.

FIGURE 3-2. Ligand blot analysis assessing the extraction of binding proteins from oviductal conditioned media prior to RIA. Concentrated extracted and non-extracted samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with [^{125}I]IGF-II radio-ligand. Molecular weight standards predict migration distances at 53, 35 and 29 kDa. Lanes 1 & 2 are oviductal conditioned media samples from monolayer and vesicle cultures respectively, both indicate the presence of IGFBPs. Lanes 3 & 4 in which IGFBPs are not detectable are the extracted replicates of lanes 1 & 2. This blot was over-exposed (15 days) to ensure that the lack of detectable IGFBPs in the extracted lanes was not the result of under exposure. Similar ligand blots in which the exposure time was reduced produced more defined IGFBP bands.


53-
35-
29-
1
2
3
4

3.5 DISCUSSION

Bovine oviductal primary cell cultures express transcripts encoding IGF-I and IGF-II and the respective polypeptides (Xia *et al.*, 1996; Watson *et al.*, 1992). Our present results have confirmed that detectable amounts of both IGF-I and IGF-II are released by bovine primary oviductal cell monolayers and vesicles. IGF-I and IGF-II have been detected in porcine oviductal fluid, and porcine oviductal primary cultures also secrete IGF-I and IGF-II (Wiseman *et al.*, 1992). The differences in IGF-I and IGF-II levels detected in oviduct conditioned media are similar to other reports as Ko *et al.*, 1991 observed a 10 fold difference in IGF-II levels over IGF-I in day 12 to 14 cyclic and pregnant sheep uterine luminal fluids. The significantly higher levels of IGF-II in vesicle cultures over monolayer cultures is particularly intriguing. Xia *et al.* (1996) observed a restricted pattern of expression of IGF-II in monolayer cultures, a pattern not observed for vesicles where IGF-II expression was uniform. It is possible that the monolayers release less IGF-II on a per cell basis than the vesicle cultures due to the presence of non-IGF-II producing cells. It is clear from studies investigating early murine development that insulin and both IGFs are capable of stimulating physiological responses and increases in cell proliferation (Schultz & Heyner, 1993; Schultz *et al.*, 1993; Simmen *et al.*, 1993; Adamson, 1993; Rao *et al.*, 1990; Harvey & Kaye, 1991, 1992; Heyner & Garside, 1994). The results

presented here confirm that immunoreactive IGF-I and IGF-II proteins are released into the culture environment by the oviduct co-culture cells.

The presence of IGF-II in bovine blastocyst conditioned media is the first report of bovine blastocysts releasing detectable levels into the media. This release of IGF-II by the blastocyst also indicates that IGF-II may potentially act in an autocrine pathway to regulate early development. If the embryo is producing IGF to act in an autocrine fashion then the receptors must be present to transduce the IGF signal. In this experiment embryos are cultured in pools free of serum and other cells during the collection of conditioned media. It is possible that the level of IGF-II released from the blastocysts represents an increased level of production stimulated by the lack of IGF in the culture. Serum and oviductal cells present in culture accounts for large amounts of IGF normally available to the embryo, and lack of these sources may force the embryo to react to this decrease by increasing production.

Hemmings *et al.* (1992) reported detectable levels of IGF-II released into conditioned media from single human blastocysts. They were able to detect up to 4 ng/ml of released IGF-II. Their data is flawed however by the failure to extract binding proteins prior to RIA. Binding proteins present in the collected samples at the time of the RIA would result in false detection of IGF due to binding of labelled IGF to binding proteins (Blum & Breier, 1994). In the present study controls were carefully completed to ensure IGFBPs were removed prior to RIA.

IGFBP extraction was confirmed by the absence of IGFBPs following ligand blotting analysis of extracted samples.

This study is the first report of IGF-I and IGF-II released from primary bovine oviduct cell cultures, and IGF-II released from bovine blastocysts. Detection of IGFs in the culture environment provides the primary components necessary for growth factor regulation of development by IGF growth factor circuits. IGF-I and IGF-II growth promoting effects are largely modulated by IGFBPs which both inhibit and potentiate IGF actions. The following two chapters charaterize IGFBP expression in cultured oviductal cells and early bovine embryos, to discern a possible role for IGFBPs in modulating maternal IGF regulatory circuits.

CHAPTER 4 DETECTION OF TRANSCRIPTS ENCODING IGFBPs IN BOVINE PREATTACHMENT EMBRYOS AND PRIMARY OVIDUCT CELL CULTURES

4.1 INTRODUCTION

IGF transport and function is modulated by interactions with up to six IGFBPs (Murphy & Baron, 1993; Heyner *et al.*, 1993; Clemmons, 1993; Jones & Clemmons, 1995). The direct role of the specific IGFBPs in modulating embryonic IGF action is unknown due in part to the various inhibitory and potentiating actions of IGFBPs. In addition, the role of IGF on regulation of early bovine embryo development is not established. However, the characterization of expression of the IGFBPs may help to elucidate possible functions by determining which specific IGFBPs are present. Preattachment bovine embryos release immunoreactive IGF-II, and the production of IGFBPs may regulate possible autocrine interactions with IGF, IGFBPs and the receptors on the surface of the embryo. In the mouse preimplantation embryo transcripts encoding IGFBP-2, -3, -4 and -6 have been detected by RT-PCR (Schultz *et al.*, 1993). The present analysis will allow for the comparison between the expression of IGFBPs in the mouse and cow.

Levels of IGFBPs in the *in vivo* environment of the embryo may be largely influenced by the expression of oviductal IGFBPs. The present study was

undertaken to investigate further the regulatory interactions of these putative bovine maternal paracrine circuits by characterizing the expression of mRNAs encoding IGFBPs in bovine primary oviduct cultures and early preattachment embryos.

4.2 MATERIALS AND METHODS

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA Isolation

Total RNA was extracted according to Temeles *et al.* (1994). Bovine zygotes were pooled into the following groups: 1) 1-cell (zygotes); 2) 2-5 cell stage 3) 6-8 cell stage; 4) day 6 morulae; and 5) day 8 blastocysts. Pools of 50-100 bovine embryos were solubilized at room temperature in 100 µL of 4 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.4, 1 M 2-β mercaptoethanol solution in the presence of 20 µg of *E. coli* rRNA. Oviductal RNA was collected employing the same method without the *E. coli* rRNA. Following vigorous vortex mixing the samples were either frozen and stored at -70°C or were fully processed by precipitating the RNA by addition of 8 µl of 1 M acetic acid, 5 µl of 2 M potassium acetate and 250 µl of 100% ethanol. The samples were precipitated overnight at -20°C. The samples were subjected to centrifugation at 10,000 x g for 20 min at room temperature. The pellets were washed twice with cold 70% ethanol and

were air dried prior to re-suspension in 20 μl of re-suspension buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$). Genomic DNA was degraded by incubating the samples with 1 unit of RQI DNase (Promega, Biotec) for 30 min at 37°C. The samples were re-extracted with phenol and re-precipitated by adding 5 μl of 3M potassium acetate, pH 5.2 and three volumes of cold 100% ethanol for 24h at -20°C. The total RNA was collected by centrifugation, the pellets were washed with cold 70% ethanol and following air drying the samples were dissolved in 10 μl of autoclaved MilliQ water. The embryo total RNA was then used for reverse transcription. Oviductal RNA was quantified employing spectophotometry and aliquots of 1 μg of oviduct primary culture total RNA were used for reverse transcription.

Reverse Transcription

RNA was reverse-transcribed by oligo (dT) priming and Superscript Reverse Transcriptase (GIBCO BRL, Burlington, ON, Canada) (Harvey *et al.*, 1995; Watson *et al.*, 1992; Watson *et al.*, 1994). The RNA samples were incubated with 1 μg of Oligo $(dT)_{12-18}$ primer (GIBCO BRL) for 10 minutes at 70°C. After cooling on ice, RNA was incubated in 1st Strand Buffer (GIBCO BRL) containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs and 200 Units of Superscript Reverse Transcriptase (GIBCO BRL).

Reverse transcriptions (RT) were incubated at 43°C for 1.5 h. The reaction was terminated by heating at 94°C for 4 min and flash cooling on ice. The cDNA was further diluted with sterile distilled water to a concentration of 2 embryo equivalents/µl or the equivalent of 40 ng of oviduct RNA/µl.

Amplification of Binding Protein cDNAs

Polymerase chain reaction (PCR) was performed as described previously (Harvey *et al.*, 1995; Watson *et al.*, 1992; Watson *et al.*, 1994). Aliquots of embryo and oviduct cDNA (5 µl) were amplified with 1 U of *Taq* DNA Polymerase (GIBCO BRL) in a final volume of 50 µl containing 10 X *Taq* reaction buffer (200 mM Tris-HCl, pH 8.4 and 500 mM KCl), plus 1.5-2 mM $MgCl_2$, 0.2-0.24 mM dNTPs and 2 µM of each sequence-specific primer. The mixture was overlaid with mineral oil and then amplified by PCR for up to 40 cycles in a DNA thermal cycler (Perkin Elmer Cetus 480; or Thermolyne, Amplitron; VWR Scientific) with each cycle consisting of denaturation at 94°C for 1 min, re-annealing of primers to target sequences at 56°C-58°C for 30 sec, and primer extension at 72°C for 1 min. PCR products (20 µl) were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide.

PCR Primers

Primer pairs were obtained from the Core Molecular Biology Facility, London Regional Cancer Center, The University Of Western Ontario. The possibility of genomic DNA contamination was assayed for by PCR using β-actin primers that bracket an intron and produce a predicted 243 bp fragment for the cDNA and a larger DNA fragment (due to the presence of the intron) if genomic DNA is present (Harvey *et al.*, 1995; Watson *et al.*, 1992; Watson *et al.*, 1994). The larger genomic DNA product was not detected in any of the cDNA samples employed in this study. Primer pairs were derived from published human, and bovine cDNA sequences (Hahnel & Schultz, 1994; Spratt *et al.*, 1991; Shimasaki *et al.*, 1991; Bourner *et al.*, 1992) and the sizes of the expected PCR products are shown in Table 4-1. To confirm identity, each DNA product was ligated into pCR™II vector and transfected into One Shot® competent bacteria cells employing the TA cloning® kit (Invitrogen, Corp. San Diego, CA). Bacteria colonies were grown on Amp⁺/βgal agar plates and suspected positive colonies (white colonies) were identified and transfered into 5 μl of LB broth. Presumptive positive colonies were then screened by PCR using 2 μl of the LB broth employing the specific primers used to produce the cloned amplicon fragment. Clones determined to contain the desired DNA insert were cultured overnight by transferring the remaining 3 μl of LB broth to 10 ml of Amp⁺/LB broth. Plasmid

DNA was isolated from overnight cultures (see TA cloning^{R} kit) and 1-2 μg of plasmid DNA was sequenced by dideoxy sequencing employing base-specific termination of enzyme-catalyzed primer-extension reactions (Sanger *et al.*, 1977) using a T7 sequencing kit (Pharmacia Biotech).

4.3 RESULTS

RT-PCR assays were repeated a minimum of three times with embryo and oviduct samples derived from replicate cultures. β-actin amplicons representing the expected size products (243bps) from cDNA amplification were detected in all embryo and oviduct cell samples (Figure 4-1). The genomic DNA β-actin amplicon was detected in RNA samples that were not DNA digested (Figure 4-1, lane 1). Figure 4-2 displays the typical detection pattern for IGFBP mRNAs in cultured bovine preattachment embryos. Figure 4-3 summarizes the expression pattern for these mRNAs in both oviductal monolayer and vesicle primary cultures.

IGFBP -2, -3, and -4 transcripts were detectable in all stages of bovine preattachment development. Transcripts encoding IGFBP-5 were not detected in early cleavage stage embryos or morulae but a weak signal was consistently observed in blastocyst samples. Transcripts for IGFBP-1 were not detected in any preattachment embryo stage (Figure 4-2) although an expected size product

was detected in bovine liver RNA samples (Figure 4-4). The expected size IGFBP-6 amplicon (345 bp) was not detected in any preattachment embryo stage. Instead a much smaller (166 bp) amplicon was consistently observed in all embryo samples (Figure 4-2). This DNA product was, however, not observed in control bovine oviduct samples (Figure 4-3). Sequence analysis indicated that, of the first 84 bp of the 166 bp amplicon (Figure 4-5), 96% were identical to the reported sequence for an *E. coli* aceE gene encoding the E1 component of pyruvate dehydrogenase. This analysis was repeated several times on different bovine embryo cDNA samples and at no time was the expected IGFBP-6 PCR product detected.

Table 4.1 IGFBP PCR primer sequences

IGFBP	Primer sequences	Amplicon Size (bp)	Amplicon Identity
BP-1	5'primer=5'CGAGCCCTGCCGAATAGAAC 3'primer=5'CATCTGGCAGTTGGGGTC	239*	-
BP-2	5'primer=5'ACTGTCACAAGCATGGCCTG 3'primer=5'TCCTCCTGCTGCTCATTGTAG	186	99.2%(bovine)
BP-3	5'primer=5'ACTTCTCCTCTGAGTCCAAGC 3'primer=5'CGTACTTATCCACACACCAGC	210	100% (bovine)
BP-4	5'primer=5'CTGTGCCCCAGGGTTCCTGC 3'primer=5'TCACCCCCGTCTTCCGGTCC	222	100% (bovine)
BP-5	5'primer=5'GCTCAAGCCAGCCCACGCAT 3'primer=5'GTCGAAGCCGTGGCACTGAA	215	96.1%(human)
BP-6	5'primer=5'GACGAGGCGCCTTTGCGGGC 3'primer=5'GGAGGAGCGGCACTGCCGCT	345*	-

* size of predicted human amplicon

Figure 4-1. RT-PCR controls employing specific amplification of an intron spanning region of the β-actin gene. Lanes (L) bands from top to bottom: 396bp, 344 bp, 298 bp, 220/201bp), (1) genomic β-actin product (≅408 bps) detected in oviduct RNA sample without DNA digestion (no RT), (2-6) cDNA β-actin amplicon detected in oviduct cDNA (RT samples) from DNA digested RNA, (7) oviduct isolated RNA sample after DNA digestion (no RT), (8) negative control (no cDNA).


396
344
298
220
201
L 1 2 3 4 5 6 7 8

FIGURE 4-2. Detection of mRNAs encoding IGFBPs in preattachment bovine embryos by RT-PCR. Lanes are (L) ladder (bands from top to bottom: 506 bp, 396bp, 344 bp, 298 bp, 220/201 bp), (1) negative control (no cDNA), (2) oviduct RT control, (3) 1C zygotes, (4) 2-5C embryos, (5) 6-8C embryos, (6) morula, (7) day-8 blastocysts. Transcripts encoding IGFBP -2, -3, and -4 were detected throughout preattachment development, while IGFBP-5 mRNAs were only detected in blastocyst stage embryos. mRNAs encoding IGFBP-1 and -6 were not detected in any preattachment embryo stage, however a smaller than expected PCR amplicon was detected in all embryo samples. The identities of each amplicon were determined by DNA sequence analysis.

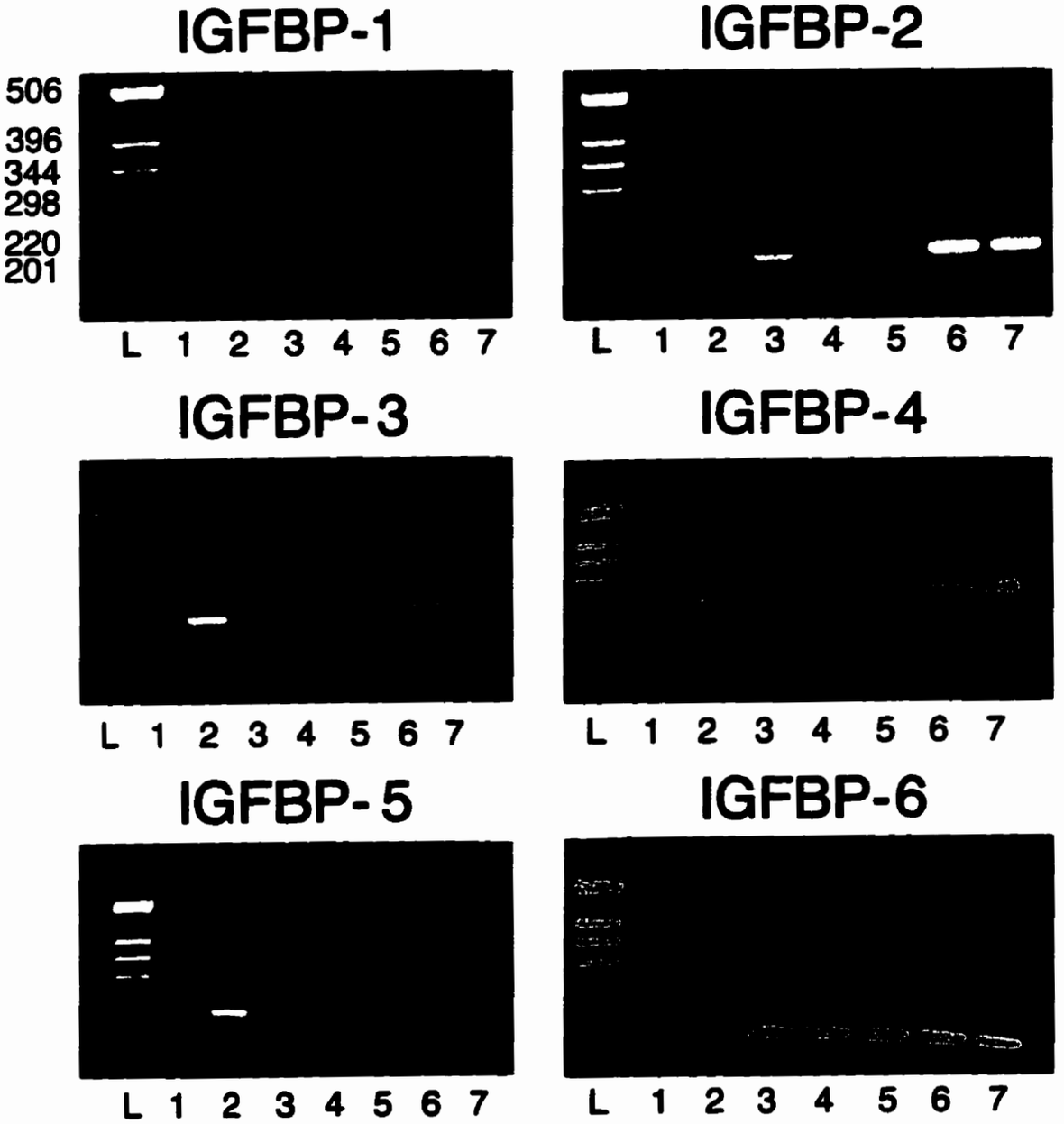

IGFBP-1
506
396
344
298
220
201
L 1 2 3 4 5 6 7
IGFBP-2
L 1 2 3 4 5 6 7
IGFBP-3
L 1 2 3 4 5 6 7
IGFBP-4
L 1 2 3 4 5 6 7
IGFBP-5
L 1 2 3 4 5 6 7
IGFBP-6
L 1 2 3 4 5 6 7

FIGURE 4-3. Detection of IGFBP transcripts in non-cultured fresh oviduct samples (F), day-2 vesicle cultures (D2V), day-8 vesicle cultures (D8V), day-2 monolayer cultures (D2M) and day-8 monolayer cultures (D8M) by RT-PCR. Lanes are (L) ladder (bands from top to bottom: 506 bp, 396 bp, 344 bp, 298 bp, 220/201 bp), Lanes 1-6 correspond to PCR products encoding IGFBPs 1-6, respectively. Transcripts encoding IGFBPs 2-5 were detected in both vesicle and monolayer primary cultures throughout an 8-day culture interval. The detection of IGFBP-6 mRNAs occurred inconsistently in only a single culture replicate (for example D2V). The identities of each amplicon were determined by DNA sequence analysis.


F
D2V
D8V
506
396
344
298
220
201
L 1 2 3 4 5 6
L 1 2 3 4 5 6
L 1 2 3 4 5 6
D2M
D8M
L 1 2 3 4 5 6
L 1 2 3 4 5 6

Figure 4-4. Detection of IGFBP-1 mRNA in bovine liver by RT-PCR. Lanes are (L) ladder (bands from top to bottom: 396bp, 344 bp, 298 bp, 220/201 bp), (1) bovine liver (239bps), (2) oviduct (3) negative control (no cDNA).

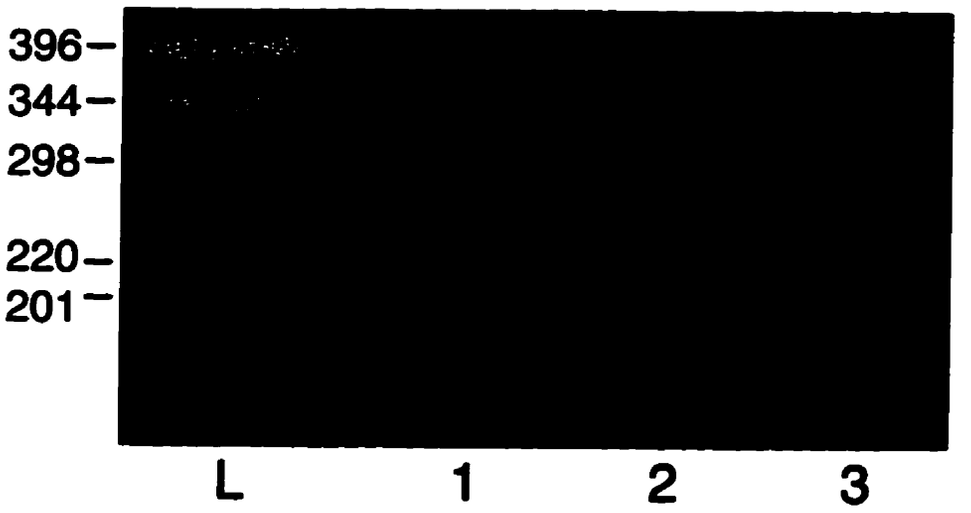

396
344
298
220
201
L
1
2
3

Figure 4-5. Nucleotide sequences of PCR amplicon fragments of bovine IGFBP 2-5. PCR products were amplified to verify their identity, against published IGFBP sequences. IGFBP-6 amplicon fragment sequence shares similarity to *E. coli* aceE gene encoding the E1 component of pyruvate dehydrogenase and has no homology to IGFBP-6. The RGD sequence in IGFBP-2 is indicated by the underlined nucleotides and the primers employed are in boldface.

IGFBP-2 SEQUENCE (186 bps, 99.2% @ bovine)

GGAAGAATTC ACTGTGACAA GCATGGCCTG TACAACCTCA AACAGTGCAA
GATGTCTCTG AACGGGCAGC GTGGGGAGTG CTGGTGTGTG AACCCCAACA
CCGGGAAGCT GATCCAGGGA GCCCCCACCA TCCGGGGAGA CCCCGAGTGT
CATCTCTT**CT ACAATGAGCA GCAGGAGGAT CCTTCC**

IGFBP-3 SEQUENCE (210 bps, 100% @ bovine)

ACTTCTCCTC TGAGTCCAAG CGTGAGACAG AATACGGGCC CTGCCGCCGG
GAAATGGAAG ACACGCTGAA CCACCTCAAG TTCCTGAACA TGCTCAGCCC
CAGGGGCATC CACATTCCCA ACTGCGACAA GAAGGGCTTC TACAAGAAAA
AGCAGTGCCG CCCTTCCAAG GGCAGGAAGC GGGGTTTCT**G CTGGTGTGTG**
GATAAGTACG

IGFBP-4 SEQUENCE (222 bps, 100% @ bovine)

GGAAGAATTC TGTGCCCCAG GGTTCCTGCC AGAGTGAGCT GCACCGGGCG
CTGGACGGCT GGCCGCCTCA CAGAGCCGCA CCCACGAAGA CCTTTACATC
ATTCCCATCC CCAACTGCGA CCGCAACGGC AACTTCCACC CCAAGCAGTG
CCACCCGGCC CTGGATGGGC AGCGCGGCAA GTGCTGGTGT GT**GGACCGGA**
AGACGGGGGT GAGGATCCTT CC

IGFBP-5 SEQUENCE (215 bps, 96% @ human)

GGAAGAATTC GCTCAAGCCA GCCCACGCAT GGTGCCCCGC GCCGTGTACC
TGCCCAACTG TGACCGCAAA GGGTTCTACA AGAGAAAGCA GTGCAAACCT
TCCCGTGGCC GCAAGCGTGG CATCTGCTGG TGCGTGGACA AGTACGGGAA
TGAGCTGCCG GGCATGGAGT ACGTGGACGG GGACT**TTCAG TGCCACGGCT**
TCGACGGATC CTTCC

PCR PRODUCT AMPLIFIED BY IGFBP-6 PRIMERS

GGAAGAATTC GACGAGGCGC CTTTGCGGGC GTCGTGAAGG AACTGAAAGA
CAACGTTGGC GATAAAGTGA AAACTGGCTC GCTGATTATG ATCTTCGAAG
TTAAAGGCGC AGTGCGGCGG CTCCGGCGAA ACAGG**AAGCG GCAGTGCCGC**
TCCTCCGGAT CCTTCC

The identity of the remaining IGFBP amplicons were confirmed by sequence analysis comparing the bovine embryo IGFBP amplicon sequences to published bovine and human IGFBP cDNA sequences (Table 4-1, Figure 4-5) (Hahnel & Schultz, 1994; Spratt *et al.*, 1991; Shimasaki *et al.*, 1991; Bourner *et al.*, 1992). Comparison of the embryonic IGFBP-2 DNA product with those of published sequences revealed a 99.2% sequence identity to the bovine IGFBP-2 sequence. Embryonic IGFBP-3 and -4 products displayed a 100% sequence identity to their respective bovine cDNA sequences and the IGFBP-5 DNA product displayed a 96.1% sequence identity to that of the human cDNA (Figure 4-5).

Analysis of IGFBP transcript distribution in oviduct monolayer and vesicle primary cultures displayed consistent expression patterns for IGFBP -2, -3, -4, and -5 over an 8-day culture interval (Figure 4-3). The mRNAs encoding IGFBP-1 were never detected in any oviduct cell culture sample while a weak PCR signal of expected size (345 bp) encoding IGFBP-6 was detected in only a single monolayer and vesicle culture sample.

4.4 DISCUSSION

We have demonstrated that bovine oviduct monolayer and vesicle cultures both express transcripts encoding IGFBPs 2-5 throughout an 8-day culture interval. In contrast, IVMF bovine zygotes express mRNAs encoding

IGFBPs 2-4 through to the blastocyst stage. mRNAs encoding IGFBP-5 were detected in bovine blastocysts while transcripts encoding IGFBP-1 were not detected in oviductal cultures or early embryos. IGFBP-6 amplicons were inconsistently detected in oviduct cultures and IGFBP-6 mRNA was not detected in early bovine embryos. Each bovine IGFBP DNA product displayed a 96% or greater sequence identity to published cDNAs (Hahnel & Schultz, 1994; Spratt *et al.*, 1991; Shimasaki *et al.*, 1991; Bourner *et al.*, 1992). The 166 bp fragment derived from the IGFBP-6 primers did not display any identity to published IGFBP-6 sequences (Shimasaki *et al.*, 1991). This amplicon was also observed by Hahnel and Schultz (1994), using identical primers to investigate the expression of IGFBP-6 mRNAs during murine preimplantation development. The IGFBP-6 amplicon of expected size was detected only in murine blastocyst samples, however, the 166 bp product was observed in all preimplantation stage embryo samples (Hahnel & Schultz, 1994). Our result, indicating a shared homology with a *E. coli* aceE gene encoding the E1 component of pyruvate dehydrogenase is intriguing and is worthy of further investigation, especially since this DNA product was not detected in bovine oviduct samples. It is possible that this amplicon represents an embryo specific gene product.

Clearly, differences in the expression of mRNAs encoding the IGFBPs exist between murine and bovine early embryos as IGFBP-5 transcripts were confined to bovine blastocysts and were not detected at any preimplantation

murine stage. IGFBP-6 mRNAs were not detected in the cow but were detected at the blastocyst stage of murine development (Hahnel & Schultz, 1994). The significance of these species differences awaits further investigation. No differences in IGFBP mRNA expression was detected in oviduct vesicle or monolayer primary cultures over an 8-day culture interval. The inconsistent detection of IGFBP-6 amplicons in both monolayer and vesicle cultures raises the possibility that IGFBP-6 expression may be low, transitory, or linked to ovarian cycles. This possibility has been investigated in the porcine ovary (Samaras *et al.*, 1993; Grimes *et al.*, 1994; Gadsby *et al.*, 1996), human oviduct (Giudice *et al.*, 1992) and rat (Yallampalli *et al.*, 1993), mouse (Markoff *et al.*, 1995) and bovine (Geisert *et al.*, 1991) uterus.

The expression of IGFBP mRNA by bovine blastocysts raises the possibility that bovine embryonic IGF circuits are modulated during early embryo development. IGFBP transcripts present in the oviduct suggest that the oviduct may be conditioning the embryonic environment with these regulatory IGFBPs. Chapter 5 investigates the release of IGFBPs from cultured oviductal cells to determine the presence of IGFBPs in the *in vitro* co-culture environment.

CHAPTER 5 RELEASE OF IGFBPs FROM BOVINE PRIMARY OVIDUCT CELL CULTURES

5.1 INTRODUCTION

IGF-I and IGF-II are both present in the *in vitro* culture environment (Chapter 3). In addition mRNAs encoding IGFBPs 2-5 are present in primary oviductal cultures (Chapter 4). The embryotropic actions of the IGF family may be selectively modulated by IGFBPs released into the culture system by the oviductal cells. This chapter focuses on characterizing the release of IGFBP polypeptides from the oviductal cells into conditioned media. The presence of IGFBPs in the culture environment would support the notion of a direct influence of these molecules in modulating IGF actions on the developing embryo.

5.2 MATERIALS AND METHODS

Detection of Oviductal IGFBPs by Western Ligand Blotting

Oviductal monolayer and vesicle conditioned media were collected from serum-free cultures as described in chapter 3, and were concentrated by centrifugal ultrafiltration using a membrane with a molecular weight cutoff of 10 kDa (Centricon-10, Amicon, Danvers, MA). Concentrated samples were subjected to electrophoresis using a non-reducing 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were then electrophoretically transferred to a

nitrocellulose membrane (Bio-Rad, Mississauga, Ont) for 2 h at a constant current of 250 milliamps. The membrane was washed for 30 min in Tris-NaCl (pH 7.4), 0.5 mg/mL sodium azide (Sigma) and 3% Nonidet P-40 (Sigma) before blocking for 2 h in Tris-NaCl (pH 7.4), 1% BSA (Sigma) all at 4°C. The membrane was washed twice for 20 min in Tris-NaCl (pH 7.4) + 0.1% Tween-20 (Sigma) and was incubated for 20 h with 400,000 cpm [^{125}I]IGF-II, (for ligand control 100 ng/ml unlabelled IGF-II was added to incubating solution) in Tris-NaCl (pH 7.4), 0.1% Tween-20, and 1% BSA at 4°C (Bradshaw & Han, 1993; Hill *et al.*, 1989). Following the incubation the membrane was taken through a series of 15 min washes, two in Tris-NaCl (pH 7.4) + 0.1% Tween-20 followed by 3 washes in Tris-NaCl (pH 7.4) at 4°C. The membrane was air dried at room temperature and exposed to X-ray film (XAR, Eastman, Kodak, Rochester, NY) with intensifying screens at -70°C for 3-7 days.

Western immunoblots were prepared on the same samples described above. Membranes were initially washed for 30 min in 10 mM Tris-HCL containing 0.15 M NaCl, 0.3% NP-40 (v/v) and 0.5 mg/ml sodium azide (pH 7.4). The membrane was then blocked for 1 h in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCL, pH 7.4) containing 0.05% Tween 20 (v/v) (TTBS) supplemented with 4% BSA (wt/v) and was washed 3 X 10 min in TTBS. Membranes were incubated for 20 h at 4°C in TTBS + 1% BSA (wt/v) with one of the following antibodies: anti IGFBP-2 (rabbit polyclonal antiserum against

bovine IGFBP-2 (Bourner *et al.*, 1992), dil. 1:1000, Upstate Biotechnology Inc, UBI, Lake Placid, NY), anti IGFBP-4 (rabbit polyclonal antiserum against human IGFBP-4 (Camacho-Hubner *et al.*, 1992), dil. 1:250, UBI) and anti IGFBP-5 (rabbit polyclonal antiserum against human IGFBP-5, dil. 1:100, Austral Biologicals, San Ramon, California). Following incubation with primary antiserum the membranes were washed with TTBS (3 X 10 min), and then incubated with anti-rabbit IgG biotin conjugates (Sigma, dil. 1:30) in 1% BSA (wt/v) TTBS for 2 h. Membranes were washed 3 X 10 min in TTBS, ExtrAvidin (Sigma, 1:30 in PBS) for 1 h, and 3 X 10 min in TTBS. Bands were visualized using a 3-3' diaminobenzidine (DAB) tetrahydrochoride (Sigma)/ 3% hydrogen peroxide reaction. The reaction was then quenched in 50 mM Tris-HCL (pH 7.5) and the membranes were air dried.

IGFBP-2 Immunofluorescence

Oviduct cell vesicles and monolayers were prepared for immunofluorescence labelling. Vesicles were collected and isolated in the same manner as for the conditioned medium procedure. However, the monolayers were grown on glass cover-slips for easier processing. Round glass cover-slips were soaked in 70% ethanol prior to coating with lysine (0.1% poly-L lysine in Tris-HCl, pH 8.5) for 15 min, and were air dried overnight under a sterile culture flow hood. The cover-slips were then added to each well of the 24-well plates

and monolayers were grown on them, and the same procedures previously described for establishing these cultures were applied. Monolayers were grown to confluency and transferred through a graded methanol series. Monolayers were transferred to 125mm dishes on ice, and were taken through the following series; 1:1 PHEM (60mM pipes, 25mM hepes, 10 mM EGTA, 1mM Mg $Cl_2 \cdot 6H_2O$, pH 6.9, Schliwa & VanBlerkom, 1981):MeOH (methanol), 1:2 PHEM:MeOH, 100% MeOH, 1:2 PHEM:MeOH, 1:1 PHEM:MeOH for 3-4 min per treatment. Vesicle cells were fixed through the same series by moving the cells into the different solutions (200μl) in 4-well culture dishes. The fixed cells were then placed in blocking solution (0.1% Triton X-100, 0.1 M lysine, 1% goat serum in PHEM buffer) for 45 min at 4°C. The cells were removed from the blocking solution and were washed for 10 min in PHEM buffer prior to overnight incubation with IGFBP-2 primary antiserum (1:200 dilution, UBI, Lake Placid, NY). Unbound primary antibody was removed by 2 washes of 10 min each and a final 6h wash in PHEM buffer. The cells were placed in a 1:50 dilution of FITC conjugated goat anti-rabbit secondary antibody, and unbound secondary antibody was removed in 3 washes of PHEM with the last wash overnight. The cells were then mounted in a small volume of FITC-guard (Sigma) and were visualized on a confocal laser microscope and a Zeiss ICM 405 fluorescence microscope at 160 X.

Preabsorbed ligand control was obtained by incubating IGFBP-2 ligand

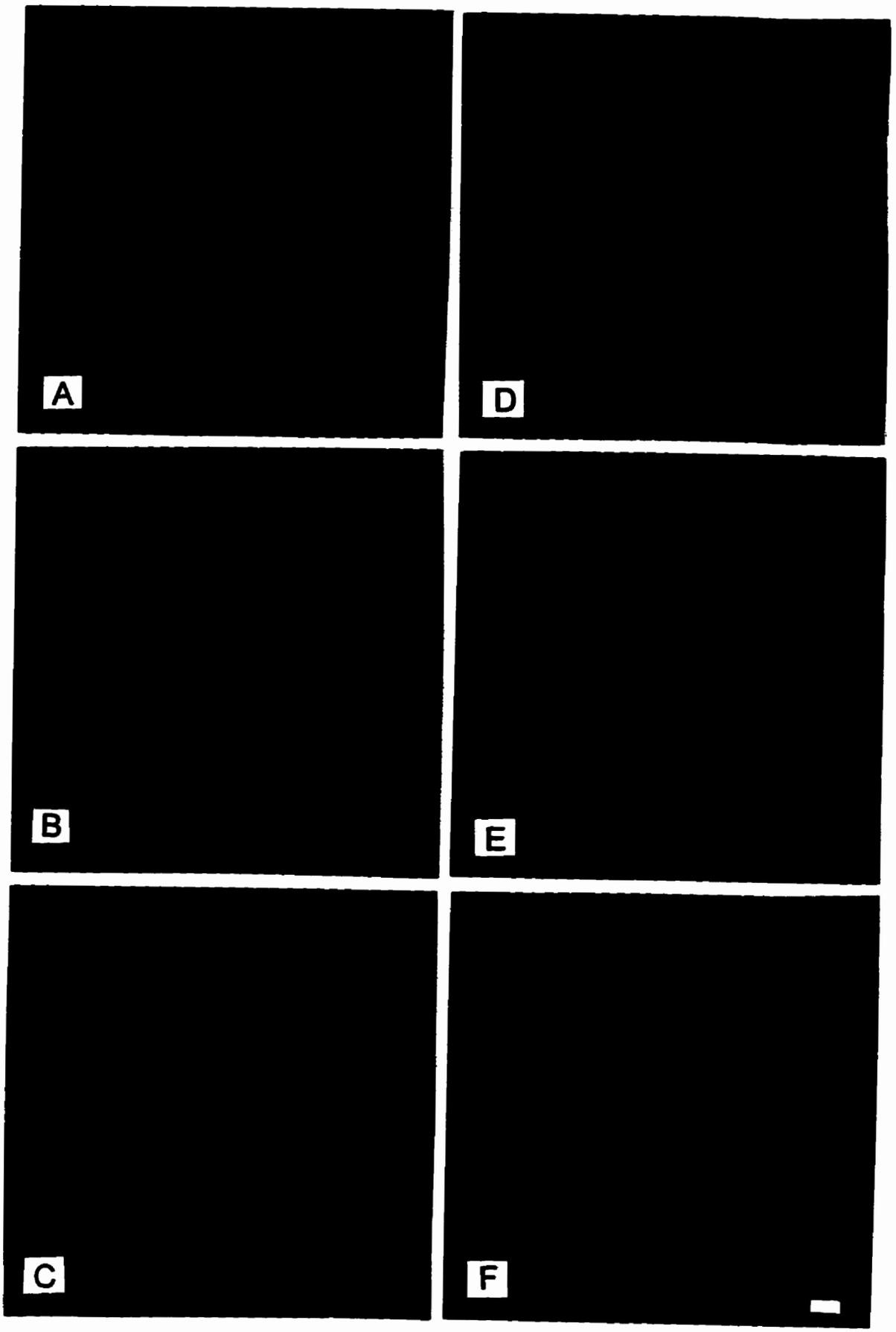
(200 ng, Austral Biologicals, San Ramon, Cal.) with the primary antibody (400 µl, 1:200 dil.) for three days at 4°C, the mixture was then centrifuged for 30 min at 13 000 x g. The upper 200 µl was collected. The non-absorbed primary antibody was subjected to the same procedure but incubation with the ligand was omitted. In addition slides were processed without the addition of the primary antibody present, to control for the possibility of the secondary antibody alone producing signal.

5.3 RESULTS

Detection of IGFBP-2 in cultured monolayer and vesicle cells

Immunofluorescence results employing IGFBP-2 primary antiserum (1:200 dilution, UBI, Lake Placid, NY) applied to preparations of primary monolayer and vesicle cell cultures revealed a positive signal for IGFBP-2 in both primary cultures (Figure 5-1). Three replicate day 8 monolayer and vesicle cell cultures were analyzed. In monolayer cultures the majority of cells produce IGFBP-2 however, not every cell was positive for IGFBP-2. In vesicle cultures it is difficult to detect IGFBP-2 in single cells, giving the appearance of uniform expression. Controls, both preabsorbed antiserum and secondary antiserum alone demonstrate the specificity of the antibody localization.

Figure 5-1. Immunofluoresence localization of IGFBP-2 proteins in oviductal monolayer (A, B, C) and vesicle cell cultures (D, E , F). B and E represent pre-absorbed controls, C and F are secondary antibody controls. IGFBP-2 localization was observed in 3 replicate vesicle and monolayer cultures. All cultures were examined under equal magnification; Bar=10μm.


A
D
B
E
C
F

Detection of IGFBPs in bovine oviductal cell conditioned media

Western ligand analysis of conditioned media prepared from monolayer and vesicle cultures over an 8-day culture period revealed four IGFBPs of approximate molecular weights 24 kDa (IGFBP-4), 31 kDa (IGFBP-5), 36 kDa (IGFBP-2) and a broad band extending from 46 to 53 kDa (IGFBP-3; Figure 5-2, lanes 1 and 2). This analysis was repeated four times employing conditioned media collected from replicate monolayer and vesicle cultures established from separate oviduct collections. No differences in the banding patterns was observed between IGF-I and IGF-II radio-labelled ligand blots (Figure 5-3) suggesting IGFBP-6 is not represented in the banding pattern. [^{125}I]IGF-II produced a more intense signal and was therefore used preferentially for subsequent ligand blots. The specificity for IGF binding displayed by these polypeptides was verified by control analysis consisting of competitive binding assays employing cold IGF-II to displace binding of radio-labelled IGF-II. In all cases this procedure eliminated the detection of any IGFBP signal in these samples (Figure 5-4, lanes 3,6). Furthermore, media was collected from the final washes and no detectable levels of IGFBPs were observed in these controls (Figure 5-4, lanes 2,5). Identities of the binding proteins were indicated from the relative molecular sizes and were confirmed by immunoblotting using IGFBP specific antisera (Figure 5-2,lanes 3-8). A single band of 36 kDa was detected employing a rabbit polyclonal antiserum raised against bovine IGFBP-2 (UBI) in

both oviductal monolayers and vesicles (Figure 5-2, lanes 3 and 4, arrow). Likewise bands of appropriate molecular weights for IGFBP-4 (Figure 5-2, lanes 5 and 6, arrow) and IGFBP-5 (Figure 5-2, lanes 7 and 8, arrow) were detected in oviductal monolayer and vesicle cultures employing rabbit polyclonal antisera specific for human IGFBP-4 (UBI) and human IGFBP-5 (Austral Biologicals) respectively.

FIGURE 5-2. Western ligand blot and immunoblot analysis of 24 h conditioned media collected from oviductal cell monolayer (1,3,5,7) and vesicle (2,4,6,8) primary cultures. Concentrated samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with [^{125}I]IGF-II radio-ligand. A representative autoradiograph reveals 4 bands with IGF-II binding affinity at mol wt 24 kDa, 31 kDa, 36 kDa and a broad band at 46-53 kDa, in both monolayer and vesicle cultures (lanes 1,2). Western immunoblot analysis employing polyclonal antisera against: IGFBP -2 (lanes 3,4), -4 (lanes 5,6), and -5 (lanes 7,8), confirms the identity of the 36 kDa, 24 kDa and 31 kDa bands (arrows) respectively; identified by ligand blot analysis.

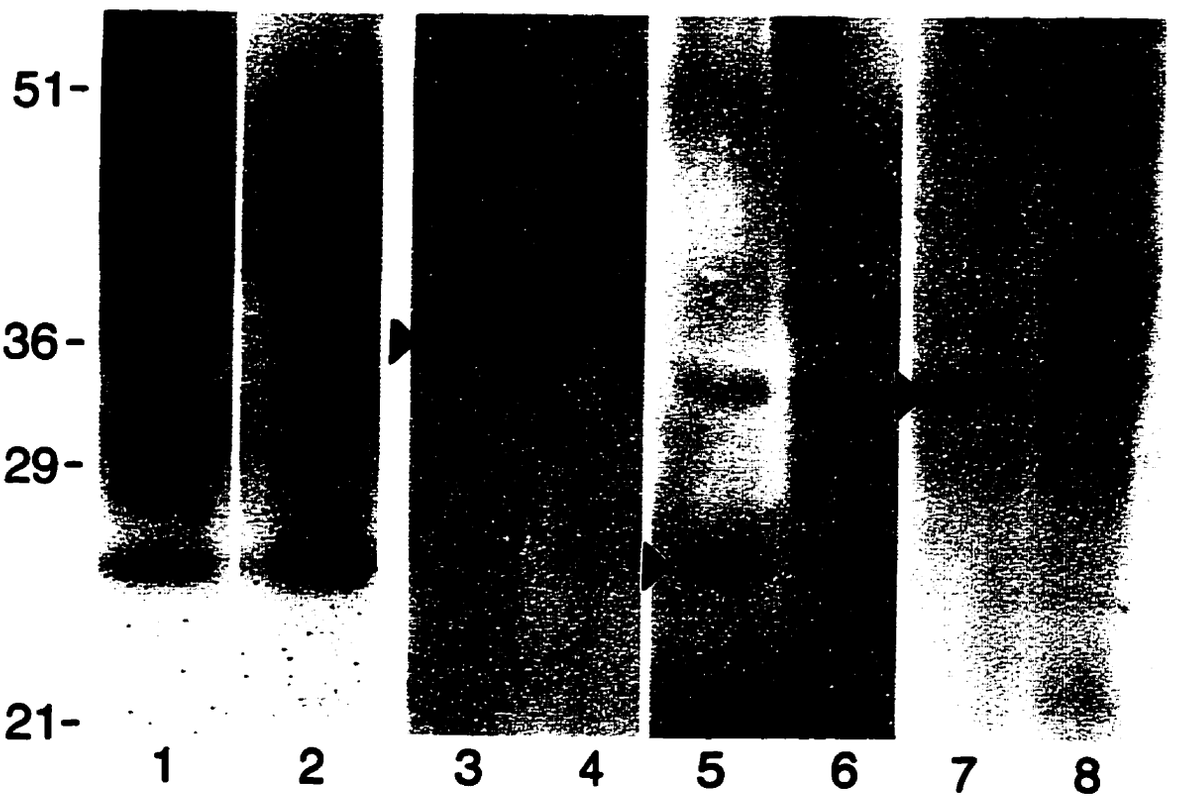

51-
36-
29-
21-
1
2
3
4
5
6
7
8

FIGURE 5-3. Comparison of IGFBP detection patterns produced by ligand blot technique employing either [^{125}I]IGF-I or [^{125}I]IGF-II. Samples of conditioned medium were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with [^{125}I]IGF-I or [^{125}I]IGF-II radio-ligand. Molecular weight standards represent the migration of proteins of 51 kDa, 36, 29 and 21. A representative autoradiograph reveals 4 bands with IGF binding affinity at molecular weight 24 kDa, 31 kDa, 36 kDa and a broad band at 46-53 kDa, in monolayer (lanes 2,5) vesicle cultures (lanes 3,6) and bovine serum (lanes 1,4). The banding patterns produced by IGF-I and IGF-II are identical, with IGF-II producing a autoradiograph of greater intensity then IGF-I.

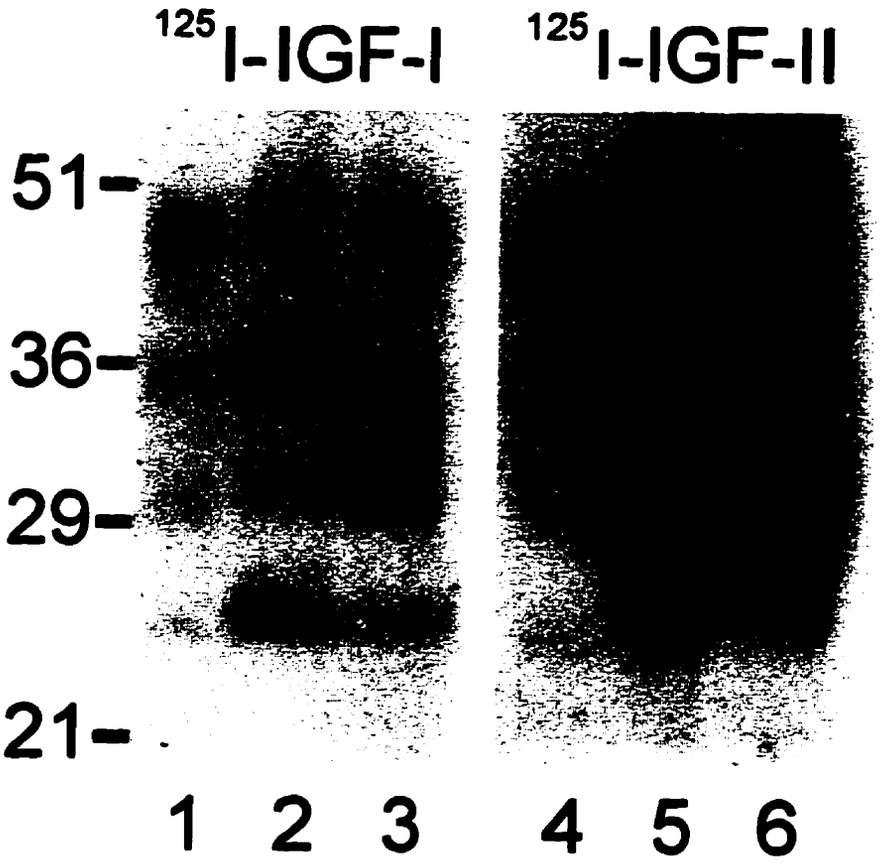
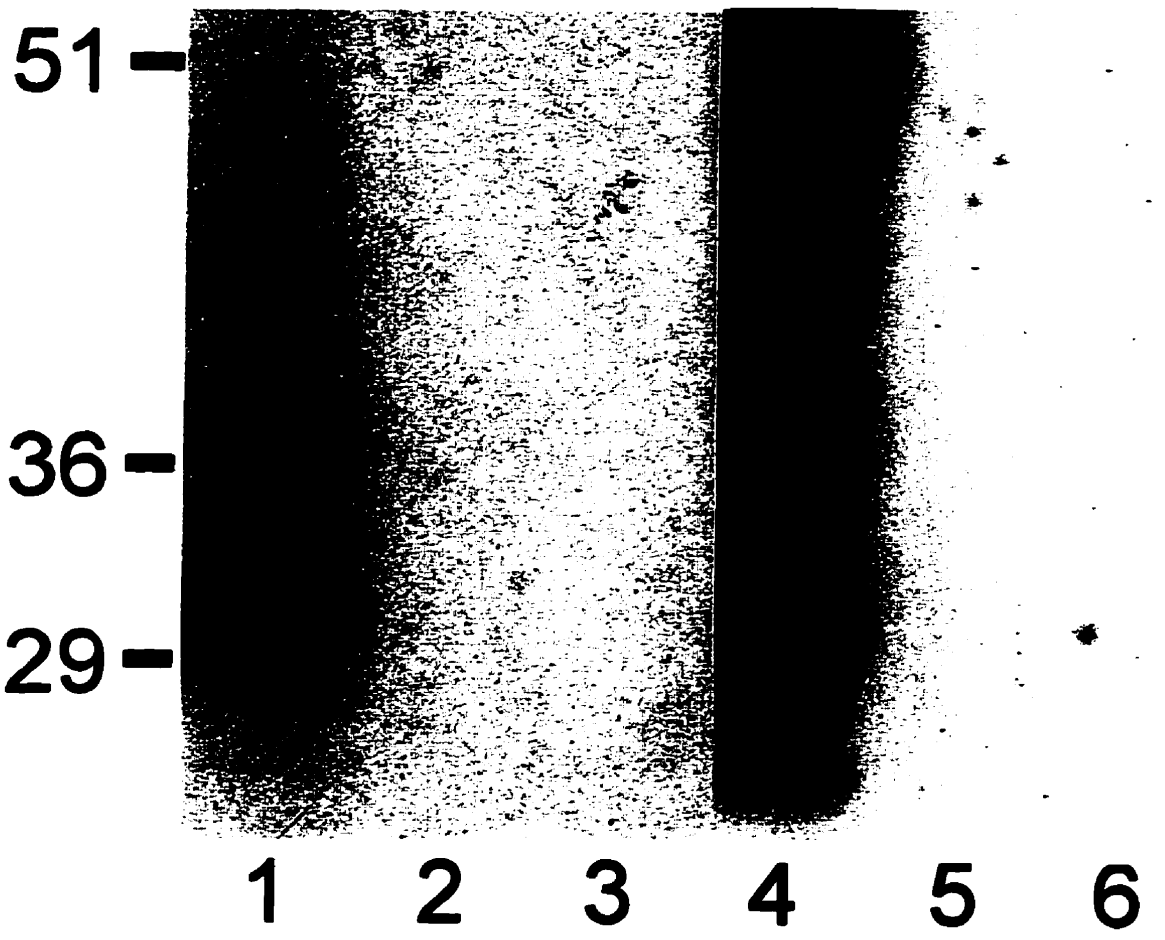

125I-IGF-I
125I-IGF-II
51
36
29
21
1 2 3 4 5 6

FIGURE 5-4. [^{125}I]IGF-II ligand blot controls for the removal of serum from cultures prior to establishment of serum-free cultures. To control against the possibility of IGFBPs from the culture, oviduct cells were washed 5 times and the last wash was processed as a control. Molecular weight standards represent the migration of proteins of 51 kDa, 36, and 29. Lanes 1, 2 and 3 represent vesicles cultures and lanes 4, 5 and 6 represent monolayer cultures. Lanes 1 and 4 are banding patterns produced by 24h oviductal cell conditioned media, and lanes 2 and 5 correspond to the final washes. Lanes 2 and 5 indicate that binding proteins were not present in the final wash media, therefore, IGFBPs present in lanes 1 and 4 are the result of IGFBP release.

Lanes 3 and 6 represent the same conditioned media samples run in lanes 1 and 2 respectively, and the [^{125}I]IGF-II binding was subjected to competitive binding by the addition of 100 ng/ml cold IGF-II. Lanes 3 and 6 demonstrate that the addition of cold IGF-II successfully competed for IGFBP binding reducing the intensity of the autoradiograph and indicating that [^{125}I]IGF-II occurs specifically.


51
36
29
1
2
3
4
5
6

5.4 DISCUSSION

Co-culture systems employing bovine oviductal cells remain an effective method of facilitating early development of bovine embryos. Xia *et al.* (1996) detected IGF-I and IGF-II proteins in both monolayer and vesicle cultures. In 8-day monolayers a restricted pattern of IGF-II localization was observed, in which some cells failed to show a signal for the IGF-II. Monolayer cultures display this restricted pattern for IGFBP-2 protein also with some cells not expressing IGFBP-2. Xia *et al.* (1996) report expression of IGF-II in all cells within compact vesicles. In the present study IGFBP-2 immunofluorescence is also detected in every cell. Detection of peptides by immunofluorescence in vesicle cultures is more difficult than in the monolayer because the signal is formed from several cell layers. Detection of IGFBP, by immunofluorescence, in the bovine oviductal cells is the first evidence reporting IGFBP polypeptides present in these cultures. This method suggests local production of the IGFBPs but does not indicate release of IGFBPs into the culture medium. Of the available IGFBP antiserum only IGFBP-2 is bovine specific. Available antiserum for the remaining IGFBPs have greater cross reactivity due to regions of high homology between the IGFBPs. Immunofluorescence employing these antisera may result in the detection of several IGFBPs. Western analysis provides a method of detecting IGFBPs released by the cells into conditioned media, and provides separation by molecular weight. Western ligand blots and immunoblots were employed to

characterize all of the IGFBPs released by these cultures.

Western ligand blot analysis detected four IGFBPs of approximate molecular weights 24 kDa, 31 kDa, 36 kDa and a broad band extending from 46 to 53 kDa in oviductal monolayer and vesicle conditioned media samples. The IGFBPs are bovine oviductal products since serum was removed prior to collection of conditioned media and ligand blots of control wash media did not result in the detection of any IGFBP signal. Furthermore, the IGFBP signal was eliminated when the blots were co-incubated with unlabelled IGF-II. Bands of proteins at 24, 31, 36, and 46-53 kDa correspond in molecular weight to IGFBP -4, -5, -2 and -3 respectively (Murphy & Baron, 1993; Clemmons, 1993; Jones & Clemmons 1995; Bradshaw & Han, 1993; Hill *et al.*, 1989; Guidice *et al.*, 1992; Yallampalli *et al.*, 1993). The 46-53 kDa band appears to represent a doublet consisting of the non-glycosylated and the glycosylated form of IGFBP-3, and runs at the same size as the dominant band present by Western ligand analysis of steer serum. The identity of the 24, 31, and 36 kDa protein was confirmed by immunoblot methods. Bands at molecular weight range 29-30 kDa detected in the ligand blots and in the IGFBP-4 Western immunoblot may represent glycosylated forms of IGFBP-4 (Olney *et al.*, 1993) or cross reactivity of antiserum with IGFBP-2 (Camacho-Hubner *et al.*, 1992). Since transcripts encoding IGFBP-1 were not detected and IGFBP-6 mRNAs were not consistently detected in these primary cultures it is unlikely that the 29-30 kDa

polypeptides are related to IGFBP-1 or IGFBP-6. Furthermore, no differences in the banding patterns was observed between IGF-I and IGF-II radio-labelled ligand blots (Figure 5-3) suggesting IGFBP-6 is not represented in the banding pattern. IGF-II is reported to have a 10 fold higher affinity for IGFBP-6 than that displayed by IGF-I (Roghani *et al.*, 1989).

In summary IGFBPs 2-5 are readily detected in oviductal cell conditioned media. This supports the potential regulation of IGF actions on embyros by IGFBPs present in the culture environment.

CHAPTHER 6 ANALYSIS OF BOVINE PARTHENOGENETIC EMBRYOS FOR THE EXPRESSION OF IGFBPs

6.1 INTRODUCTION

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 (Surani, 1994). IGF-II is an imprinted gene in which expression stems from only the paternal allele (Surani, 1994; DeChiara *et al.*, 1991). Imprinting of IGF-II has been hypothesized to function as a regulatory mechanism to decrease the available copies of IGF-II, since over-expression of IGF-II can be detrimental, as observed in gene "knock-out" studies (Filson *et al.*, 1993; Lau *et al.*, 1994; Wang *et al.*, 1994; Rappolee *et al.*, 1992; Vu & Hoffman, 1994). The IGF-II receptor is also believed to be regulated by imprinting in an opposite fashion to IGF-II. Expession of IGF-II receptor is the result of the maternal allele only (Stoger *et al.*, 1993). The present study employed a bovine parthenogenetic model to contrast expression of IGFBP mRNAs between these embryos with *in vitro* fertilized zygotes. Although imprinting of the IGF-II gene is well established by birth (Surani, 1994) a debate continues regarding the state of the imprint during preimplantation development. Separate reports suggest that IGF-II is imprinted

at this early stage (Rappolee *et al.*, 1992) and conversely that it is not imprinted (Latham *et al.*, 1994). These studies were concerned with gene regulation at the level of transcription, reporting the presence or absence of IGF-II mRNA. In the present study I have also measured levels of IGF-II secretion into conditioned media by parthenogenotes and contrasted these levels with those observed for IVMF embryos.

Before useful comparisons can be made between the bovine parthenogenetic model and *in vitro* fertilized embryos it is necessary to develop reliable methods of producing parthenogenetic embryos. Clearly chromosomal complement will impact dramatically on patterns of gene expression. Many treatment regimes have been employed in an attempt to produce diploid embryos including chemical agents capable of blocking polar body extrusion such as cytochalasin D or B (Balakier and Tarkowski, 1976; Kubiak *et al.*, 1991; Fukui *et al.*, 1992; Minamihashi *et al.*, 1993;) cycloheximide (Presicce *et al.*, 1994a,b; Hagemann *et al.*, 1995) and 6-dimethylaminopurine (DMAP) (Fulka *et al.*, 1991; Szollosi *et al.*, 1993; Navara *et al.*, 1994; Susko-Parrish *et al.*, 1994).

In order to determine the effectiveness of the diploidization procedure, the chromosomal complements of parthenogenetic bovine embryos arising from standard oocyte activation and diploidization methods were analyzed.

6.2 MATERIALS AND METHODS

Oocyte Activation and Diploidization

Bovine oocyte activation and diploidization procedures employing ethanol, ionomycin, cytochalasin D and DMAP were applied as outlined in Minamihashi *et al.* (1993) and Susko-Parrish *et al.* (1994). The treatments included; 1) oocyte activation with ethanol (7% for 5 min) followed by cytochalasin D treatment (5 μg/ml; Sigma) for 6 h; 2) activation with ethanol and treatment with DMAP (1.9 mM, Sigma) for 6 h and 3) activation with ionomycin (5 μM, Sigma) for 5 min and treatment with DMAP (1.9 mM) for 6 h. Treatments involving oocyte activation alone were applied as a double activation method with an initial treatment with ionomycin or ethanol for 5 min separated by a 4 h culture interval before a second 5 min treatment with ionomycin or ethanol (Susko-Parrish *et al.*, 1994).

Following the activation/diploidization protocol, oocytes were washed and placed directly into culture drops. Parthenogenotes were co-cultured in 50 μl drops of TCM-199 medium + 10% SS under oil with up to 40 oviductal vesicles added per drop (Xu *et al.*, 1992; Harvey *et al.*, 1995). To sustain development through to the blastocyst stage, 50 μl of TCM-199 + 10% SS was added to each culture drop following 48 h. All of the collected COCs were utilized in this study and no oocyte selection strategy was employed.

Chromosomal Analysis

Parthenogenetic bovine embryos were removed from culture at either the 2-cell or blastocyst stages. Parthenogenotes were first incubated in 0.05 μg/ml of colcemid in TCM-199 + 10% SS for 12h. They were then exposed to a hypotonic 1% Na Citrate solution for 3 min to promote nuclear swelling and cell spreading. Embryos were spread on clean glass slides with MeOH (methanol):AcAcid (acetic acid) (1:1) while blowing gently with the slide placed under a lamp. The embryos were fixed on the slides overnight at 4°C in MeOH:AcAcid (3:1) and were then air dried and stained in 4% Giemsa for 4 min to reveal chromosomal complements (King *et al.*, 1979; Plante and King, 1996). Metaphase spreads were counted under low power while chromosomes were examined at both 400 and 1000 X under oil to determine the chromosome composition for each metaphase spread.

Detection of IGFBP mRNA

RNA was isolated from 3 pools of 50 parthenogenetic blastocysts and subjected to RT-PCR analysis using primers specific for the six IGFBPs (see chapter 3). Parthenogenetic embryos employed in the RT-PCR studies were activated in ethanol and diploidized by DMAP incubation, as described.

Release of IGF-II from Parthenogenetic Embryos

RIA technique was used to determine the amount of IGF-II released per embryo into conditioned media over a 24h period (see chapter 4)

Statistical Analysis

Cleavage frequency and development to the blastocyst stage was analyzed by Chi-square test. The significant value was $p<0.05$. Release of IGF-II levels was analyzed by a paired t test, significant at $p<0.05$.

6.3 RESULTS

Production of Bovine Parthenogenetic Embryos

Development following Oocyte Activation and Diploidization Treatments

Three oocyte activation and diploidization treatments were compared to assess their influence on chromosomal complement and development to the blastocyst stage. Oocytes were matured for 24 h prior to artificial oocyte activation to limit a possible influence of spontaneous oocyte activation on the outcomes. In total, 593 oocytes representing three experimental replicates were randomly assigned to the experimental groups. The proportion of cleaved parthenogenotes varied significantly between treatments ($p<0.05$) with 68.0 $\pm$ 1.8% (mean $\pm$ SEM, n=3) of the oocytes treated with ethanol and DMAP dividing (Figure 6-1). The proportion of cleaved embryos did not vary significantly

between the ethanol/cytochalasin D or ionomycin/DMAP treatments (Figure 6-1). Blastocyst formation (18.4 ± 2.5%) did not vary significantly between the treatments (Figure 6-3). Once again however, the ethanol/DMAP treatment group displayed the highest frequency of development with 22.3 ± 3.0% of treated oocytes progressing to the blastocyst stage (Figure 6-1).

Development Following Oocyte Activation Without Diploidization

A comparison between the two oocyte activation treatments alone without cytochalasin D or DMAP treatment was made to assess the potential for development of activated oocytes to the blastocyst stage. In total, 203 oocytes representing three experimental trials were randomly assigned to the two treatment groups. The oocytes were subjected to a double activation procedure to enhance the cleavage rate of non-diploidized activated oocytes. The proportion of oocytes that cleaved following activation with ionomycin (34.7 ± 5.3%) or ethanol (39.4 ± 8.5%) did not vary significantly. This frequency of cleavage was substantially lower than that observed for the cytochalasin D/DMAP treatment groups. Development to the blastocyst stage was not observed in the oocyte activation alone treatment groups.

Chromosomal Complement of Blastocysts

In total, 44 blastocysts were examined for chromosomal analysis from the ethanol/cytochalasin D (n=21), ethanol/DMAP (n=11) and ionomycin/DMAP (n=12) treatments (Figure 6-2 A,B,C,D). 219 metaphase chromosomal spreads were examined from the 44 blastocysts representing a mean of 4.97 metaphase spreads per embryo. The number of metaphase spreads per blastocyst varied from 1 to 21. The number and percentage of chromosomal complements displayed by parthenogenetic blastocysts derived from all three oocyte activation and diploidization treatments are shown in Table 6-1. The majority of embryos displayed tetraploid and mixoploid chromosomal complements (Figure 6-2, A,C) consisting of primarily diploid/triploids and diploid/tetraploids. Consistent diploid complements were only observed in 5/44 (11.4%) of the evaluated blastocysts. An example of an octaploid chromosomal complement is displayed in Figure 6-2 B. The analysis suggests that these treatment regimes result in high rates of aberrant chromosomal complements.

Chromosomal Complements of 2-Cell Stage embryos

Since parthenogenetic blastocysts displayed low frequencies of diploid chromosomal complements it was important to address the timing of the events that resulted in these aberrant chromosomal complements. For this reason, we examined the chromosomal complement of 2-cell embryos treated with ethanol

or ionomycin alone. Since the oocytes in these treatment groups were not subjected to cytochalasin D or DMAP treatments we were able to also investigate whether the aberrant chromosomal complements arose, in part, due to the these treatments or could arise simply as a consequence of artificial oocyte activation.

A total of 24 2-cell stage parthenogenotes were analyzed from the ethanol alone (n=12) and ionomycin alone (n=12) treatments. In total, 57 chromosomal spreads were examined from the 24 embryos representing a total of 2.38 spreads per embryo. The results are displayed in Table 6-2. The number of metaphase spreads per 2-cell embryo varied from 0 to 9 (Figure 6-2 D). The majority of 2-cell parthenogenotes (16/24 66.7%) displayed a haploid chromosomal complement (Table 6-2). The mixoploid embryos consisted of haploid/diploids (Figure 6-2 D). Since development to the blastocyst stage was not observed in these treatment groups it was not possible to explore the influence of culture on the progression of aberrant chromosomal complements.

Expression of IGFBP mRNA in Parthenogenetic Embryos

Bovine parthenogenetic blastocysts express transcripts encoding IGFBP -2, -3, -4 and -5 (Figure 6-3). Transcripts for IGFBP-1 were not observed and a similar 166 bp amplicon (as found in IVMF blastocysts) was amplified with the IGFBP-6 primers. The expression pattern of IGFBP mRNA was identical to the

pattern detected with IVMF embryos (Figure 4-1).

Release of IGF-II from parthenogenetic embryos

RIAs were conducted to measure the level of IGF-II released into conditioned media from parthenogenetic and fertilized blastocysts. Blastocysts produced following fertilization released significantly greater amounts (mean ± SEM) of IGF-II (36.2 ± 3.9 pg/embryo) compared to parthenogenetic embryos (9.6 ± 2.8 pg/embryo, $p<0.05$, n=6, Figure 6-4).

Table 6-1. Chromosomal composition of parthenogenetic blastocysts

Treatment	Haploid (%)	Diploid (%)	Tetraploid (%)	Octaploid (%)	Mixoploid (%)
Etoh/CytoD	1/21 (4.8)	3/21 (14.3)	6/21 (28.6)	1/21 (4.8)	10/21 (47.6)
Etoh/DMAP	0/11 (0)	1/11 (9.1)	4/11 (36.4)	1/11 (9.1)	5/11 (45.5)
Iono/DMAP	0/12 (0)	1/12 (8.3)	8/12 (66.7)	0/12 (0)	3/12 (25.0)
Total	1/44 (2.3)	5/44 (11.4)	18/44 (40.9)	2/44 (4.5)	18/44 (40.9)

Table 6-2 Chromosomal composition of 2-cell parthenogentic embryos

Treatment	Haploid (%)	Diploid (%)	Tetraploid (%)	Octaploid (%)	Mixoploid (%)
Ionomycin	8/12 (66.7)	2/12 (16.7)	1/12 (8.3)	0/12 (0)	1/12 (8.3)
Etoh	8/12 (66.7)	2/12 (16.7)	0/12 (0)	0/12 (0)	2/12 (16.7)
Total	16/24 (66.7)	4/24 (16.7)	1/24 (4.2)	0/24 (0)	3/24 (12.5)

FIGURE 6-1. Development of Bovine Parthenogenotes Following Oocyte Activation and Diplodization Treatments. In total, 593 oocytes (three experimental replicates) were randomly assigned to experimental groups consisting of 1) oocyte activation with ethanol (7% for 5 min) followed by cytochalasin D treatment (5 mg/ml; Sigma) for 6 h; 2) activation with ethanol and treatment with dimethylaminopurine (DMAP, 1.9 mM, Sigma) for 6 h and 3) activation with ionomycin (5 mM, Sigma) for 5 min and treatment with DMAP (1.9mM) for 6 h. The mean $\pm$ SEM for the proportion of cleaved parthenogenotes (48 h following treatment) and blastocysts (day 8 of culture) was determined for each group. Oocytes activated by ethanol/DMAP treatment displayed a significantly higher cleavage frequency* of 68.0 $\pm$ 1.8% ($p<0.05$). Blastocyst formation did not vary significantly between the treatments.

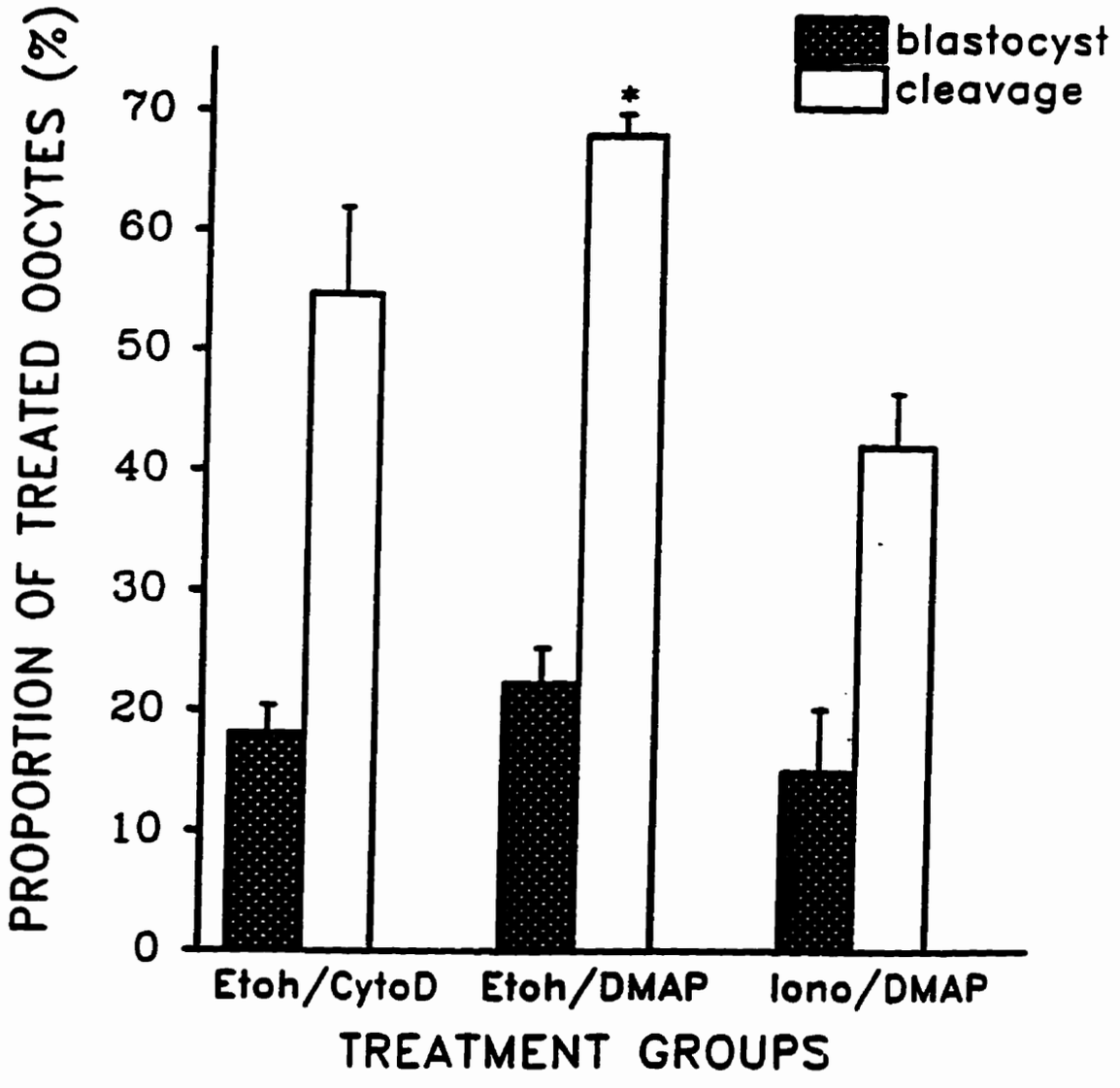

blastocyst
cleavage
PROPORTION OF TREATED OOCYTES (%)
70
60
50
40
30
20
10
0
*
Etoh/CytoD
Etoh/DMAP
Iono/DMAP
TREATMENT GROUPS

Figure 6-2. Parthenogenetic Bovine Embryo Chromosomal Complements. Metaphase chromosomal spreads representing various chromosomal complements of bovine parthenogenetic blastocysts and 2-cell embryos. Representative chromosomal composition of blastocysts including A) tetraploid metaphase spread; under oil with 100X objective, B) octaploid metaphase spread; 100X objective, C) mixoploid complements, 40X objective. 2D) Metaphase spread of a 2-cell multi-nucleated parthenogenote containing 3 nuclei consisting of haploid, diploid and tetraploid nuclei; 40X objective.

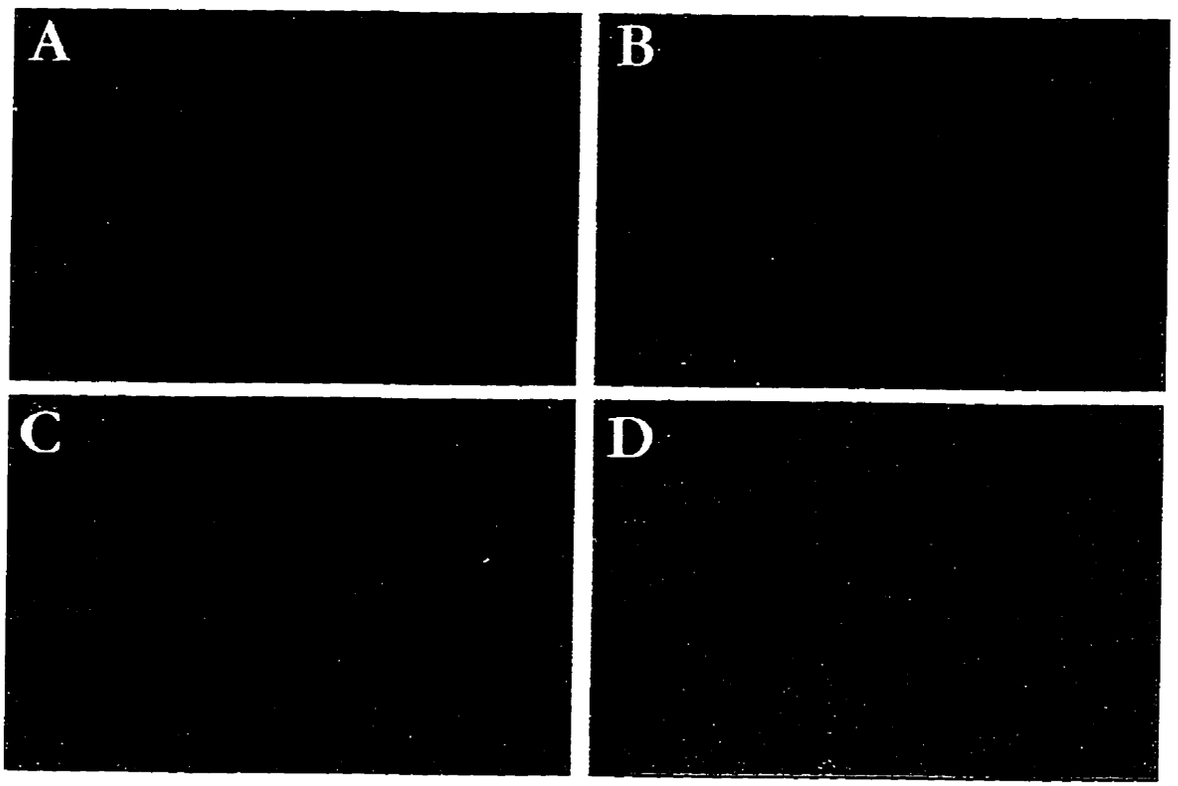

A
B
C
D

FIGURE 6-3. Detection of mRNAs encoding IGFBPs in parthenogenetic bovine embryos by RT-PCR. Lanes are (L) ladder (bands from top to bottom: 516/506 bp, 394bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp), (1-6) represent IGFBPs 1-6. Transcripts encoding IGFBP -2, -3, -4 and 5 were detected. mRNAs encoding IGFBP-1 and -6 were not detected in any preattachment embryo stage, however a smaller than expected PCR amplicon was detected for IGFBP-6 in all embryo samples.

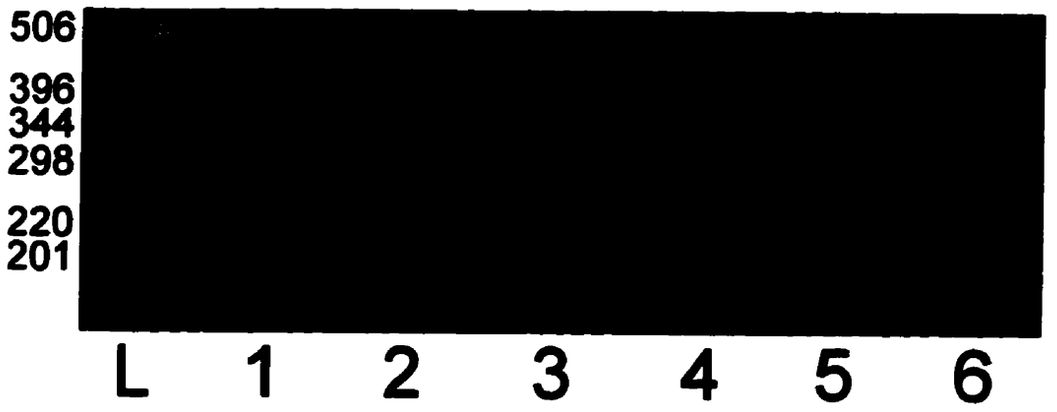
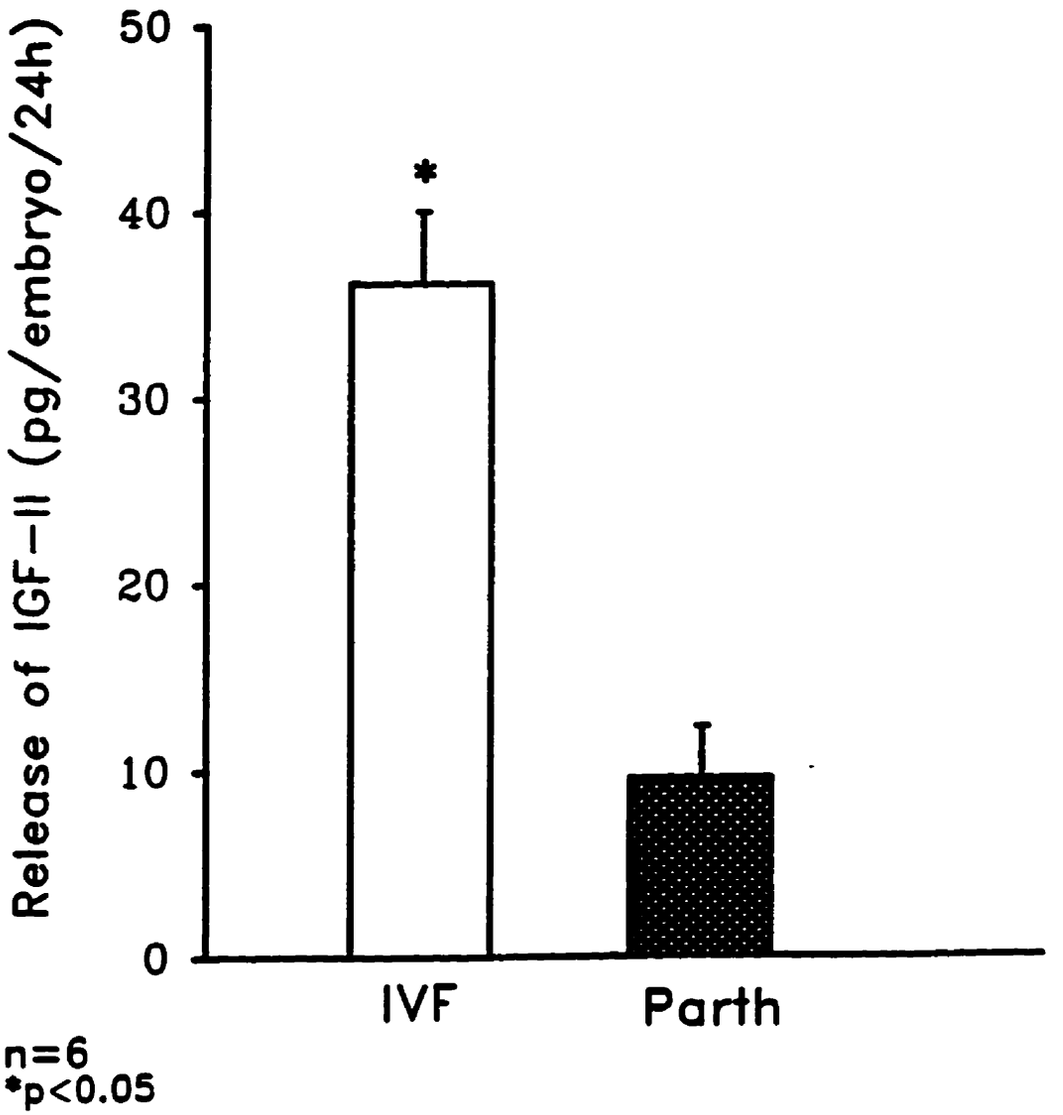

506
396
344
298
220
201
L
1
2
3
4
5
6

FIGURE 6-4. Release of IGF-II from preattachment bovine IVF blastocyst and parthenogenetic blastocysts, over 24 h into serum-free medium. Following the extraction of binding proteins IGF levels were measured by RIA. Significantly greater levels ($p<0.05$) of IGF-II release was detected from IVF embryos compared to parthenogenetic embryos. IGF-I release was below the detectable level of the assay.


Release of IGF-II (pg/embryo/24h)
50
40
30
20
10
0
*
IVF
Parth
n=6
*p<0.05

6.4 DISCUSSION

Bovine parthenogenetic embryos contain genetic material derived only from the maternal parent (Surani, 1994). These embryos are useful for determining genes that are imprinted, if the silenced allele is from the maternal parent. IGF-II is believed to be an example of a gene that undergoes such imprinting (DeChiara *et al.*, 1991). RIA results suggest that IGF-II release is significantly less from parthenogenetic than from fertilized blastocysts. Parthenogenetic embryos display a reduced cell number compared to fertilized embryos at the blastocyst stage. The reduction is about half the cell number found in normal blastocysts (Du *et al.*, 1996; data not shown). The results presented are applied on a per embryo basis and thus variations in IGF release may be reduced if the data were subjected to per cell analysis. However, if corrected for cell number the amount of IGF-II released by parthenogenetic embryos would still be half the amount released by fertilized blastocysts. In conclusion parthenogenetic embryos release less IGF-II then IVMF embryos. The fact that parthenogenetic embryos release any IGF-II is of interest. If imprinting influences gene expression (Rappolee *et al.*, 1992), it is possible that the IGF-II released is the result of a leaky imprint that allows the production of a few copies of mRNA, aberrant gene expression due to chromosomal ploidy or may be due to the release of membrane bound IGF-II from embryos accumulated during IVC. Alternatively, the IGF-II gene may not utilize the imprint

until later stages of embryo development (Latham *et al.*, 1994), or the lower IGF-II release is the result of lower overall transcription in less healthy parthenogenetic embryos.

The pattern of expression of IGFBP mRNA determined for the parthenogenotes was identical to that found for IVMF embryos. This suggests that none of the IGFBPs expressed are silenced at the maternal allele by imprinting, at least at the transcript level.

This study employed parthenogenetic bovine embryos primarily to contrast parthenogenotes and fertilized embryos. However, analysis of chromosomal complements of parthenogenotes demonstrated that expected diploid chromosomal ploidys are not often observed. Nevertheless, these embryos were still of use to provide an initial analysis of IGFBP mRNAs and IGF-II release. The majority of parthenogenotes displayed polyploid and mixoploid chromosomal complements. A high proportion of parthenogenetically activated bovine oocytes cleave following activation with ethanol or ionomycin and diploidization with cytochalasin or DMAP treatments. The cleavage frequency was highest when ethanol activation was combined with DMAP treatment but no difference in development to the blastocyst stage was observed. The application of diploidization treatment clearly influenced cleavage and development to the blastocyst stage since oocyte activation alone resulted in reduced cleavage frequency and no development to the blastocyst stage. All three oocyte

activation and diploidization treatments were linked to high frequencies of abnormal chromosomal complements. Although higher proportions of oocytes activated alone displayed an expected (haploid) chromosomal complement at the 2-cell stage, multi-nucleated cells were observed and development was not supported to the blastocyst stage in these groups. The results suggest that haploid oocytes display a reduced developmental capacity and that although the diploidization treatments, in part, restore this developmental capacity they also result in the establishment of abnormal chromosomal complements.

Total cell numbers for parthenogenetic blastocysts are reported to be substantially lower than those for fertilized (IVF) blastocysts (Du *et al.*, 1996; de la Fuente personnel communication); however, the ratios of trophectoderm to total cell number may be similar for both embryo types suggesting that parthenogenetic embryos are delayed in their developmental program. The appearance of multi-nucleated blastomeres are reported in bovine parthenogenotes (Plante and King, 1996) and in fertilized human zygotes (Hardy *et al.*, 1993). These cells may arise by either cell fusion, nuclear amitotic splitting or acytokinesis (Hardy *et al.*, 1993). A clear significance of these cells on developmental fate is not resolved, but it seems apparent that their presence is associated with reduced embryo viability.

A spontaneous oocyte activation rate of as high as 46% has been previously reported by King *et al.*, (1988) and Minamihashi *et al.*, (1993) for

bovine cultured oocytes and of these 74.4% were haploid displaying low rates of development to the blastocyst stage (Plante and King, 1996). Artificial activation of bovine oocytes by ethanol (Fukui *et al.*, 1992; Minamihashi *et al.*, 1993) or ionomycin (Susko-Parrish *et al.*, 1994; Hagemann *et al.*, 1995) is reported to increase the levels of oocyte activation. When coupled with diploidization treatments rates of development to the blastocyst stage are increased (Fukui *et al.*, 1992; Minamihashi *et al.*, 1993; Susko-Parrish *et al.*, 1994). There are some reports of limited viability of parthenogenetic bovine blastocysts following embryo transfer as determined by a delay in return to estrous (Fukui *et al.*, 1992; Susko-Parrish *et al.*, 1994). Studies employing chimeric embryos produced by combining parthenogenetic and normal embryonic blastomeres have shown that parthenogenetic cells can differentiate into precursors of all cell lineages (Stevens *et al.*, 1978; Nagy *et al.*, 1989).

Oocyte activation by ethanol or ionomycin treatment is likely mediated by stimulating transient Ca^{++} currents that mimic those created by the spermatozoa (Collas *et al.*, 1993; Swann and Ozil, 1994). This induction of Ca^{++} fluxes promotes the resumption of meiosis and extrusion of the second polar body but not pronuclear formation (Susko-Parrish *et al.*, 1994). Treatment with a diploidization agent is required to suppress second polar body extrusion (cytochalasin treatment) or inhibit the second reduction division so karyokinesis does not occur (DMAP) (Kubiak *et al.*, 1991; Fukui *et al.*, 1992; Rime *et al.*,

1989; Szollosi *et al.*, 1991; Susko-Parrish *et al.*, 1994). Both of these treatment regimes are effective at stimulating oocyte activation, initiation of early cleavage divisions and development to the blastocyst stage. However, our results clearly suggest that the majority of parthenogenetic bovine embryos produced by these procedures do not develop diploid chromosomal complements. This result has important implications for future genetic analysis of bovine parthenogenetic embryos and may also provide some insight into the limited developmental potential observed for these embryos following embryo transfer. Fukui *et al.* (1992) reported that of 13 parthenogenetic morulae/blastocysts analyzed only 1 displayed a diploid chromosomal complement.

This high frequency of chromosomal abnormalities in the bovine parthenogenote appears to be at variance with the mouse parthenogenote. Balakier and Farkowski (1976) investigated the production of parthenogenetic mouse embryos by applying heat shock oocyte activation and cytochalasin B diploidization. They reported that all of the 15 analyzed murine parthenogenotes displayed a diploid chromosomal complement. Up to 95% of activated murine oocytes cleave and up 86% of oocytes can proceed to the morulae/blastocyst stages (Cuthbertson, 1983). Although aneuploidy was noted in up to 18.8% of ethanol activated murine oocytes (Kaufman, 1982) it seems clear that the percentage of diploid parthenogenetic murine embryos is much greater than that for bovine parthenogenotes.

Insight into the origin of these species differences may be provided by examining the role of the fertilizing sperm in regulating the initial cleavages. Schatten *et al.* (1991) showed that the centrosome is maternally inherited from cytoplasmic sites in parthenogenetic murine embryos. A different pattern has emerged for the cow, suggesting that the sperm and therefore a paternal factor is responsible for centrosome formation in this species and perhaps also the human (Navara *et al.*, 1994). Fertilized bovine oocytes display microtubule arrays that form an aster associated with the penetrated sperm head (Long *et al.*, 1993; Navara *et al.*, 1994). While parthenogenetically activated bovine oocytes display a disorganized microtubule pattern (Navara *et al.*, 1994) that may provide a basis for the origin of the abnormal chromosomal complements.

Furthermore, chromosomal complement appears to have an important influence on developmental rate. Henery and Kaufman (1992) reported that haploid murine parthenogenotes displayed a longer mitotic doubling time than diploid parthenogenotes or fertilized zygotes. Kawarsky *et al.*, (1996) observed a similar phenomenon for *in vitro* fertilized bovine zygotes with haploid and polyploid embryos displaying slower rates of development than mixoploid and diploid embryos. Plante and King (1996) reported abnormal morphology and low cell numbers in parthenogenetic bovine embryos arising from spontaneously activated "aged" oocytes.

Oocyte maturation is regulated by maturation promoting factor MPF

(Vande Woude, 1994; Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). C-mos is involved in MPF regulation and recent results provided by gene "knock-out" studies for c-mos suggest that inactivation of the c-mos gene product results in spontaneous oocyte activation (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). MPF is a heterodimer of cyclin B and cyclin-dependent-kinase $p34^{cdc2}$. Inactivation of MPF is prevented by cytostatic factor CSF. Therefore, CSF maintains MPF activity arresting the oocyte in metaphase MII. MPF and CSF activities are lost following fertilization or parthenogenetic activation (Zernicka-Goetzm *et al.*, 1995). Metaphase II arrested oocytes fused to parthenogenetically activated mouse eggs remain arrested while those fused to fertilized eggs become activated. This has clearly revealed an important difference between fertilized and parthenogenetically activated oocytes and suggests that cytoplasmic maturational events can impact upon early development.

Finally, parthenogenetic activation is commonly employed to provide oocyte recipients for nuclear transplantation experiments (Du *et al.*, 1995; Stice *et al.*, 1996; Campbell *et al.*, 1996). It is thus important to characterize any influence of oocyte activation on oocyte and embryo viability. As the possible research applications for the parthenogenetic model continue to increase it is essential to investigate the possible variation from normal genetic and molecular mechanisms that artificial oocyte activation procedures may place on the

ensuing embryos. Future studies utilizing bovine parthenogenetic embryos produced by these standard methods should consider the impact of chromosomal complement on experimental outcomes. Our results may contribute to an understanding of the processes that underlie the reduced development of bovine parthenogenotes *in vitro* and following embryo transfer. In addition they highlight a possible difference between mouse and bovine oocytes in terms of their capacity to independently support their early developmental programs.

CHAPTER 7 CONCLUSIONS WITH GENERAL DISCUSSION

The overall objective of my study was to characterize the expression of mRNAs and polypeptides encoding IGFBPs in bovine primary oviductal cultures and preattachment embryos. The results were extended to include an analysis of IGFBP mRNA expression and IGF-II release from parthenogenetic embryos, in order to examine the contribution of the female genome to the production of these components of the IGF system. The study of parthenogenetic embryos included a complete analysis of chromosomal complements of parthenogenetic embryos following three standard artificial activation and diploidization methods.

The results of these studies can be summarized as follows:

1) Development of bovine embryos to the blastocyst stage is dependent on the culture environment. Embryo co-culture on bovine oviductal cell vesicles (21.5 ± 1.2%) vs no cells (3.3 ± 1.4%) significantly increased development of blastocysts. These results suggest that the oviduct cells condition the culture to provide a suitable environment for supporting early bovine development.

2) Detectable levels of immunoreactive IGF-I and IGF-II are released from primary bovine oviduct cell cultures, and IGF-II detectable levels are released from bovine blastocysts. This result confirms the presence of both IGF-I and IGF-II in the co-culture environment. The release of IGF-II from bovine blastocysts

suggests that the embryo may release IGF-II in support of development in an autocrine fashion.

3) Bovine oviduct monolayer and vesicle cultures both express transcripts encoding IGFBPs 2-5 throughout an 8-day culture interval. In contrast, IVMF bovine zygotes express mRNAs encoding IGFBPs 2-4 through to the blastocyst stage. mRNAs encoding IGFBP-5 were detected in bovine blastocysts while transcripts encoding IGFBP-1 and IGFBP -6 were not detected.

4) Bovine oviduct monolayer and vesicle cultures both produce and release IGFBPs 2-5 into conditioned media.

5) Parthenogenetic bovine blastocysts express mRNAs encoding IGFBPs 2-5, in an identical fashion displayed by IVMF bovine blastocysts. This IGFBP expression pattern suggests that the IGFBPs maternal alleles are active and not subjected to genomic imprinting during the preattachment development period. IGF-II released from parthenogenetic embryos is significantly lower then IVMF embryos.

The detection of IGF-I, IGF-II and IGFBPs 2-5 in the culture environment are important findings because they suggest that IGF paracrine and autocrine regulatory circuits are present and may contribute to the events that regulate

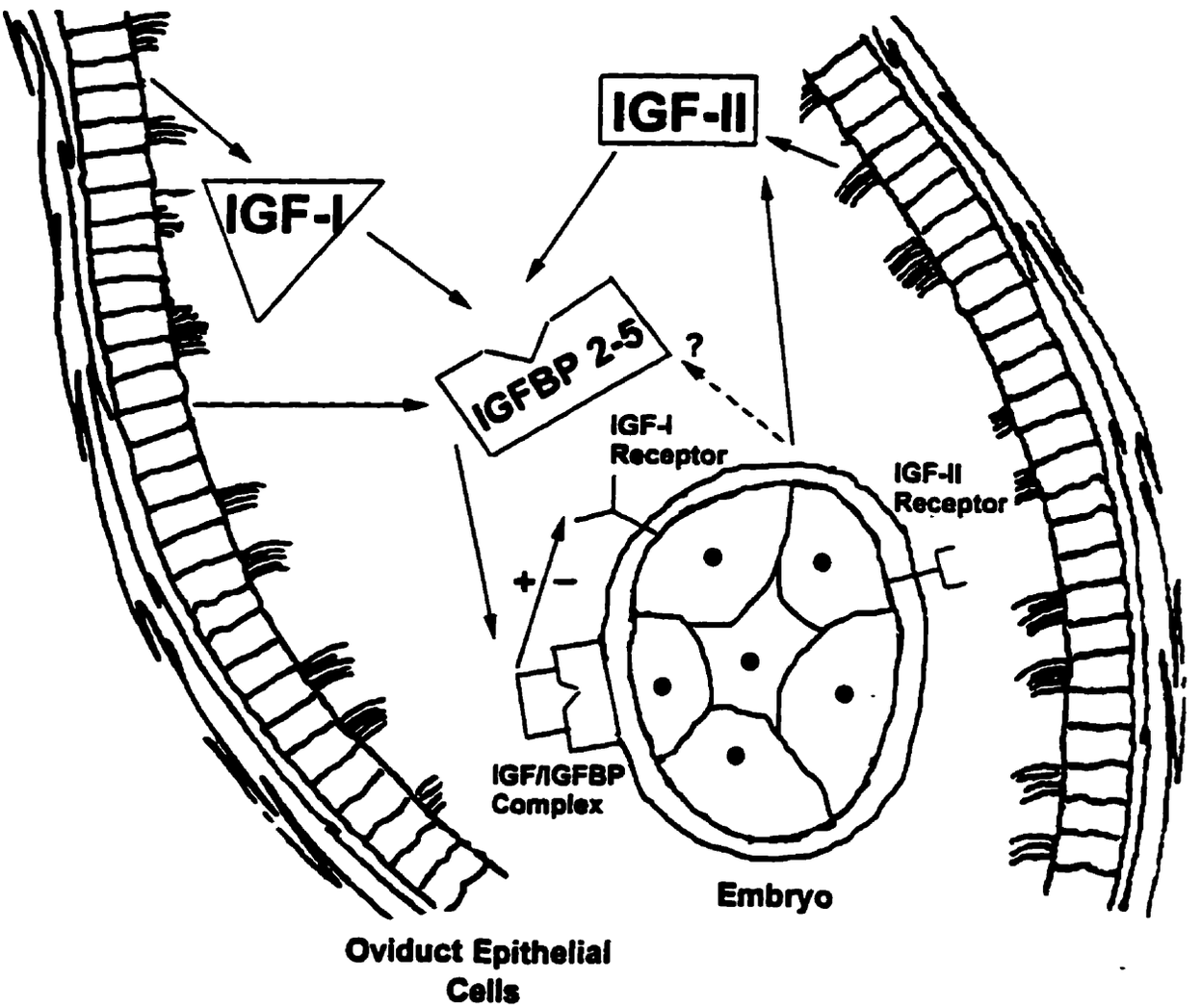
early development (Figure 7-1). The next step is to measure the direct influences of these ligands on early development and determine the IGF/IGFBP dynamics that oversee their actions. Therefore, the analysis of such a culture system is far from complete by this study of the IGFs alone. The progression towards a chemically defined culture system forces the focus of investigation onto determining the factors necessary to support the early developmental program.

Our knowledge of bovine embryo culture is enhanced by characterizing the IGF circuit as a possible molecular mechanism supporting bovine development *in vitro*. The majority of research on preimplantation embryos has involved the mouse. Early development in the mouse is not representative of all mammals and species differences exist between mouse, cow and human. One advantage of the mouse system is the ease of housing and attaining early embryos from such a small mammal. Human embryos cannot by utilized for research because of ethical concerns. For these reasons bovine embryos produced *in vitro* from slaughter house materials represents a valuable source of early embryos. The ability to produce bovine embryos effectively allows the study of early developmental events. Current research is directed towards producing chemically defined conditions capable of supporting bovine embryo development. In my study the detection of IGF-I, IGF-II and IGFBPs in our *in vitro* system suggests that the IGF family of growth factors should be included in defined media for optimal embryo production. Improving our ability to produce

bovine embryos will advance basic research involving embryonic gene expression and regulation, leading to both greater success in the commercial application of embryo transfer, and uncovering some of the mystery that still exists regarding human infertility.

In conclusion the detection of IGF-I, IGF-II and IGFBPs 2-5 in the co-culture environment strongly supports the prescence of a paracrine IGF circuit existing between the maternal oviduct cells and the developing embryo. The release of IGF-II, and the detection of IGFBPs 2-5 mRNA in the bovine embryo suggests that IGFs are contributing to the developmental regulation of embryos in an autocrine fashion.

FIGURE 7-1 A schematic representation of putative paracrine IGF interactions between primary oviductal cells and developing bovine embryos.


IGF-II
IGF-I
IGFBP 2-5
?
IGF-I
Receptor
IGF-II
Receptor
+
–
IGF/IGFBP
Complex
Embryo
Oviduct Epithelial
Cells

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