DISSERTATION

REGULATION OF PLACENTAL DEVELOPMENT

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CASEY D. WRIGHT ENTITLED REGULATION OF PLACENTAL DEVELOPMENT BE ACCEPTED AS FULFULLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

REGULATION OF PLACENTAL DEVELOPMENT

Bovine peri-attachment factor (bPF) mRNA is a novel transcript originally detected in early conceptus and adult kidney. Two known homologs of bPF are murine protein G90, which has been detected in embryonic and adult mouse brain, adult mouse intestine, kidney and testis and a human hypothetical protein detected in placenta. Our first objective was to screen genomic DNA from several species by Southern hybridization for PF homologs. Our second objective was to examine cow, sheep, horse, rat, mouse and porcine conceptus and adult tissues for expression of PF/G90 mRNA. Finally, our third objective was to localize PF within the cell following expression in mammalian cell lines. Southern hybridization revealed genomic PF homologs in the human, dog, cow, pig, rabbit, yeast, and horse. Northern hybridization and/or RT-PCR analysis detected PF in bovine (day 14, 17, 21 and 28), ovine (day 13 and 15), porcine (day 10 and 12) and equine (day 14, 16, 20 and 30) conceptus tissue, coinciding with the stage of conceptus development when bPF was previously detected. Analysis of adult sheep tissues detected PF in kidney and lung. Interestingly, no PF could be detected in rat or mouse adult tissues. Screening of day 135 fetal ovine fetal tissues revealed detectable PF mRNA in the lung but not in the heart. Western analysis of a PF fusion protein detected PF in both cytoplasmic and

nuclear fractions of stably transfected CHO and Cos-7 cells. The Southern hybridization data indicates a conserved gene for PF exists across several species of different phylogenetic orders. Results of bovine conceptus tissue expands the previous temporal expression window of PF mRNA to day 28, and results with ovine and equine conceptus tissues are similar to those reported for cattle; additionally ovine PF was detected in adult and fetal lung. The predicted nuclear targeting sequence of PF is apparently functional as evidenced by the immunoblot analysis. Given the detection of PF fusion protein in the nucleus and absence of DNA and RNA binding domains, we hypothesize that PF acts as a co-activator or co-repressor to modify transcription within the nucleus.

While several proteins have been implicated in transcriptional regulation of PL in *in vitro* studies, little is known about the transcriptional regulation of oPL *in vivo*. The purpose of this research was to determine the promoter sequence necessary to give cell specific expression of oPL in BNCs. Ovine PL promoter deletion constructs were made to drive EGFP expression, and stably transfected into ovine fetal fibroblasts for nuclear cloning and embryo transfer. A 47 dGA cell line was chosen for nuclear cloning for advantages in cell culture stability before nuclear transfer and for appropriate growth, quality, and number of blastocysts derived from this cell line. Transgenic tissues were evaluated under fluorescence microscopy, where it was discovered that auto-fluorescence within placental tissue was going to make specific EGFP expression difficult to determine. Unsuccessful attempts were made to detect EGFP by immunohistochemistry and *in situ* hybridization; however, a positive control could

be detected. The exact reason for the inability to detect EGFP and the erratic detection of the neomycin positive control is unknown. Our fixation and immunohistochemistry and *in situ* hybridization techniques have been used successfully in previous research; therefore, we believe there was most likely a problem with the tissue. With the lack of success in analyzing oPL transcriptional regulation by using transgenic nuclear cloning, we focused on culture of placental cells *in vitro* and subsequent infection of the cells with adenovirus and lentivirus that carried CMV promoter driven GFP cassettes. Infection of multiple cell types including BNCs was successful with both adenovirus and lentivirus.

Ovine growth hormone was previously reported to be expressed in placental tissue for a brief (20 days) period during maximal placental growth and development. Our objectives here were to define the cellular source of oGH in the placenta, and study the impact of exogenous GH during the normal window (35-55 dGA) of placental expression. The results of *in situ* hybridization indicate that oGH is expressed by uterine epithelium and no tissues of fetal origin. For GH treated ewes, serum GH and IGF-I concentrations were increased approximately 10-fold ($P < 0.001$) by day 5 of treatment and the increase was maintained throughout the treatment period. Serum progesterone concentrations were unaffected by treatment. Uterine, uterine fluid, placental and fetal weights were unaffected by treatment for both day 55 and 135 groups. Fetal length, liver weight, and liver weight per kg of body weight were not changed by maternal GH treatment. Maternal GH administration did not significantly alter GH (caruncle), or oPL (cotyledon) mRNA concentration as detected by Northern hybridization.

In the cotyledon, IGF BP-1 and BP-4 were significantly ($P < 0.05$) increased, while IGF BP-2 was significantly decreased. The expression of IGF BP-3 was unaffected by treatment. Within the caruncle, IGF BP-1 was decreased, while IGF BP-3 and IGF BP-4 were increased, and IGF BP-2 was unchanged due to GH treatment. In contrast to pigs, these data indicate that maternal serum concentrations of GH and IGF-I have no significant effect on placental and fetal growth. Although we did not impact placental or fetal growth, exogenous GH administration does impact the abundance of mRNA encoding IGF binding proteins within the placenta. Increase in caruncular IGF BP-3 is likely a response to increased maternal serum IGF-I. The increase in IGF BP-4 may also be due to increased IGF-I as IGF BP-4 is a negative regulator of IGF-I action. The decrease in IGF BP-1 is possibly the result of increased maternal insulin in response to GH treatment as insulin decreases IGF BP-1 transcription. While we cannot explain the changes in transcription within the cotyledon, they are important in trying to explain the role of the IGF system at the maternal-fetal interface in growth and differentiation.

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about farming, the kids, and occasional comedy over the phone has made the days go faster. For my older siblings, they were my inspiration to seek advanced degrees. They showed me that there is more than feeding and weighing animals in this world for kids of agriculture background. Hopefully what I have accomplished here can compare to what they have accomplished.

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CHAPTER I

INTRODUCTION

The development of the placenta, from elongation and implantation of the embryo through the completion of placentation, requires a diverse group of cytokines, growth promotants, cell adhesion molecules, angiogenic molecules, and hormones. Most all of these molecules are regulated by a plethora of transcriptional enhancers or repressors to provide spatial and temporal expression to ensure proper placental development. The placenta is a multifunctional organ that plays a critical role in the transfer of nutrients and waste products between the maternal and fetal circulations. Additionally, the placenta plays an immunoprotective role as a barrier against pathogens and protecting the fetus from maternal immune response. Throughout gestation, hormones are synthesized by the placenta and secreted into both maternal and fetal circulation. These hormones play critical roles in directing the development and growth of a healthy fetus.

The impact of aberrations in the development of the placenta and subsequent impact on fetal growth and development are far reaching. Failure of the early conceptus to communicate its presence and attain attachment to the maternal system can lead to early wastage or loss of the pregnancy. For humans and domestic livestock, the lack of appropriate fetal growth and

development can lead to increases in neonatal morbidity and mortality. The deleterious effects extend into the adult life of these unfortunate neonates, as they have increased incidences of disease awaiting them as they age. Increases in ischemic heart disease, stroke, diabetes, and hypertension are only a few of the problems awaiting these individuals.

In agriculture, there is a large premium placed on the growth rate of domestic livestock after birth. However, the growth of the fetus *in utero* has dramatic impacts on the incidence of dystocia, as well as the mortality associated with growth restricted neonates. In addition, domestic livestock species can also suffer suboptimal health status during postnatal growth as a function of less than optimal growth and development during gestation. The pressure to increase production, particularly in dairy cattle increases early embryonic wastage such that merely initiating a successful pregnancy is problematic. This failure in getting livestock pregnant consistently, and gestating the fetus appropriately, puts a large financial burden on the domestic livestock industries.

Research into the understanding of appropriate placental and fetal development will lead to ensuring the postnatal growth and health of many species. The following review of literature is intended to describe normal and abnormal growth and development of the sheep placenta and how the ewe can be used to study placental abnormality in humans. Particular attention is given to the roles of peri-attachment factor, ovine placental lactogen, ovine placental, and growth hormone in orchestrating growth and development of the placenta. The goal of the research presented later in this dissertation is to gain insight into the

roles of selected proteins in establishment, development, growth and maintenance of a successful pregnancy. Further understanding of aberrations during placental and fetal growth will allow for development of treatments that can help avoid and/or alleviate perinatal and postnatal development and growth problems as a result of placental abnormality.

CHAPTER II

REVIEW OF LITERATURE

The hatched blastocyst has to pass through a number of transformations and cell differentiation steps to develop into a placenta to support the developing fetus. Starting with blastocyst elongation, the early trophoblast must gain apposition of the maternal epithelium and produce chemical signals to make the maternal system aware of the impending pregnancy. Subsequently, the trophoblast must continue its growth and differentiation to establish a placental vasculature capable of sufficient nutrient transfer and efficient removal of waste products to maintain fetal growth and development.

CONCEPTUS DEVELOPMENT

Early embryonic development, implantation and maintenance of the pregnancy are critically dependent on successful embryo-maternal communication leading to maternal recognition of pregnancy. Early embryo development (days 15-17 post coitus) has been defined as the critical period (Binelli et al., 2001) when embryo-maternal communication initiates antiluteolytic pathways. Early studies of embryonic wastage (Boyd et al., 1969) revealed up to 30% of pregnancies failed by this critical window. More recent studies have estimated embryonic loss in a range of 28-40% (Binelli et al., 2001; Fricke et al., 2003; Roche et al., 1981; Zavy and Geisert, 1994). Embryonic loss in dairy cattle

has been estimated as high as 55% (Lucy, 2001) and has been increasing steadily since the 1950's. Those cattle that require multiple fertilization attempts to conceive i.e. repeat breeders, have a 47% failure of conception at the critical window of maternal recognition of pregnancy (Ayalon, 1978). For heifers classified as repeat breeders, 72% of embryos are abnormal (Zavy and Geisert, 1994). Clearly little progress has been made towards solving the problem of embryonic wastage as we have failed to increase reproductive efficiency. When trying to explain embryonic loss, only 10.4% of abnormal embryos can be explained by chromosomal abnormalities (Zavy and Geisert, 1994). This leaves a significant portion of embryonic loss to be explained by physiological malfunction. The most researched protein during ruminant maternal recognition of pregnancy is interferon-τ, and its role in initiating antiluteolytic pathways is clearly defined (Hansen et al., 1999; Thatcher et al., 1995). Though interferon-τ plays a critical role in maintenance of the pregnancy, other cytokines and growth factors also play critical roles in early embryonic growth, development and survival (Martal et al., 1997).

Using differential display, transcriptional changes between day 15.5 and 17.5 bovine conceptus tissue were detected for four different genes (Glover and Seidel, Jr., 2003). Allograft inflammatory factor 1, ligand of eph-like receptor kinase 5, interferon-τ and a novel transcript named peri-attachment factor (Glover and Seidel, Jr., 2003). Bovine peri-attachment factor (bPAF) expression was 10 fold higher in day 17.5 conceptus versus day 15.5. At 30 days of gestation, bPAF was no longer detectable in conceptus tissue. A full length

cDNA (ascension no. AY027656) for bPAF was cloned from a day 25 cDNA library (Glover and Seidel, Jr., 2003). Analysis of the predicted amino acid sequence revealed 4 putative protein kinase C (PKC) phosphorylation sites, two casein kinase II phosphorylation sites, and a nuclear targeting sequence with no apparent DNA or RNA binding domain (Glover and Seidel, Jr., 2003). Northern hybridization screening of adult bovine tissues detected slight expression of bPAF in kidney. The authors reported only one known homolog to bPAF, a hypothetical human protein (hHP; Genbank, XM_166586) which shared 83% nucleotide sequence identity and 77% amino acid identity with bPAF (Glover and Seidel, Jr., 2003).

Further search of nucleotide databases revealed a mouse homolog to bPAF. A novel transcript was detected in mouse small and large intestine named G90 (Krause et al., 1999). The authors reported no complete open reading frame within the G90 transcript (Krause et al., 1999). However within the reported sequence, an open reading frame exists that would code for a peptide similar to bPAF and is identical to a peptide coded from a cDNA (Genbank BY 731406) cloned from the mouse eye (Okazaki et al., 2002). A blast search of the rat genome identified a rat sequence with high homology to these PAF homologues as well. All of these peptides have remarkable conservation ranging from 66 to 84%, and within the predicted nuclear targeting sequence with only one amino acid switch (R97K) amongst all of the species. The G90 gene transcript appears to be contained in 2 exons separated by a single intron (Krause et al., 1999), with the predicted protein transcribed within exon 2. This

matches the structure of hHP as predicted by alignment of the transcript with human genomic sequence. Mouse G90 is located on chromosome 6 (Krause et al., 1999), while hHP is located on chromosome 7.

Bovine PAF is expressed in early conceptus development, a time when critical events like maternal recognition and early conceptus elongation are occurring. Given that bPAF is predicted to have a nuclear target sequence with no obvious DNA or RNA binding motifs, bPAF may play a role as a co-repressor or co-activator in early conceptus development. Further investigation of the expression, localization and protein characterization are needed to understand what potential role this novel protein may have in early conceptus transcriptional regulation.

The ovine blastocyst arrives in the uterus by the 5th day of gestation and enters a phase of rapid elongation (Davies and Wimsatt, 1966). Of the membranes expanding from the blastocyst, the chorion and allantois develop into the placenta. The chorion is epithelial in nature and is derived from the trophectoderm and represents the leading edge of placental elongation and villous development (Leiser and Kaufmann, 1994). The allantois develops from the embryonic hindgut and its associated mesenchyme is richly vascularized (Leiser and Kaufmann, 1994). The allantois and chorion grow outward from the embryonic disc as bi-fold sacs. Initially the chorion is loosely associated to maternal epithelium (Boshier, 1969). By the 17th day the chorion surface begins to adhere to the maternal caruncular epithelium (Davies and Wimsatt, 1966).

There are a plethora of molecules, cytokines, hormones and growth factors that play roles in apposition of maternal epithelium, implantation (Carson et al., 2000), and maternal recognition of pregnancy (Zavy and Geisert, 1994). The expression of integrins on the conceptus surface and the apical surface of uterine epithelium has been reported (Johnson et al., 1999). Alpha(v)beta(3) integrin is capable of binding fibronectin, oncofetal fibronectin, vitronectin, osteopontin, tenascin, and von Willebrand's factor (Bowen and Burghardt, 2000). Osteopontin has been shown to be a major uterine secretion during pregnancy (Johnson et al., 1999). Additionally, a mucin family member, glycosylation-dependent cell adhesion molecule 1-like (GlyCAM-1-like) protein is produced by both the trophectoderm and endometrium, and appears to be regulated by progesterone (Spencer et al., 1999a). By the 20th day, the embryonic membranes have grown sufficiently to contact maternal epithelium throughout the entire uterine horn (Boshier, 1969; Davies and Wimsatt, 1966). The chorionic epithelium contacting the endometrium is a pseudostratified columnar epithelium supported by a stromal mesenchyme that lacks considerable cellularity (Davies and Wimsatt, 1966).

PLACENTAL DEVELOPMENT

Chorionic binucleate cells (BNC) are evident by day 19, comprising 3.7% of the cells present and increase in number from this point forward as the embryonic membranes continue to develop adherence to the uterine epithelium (Boshier, 1969). By the 4th week of gestation, the cellular density immediately associated to caruncles is increased and primary microvillous interdigitation of

the maternal epithelial crypts is evident (Boshier, 1969; Davies and Wimsatt, 1966; Stegeman, 1974). By the 5th and 6th weeks of gestation, the number of BNC is increased within the apical regions of the maternal crypts (Boshier, 1969). As gestation advances the primary villi branch to form secondary villi, and eventually tertiary villi and continue to increase in number until approximately the 16th week of gestation (Stegeman, 1974). Development of the placental vascular system to support the developing fetus is quite advanced by the 10th week.

Early vascularization is evident in the allantoic mesenchyme. As the allantois and chorion merge, the allantoic vascular mesenchyme fuses with the chorion to complete embryonic circulation (Leiser and Kaufmann, 1994). Although blood vessels can be detected earlier than the 5th week of gestation, it is not until the 7th to 10th week that vessel diameter and number are noticeably increasing (Stegeman, 1974). Vessel number and diameter will continue to increase until the 15th week of pregnancy (Stegeman, 1974). The regulation of vascular development within the placenta is driven by a large family of vascular endothelial growth factors (VEGF). This family of growth factors includes three splice variants of placental growth factor (PlGF) as well as VEGF-A, -B, and -C. The VEGF ligands bind to a family of receptors, VEGFR-1, -2, and -3, to regulate angiogenesis within the placenta (Regnault et al., 2002b; Regnault et al., 2003). Once a point of sufficient vascularization within the maternal-fetal interface has been reached, the placenta begins a period of maximal growth and development, which places an increased demand for nutrient uptake and metabolism.

PLACENTAL GROWTH

The placenta plays a critical role in delivering nutrients to the developing fetus. Oxygen, glucose, and amino acids must be delivered to the fetus, while at the same time waste products of fetal growth must be delivered to the maternal-fetal interface for removal from fetal circulation. Beyond the nutrient requirements of the fetus, the placenta itself imposes a demand for nutrients to support its own growth and development (Meschia et al., 1980).

During the course of gestation, the metabolic requirements of the uterus, placenta, and fetus increase dramatically. These demands can be met by increasing the blood flow to the uterus or by increasing the capacity to extract nutrients from the maternal circulation (Morriss, Jr. et al., 1974). During the first 50 days of gestation, the nutrient demand for the uterus and its developing contents are considerably less than at later time points in gestation (Morriss, Jr. et al., 1974). Studies of mid-gestation growth of the placenta show that at 40-50 days, the placenta begins a maximum growth rate phase ending by day 80 when placental total mass declines towards parturition (Ehrhardt and Bell, 1995). On a dry mass basis, the placenta exhibits the same initial growth phase followed by a plateau in mass at approximately day 80 of gestation (Ehrhardt and Bell, 1995). *In vitro* analysis of placentomes verified these data showing increases in DNA, RNA, protein accretion rates starting at day 40 and continuing before reaching a plateau at day 100 (Ehrhardt and Bell, 1995).

In late gestation, the fetal lamb receives only 55% of the oxygen uptake by the utero-placental unit (Meschia et al., 1980; Sparks et al., 1983). Greater than

70% of the glucose taken up by the uterus is utilized in the utero-placental unit as well (Meschia et al., 1980), resulting in a tremendous amount of lactate being dumped into the fetal and maternal circulation (Meschia et al., 1980; Sparks et al., 1983). The rates of uptake and metabolism of oxygen and glucose approximates the levels reached in the very metabolically active brain (Sparks et al., 1983). In contrast to glucose, the utilization of amino acids appears to be predominantly by the fetus (Meschia et al., 1980). The periods of increased growth rate for the placenta between days 40 and 80, followed by the fetus at days 120 to 150 (Mellor and Matheson, 1979), correlate well with documented increases in uterine blood flow (Clapp, 1978; Rosenfeld et al., 1974) and placental blood flow (Rosenfeld et al., 1974). Blood flow within the placenta increases to a maximum flow rate of 1000ml/min at the beginning of the last month of gestation (Rosenfeld et al., 1974).

Comparison of Sheep and Human Placental Structure

Eutherian mammals develop a placenta where fetal membranes become intimately associated with the uterine endometrium for the purpose of a materno-fetal circulatory exchange (Leiser and Kaufmann, 1994). Across the mammalian species, an extraordinary amount of variability exists in the placental structures to meet the particular nutrient exchange requirements for the individual species (Leiser et al., 1997; Leiser and Kaufmann, 1994). Although many different placental structures are observed, there exist several important similarities between the human and sheep placenta, which make the sheep an appropriate model for studying placental function in humans.

Both humans and sheep have a placenta which initially is derived of chorion, which then merges with the allantois to form a chorioallantoic placenta for the duration of pregnancy (Leiser and Kaufmann, 1994). Although ruminants have a cotyledonary placental shape with multiple attachments (Boshier, 1969; Stegeman, 1974) and humans have a discoidal placental shape with a single large attachment (Leiser and Kaufmann, 1994), the interdigitation of maternal and fetal tissues takes the form of a villous tree for both species (Leiser and Kaufmann, 1994). These interdigitations appear in a parallel arrangement with alternating fetal-maternal vascular trees (Krebs et al., 1997; Leiser et al., 1997). The similarities in the villous structure leads to maternal and fetal blood flow relationships that are strikingly similar for the human and sheep (Leiser et al., 1997; Leiser and Kaufmann, 1994). Both species have a villous placental type particularly reflected within the fetal villous tree where both species show stem, intermediate and terminal vessels (Kaufmann et al., 1979; Leiser et al., 1997; Sen et al., 1979). The similarities within the terminal vessels is of particular importance as this region of the villous tree is responsible for the majority of exchange between the maternal and fetal circulations (Leiser et al., 1997).

The similarities in fine structure of the placenta shared by humans and sheep, makes the sheep a useful model for study of human placental development. Not surprising, several research groups have developed different models to study the various aspects of human placental growth and development using the sheep as a model (Anthony et al., 2003).

INTRAUTERINE GROWTH RESTRICTION

Infants gestated under sub-optimal conditions often are presented at birth as small for gestational age. Intrauterine growth and its aberrations are of major concern in modern obstetrics because birth weight is the strongest known indicator of perinatal mortality (Pollack and Divon, 1992). A distinct classification of some of these infants is those suffering from intrauterine growth restriction (IUGR) which affects up to 7% of all pregnancies (Brar and Rutherford, 1988). Causes of IUGR include fetal genetics, fetal infection, maternal nutrient status, maternal hypoxia, maternal disease (i.e. pre-eclampsia), gestation at high altitude and placental insufficiency, including multiple fetuses (Battaglia, 1970; Keyes et al., 2003; Pollack and Divon, 1992; Robinson et al., 2000).

Complications that arise during pregnancy include intrauterine demise, intrapartum fetal distress, and perinatal hypoxia (Regnault et al., 2002a). Neonatal complications for these infants include meconium aspiration, hypoglycemia, pulmonary hemorrhage, cognitive dysfunction and cerebral palsy (Battaglia, 1970; Pollack and Divon, 1992; Regnault et al., 2002a). Systemic developmental consequences for the fetus associated with IUGR include malformation and/or malfunction of the renal (Lumbers et al., 2001), cardiac (Murotsuki et al., 1997b; Murotsuki et al., 1997a), intestinal (Avila et al., 1989), and respiratory (Joyce et al., 2001; Rees et al., 1991) systems. Furthermore these infants are predisposed to health problems later in adult life. Recent epidemiologic evidence indicates IUGR infants suffer increased incidences of coronary heart disease, ischemic heart disease, type II diabetes, hypertension

and stroke as adults (Barker et al., 1989a; Barker et al., 1989b; Barker et al., 1990; Barker et al., 1993; Barker et al., 2002; Barker and Osmond, 1986; Poulter et al., 1999). In addition this cohort of adults showed trends towards higher incidences of lung disease and lung cancer (Barker et al., 1989b). The assembled findings of David Barker led to the hypothesis that the physiological, neuroendocrine and metabolic adaptations of the fetus to survive intrauterine growth restriction have a permanent effect on the development, differentiation, and proliferation of tissues such that pathological consequences are seen later in adult life.

The impetus for the research presented later in this dissertation is based on evidence from an ovine model of IUGR. Using a model of maternal hyperthermia, ewes are exposed to an ambient temperature cycle of 40°C for 12 hr, 35°C for 12 hr at 30-40% humidity from day 40 to day 90 of gestation leading to a 0.6°C increase in core body temperature (Regnault et al., 1999). This model produces placental insufficiency resulting in decreased placental and fetal weight with concurrent changes in placental vascular growth factors and their receptors, ultimately leading to intrauterine growth restriction (Regnault et al., 1999; Regnault et al., 2002b; Regnault et al., 2003). Within 30 days of this treatment, oPL, progesterone, and prolactin are significantly reduced (Regnault et al., 1999). Decreased levels of human placental lactogen have been associated with fetal distress as well (Letchworth, 1973). Additional ongoing research in this lab has shown that placental growth hormone is also decreased in the same model of maternal hyperthermia (Regnault and Anthony unpublished data).

PLACENTAL LACTOGEN

In eutherian mammals, the placenta is responsible for directly mediating or modulating the maternal environment required for maintenance of normal fetal growth and development (Anthony et al., 1995). The placenta of numerous species produces hormones that are structurally and functionally similar to the pituitary hormones prolactin (PRL) and growth hormone (GH). The majority of these hormones have a molecular weight of 20,000 to 25,000 and regulate various maternal processes including mammary gland differentiation, steriodogenesis, somatic growth, and intermediary metabolism (Ogren and Talamantes, 1988). The most extensively studied member of the PRL-like hormones are the placental lactogens (PL); species studied include human, monkey, baboon, mouse, rat, hamster, sheep, cow and goat (Ogren and Talamantes, 1988). Placental lactogens can be divided into two groups: 1) those with structure more similar to that of PRL and GH of the same species, and 2) those with structure that appears different from GH and PRL (Ogren and Talamantes, 1988)

Placental Lactogen Structure

Placental lactogens that are structurally related to GH and PRL as a group are single-chain polypeptides with molecular weights of 22,000 to 25,000. These proteins are characterized by two or three disulfide bonds in positions analogous to those present in GH and PRL (Ogren and Talamantes, 1988). Included in the GH/PRL related group are human PL (hPL), mouse PL-II (mPL), rat PL-II (rPL-II), ovine PL (oPL), baboon PL, and caprine PL. Those placental lactogens with less

structural identity with PRL and GH include bovine PL (bPL), mouse PL-I (mPL-I), and rat PL-I (rPL-I) (Ogren and Talamantes, 1988) all of which are glycosylated to varying degrees.

RUMINANTS

Ovine placental lactogen (oPL) has been purified and characterized from sheep cotyledonary tissue. The purified protein has an approximate molecular weight of 22,000-24000 as determined by gel filtration on Sephadex G-100 and has an isoelectric point of 8.8 (Chan et al., 1976; Reddy and Watkins, 1978b). More recently, immunohybridization of day 100 cotyledon tissue showed native oPL has a molecular weight closer to 22,000 and an isoelectric point of 9.2 (Warren et al., 1990a). Ovine PL is a nonglycosylated single chain polypeptide of 198 amino acids preceded by a 38 amino acid signal sequence (Colosi et al., 1989; Warren et al., 1990a). Sequence analysis reveals that oPL is structurally more similar to ovine prolactin (oPRL) than it is ovine growth hormone (oGH), 49% and 28% sequence identity, respectively (Warren et al., 1990b).

Bovine PL, comprised of 200 amino acids, has been shown to have isoforms ranging in molecular weight from 30,000 to 32,000 (Bolander Jr. and Fellows, 1976; Byatt et al., 1992; Murthy et al., 1982). As with oPL, bPL has an amino acid sequence that more closely resembles bPRL than bGH, 51% and 20% sequence identity respectively (Anthony et al., 1995; Forsyth, 1994). While the homology in amino acid sequence for bGH and oGH or bPRL and oPRL are 98%, the homology between bPL and oPL is only 66% (Anthony et al., 1995). The difference in the ruminant PLs is thought to be a result of positive selection

for PL leading to divergence in PL between the two species (Wallis, 1993). As suspected by Shuler et al., bovine PL is glycosylated, which does not appear to affect its biological activity (Byatt et al., 1992; Schuler et al., 1988), as deglycosylated bPL produced similar activity in Nb2 lymphoma cell bioassays (Byatt et al., 1992). It was originally thought that the isoforms of bPL detected were due to variable glycosylation of a single transcript of bPL. However, more recent studies have shown that the variable molecular weights of bPL may be due to alternative splicing of the bPL transcript (Kessler and Schuler, 1991).

NON-RUMINANTS

Human PL, like ovine PL, is not glycosylated and is secreted as a polypeptide chain of 191 amino acids with a molecular weight of approximately 22,000 (Beckers et al., 1998; Handwerger, 1991). As compared to the species already discussed, hPL shares more sequence identity with hGH (85%) than it does with hPRL (Forsyth, 1994).

For rodents, the hamster, mouse and rat produce various molecular weight isoforms of placental lactogen, that are structurally related (Southard and Talamantes, 1987). Originally it was discovered that rats produced rPL-I early in pregnancy from days 8-14 followed by production of rPL-II starting at day 12 and continuing throughout the remainder of gestation (Robertson et al., 1982). All of the mature rat placental lactogens are preceded by a 30 amino acid secretion signal sequence (Robertson et al., 1996). Rat PL-I is a glycosylated 200 amino acid polypeptide with a molecular weight of approximately 40,000, while rPL-II is a non-glycosylated 192 amino acid peptide with a molecular weight of 20,000,

with isoforms ranging from 6.0 to 6.4 (Duckworth et al., 1986; Robertson et al., 1982; Robertson et al., 1996). A variant of rPL-I (rPL-I_v) is a 195 amino acid peptide with a molecular weight of 30,000-34,000 and is glycosylated like rPL-I (Robertson et al., 1996). Removal of the post-translational glycosylation of rPL-I_v reveals a native protein with a molecular weight of 27,000 (Robertson et al., 1996). Rat PLs share sequence identity with rat prolactin; however, rPL-I and –II have a much higher (200-500 fold) mitogenic activity than rPL-I_v (Robertson et al., 1996). An Arg173Ser amino acid switch appears to block the interaction of rPL-I_v with the PRL receptor in Nb2 lymphoma cells (Robertson et al., 1996).

In mice, PL-I is a mixture of glycosylated proteins ranging in molecular weight from 29,000 to 42,000 (Colosi et al., 1987b). When deglycosylated, mPL-I has a molecular weight of 28,000 to 29,000 (Colosi et al., 1987a). The amino acid sequence of mPL-I is 224 residues with a mature secreted peptide of 194 amino acids (Colosi et al., 1987b). Murine PL-I shares a 44% amino acid identity with mPL-II and is more structurally similar to mPRL (33%) than mGH (21%). Mouse PL-II is a 192 amino acid polypeptide that is not glycosylated (Colosi et al., 1987b; Forsyth, 1994). Colosi et al. speculated that the heterogeneity of mPL-I is due to variable glycosylation of one translated product (Colosi et al., 1987a). After cloning the sequence of mPL-I, and using scheme of restriction enzyme digestion, Colosi et al reversed this hypothesis to say multiple genes for mPL-I may exist (Colosi et al., 1987b). Later it was shown that multiple copies of mPL-I resided on chromosome 13 (Jackson-Grusby et al., 1988) supporting the notion of multiple mPL-I genes. More recently it was reported three different

transcripts for mPL-I (α, -β, and -γ), which share 98% nucleotide sequence identity, are produced from 3 different genes (Wiemers et al., 2003).

Synthesis and Secretion of Placental Lactogen

As reviewed by Ogren and Talamantes (Ogren and Talamantes, 1988), the placental lactogens are synthesized and secreted from cells within the fetal component of the placenta. Members of the placental lactogen family of proteins have been studied in several species to elucidate the structure and secretion of these proteins.

Humans

Production of hPL has been detected by immunoflourescence in the syncytiotrophoblast layer of the placenta (Tabarelli et al., 1983). *In situ* hybridization evidence shows that hPL is localized to the syncytial layer of the placenta within 5 days following implantation (Beckers et al., 1998). Human PL is detectable in maternal serum by the sixth week of gestation and increases in concentration to the 30th week when peak concentrations are 5-7μg/ml, which exceeds the production of any other protein hormone (Beckers et al., 1998; Handwerger, 1991). Quantitatively, hPL compromises 10% of soluble protein production by the placenta (Beckers et al., 1998).

Ruminants

The oPL gene has been structurally characterized, and 4.5-kb of the promoter has been sequenced; where maximal activity of the oPL promoter was seen in the proximal 383-bp in BeWo (human) and Rcho-1 (Rat) choriocarcinoma cell lines (Liang et al., 1999). DNAse protection and eletrophoretic mobility shift

assays revealed that GATA-2 (2 sites), AP-2α, and Purα interact with there putative sites within the proximal oPL promoter (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001). Mutation of the GATA, AP-2α, and Purα sites and transfection analysis of the sites revealed that GATA, AP2α and Purα sites are functional, and serve to increase promoter activity (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001). In humans, several transcription factors have been shown to impact transcription of human placental lactogen in heterologous cell culture (Jiang et al., 1995; Jiang and Eberhardt, 1994; Lytras and Cattini, 1994; Rogers et al., 1986). Although many of these transcriptional regulators of hPL are enhancers of transcription, additional elements upstream of the gene regulate its expression in a tissue specific manor (Nachtigal et al., 1993). Although these studies have provided insight into the transcriptional regulation of oPL and hPL *in vitro*, there has been no attempt to study the tissue specific regulation of PL *in vivo*.

In sheep and cattle, PL production has been localized to the binucleate cells of the chorion (Duello et al., 1986; Kappes et al., 1992; Martal and Djiane, 1977; Reddy and Watkins, 1978a; Verstegen et al., 1985). Ovine PL is detectable in maternal serum by day 40-50 and increases in concentration as gestation advances, and then plateaus at day 120 to parturition (Handwerger et al., 1975; Handwerger et al., 1977; Kappes et al., 1992; Rueda et al., 1995). Similar secretion patterns for oPL have been seen with *in vitro* studies of cultured cotyledonary explants (Soares et al., 1999). Fetal serum concentrations of oPL also peak at approximately day 90 however the concentrations are 10-fold lower

than those seen in maternal serum (Chan et al., 1978; Kappes et al., 1992). In the last third of gestation concentrations of oPL in maternal serum is increased in a incremental fashion depending on the number of fetuses being gestated (Gootwine, 2004; Handwerger et al., 1975). It is implied that serum oPL concentrations are correlated to placental mass, and hence increases in fetal number will inherently increase the maximum circulating oPL concentrations (Gootwine, 2004). In cattle, maternal serum levels of bPL are detectable at the end of the first trimester of gestation, and fluctuate in concentration never exceeding 3.5 ng/ml and are unaffected by fetal genetics (Hossner et al., 1997). Fetal serum bPL concentrations at 85 days of gestation appear to be 10 fold higher than those seen in maternal serum, and steadily decline toward parturition (Holland et al., 1997).

RODENTS

The synthesis of mPL-I and -II have been co-localized to the same trophoblastic giant cells simultaneously on gestational day 9 tissue sections, as well as *in vitro* cultured cells (Faria et al., 1991; Yamaguchi et al., 1994). In rats, PL-I is secreted from day 11-14, while rPL-II is first detected in maternal serum on day 12 and increases steadily to parturition (Duckworth et al., 1986; Robertson et al., 1982; Robertson and Friesen, 1981). *In situ* hybridization and immunocytochemistry studies have shown that rPL-I and -II are produced in trophoblast giant cells (Faria et al., 1990; Tabarelli et al., 1983), initially in the basal zone followed later by expression in both basal and labyrinth zones of the placenta (Duckworth et al., 1990). Rat PL-I can be detected on tissue sections at

day 7, while rPL-II is detectable at day 11 (Faria et al., 1990). Interestingly, the temporal production of rPL-I followed by rPL-II may be due to an *in vivo* signal acting on giant cells to switch from rPL-I to rPL-II synthesis (Soares et al., 1985).

Unlike other rPLs, rPL-I_v was detected in cytotrophoblasts of the placenta and is secreted in a temporal fashion similar to rPL-II (Robertson et al., 1996). Review of maternal serum concentrations presented by Robertson et al. (1996) agrees with early reports of rPL-I; however their data suggests that rPL-II may circulate at a basal concentration while rPL-I_v increases steadily until parturition. At day 20 of gestation, fetal serum concentrations of rPL-II reach 28 ng/ml which is 14 fold less than maternal serum concentrations (Freemark et al., 1993). Near term, rPL-I_v is circulating in maternal serum at 4 fold higher concentrations than rPL-II. It is unclear if serum concentration of rPLs are correlated to litter size as pup number has no effect on the observed maternal serum concentrations of rPLs (Klindt et al., 1982; Robertson and Friesen, 1981). Robertson and Friesen, reported a correlation of fetal number and rPL concentration; however, Klindt et al. reported that rPL concentrations were similar for rats with 7 to 14 pups (Klindt et al., 1982; Robertson and Friesen, 1981). The discrepancy between the reports may be due to the episodic secretion pattern of rPL (Klindt et al., 1982). Hamsters, which also have two PL forms, show a similar pattern of haPL-I and haPL-II secretion as rats, with PL-I reaching maximum concentration during mid-gestation while PL-II secretion starts late in gestation and increases to parturition (Ogren and Talamantes, 1988).

Biological Actions of Placental Lactogen

Although placental lactogens have been evaluated in maternal intermediary metabolism, fetal growth, mammary growth and ovarian function, their role in pregnancy is not clearly understood. Traditional endocrine ablation experiments with placental lactogen are not feasible. Complimentary DNA sequences for both ovine and bovine placental lactogen have been cloned and large-scale preparation of recombinant proteins has aided in elucidating the biological actions of placental lactogen (Colosi et al., 1989; Krivi et al., 1989). However, interpretation of changes due to increased oPL infusion are difficult since the serum concentrations of endogenous oPL are approximately 10 fold (fetal) and 100 fold (maternal) higher than the Kd of receptors for oPL (Fowlkes and Freemark, 1993; Kappes et al., 1992; Pratt et al., 1995) and infused oPL would have little to no effect on an already saturated system.

RUMINANTS

Early research in hypophysectimized rats revealed that bPL had somatogenic and lactogenic actions when compared to bGH and bPRL (Bolander, Jr. and Fellows, 1976). More recently the findings of Bolander and Fellows were verified in normal rats given recombinant bPL and bGH (Byatt et al., 1991). Rats given bPL gained weight faster on a daily basis, than rats treated with bGH and these results are supported by higher food intake and increased levels of serum IGF-I for the bPL-treated rats (Byatt et al., 1991). *In vitro* studies with preadipocytes showed that bPL is lypolytic and its actions on adipocytes are mediated through the bovine GH receptor (Vashdi et al., 1992).

Bovine PL has luteotropic properties, as administration to dairy heifers increased corpus luteum diameter and serum IGF-I (Lucy et al., 1994). Radioreceptor assay results showed that the effects of bPL could be mediated through either GH and PRL receptors or a combination of the receptors as speculated in the IGF-I results (Byatt et al., 1991). More recently, in a heterologous system it was shown that bPL cannot act through a homodimer of bGH receptors (Warren et al., 1999), however as reviewed by Anthony et al., evidence suggests that bPL may act through hetero or homodimers of bGH and bPRL receptors in a tissue specific manner (Anthony et al., 1995). Additionally Anthony et al. hypothesized that bPL could bind a single monomer of the bovine GH receptor (Anthony et al., 1995). This may be indicative of oPL antagonism of oGH action by occupying a single oGH receptor to block oGH signal at the membrane, thereby directing nutrients away from the maternal system towards the pregnancy.

In rabbit mammary explants, oPL can stimulate lactogenic activity to the same degree as oPRL as measured by increased β-casein (Servely et al., 1983). The actions of oPL in this tissue appear to be through the PRL receptor as addition of a PRL receptor antibody completely abolishes the effects of oPL (Servely et al., 1983). The increased β-casein production has also been seen in sheep with a concurrent elevation in milk production (Kann et al., 1999; Leibovich et al., 2001). The lactotropic effects of oPL appear to be slow in generation as increases in milk yield is not detectable after 5 days of treatment (Bassett et al., 1998; Min et al., 1997).

The effects of oPL on the corpus luteum are similar to that of oPRL. In rats treated with bromoergocryptine (prolactin secretion inhibitor), oPL could maintain progesterone production in corpus luteum explants treated with prostaglandin $F_{2\alpha}$ (Chan et al., 1980; de la Llosa-Hermier MP et al., 1983). Similar results have been seen in ovine luteal cultures as well (Wierzchos et al., 2000). The luteoprotective effects of oPL are maintained through the functional life of the corpus luteum and well past the time when the placenta becomes the major source of progesterone (Gregoraszczuk et al., 2000).

Ovine PL administration to rats stimulates somatogenic actions including increases in amino acid transport, increases in hepatic IGF-I expression and serum IGF-I concentrations, and increases in glycogen synthesis, lipolysis, and protein accretion (Freemark and Handwerger, 1983; Freemark and Handwerger, 1986; Hurley et al., 1977; Singh et al., 1992). Additionally, evidence suggests that oPL stimulates glycogen synthesis in cultured fetal ovine hepatocytes (Freemark and Handwerger, 1986). Similar effects on IGF-I, glycogen synthesis, lypolysis and protein accretion have been seen in growing lambs (Leibovich et al., 2001). Administration of oPL to growing lambs also increased feed intake and daily gain (Min et al., 1996). For adult ewes, only the increase in IGF-I have been documented (Kann et al., 1999). Increases in fetal concentrations of oPL have been correlated to increased fetal weight in sheep, and fetal infusion of exogenous oPL late in gestation appears to increase fetal organ mass and serum IGF-I in fetal circulation (Schoknecht et al., 1991; Schoknecht et al., 1996).

Fetal infusion of oPL also verified earlier research in rats where glycogen synthesis in the liver is elevated (Freemark and Handwerger, 1984; Schoknecht et al., 1996). It appears also that oPL may stimulate the fetal production of IGF-II as hypophysectomy of fetal lambs leads to marked reduction of IGF-I with no decrease in IGF-II. Only after birth do IGF-II levels fall, presumably due to the loss of placental lactogen or some placental factor (Mesiano et al., 1989). The actions of oPL on the fetal liver appear to be through a yet undefined receptor, that is neither the oGH or oPRL receptor (Pratt et al., 1995) and has an apparent molecular weight of 44,000 (Freemark and Comer, 1989).

NON-RUMINANTS

During gestation intermediary metabolism of a woman goes through a transition. Early in gestation, body fat accumulates and by mid-gestation the mother shifts metabolic conditions to hyperglycemia, hypertriglyceridemia, and hyperinsulinemia (Handwerger and Freemark, 2000). This metabolic status is thought to insure a continuous supply of nutrients to the developing fetus. Culture of neonatal pancreatic islet cells with PL shows a direct stimulation of insulin secretion in mice, rats, and humans, supporting the hyperinsulinemia seen during pregnancy (Brelje et al., 1993). Hypertriglyceridemia seen during pregnancy is likely due to the stimulation of lypolysis in adipocytes (Handwerger and Freemark, 2000).

Human PL has many of the same somatogenic effects of ovine and bovine PL. Increases in glycogenesis and amino acid transport in fetal tissues (Handwerger, 1991) have been reported for hPL. In heterologous studies with

rat embryos, the addition of hPL increased growth rates of embryos, and addition of antibodies against IGF-I and –II could equally ablate the enhanced growth (Karabulut et al., 2001). That effects of hPL on rat embryo growth are at least partially directed through IGF is supported by increases of IGF-II secretion by rat embryo fibroblasts (Handwerger, 1991). As seen with oPL, hPL is luteoprotective in cultured rat luteal tissue as evidenced by decreasing (50%) 20α-dihydroprogesterone accumulation seen following exposure to PGF2α (de la Llosa-Hermier MP et al., 1983). Interestingly, almost 40 years ago it was reported that hPL had a stimulatory effect on erythropoiesis in mice (Jepson and Friesen, 1968). However, the erythropoietic effects of hPL have not received much attention since. Rodents have similar biological actions as humans and ruminants, although the rPL-I variant does not possess the mitogenic properties of rPL-I detected in the Nb2 lymphoma cell bioassay (Robertson et al., 1996).

PLACENTAL GROWTH HORMONE

In humans, growth hormone (GH) is part of a gene cluster that contains two copies of the GH gene and two copies of the placental lactogen (hPL) gene that produce functional proteins and one copy of hPL that is a pseudogene (Barsh et al., 1983; DeNoto et al., 1981; Hirt et al., 1987; Seeburg, 1982). The GH/PL gene cluster is located in a 66,500-bp region of chromosome 17 (Chen et al., 1989; George et al., 1981). All five genes are arranged sequentially (hGH-N, hPL-L, hPL-A, hGH-V and hPL-B in order) in the gene cluster, share a high sequence identity (91-99%), and are presumably the result of tandem

duplications of the same gene (Barsh et al., 1983; Chen et al., 1989; Hirt et al., 1987), as speculated by others (Niall et al., 1971; Seeburg, 1982).

The expression of hGH-V has been localized to sycytiotrophoblasts within the placenta of women (Scippo et al., 1993). The hGH-V gene encodes a peptide similar to hGH-N with only 13 amino acid substitutions within the mature peptide (Seeburg, 1982). The protein is present in the placenta in a native and glycosylated form (Frankenne et al., 1988; Frankenne et al., 1990). Human GH-V appears in maternal serum in the 5th week of gestation, and increases towards parturition, reaching concentrations several-fold higher than pituitary GH (Evain-Brion et al., 1994; Frankenne et al., 1988). A decrease in pituitary GH coincides with the increasing placental GH, indicating that placental GH appears to control the GH status within the maternal system (Evain-Brion et al., 1994; Frankenne et al., 1988). The increase in GH-V secretion appears to be a function of increased syncytiotrophoblast differentiation (Alsat et al., 1998). Human GH-V does not cross into fetal circulation as it is not detectable in cord blood at term; while pituitary GH (hGH-N) is detected (Frankenne et al., 1988).

Sheep also have a duplicate copy of the GH gene (Valinsky et al., 1990). While there are two genes for ovine growth hormone (oGH-1 and oGH-2), only oGH-2 has multiple alleles (oGH-2N and oGH-2Z (Gootwine et al., 1993; Valinsky et al., 1990). The sequence of the oGH-1 transcript was originally report by Orian et al. (Orian et al., 1988). The transcripts of the oGH-2N and -2Z alleles are more similar to the ovine GH sequence reported by Byrne et al. (Byrne et al., 1987). The oGH-2 genes encode proteins similar to pituitary

derived oGH-1 where only three amino acids are switched in the Z allele versus the N allele (Lacroix et al., 1996). Cleavage of a 26 amino acid secretion signal leaves a 191 amino acid mature peptide with a molecular weight of 22,000 (Byrne et al., 1987; Lacroix et al., 1996; Orian et al., 1988).

The expression of oGH-2 has been detected in the sheep placenta (Lacroix et al., 1996; Lacroix et al., 1999) and it has been inferred that both the N and Z alleles of the gene are expressed (Ofir and Gootwine, 1997). However, distinct localization of oGH expression in the placenta is still lacking. The expression of oGH in the placenta is detected from approximately day 35 to 55 of gestation (Lacroix et al., 1996; Lacroix et al., 1999). Lacroix et al. reported immunodetection of GH in maternal syncytial and stromal cells and the fetal trophoblast in day 40 to 45 placentomes (Lacroix et al., 1996). More recently, this group reported *in situ* hybridization of oGH in only the syncytium and trophoblast (Lacroix et al., 1999). Although both reports from the Lacroix group agree about detection of oGH within the placenta, the conclusions drawn about the localization of this expression are by no means definite. The magnification of images presented in these reports is far too low to distinguish the true cellular localization of oGH. Further research to determine the true cellular origin of oGH in the placenta is needed.

Biological Actions of Placental Growth Hormone

In women, it appears that placental growth hormone alters maternal metabolism to make nutrients available to the pregnancy. Analysis of serum samples of women resulted in high correlations of maternal serum placental

growth hormone and IGF-I (Alsat et al., 1998; Caufriez et al., 1990). Intrauterine growth restricted pregnancies demonstrate decreased hGH-V and decreased IGF-I (McIntyre et al., 2000; Mirlesse et al., 1993). This indicates that placental growth hormone has similar biological action to pituitary derived GH by stimulating IGF-I expression. McIntyre et al. reported that 40% of the birth weight difference between normal and IUGR pregnancies would be explained by hGH-V and IGF-I concentrations (McIntyre et al., 2000). Purification of recombinant hGH-V has allowed for some heterologous studies with hGH-V (Igout et al., 1993). Recombinant hGH-V has somatogenic and lactogenic activity in heterologous studies as indicated by stimulation of tibial epiphyseal plate growth in hypophysectimized rats and proliferation of Nb2 lymphoma cells (Alsat et al., 1998).

Studies in sheep to elucidate the biological actions of placental growth hormone have produced little understanding of the role of growth hormone within the placenta. Administration of GH during elongation and early placentation has no effect on placental development (Spencer et al., 1999b). Only endometrial gland proliferation was stimulated and this effect was seen only with concurrent interferon tau administration (Spencer et al., 1999b). Wallace et al. reported an increase in placental weight due to GH infusion (day 35 to 80); however, this may have been due to increased fluid weight resulting from polyhydraminios instead of exogenous GH (Wallace et al., 2004). Additionally, the increase in placental weight reported by Wallace et al. was only seen in adolescent ewes maintained on a high dietary intake (Wallace et al., 2004). This feeding regimen for

adolescent ewes is a model of placental and fetal growth restriction, where interestingly, maternal serum GH levels are decreased while IGF-I levels are increased. (Wallace et al., 1997). This indicates some type of alteration of the normal GH/IGF-I system in which GH and IGF-I concentrations are positively correlated. Therefore, caution should be used when interpreting the results of maternal oGH infusion in this model. Infusion of GH maternally may have resulted in increases in placental weight; however, this may represent a resetting of the normal GH/IGF-I system, thus providing a repartitioning of nutrients toward the pregnancy to normalize placental growth.

Administration of GH during mid-pregnancy results in increased maternal serum non-esterified fatty acids, IGF-I, and glucose, but no change in fetal growth (Jenkinson et al., 1999). Infusion of GH in the last 50 days of gestation can increase fetal weight by 10% (Jenkinson et al., 1999); however, several reports showed no effect of oGH administration on fetal or placental weight (Currie et al., 1996; Harding et al., 1997; Stelwagen et al., 1994). Stelwagen et al. and Harding et al. may not have produced an effect by giving comparatively low doses (0.1mg/kg body weight); however Currie et al. administered the same concentration of oGH (0.15mg/kg of body weight) as Jenkinson et al., in a similar time frame of gestation. Interestingly, all of the studies discussed here do not administer exogenous GH concurrent with growth hormone expression (~35 to 55 days of gestational age; dGA) within the placenta (Lacroix et al., 1996; Lacroix et al., 1999). It has been shown that administration of recombinant porcine GH (rpGH) to gilts during early to mid-gestation will increase fetal and placental

weight (Sterle et al., 1995). Increases in fetal weight and length have also been reported for underfed gilts given rpGH during midgestaton (Gatford et al., 2000). It remains to be seen what the effects of GH administration in sheep will have, particularly if the GH administration is concurrent with the normal expression window of GH within the placenta.

SUMMARY

The development of the placenta, from elongation and implantation through completion of placentation, requires a diverse group of cytokines, growth promotants, cell adhesion molecules, angiogenic molecules, and hormones. Peri-attachment factor, ovine placental lactogen and ovine placental growth hormone are all expressed in the developing conceptus (PAF) and placentomes (oPL and oGH) of the placenta.

Peri-attachment factor is expressed in a temporal window when maternal recognition of pregnancy and apposition of maternal epithelium is occurring. During this time a number of molecules are expressed in a spacial and temporal fashion. Peri-attachment factor has a predicted nuclear targeting sequence, and should it localize to the nucleus it could have an integral role in regulating the transcription of genes important to the early conceptus.

Ovine placental lactogen has been shown in heterologous studies to be mammotropic, luteotropic, somatogenic and to alter maternal metabolism to provide nutrients for the pregnancy. While several studies have looked at the transcriptional regulation of PL *in vitro*, there has been no attempt to study the transcriptional regulation of PL *in vivo*. Further analysis of the regulation of oPL

will lead to a better understanding of the roles of this hormone in the placenta and its impacts on placental and fetal growth. Addition of exogenous growth hormone has led to mixed results that have not given insight into the potential role of oGH in the placenta. Furthermore, no one has administered GH during its normal expression window in the placenta, so results to date have to be viewed with some reservation.

CHAPTER III

EXPRESSION OF PERI-ATTACHMENT FACTOR

INTRODUCTION

Reproductive efficiency has a dramatic effect on the economics of livestock production. Early embryonic wastage is a large component of total reproductive failure. This mortality leads to increased number of days open and impedes genetic gains. Conservative estimates for the losses incurred by the beef and dairy industries, exceed ~$1.8 billion annually, based on lost weight gain, milk production and replacement of females (United States Department of Agriculture, 2003). Research targeting early embryonic growth and development could greatly increase the profits gained for both the beef cattle and dairy industries.

Early embryonic development, implantation and maintenance of the pregnancy are critically dependent on successful embryo-maternal communication leading to maternal recognition of pregnancy. Early embryo development (days 15-17 post coitus) has been defined as the critical period (Binelli et al., 2001) when embryo-maternal communication initiates antiluteolytic pathways in cattle. Early studies of embryonic wastage (Boyd et al., 1969) revealed that up to 30% of pregnancies failed by this critical window. More recent studies have estimated embryonic loss in a range of 28-40% (Binelli et al.,

2001; Fricke et al., 2003; Roche et al., 1981; Zavy and Geisert, 1994). The embryonic loss in dairy cattle has been estimated as high as 55% (Lucy, 2001) and has been increasing steadily since the 1950's. Those cattle that require multiple fertilization attempts to conceive i.e. repeat breeders, have a 47% failure of conception at the critical window of maternal recognition of pregnancy (Ayalon, 1978). For heifers classified as repeat breeders, 72% of embryos are abnormal (Zavy and Geisert, 1994). Clearly little progress has been made towards solving the problem of embryonic wastage as we have failed to increase reproductive efficiency. When trying to explain embryonic loss, only 10.4% of abnormal embryos can be explained by chromosomal abnormalities (Zavy and Geisert, 1994). This leaves a significant portion of embryonic loss to be explained by physiological malfunction. The most researched protein during ruminant maternal recognition of pregnancy is interferon-τ, and its role in initiating antiluteolytic pathways is clearly defined (Hansen et al., 1999; Thatcher et al., 1995). Though interferon-τ plays a critical role in establishment of the pregnancy, other cytokines and growth factors also play critical roles in early embryonic growth, development and survival (Martal et al., 1997).

Using differential display, transcriptional changes between day 15.5 and 17.5 bovine conceptus tissue were detected for four different genes (Glover and Seidel, Jr., 2003). Increased expression was detected at day 17.5 for allograft inflammatory factor 1, ligand of eph-like receptor kinase 5, interferon-τ and a novel transcript named peri-attachment factor (Glover and Seidel, Jr., 2003). Bovine peri-attachment factor (bPAF; since renamed bPF) mRNA concentration

was 10 fold greater in day 17.5 conceptus as compared to day 15.5. Bovine PF expression was no longer detectable in day 30 bovine conceptus tissue. A full length cDNA (accension no. AY027656) for bPF was cloned from a day 25 cDNA library (Glover and Seidel, Jr., 2003). Analysis of the predicted amino acid sequence revealed 4 putative protein kinase C (PKC) phosphorylation sites, two casein kinase II phosphorylation sites, and a nuclear targeting sequence with no apparent DNA or RNA binding domain (Glover and Seidel, Jr., 2003). Northern hybridization sceening of adult bovine tissues detected slight expression of bPF in kidney. The authors reported only one known homolog to bPF, a hypothetical human protein (hHP; Genbank, XM_166586) which shared 83% nucleotide sequence identity and 77% amino acid identity (Glover and Seidel, Jr., 2003).

Further search of nucleotide databases revealed a mouse homolog to bPF, a transcript detected in mouse small and large intestine named G90 (Krause et al., 1999). The authors reported a complete open reading frame did not exist within the G90 transcript (Krause et al., 1999). However within the reported sequence, an open reading frame exists that would code for a peptide similar to bPF and is identical to a peptide coded from a cDNA (Genbank BY 731406) cloned from the mouse eye (Okazaki et al., 2002). A nucleotide search of the rat genome with mouse G90 sequence identified a rat sequence with high homology to these PF homologues as well. All of these peptides have remarkable conservation ranging from 66 to 84%, and within the predicted nuclear targeting sequence there is only one amino acid switch (R97K) amongst all of the species.

Bovine PF is expressed in a critical window when the embryo is communicating its presence to the maternal system and initiating early conceptus growth and development. Given that bPF is predicted to have a nuclear target sequence with no obvious DNA or RNA binding motifs, bPF may play a role as a co-repressor or co-activator in early conceptus development. Further investigation of the expression, localization and protein characterization is needed to understand what potential role this novel protein may have in early conceptus transcriptional regulation. The aim of the following research was to determine the presence and expression of bPF homologues, and to determine the cellular localization of translated bPF.

MATERIALS AND METHODS

Southern Hybridization with PF cDNA

To screen for the presence of PF homologues in species other than cattle, Southern hybridization analysis of genomic DNA from several species was performed. A Zoo Blot (Seegene, Rockville, MD, USA) was purchased to screen several species at once. The Zoo Blot membrane had 10 μg of genomic DNA from human, rat, mouse, dog, cow, pig, rabbit, chicken, frog (Xenopus), fish (Zebrafish), C. elegans, and yeast. All genomic DNA samples on the Zoo Blot were digested with EcoRI prior to electrophoresis and transfer. In addition to these species, equine genomic DNA was harvested from the white buffy coat of 10 ml of whole blood collected in EDTA vacutainer tubes. The white buffy coat was digested overnight with proteinase K in digestion buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS in 1 x PBS) at 50°C in a

shaker. Genomic DNA was precipitated with 0.5 vol of 7.5 M ammonium acetate and 2 volumes of cold 100% ethanol. The solution was inverted 10-12 times, followed by centrifugation at 20,000 x g for 10 min. The resulting pellet was washed with 70% ethanol, allowed to dry then resuspended in TE (10 mM Tris-HCl and 1 mM EDTA pH 8.0) with 0.1% SDS and 1μg/ml of ribonuclease A (RNase A; Sigma, St. Louis, MO, USA) and incubated for 1 hour. The DNA sample was purified by phenol:chloroform:isoamyl alcohol extraction followed by centrifugation at 10,000 x g for 10 min. The upper phase was removed and precipitated overnight with 0.1 volume of 4 M NaCl, and 3 volumes of 100% ethanol at 20°C. The precipitated DNA was collected by centrifugation at 20,000 x g for 15 min at 4°C. The genomic DNA was resuspended in TE and concentration and purity were determined by absorbance at 260 nm and 280 nm.

For southern analysis, 10 μg of genomic horse DNA was digested with EcoRI, electrophoresed on a 1% agarose gel and denatured for 1 hr with 1.5 M NaCl and 0.5 M NaOH buffer. The gel was then neutralized with a 3.0 M NaCl and 0.5 M Tris buffer at pH 7.5 for one hour. Following neutralization the gel was transferred to a nitrocellulose membrane overnight by capillary transfer with 20 x SSC (3 M NaCl and 0.3 M $NaC_6H_5O_7 \cdot 2H_2O$), and then crosslinked with UV irradiation for 12 seconds. To generate a cDNA template for Southern analysis, primers (forward 5'-atcagcaccccagtctcctc-3' and reverse 5'-ctctcgggctattgcttgtc-3') were used in PCR with an annealing temperature of 64°C to produce a 381-bp cDNA corresponding to the predicted open reading frame of PF. A full length bPF cDNA was the template for PCR (Glover and Seidel, Jr., 2003). The cDNA

was radiolabeled to a minimum specific activity of $5x10^8$ CPM per μg of DNA. The membrane was blocked with pre-hybridization buffer (6x SSPE, 50% deionized formamide, 5x Denhardt's solution, 1% SDS, 2 mg/ml herring sperm DNA, 50 mg/ml yeast tRNA) for 4 hr before addition of $4x10^6$ CPM/ml of labeled bPF template. The membrane was hybridized overnight then washed with 2 x SSC and 0.1% SDS for 15 min at room temperature followed by 15 min at 42°C, then two washes in 0.1 x SSC and 0.1% SDS for 30 min at 60°C. Following washes to remove unbound radiolabel and nonspecific binding, the membrane was exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA) for 72 hr. The screen was scanned using a Storm Imager (Molecular Dynamics) to visualize hybridization of PF.

Tissue Collection

To assess the temporal expression of bPF in bovine embryos, several time-mated mature crossbred cows were flushed at different time points (days 14, 17, 21, and 28) in gestation. Conceptus tissues were sterily flushed from the uterus in 1 x phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 pH 7.3) supplemented with 0.25% bovine serum albumin. The tissue was snap frozen in liquid nitrogen for tcRNA harvest. The fetus of one day 28 pregnancy was isolated from trophoblast to assess the differential expression of the two tissues. To screen other species for expression of PF homologues, conceptus tissue was collected from sheep, pigs, and horses at during early gestation. Following euthanization (20 mg/kg sodium pentobarbital; Sigma, St. Louis, MO, USA) and hysterectomy conceptus tissue

from ewes at 13 and 15 days of gestational age (dGA) was flushed through the uterine tip with 1 x PBS, collected and snap frozen in liquid nitrogen. For equine tissue, the conceptus was sterily flushed from mares at days 14, 16, 20 and 30 of gestation. As with the bovine tissue, one equine fetus at day 30 was isolated from the trophoblast tissue for assessment of differential expression. Conceptus tissue from pigs was generously supplied by Dr. Matthew Wheeler (University of Illinois) from gestational day 10 and 12 sows. The sows were euthanized, hysterectomized and conceptii were flushed from the uterus. Previously collected adult tissues from mouse, rat and sheep were also screened for expression of a PF homolog. Additionally, ovine fetal heart and lung from a 135 days of gestation (dGA) pregnancy were screened for PF.

Preparation of Total Cellular RNA and Detection of PF Expression

Total cellular RNA was isolated from all adult tissues, ovine fetal tissues, and day 28 bovine and day 20 and 30 equine conceptus tissues by methods previously described (Kappes et al., 1992; Warren et al., 1990b). An aliquot of the stock tcRNA samples was diluted to a concentration of 1 μg/μl ± 0.2 μg/μl for use as a working solution for analysis. For the earlier conceptus tissues, RNeasy Mini columns (Qiagen, Valencia, CA, USA) were used according to manufacturer's protocol for tcRNA isolation. The concentration and purity of the samples was then determined by absorbance at 260 nm and 280 nm. Samples with sufficient tcRNA were electrophoresed on 1.2% agarose-formaldehyde gels for Northern hybridization analysis as previously described (Kappes et al., 1992; Warren et al., 1990b). The same radiolabeled cDNA for the bPF open reading

frame used in the Southern analysis was used for Northern analysis. For samples lacking sufficient tcRNA (day 14 and 17 bovine conceptus and day 10 and 12 porcine conceptus) RT-PCR was used to detect PF expression. Prior to RT-PCR, the tcRNA samples were treated with DNase I (Roche, Indianapolis, IN, USA) to remove genomic DNA contamination. The DNase I reaction (100μl volume) was 10 mM $MgCl_2$, 50 mM Tris, and 10 U of DNase I and tcRNA samples were incubated at room temperature for 2 hours. The reaction mix was then purified by addition of phenol:chloroform:isoamyl alcohol (0.5 volume to 0.5 volume) and centrifugation at 10,000 x g for 10 min. The upper aqueous phase was transferred to a new tube and precipitated overnight by adding 0.1 volume of 4 M potassium acetate and 3 volumes of 100% ethanol. The pellet was washed with 70% ethanol and allowed to dry. The tcRNA was hydrated in diethylpyrocarbonate treated water (depc-treated) and absorbance at 260 nm and 280 nm was used to determine concentration and purity. For the reverse transcriptase reaction, 2 μg of tcRNA was used in the SuperScript First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). The primers used to generate the bPF open reading frame cDNA were used on RT product. The PCR conditions were the same as when generating the bPF open reading frame cDNA. The reaction was run for 40 cycles and the entire reaction mix for each sample was electrophoresed on a 1% agarose gel. The gel was denatured and neutralized as described above and the samples were transferred to a nitrocellulose membrane and cross-linked. Hybridization was carried out as described for the

Southern hybridization analysis, with $4x10^6$ CPM/ml of radiolabeled bPF cDNA. The membranes were exposed to X-ray film to visualize hybridization.

Mammalian Expression of bPF-V5-His Protein

For mammalian expression analysis of PF, the bPF open reading frame cDNA was ligated into pcDNA 3.1 V5-His TOPO (Invitrogen, Carlsbad, CA, USA) mammalian expression plasmid (bPF-V5-His) by direct PCR cloning according to manufacturer's recommendations. The plasmid was then transformed into DH5α E. *coli* cells for propagation and sequencing (Macromolecular Resources; Colorado State University). The mRNA sequence of the bPF-V5-His fusion protein is 639-bp in length minus the poly-A tail. To make stable cell lines, Cos-7 and Chinese hamster ovary (CHO) cell lines were chosen for lipid mediated transfection. The maintenance media for CHO cells was F12K (Mediatech, Herndon, VA, USA) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 2.5 mg/L Amphotericin B, 50 mg/L gentamicin sulfate, and 10% fetal bovine serum (FBS). The maintenance media for Cos-7 cells was Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) supplemented with 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 2.5 mg/L Amphotericin B, 50 mg/L gentamicin sulfate and 10% FBS. Both cell lines were maintained at 37°C with 5% CO_2. The cells were transfectected with 20 μg of plasmid DNA using Polyfect (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The cells were cultured for an additional 48 hours and then selection antibiotic (G418; Gemini Bio-Products, Woodland, CA, USA) was added to the culture media at a concentration of 500 μg/ml.

Colonies resistant to G418 were harvested from the culture plates using cloning columns. The cells were allowed to become semi-adherent by incubation with 0.25% Trypsin-EDTA media (Sigma, St. Louis, MO, USA), and then each colony was aspirated into the column and transferred to a separate culture well. The cells were maintained on selection media throughout colony selection and culture of clonal cell lines. When sufficient cells were cultured, tcRNA was harvested from the clonal cell lines to screen for the bPF-V5-His fusion protein transcript. One 15 cm plate of cells was collected for tcRNA analysis from each clonal cell line. The cells were detached by incubation with 0.25% Trypsin-EDTA media for 10-15 min and collected by centrifugation at 2000xG for 10 min. TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) was used to isolate tcRNA from the clonal cell lines. The cells were resuspended in 2 ml of TRI-Reagent and pipeted vigorously to break up the cells and then incubated at room temperature for 10 min. After incubation, 200 μl of BCP (1-bromo-3-chloropropane; Molecular Research Center) was added and mixed vigorously. The samples were allowed to phase separate for 15-20 min at room temperature then centrifuged at 11,000 x g for 15 min at 4°C. The upper phase was transferred to a new tube, and tcRNA was precipitated overnight by adding 3 volumes of 100% ethanol at 20°C. The precipitated tcRNA was collected by centrifugation at 20,000 x g for 20 min at 4°C. The RNA pellet was washed with 70% ethanol and allowed to dry. The tcRNA was resuspended in depc-treated water and concentration and purity were determined by absorbance at 260 nm and 280 nm. For Northern hybridization, 15 μg of tcRNA was electrophoresed

and transferred to a nylon membrane. The membranes were blocked with pre-hybridization solution (6x SSPE, 50% formamide, 1% dextran sulfate, 1% SDS, 30 μg/ml herring sperm DNA, and 50 μg/ml yeast tRNA) and hybridized overnight with the bPF open reading frame cDNA as a radiolabel at $4x10^6$ CPM/ml. The membranes were exposed to Phosphor screens for 72 hours then scanned with the Storm Imager.

Separation of Nuclear and Cytoplasmic Protein

One Cos-7 and CHO clonal cell line transcribing the appropriate mRNA for bPF-V5-His was cultured for western analysis of nuclear and cytoplasmic fractions. For nuclear protein separation, 6 culture plates (15 cm) of CHO and Cos-7 cells were used. Culture media was aspirated from cells and replaced with 0.25% trypsin-EDTA media for 10-15 min. The cells were washed from the plates and collected by centrifugation at 1500 x g for 10 min. The cells were washed in 5 packed cell vol of hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT). The cells were collected by centrifugation at 1850 x g for 5 min and resuspended in hypotonic buffer (3 vol of the original packed cell volume) and incubated on ice for 10 min. The swollen cells were homogenized in a Dounce homogenizer with twenty strokes of a type B pestle. The homogenate was centrifuged at 3300 x g for 15 min to collect intact nuclei. The supernatant was collected as the cytoplasmic fraction of proteins. The nuclear pellet was resuspended in 0.5 vol of low salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) followed by dropwise addition of high salt buffer

(substitute 1.2 mM for 0.02 mM KCl in low salt buffer). The suspension of nuclei was incubated on ice for 30 min with shaking at 4°C. The nuclear membrane and debris were pelleted by centrifugation at 15,000 x g at 4°C for 30 min. The extracted nuclear protein in the supernatant was dialyzed overnight in 6,000 MW (Spectra/Por; Spectrum Laboratories, Rancho Dominguez, CA, USA) dialysis tubing against 500 ml of dialysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). Precipitant from dialysis was removed by centrifugation of the solution at 15,000 x g for 15 min at 4°C. The nuclear and cytoplasmic protein fractions were quantified versus BSA standard concentrations using a Bradford protein assay (Bio-rad, Hercules, CA, USA).

Construction of His-Express-bPF Plasmid

To construct a prokaryotic expression system for bPF, the open reading frame ligated into pcDNA3.1 V5-His TOPO was digested out with *Bam*HI and *Bst*BI and ligated into prSET B (Invitrogen). The prSET B plasmid was digested with *Bam*HI and *Bst*BI to receive the bPF insert. For the ligation reaction (10 μl volume; 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP with 3 U of T4 ligase; Promega, Madison, WI, USA) the plasmid to insert ratio was 1 M to 3.75 M in a volume of 10 μl. The ligation was incubated at room temperature for 3 hours then the plasmid was transformed into DH5α cells and incubated overnight. Colonies were cultured in LB broth with 50 μg/ml ampicillin to replicate the plasmids for sequence confirmation. Positive clones of prSET B containing bPF (His-Express-bPF) with the correct sequence were then transformed into

BL21 (DE3) pLysS cells (Invitrogen, Carlsbad, CA, USA). The transformants in BL21 cells were plated on LB-amp agar plates treated with 35 μg/ml chloramphenicol and incubated overnight.

Expression of His-Express-bPF Fusion Protein

Four positive clones of His-Express-bPF cells were grown in 2 ml of LB broth with 50 μg/ml ampicillin and 35 μg/ml chloramphenicol overnight at 37°C with shaking at 225-250 rpm for pilot expression as recommended by the manufacturer. The soluble protein collected from the pilot expression was quantified using Bradford protein analysis. For preparation of a large quantity of His-Express-bPF fusion protein, a 500 ml culture of BL21 cells was inoculated with 1 mM IPTG and cultured an additional 4 hours to reach the optimal expression of the His-Express-bPF fusion protein seen in the pilot expression. The cells were resuspended in binding buffer (10 mM $Na_2HPO_4 \cdot 2H_2O$, 10 mM $NaH_2PO_4 \cdot H_2O$ 0.5 M NaCl, and 20 mM Imidazole pH 7.4) then lysed by sonication with a Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT, USA) for purification with a HisTrap HP Kit according to protocol (Amersham, Piscataway, NJ, USA). Soluble protein was loaded on the column at a flow rate of 0.1 ml/min. The column was washed with 20 ml of binding buffer and then bound protein was eluted off with 5 ml of elution buffer (substitute 500 mM for 20 mM imidazole in binding buffer) according to the manufacturer's recommendations. The flow through, wash and elution fractions were collected separately and quantified by Bradford assay.

Western Hybridization Analysis

For each sample 50 μg of total protein was combined with NuPAGE 4x load dye (1 M glycerol, 140 mM Tris base, 100mM Tris HCl, 7 mM lithium dodecyl sulfate, 40 nM EDTA, 0.025% Serva Blue G250, and 0.025% Phenol Red, final concentration) supplemented to 10% β-mercaptoethanol, boiled for 4 min and loaded on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen; Carlsbad, CA, USA). The samples were electrophoresed for 1 hour at 200 volts in 1 x MOPS running buffer (100 mM MOPS, 100 mM Tris-HCl, 3.5 mM SDS, and 0.8 mM EDTA). The gels were then transferred to nitrocellulose membrane in an XCell II Hybridization Module (Invitrogen) at 30 volts for 2 hours in NuPAGE transfer buffer (500 nM bicine, 500 nM Bis-Tris, 20.5 nM EDTA, and 1 mM chlorobutanol, in methanol) as described (Regnault et al., 2002b). The gels with protein samples from the pilot expression of His-Express-bPF were then stained with Coomassie blue (0.25% Coomassie blue, 45% methanol and 7.5% acetic acid) prior to transfer. Following destain and imaging, the gels were soaked in transfer buffer then transferred to a nitrocellulose membrane. For immunoblot of bPF-V5-His, an antibody raised against the V5 epitope (Invitrogen) was used as recommended for detection of the fusion protein. For immunoblot of His-Express-bPF, an antibody raised against the Express epitope (Invitrogen) was used as recommended for western analysis. SuperSignal West Femto (Pierce, Rockford, IL, USA) was used as the chemiluminescent subtrate for detection of immunoreactive protein.

RESULTS

To screen several other species for PF homologs, the bPF open reading frame nucleotide sequence was used in Southern hybridization analysis. Genomic DNA from several species was digested with *Eco*RI and transferred to a membrane for hybridization. Figure 3.1 illustrates the results of the Southern hybridization analysis with radiolabeled bPF open reading frame cDNA. Hybridization of the radiolabeled cDNA was detected in human, dog, cow, pig, rabbit, yeast and horse. A PF homolog was not detected by Southern hybridization in rat, mouse, chicken, frog, fish or worm (C *elegans*). Interestingly, we did not detect a rodent homolog for PF, when previous reports detected a PF homolog in mouse tissues (Krause et al., 1999; Okazaki et al., 2002).

Several tissues were screened by Northern hybridization for expression of PF homologs. Tissues were collected from several species, including adult tissues and conceptus tissues from similar gestational time points when bPF was detected in bovine conceptus. As seen in figure 3.2, an ovine homolog (oPF) to bPF was detected only in conceptus (day 13, not shown and day 15), and adult kidney and faintly in lung. In addition to these tissues, oPF was detected in day 135 fetal lung. Within the adult lung only, two distinctly different transcripts were revealed by Northern hybridization. However, the larger transcript may be an artifact, as it represented a considerably longer transcript than is reported for both bPF and mouse G90/mPF full length cDNAs (Glover and Seidel, Jr., 2003; Krause et al., 1999).

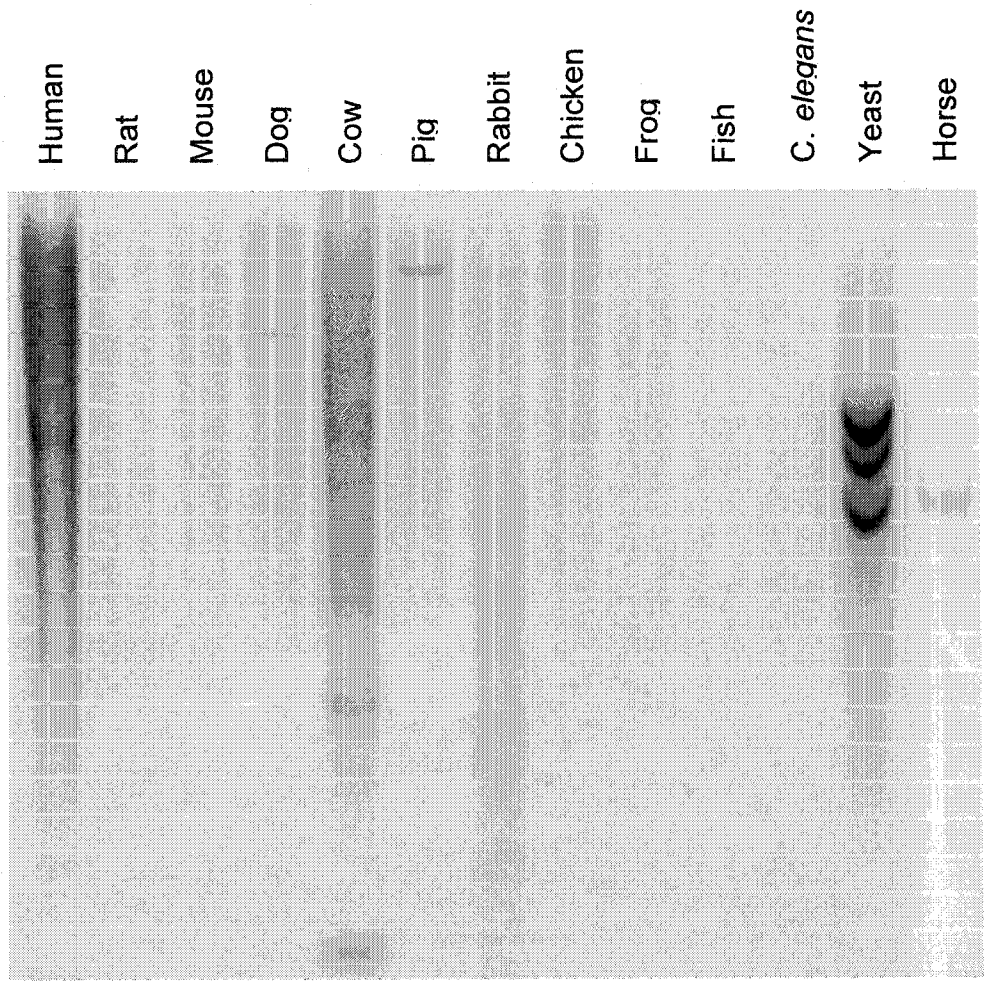

Figure 3.1: Southern hybridization of genomic DNA. Human, rat, mouse, dog, cow, pig, rabbit, chicken, frog, fish, C. *elegans*, and Yeast genomic DNA was radiolabeled with with bPF cDNA. Ten μg of DNA were loaded per lane. PAF homolog was detected in human, dog, cow, pig, rabbit, yeast, and horse.

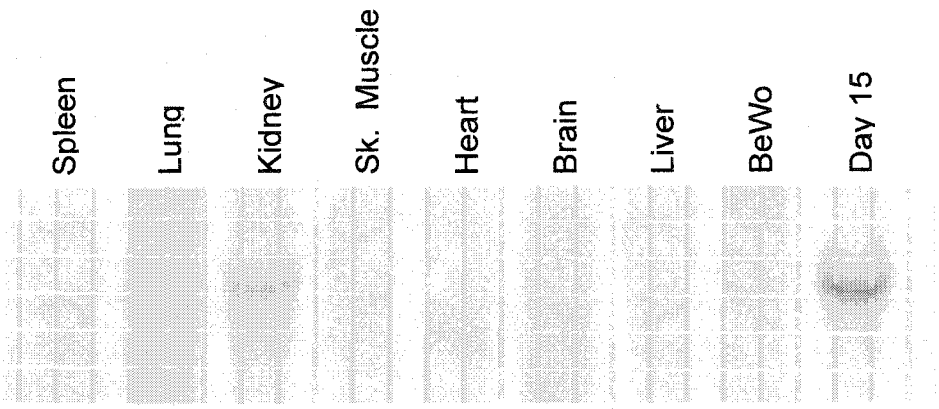

Figure 3.2: Northern hybridization of adult sheep tissues, BeWo cells, and day 15 ovine conceptus with bPF cDNA. Ten μg of tcRNA was loaded per lane. PF mRNA was detected in adult lung and kidney and day 15 ovine conceptus.

Several adult rat tissues were screened for a rodent homolog to bPF. As shown in figure 3.3, no PF was detected in the selected rat tissues. This is intriguing as a nucleotide sequence database search revealed that the rat genome has nucleotide sequence that is predicted to code for a similar protein to bPF. The homology of the predicted rat PF (rPF) to bPF and mPF are 66 and 84% respectively. Bovine PF expression was detected in day 21 and 28 trophoblast (figure 3.3) tissues which confirms earlier reports of bPF expression until day 30 of gestation (Glover and Seidel, Jr., 2003) and not in day 28 conceptus. The lack of detection in day 28 conceptus may be due to dilution of trophoblast mRNA by fetal mRNA. As shown in figure 3.3, the existence of an expressed equine PF (ePF) homolog has been detected in day 14 and 16 equine conceptus tissues.

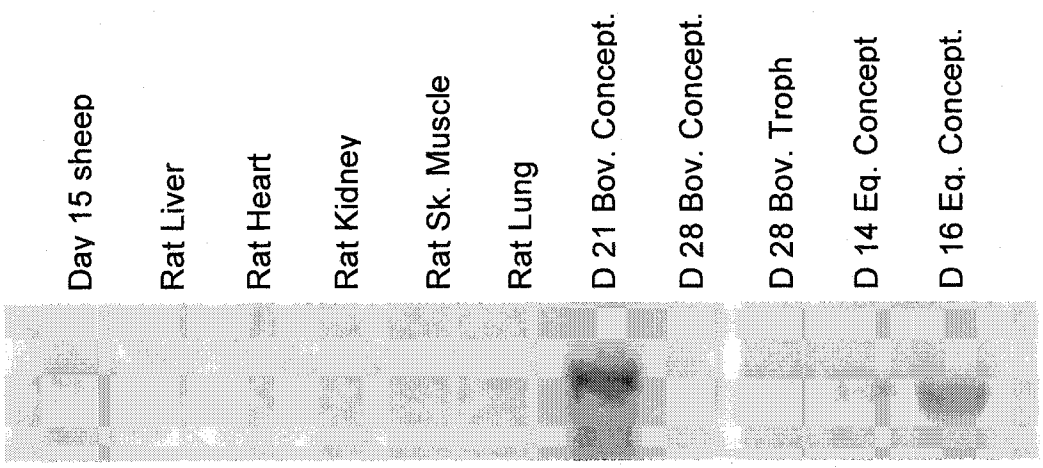

Figure 3.3: Northern hybridization of sheep, rat, cow and horse tissues with bPF cDNA. 10μg tcRNA per lane. PF mRNA was detected in day 15 ovine, day 21 bovine, day 14 and 16 equine conceptus and day 28 bovine trophoblast.

The expression window of ePF was further expanded by detection of mRNA by northern hybridization in day 20 and 30 conceptus tissues (figure 3.4). A day 30 equine fetus was separated from trophoblast tissue to elucidate the source of ePF. As shown in figure 3.4, the equine fetus does not express PF. Mouse G90/mPF was highly detectable in kidney and testis (Krause et al., 1999), however we were unable to detect any G90/mPF homolog in several adult mouse tissues including kidney (Figure 3.4).

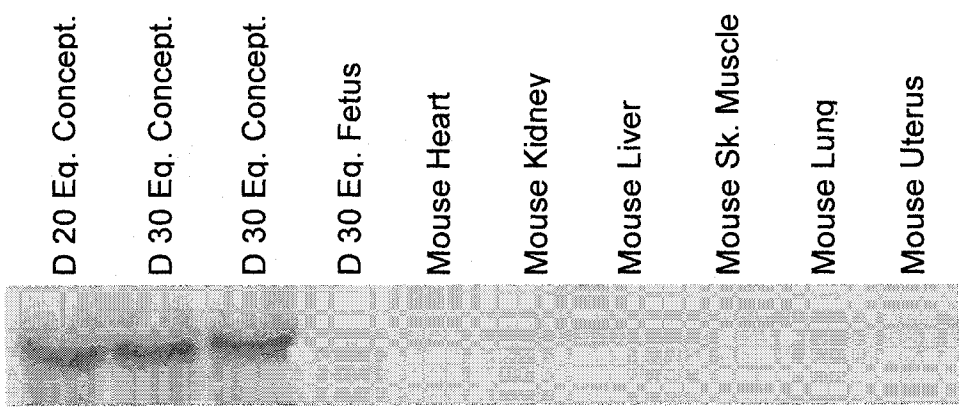

Figure 3.4: Northern hybridization of horse and mouse tissues with bPF cDNA. 10μg of tcRNA per lane. PAF mRNA was detected in day 20 and 30 horse conceptus.

The expression of PF was detected in bovine day 14 and 17, and porcine day 10 and 12 conceptus by RT-PCR and verified by southern hybridization (data not shown). However, the coding region of PF is exclusive to one of two exons as revealed by the genomic sequence of the mouse (Krause et al., 1999) and

human genes. Given that the 5' and 3' untranslated regions of the cDNAs PF homologs are not highly conserved, the primers for RT-PCR were designed against the ORF where conservation is highest. Therefore, it cannot be ruled out that results of the Southern hybridization may be due to genomic DNA contamination in the PCR reaction; thus, these results should be viewed cautiously.

To determine the cellular localization of translated PF protein, cDNA for the entire predicted open reading frame of bPF was inserted in a mammalian expression vector and stably transfected into CHO and Cos-7 cells. The cells were cultured to confluence and expression of the bPF-V5-His fusion transcript was verified by Northern hybridization. Cell lines (both CHO and Cos-7) expressing the fusion protein were then cultured for western analysis. Following collection of cells, nuclear and cytoplasmic proteins were separated and the fusion protein was detected with an antibody directed against the V5 epitope. Figure 3.5 shows the results of the western analysis for transfected and control CHO and Cos-7 cells.

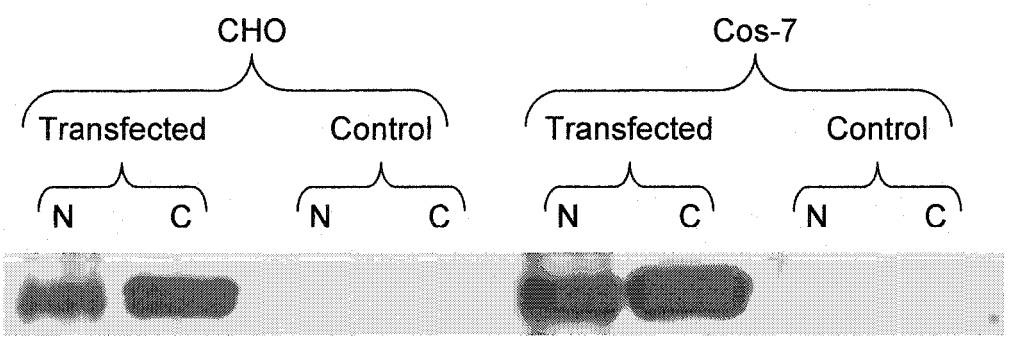

Figure 3.5: Western immunoblot of Chinese hamster ovary (CHO) and Cos-7 cell lines transfected with bPF mammalian expression vector. N = nuclear protein, C = cytoplasmic protein.

The bPF-V5-His fusion protein was detected in both nuclear and cytoplasmic protein fractions of CHO and Cos-7 cells, indicating that both cell lines are capable of translating the fusion protein. The detected protein migrated at a molecular weight of 20,000, which is larger than its predicted molecular weight of ~17,000. This may be due, in part, to net positive charge of the histidine tag residues slowing migration. Expression of the fusion protein was not detected in either non-transfected control cell lines. The detection of the fusion protein in the nucleus indicates that the predicted nuclear targeting sequence is apparently functional. In order to further characterize bPF, the open reading frame was ligated into a prokaryotic expression vector for production of a recombinant bPF fusion protein.

The prSET expression vector incorporates a histidine tag and Express epitope on the amino terminus of the inserted bPF cDNA (His-Express-bPF). The Express epitope is flanked by an enterokinase cleavage site, to liberate bPF from the histidine tag and Express epitope. The histidine tag was used in nickel column chromatography to purify the recombinant fusion protein, and an antibody directed against the Express epitope was used in western analysis for detection. Bacteria transformed with the His-Express-bPF vector were cultured and expression of the fusion protein was initiated by addition of IPTG. Protein samples collected from the pilot expression were electrophoresed and then stained with Coomassie to identify the fusion protein. The results of a pilot expression are presented in figure 3.6. By Coomassie staining, it appeared that maximal expression of the fusion protein was reached by 4 hr of incubation

following IPTG induction. The same clone of the fusion protein shown in figure 3.6 was cultured for a large scale preparation of the protein to be purified. Figure 3.7 shows the results of western analysis of fractions collected following nickel column chromatography. As seen with the mammalian expression vector, His-Express-bPF was detected at a larger molecular weight than predicted. The predicted molecular weight of the protein is ~21,000; however, we detected the protein at a molecular weight near 30,000. Again, this may be due to addition of a histidine tag, or could be a result of the preparation, and purification of the protein. We now have the ability to generate a fusion protein, which can be purified by nickel chromatography and then cleaved to give recombinant bPF to further characterize the protein and its function.

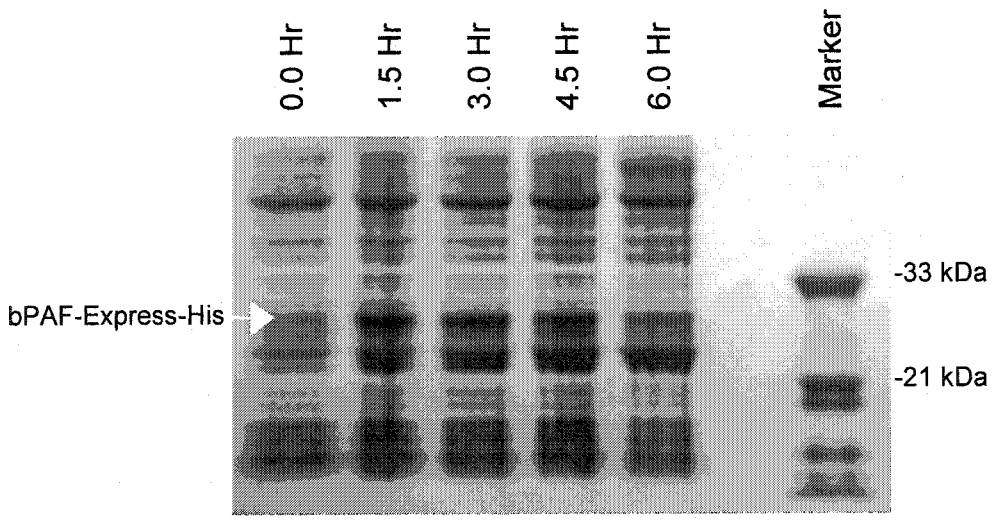

Figure 3.6: Coomassie stain of IPTG induced bPF-Express-His fusion protein. 50μg of protein per lane.

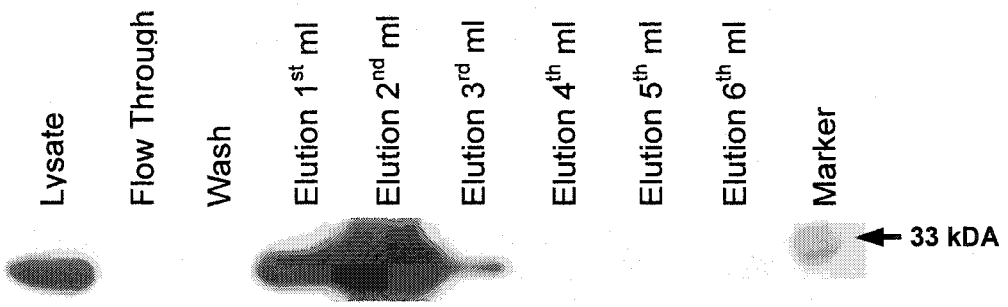

Figure 3.7: Purification of bPF-Express-His fusion protein by Ni-column chromatography. The fusion protein was detected in lysate and elution fraction 1-3.

DISCUSSION

Our objective with this research was to determine the existence of PF homologs to bPF, and their expression in species other than bovine. Additionally, our goal was to begin characterization of bPF protein and its localization within the cell. The results of the Southern hybridization identified a conserved genomic sequence of PF in multiple species of different phylogenetic orders. Interestingly, we could not detect murine G90/mPF. The sequence identity of bPF and mPF are highly conserved within the predicted nuclear targeting sequence and surrounding protein; however, this conservation does not exist further 5' of the nuclear targeting sequence. When murine G90/mPF, the predicted rat PF, bPF and hHP/hPF are aligned by amino acid sequence, it is revealed that murine G90/mPF has several apparent deletions of amino acids dispersed among highly conserved sequences (Figure 3.8). When nucleotide sequence is aligned according to amino acid alignment, the sequence identity is

77%. With these deletions, the resulting frame shifts, mPF and bPF exhibit greatly reduced sequence identity, which could decrease our ability to detect rodent PF when using bPF cDNA as a radiolabel.

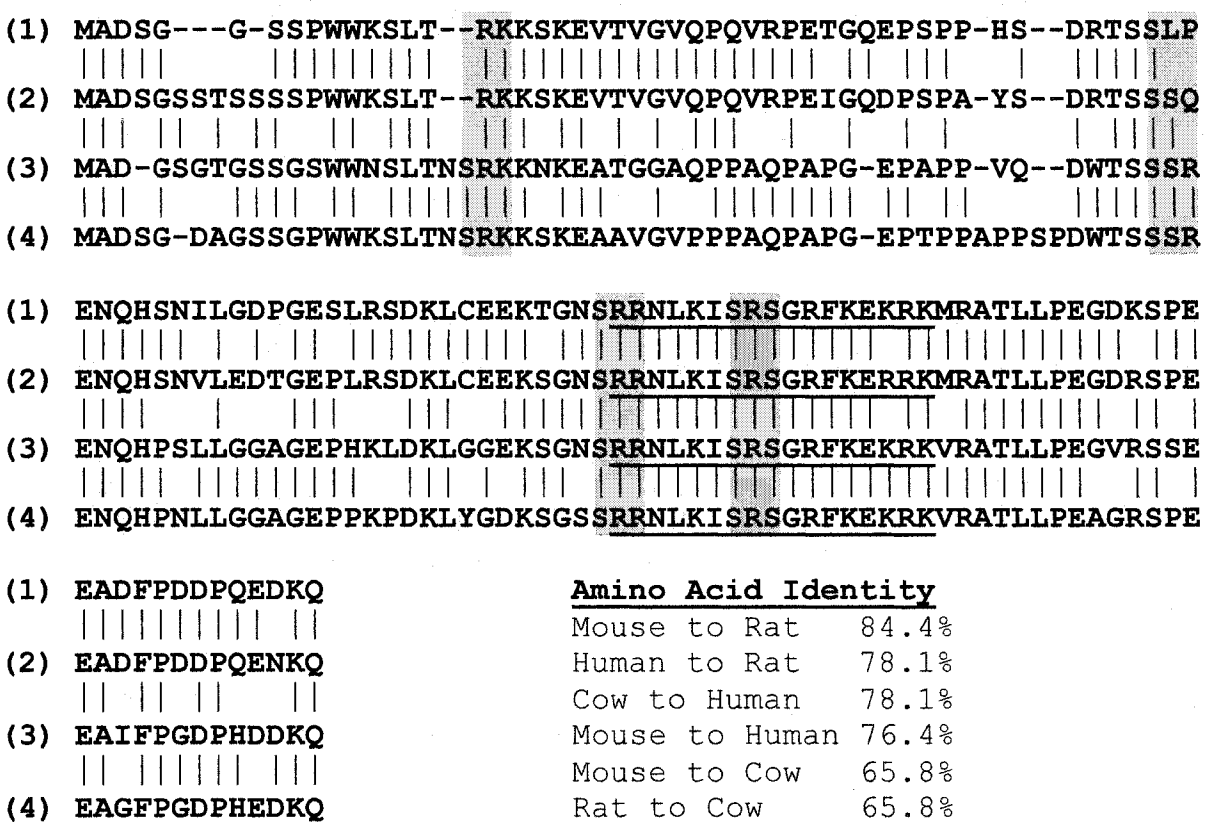

Figure 3.8: Comparison of mouse (1), rat (2), cow (3) and human (4) PF homolog amino acid sequences. The underlined portion is the nuclear targeting sequence, and the highlighted residues are PKC phosphorylation sites.

The expression of bPF in conceptus tissue has been confirmed at days 21 and 28 of gestation which agrees with previous reports of expression in bovine conceptus (Glover and Seidel, Jr., 2003). Ovine (oPF), porcine (pPF), and

equine (ePF) homologs were all detected at a comparable developmental time point of gestation to that of bPF. Additionally as seen with bPF, oPF is expressed in adult kidney. Interestingly, oPF is also expressed in the lung and the expression was detected in the day 135 ovine fetus as well. This is the first detection of PF in fetal sheep tissue. Mouse G90 has been detected in embryonic mouse brain tissues, particularly the isthmus of ventricles 3 and 4, and sensory hair cells and cochlea of the inner ear (Meunier et al., 2003). Hybridization of G90 cDNA was reported in other parts of the brain and ear; however, hybridization was weak (Meunier et al., 2003).

Krause et al. hypothesized that G90 was a regulatory RNA, as it appeared the cDNA lacked a complete coding sequence with translational start and stop sites in frame (Krause et al., 1999). Review of the cDNA sequence for G90 revealed an open reading frame (ORF) with translational start and stop sites in frame. This ORF coded for a protein similar to PF, hHP and a predicted rat PF protein (Figure 3.8). Although mouse PF/G90 amino acid has deletions, as compared to bPF, the four protein kinase C sites and nuclear targeting sequence are conserved. Furthermore, the detection of bPF fusion protein in the nucleus of cells in culture indicates that bPF is targeted to the nucleus. The translation of PF in a mammalian expression system and detection of the protein in the nucleus provides evidence that PF likely is a functional protein versus a regulatory RNA. The role of the protein within the nucleus is not clear; however it is probable that bPF interacts with other proteins in the nucleus as the protein has no apparent nucleic acid binding domains. Peri-attachment factor may act

as a co-repressor or co-activator within the nucleus to impact transcription within the cell. Figure 3.9 describes our hypothetical role of PF within the cell. Following PKC phosphorylation, PF migrates to the nucleus and acts as a co-repressor or co-activator to alter transcription.

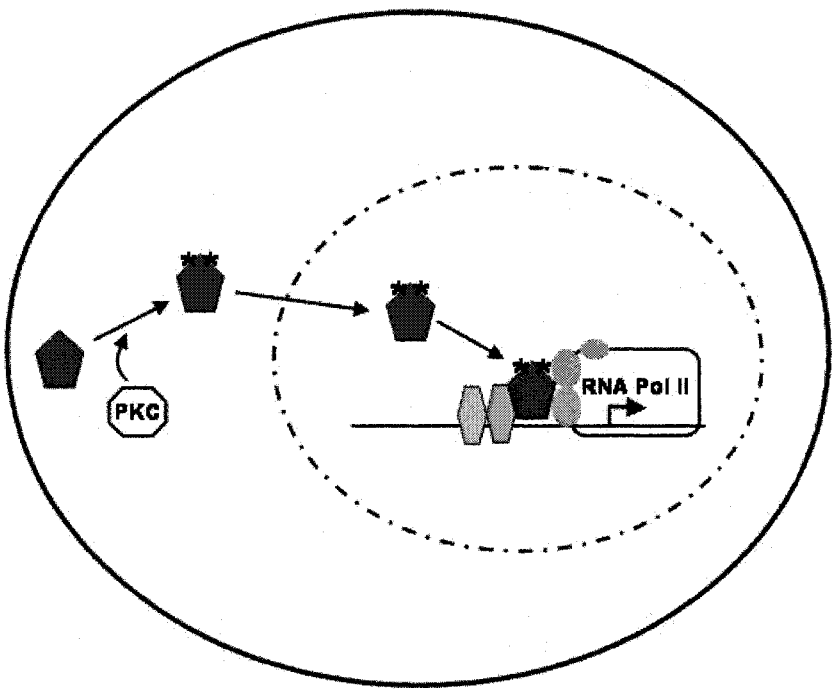

Figure 3.9: A schematic representation of the postulated role of bPF in the early bovine conceptus. Following phosphorylation by protein kinase C, bPAF translocates to the nucleus, becoming part of a functional transcription complex.

Peri-attachment factor may play a critical role in early conceptus development, given that expression of PF in conceptus is conserved across several species. Further research is needed to elucidate and characterize PF

and roles it may play within the nucleus, particularly its potential role in critical early conceptus development. Production of a recombinant PF protein as described here will greatly increase the ability to further define PF. Development of an antibody against PF will be valuable in verifying the cellular localization and compartmentalization of PF.

SUMMARY

A novel mRNA transcript was recently described in bovine conceptus tissue, and named peri-attachment factor (PF). Bovine PF (bPF) mRNA is present during early conceptus expansion (days 15 and 17 post coitus), but is absent from tissues collected after day 30 and in adult tissues. The exception to this is the detection of bPF mRNA in adult kidney. Two known homologs of bPF are murine protein G90, which has been detected in embryonic and adult mouse brain, adult mouse intestine, kidney and testis and a human hypothetical protein detected in placenta. Based on the inferred amino acid sequence, it was hypothesized that PF/G90 is a nuclear protein that may play an active role in early conceptus development. Our first objective was to screen genomic DNA from several species (human, rat, mouse, dog, cow, pig, rabbit, chicken, frog, fish, C. elegans, yeast, and horse) by Southern hybridization for PF homologs. Our second objective was to examine cow, sheep, horse, rat, mouse and porcine conceptus and adult tissues (heart, lung, liver, spleen, kidney, skeletal muscle, uterus and brain), for the existence of PF/G90 mRNA. Finally, our third objective was to localize PF within the cell following expression in mammalian cell lines.

Southern hybridization revealed genomic PF homologs in the human, dog, cow, pig, rabbit, yeast, and horse. Northern hybridization and/or RT-PCR analysis detected PF in bovine (day 14, 17, 21 and 28), ovine (day 13 and 15), porcine (day 10 and 12) and equine (day 14, 16, 20 and 30) conceptus tissue, coinciding with the stage of conceptus development when bPF was previously detected. Analysis of adult sheep tissues detected PF in kidney and lung. Interestingly, no PF could be detected in rat or mouse adult tissues, especially in the kidney where PF was detected in cow and sheep tissue. This may be due to amino acid deletions that shift nucleotide sequence in the mouse versus bovine PF. Screening of day 135 fetal ovine fetal tissues revealed detectable PF mRNA in the lung but not in the heart. Western analysis of a PF fusion protein detected PF fusion protein in both cytoplasmic and nuclear fractions of stably transfected CHO and Cos-7 cells.

The Southern hybridization data indicates a conserved gene for PF exists across several species of different phylogenetic orders. Results of bovine conceptus tissue expands the previous temporal expression window of PF mRNA to day 28, and results with ovine and equine conceptus tissues are similar to those reported for cattle; additionally ovine PF was detected in adult and fetal lung. The predicted nuclear targeting sequence of PF is apparently functional as evidenced by the immunoblot analysis. Given the detection PF fusion protein in the nucleus and absence of DNA and RNA binding domains, we hypothesize that PF acts as a co-activator or co-repressor to modify transcription within the nucleus. While nothing is known about the function of PF, it has now been

described during a critical window of conceptus development in two ruminant and two non-ruminant species. Further characterization of PF is needed before a role for this protein can be assigned.

CHAPTER IV

TRANSCRIPTIONAL REGULATION OF OVINE PLACENTAL LACTOGEN

INTRODUCTION

In eutherian mammals, the placenta is responsible for directly mediating or modulating the maternal environment required for maintenance of normal fetal growth and development (Anthony et al., 1995). The placenta of numerous species produces hormones that are structurally and functionally similar to the pituitary hormones prolactin (PRL) and growth hormone (GH). The majority of these hormones have a molecular weight of 20,000 to 25,000 and regulate various maternal processes including mammary gland differentiation, steriodogenesis, somatic growth, and intermediary metabolism (Ogren and Talamantes, 1988). The most extensively studied member of the PRL-like hormones are the placental lactogens (PL); species studied include human, monkey, baboon, mouse, rat, hamster, sheep, cow and goat (Ogren and Talamantes, 1988).

Ovine placental lactogen (oPL) has a molecular weight of 22,000 and an isoelectric point of 9.2 (Warren et al., 1990a). Ovine PL is a nonglycosylated single chain polypeptide of 198 amino acids preceded by a 38 amino acid signal sequence (Colosi et al., 1989; Warren et al., 1990a). Sequence analysis reveals

that oPL is structurally more similar to ovine prolactin (oPRL) than it is ovine growth hormone (oGH), 49% and 28% sequence identity, respectively (Warren et al., 1990b).

In rabbit mammary explants, oPL can stimulate lactogenic activity to the same degree as oPRL as measured by increased β-casein (Servely et al., 1983). The actions of oPL in this tissue appear to be through the PRL receptor as addition of a PRL receptor antibody completely abolishes the effects of oPL (Servely et al., 1983). The increased β-casein production has also been seen in sheep with a concurrent elevation in milk production (Kann et al., 1999; Leibovich et al., 2001). The effects of oPL on the corpus luteum are similar to that of oPRL. In rats treated with bromoergocryptine (prolactin secretion inhibitor), oPL could maintain progesterone production in corpus luteum explants treated with prostaglandin $F_{2\alpha}$ (Chan et al., 1980; de la Llosa-Hermier MP et al., 1983). Similar results have been seen in ovine luteal cultures as well (Wierzchos et al., 2000). The luteoprotective effects of oPL are maintained through the functional life of the corpus luteum and well past the time when the placenta becomes the major source of progesterone (Gregoraszczuk et al., 2000).

Ovine PL administration to rats stimulates somatogenic actions including increases in amino acid transport, increases in hepatic IGF-I expression and serum IGF-I concentrations, and increases in glycogen synthesis, lipolysis, and protein accretion (Freemark and Handwerger, 1983; Freemark and Handwerger, 1986; Hurley et al., 1977; Singh et al., 1992). Additionally, evidence suggests that oPL stimulates glycogen synthesis in cultured fetal ovine hepatocytes

(Freemark and Handwerger, 1986). Similar effects on IGF-I, glycogen synthesis, lypolysis and protein accretion have been seen in growing lambs (Leibovich et al., 2001). Administration of oPL to growing lambs also increased feed intake and daily gain (Min et al., 1996). Increases in fetal concentrations of oPL have been correlated to increased fetal weight in sheep, and fetal infusion of exogenous oPL late in gestation appears to increase fetal organ mass and serum IGF-I in fetal circulation (Schoknecht et al., 1991; Schoknecht et al., 1996).

Fetal infusion of oPL also verified earlier research in rats where glycogen synthesis in the liver is elevated (Freemark and Handwerger, 1984; Schoknecht et al., 1996). It appears also that oPL may stimulate fetal production of IGF-II, as hypophysectomy of fetal lambs leads to marked reduction of IGF-I with no decrease in IGF-II. Only after birth do IGF-II levels fall, presumably due to the loss of placental lactogen or some placental factor (Mesiano et al., 1989). The actions of oPL on the fetal liver appear to be through that of a yet undefined receptor that is neither the oGH or oPRL receptor (Pratt et al., 1995) and has an apparent molecular weight of 44,000 (Freemark and Comer, 1989).

The oPL gene has been structurally characterized, and 4.5-kb of the promoter has been studied; where maximal activity of the oPL promoter was seen in the proximal 383-bp in BeWo (human) and Rcho-1 (Rat) cell choriocarcinoma cell lines (Liang et al., 1999). DNAse protection and eletrophoretic mobility shift assays revealed that GATA-2 (2 sites), AP-2α, and Purα interact with there putative sites within the proximal oPL promoter (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001). Mutation of the

GATA, AP-2α, and Purα sites and transfection analysis of the sites revealed that GATA, AP2α and Purα sites are functional, and serve to increase promoter activity (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001). In humans, several transcription factors have been shown to impact transcription of human placental lactogen in heterologous cell culture (Jiang et al., 1995; Jiang and Eberhardt, 1994; Lytras and Cattini, 1994; Rogers et al., 1986). Although many of these transcriptional regulators of hPL are enhancers of transcription, additional elements upstream of the gene regulate its expression in a tissue specific manor (Nachtigal et al., 1993). Though these studies have provided insight into the transcriptional regulation of oPL and hPL *in vitro*, there has been no attempt to study the tissue specific regulation of PL *in vivo*. The goal of this research is to ascertain the promoter sequence necessary for tissue specific expression of oPL.

MATERIALS AND METHODS

Isolation of Ovine Fetal Fibroblasts and Transfection

A primary ovine fetal fibroblast cell line was isolated from a fetus at 47 days of gestational age (dGA). Fetal tissue was removed from the uterus and washed with 1 x phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) to remove debris. The tissue was manually minced and incubated for one hour in collagenase digestion solution (1 x PBS, 1250U type IV collagenase per ml). Following digestion, the fetal fibroblasts were washed in 1 x PBS, pelleted by centrifugation, resuspended in media and allowed to adhere to culture plates. The cells were maintained in

Dulbecco's Modified Eagle's Media nutrient mixture F-12 HAM (DMEM/F12) supplemented with 1.2g/L $NaHCO_3$, 5% fetal bovine serum (FBS), 5% donor horse serum (DHS), 50 mg/L gentamicin sulfate, and 2.5 mg/L Amphotericin B at 37°C and 5% CO_2. Cells were grown to approximately 80% confluence and then passed (1 to 3) by incubation with 1 x PBS, pelleting by centrifugation and resuspended in media.

Previously, 383-bp, 1616-bp and 4498-bp of the oPL promoter were ligated upstream of the EGFP coding region in the pEGFP-1 (Clonetech, Palo Alto, CA, USA) plasmid (Liang et al., 1999). For electroporation, cells were suspended in 1 x PBS at a density of 1 $x10^7$ cells per ml, 40-50 μg of plasmid was added to 400μl of cells and electroporated with 2 pulses of 450 millivolts for 80 milliseconds using a BTX Electro Cell Manipulator (Biotechnologies & Experimental Research, San Diego, CA, USA). Cells were then suspended in media and allowed to recover for 48 hours before addition of G418 (600 μg/ml; Gemini Bio-Products, Woodland, CA, USA) to initiate selection. For lipid mediated transfection, Lipofectamine (Invitrogen, Carlsbad, CA, USA) was used in serum free media with 50 μg of DNA/15 cm plate. To select colonies, cells were incubated with 1 x PBS to start cell detachment. Prior to complete detachment, colonies were aspirated with sterile pipettes. Following selection, colonies were isolated to new plates and clonal cell lines were cultured for use in nuclear cloning. The integration of the oPL promoter constructs was confirmed by PCR. Primers (forward 5'-aagaatgcggtaaaagagaa-3' and reverse 5'gttgtggctgttgtagttgt-3') were designed to amplify the proximal 322-bp of

promoter to +131-bp of EGFP for the -380 construct. Primers (forward 5'-gagtgtggtctttctgtaat-3' and reverse 5'gttgtggctgttgtagttgt-3') amplified the proximal 875-bp to +131 of EGFP for the -1.6 construct. For both constructs, genomic DNA from stable cell lines was used in PCR with 40 cycles of 95°C denaturation for 1 min, annealing at 56°C for 1 min, and extension for 1 min at 72°C.

Oocyte Maturation and Nuclear Cloning

Fresh ovine ovaries were collected from a local abattoir in 30-35°C saline supplemented with 2% penicillin-streptomycin antibiotic. Cumulus-oocyte-complexes (COC) were aspirated from 2-8 mm follicles and placed in HEPES-buffered TCM 199 media (Sigma, St. Louis, MO, USA) with 100 IU heparin and 2% FBS. The collected COC were washed in collection media then matured 24 hours in TCM 199 supplemented with 10% FBS, 10 μg/ml ovine FSH, 10 μg/ml ovine LH, and 1 μg/ml estradiol at 39°C and 5% CO_2. Following maturation, oocytes were removed from cumulus cells by gentle pipeting in Synthetic Oviductal Fluid (SOF) media with 3% fatty acid free BSA and 0.1% hyaluronidase. Oocytes were then washed in HEPES-buffered TCM 199 media and transferred to manipulation plates. Manipulation media was 1 x PBS supplemented with 5% sucrose and 3% polyvinylpyrrolidone (PVP). Stably transfected fetal fibroblasts were collected, washed with 1 x PBS and then transferred to manipulation media. For enucleation, the polar body and metaphase plate were removed, and the fibroblast was placed next to the ooplasm under the zona pellucida (Prather et al., 1989). After manipulation, the

oocytes were re-equilibrated to BSA supplemented SOF media and cultured overnight at 39°C and 5% CO_2.

Fusion and Activation

Oocytes were equilibrated in fusion media in a stepwise gradient ending in fusion media (0.3 M mannitol, 0.1 M $MgSO_4$, 0.05 mM $CaCl_2$ in sterile water). Oocytes were held in fusion media for 10 min then aligned between electrodes for fusion. Optimized fusion conditions for clones were one pulse of 20 volts for 15 μsec using the BTX Electro Cell Manipulator. After fusion the oocytes were equilibrated to SOF media (3% BSA and essential amino acids) by a stepwise gradient and cultured for 2 hours at 39°C with 5% CO_2. The oocytes were activated by addition of 5 μM calcium ionophore (A23187; Calbiochem, La Jolla, CA, USA) and 2 mM dimethylaminopurine (DMAP; Sigma, St. Louis, MO, USA) for 4 min. Reconstituted embryos were then cultured for 4 hours in SOF with 2 mM DMAP. Following the DMAP incubation, the embryos were returned to SOF media with BSA and essential amino acids for culture overnight. The following morning cleaved embryos were placed in a fresh drop of SOF media and readied for transfer to recipient ewes.

Embryo Transfer

Crossbred ewes were observed for estrus for 40-50 days prior to initiation of embryo transfer to establish normal cyclicity within the pool of recipients. Ewes that exhibited standing estrus the day of enucleation of oocytes (30-48 hours prior to transfer) were anesthetized by venous injection of sodium pentobarbitol (20 mg/kg), intubated, shorn and sterily prepared for surgical

embryo transfer. Uterine horns and ovaries were exteriorized through a midline incision slightly cranial to the mammary glands. Presence of corpora lutea was noted for each ovary and 15-20 cleaved embryos were transferred into each oviduct. Ewes were given 1 ml of banamine to alleviate surgical discomfort and observed while recovering from surgery.

Transgenic Tissue Analysis

Ewes with established pregnancies as determined by observation for return to estrus were euthanized for tissue collection at various time points of gestation (25-42 days). Conceptus tissues were frozen fresh in O.C.T. Compound (Sakura; Torrance, CA, USA) or collected in 1 x PBS then fixed in 4% paraformaldehyde overnight. After fixation, the tissues were washed in 1 x PBS and stored in 70% ethanol until paraffin embedding.

Fluoroscopy and Immunohistochemistry Analysis

Tissue embedded in O.C.T was sectioned at 10 or 12 μm and mounted on slides with Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA, USA) for visualization of fluorescence. Upon inspection of the tissue it was noted that an intense auto-fluorescence was occurring in the placental tissue such that determination of true GFP expression was going to be compromised. Paraffin embedded tissue was sectioned at 6 μm for detection of GFP. Sections were mounted on slides and counterstained with DAPI to aid in visualizing nuclei. Two antibodies directed against GFP (anti-GFP ab290 and ab6556; Abcam) were purchased to localize GFP by immunohistochemistry. Immunohistochemistry was performed as recommended for use with Vectastain Elite ABC Kit (Vector

Laboratories Inc, Burlingame, CA, USA) as previously described (Kappes et al., 1992; Limesand and Anthony, 2001). Both primary antibodies were diluted 1:400 for use in detection of GFP.

EGFP cDNA Generation

For *in situ* hybridization, a cDNA was generated from the coding region of enhanced green fluorescent protein (EGFP) plasmid where 5'-atggtgagcaagggcgaggag-3' was the forward primer and 5'-ggggaggtgtgggaggttttt-3' was the reverse primer. The reaction mix was 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.4 μM primer (forward and reverse) and 2 U of *Taq* Polymerase. The PCR product (803-bp) was generated by 40 cycles of 95°C 1 min to denature; 65°C 1 min to anneal; 72°C 1 min for extension. This PCR product was gel purified and cloned into PCR-Script Amp SK (Stratagene, Cedar Creek, TX, USA) per manufacturer's recommendations for sequence validation and amplification. The plasmid was harvested by alkaline lysis, and purification by centrifugation through a CsCl gradient (Liang et al., 1999). The plasmid preparations were sequenced by Macromolecular Resources (Colorado State University).

In Situ Hybridization

Following sequence validation, the EGFP cDNA was digested from PCR-Script Amp with *Eco*RV and *Sac*II restriction enzymes. For a positive *in situ* hybridization control, pBK-CMV plasmid (Stratagene, Cedar Creek, TX, USA) with no insert was used as template for radiolabeling. The pBK-CMV plasmid contains a neomycin resistance cassette that is common to the EGFP plasmid

used in oPL promoter constructs. The CMV driven neomycin resistance should be detectable in all cell types. Radiolabeling of cDNA for *in situ* hybridization was performed using 50-100ng of template in the DECAprime II random prime labeling kit (Ambion, Austin, TX, USA) and using α-phosphate 33-labeled cytosine tri-phosphate (αP^{33} dCTP) per manufacturer's instructions. *In situ* hybridization was performed as previously described (Kappes et al., 1992).

Viral Infection of Placental Tissue

Cotyledons (n=7) were sterily separated from the uterine caruncles of a ewe at day 100 of gestation. The cotyledons were washed and resuspended in 200 ml of 1 x PBS at 37°C, then mechanically homogenized in a Waring blender. The homogenate was centrifuged at 550 x g for 10 min to collect the tissue. The supernatant was discarded and the tissue pellets were resuspended in 25 ml of 1 x PBS. Twenty five ml of digestion buffer (1 x PBS with 1 x trypsin, 1250 U/ml of type IV collagenase, and 1 U/ml DNase I) was added and samples were incubated shaking at 37°C for 1 hr. Samples were washed 2 times in 1 x PBS and passed through 4 layers of cheesecloth. The flow through was centrifuged at 2500 x g for 10 min to collect the cells. Cell pellets were washed in 15 ml of 1 x PBS, centrifuged (2500 x g), resuspended in 1 x PBS with 4% glycerol, incubated for 10 min at 37°C, centrifuged (2500 x g) then resuspended in 1 x PBS to lyse red blood cells. The cells were centrifuged at 2500 x g and the final cell pellet was resuspended in 30 ml of 1 x PBS. The resuspended cells (4 ml) were loaded on to 25 ml Percoll (1131g/ml; Amersham, Piscataway, NJ, USA) gradients established by centrifugation at 30,000 x g for 15 min. The cells were

separated on the gradient by centrifugation at 1600 x g for 15 min. Starting with the top cell (uninucleate) layer in the gradient, 2 ml fractions were collected and washed with 1 x PBS to remove the Percoll. The fractions (n=10) were allowed to plate on separate chamber slides. The media consisted of DMEM/F12 supplemented with 1.2 g/L $NaHCO_3$, 2.5 mg/L Amphotericin B, 34 mg/L D-valine, 10% FBS, 100 U/ml penicillin and 100μg/ml streptomycin. After 48 hr of culture, the cells on the chamber slides were infected with an andenovirus (AdEasy; 1 $x10^6$ viral particles per slide) or a lentivirus (pLL3.7; $4x10^4$ particles per slide) which encoded GFP under regulation by the cytomegalovirus promoter (Rubinson et al., 2003). Infection lasted overnight, when fresh media was added to the chamber slides. After 48 hours, the cells were fixed with 4% paraformaldehyde washed with 1 x PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting media with DAPI to stain nuclei. The cells were visualized with fluorescence microscopy (Nikon Eclipse E800; Nikon, Mellville, NY, USA) for expression of GFP.

RESULTS

To assess the *in vivo* transcriptional regulation of the oPL promoter, constructs made previously with 383-bp, 1616-bp and 4498-bp of the oPL promoter were ligated upstream of the EGFP coding region in the pEGFP-1 plasmid (Liang et al., 1999). These constructs were stably transfected into ovine fetal fibroblasts for use in transgenic nuclear cloning and subsequent embryo transfer. Prior to nuclear cloning, several cell lines were evaluated for

competency in generating blastocysts following nuclear transfer. Table 4.1 describes the growth of three cell lines cultured *in vitro*.

Depicted in table 4.1, is the number of clones at different stages of growth as the percentage of the number of clones that cleaved following activation. For the cell lines tested the cleavage rates for the clones were 70.3% (47 dGA), 72.0% (60 dGA), and 89.7% (dGA). As seen in table 4.1, the 47 and 90 dGA cell lines advanced approximately 1 stage per day of culture, while the 60 dGA cell line grew rapidly to day 16 and then arrested growth at the morula stages. The 60 dGA cell line clones degenerated after 5 days of *in vitro*, never producing a blastocyst. Although the 90 dGA cell line generated the same percentage of blastocysts, the blastocysts derived from the 47 dGA cell line were healthier in appearance. The day 47 fetal fibroblast cell line was chosen for nuclear cloning for several reasons; 1) the cell line was more stable in culture than earlier age cell lines, 2) 47 dGA cells generated blastocysts at a higher rate across clutches of oocytes than later cell lines (15.2% versus 10.3% for day 90), and 3) the blastocysts produced were of higher quality and developed at a similar rate to normal blastocysts. Of 58 recipient ewes, 24 advanced in pregnancy beyond day 27, and 13 of these pregnancies supplied healthy tissue. Healthy tissue for each reporter construct (-380, 4 ewes; -1.6, 3 ewes; and -4.5, 2 ewes) was collected and embedded in paraffin. The embedded tissue was sectioned and immunohistochemistry performed; however, GFP could not be detected by two different antibodies. Increases in primary and secondary antibody concentrations failed to detect GFP protein.

Table 4.1. Growth of embryos resulting from nuclear cloning. Values presented are percentage of clones at a given developmental stage as a percent of the embryos that cleaved.

	Stage	2 cell	4 cell	8 cell	16 cell	Early morula	Compact morula	Blastocyst
Day	Cell Age							
	47	67.3	19.2	1.9	1.9			
1	60	22.2	13.9	19.4	41.7			
	90	27.9	9.8	14.8	19.7			
	47	13.5	51.9	32.7	3.8	1.9		
2	60		5.6	13.9	19.4		61.1	
	90	3.3	29.5	27.9	24.6	11.5	1.6	
	47	7.7	19.2	30.7	30.7	9.6		
3	60			19.4		16.7	2.8	
	90	4.9	14.8	34.4	16.4	26.2	1.6	
	47	5.8	13.5	13.5	32.7	13.5	15.4	
4	60			19.4		16.7	2.8	
	90	3.3	13.1	41.0	14.8	18.0	1.6	
	47	5.8	11.5	23.1	21.1	11.5	21.2	
5	60				16.7		5.6	
	90	1.6	16.4	27.9	24.6	13.1	8.2	
	47	3.8	5.8	34.6	25.0	5.8	13.5	1.9
6	60*							
	90	1.6	14.8	21.3	21.3	21.3	3.3	3.3
	47	1.9	5.8	19.2	25.0	7.7	11.5	5.8
7	60*							
	90	3.3	11.5	16.4	27.9	5.0	11.5	8.2
	47	9.6	13.5	19.2	17.3	5.8	5.8	11.5
8	60*							
	90		14.8	18.0	9.8	14.8	8.2	11.5

* Embryos degenerated following five days of *in vitro* culture. The n for clones from each cell line tested were n=52, n=36, n=61 respectively for day 47, 60 and 90 cell lines.

The method for detection of GFP shifted to *in situ* hybridization with a cDNA directed against the EGFP mRNA. Analysis of *in situ* hybridization revealed no detectable GFP, however sporadic hybridization was detectable in the control slides. Surprisingly, hybridization of the pBK-CMV cDNA was not hybridized to all cells. The control radiolabeled cDNA from pBK-CMV plasmid hybridized to no particular cell type among the different transgenic constructs (-380-bp, -1,600-bp, and -4,500-bp), however the intensity and localization of the hybridization was inconsistent among the samples.

The inconsistency of the transgenic nuclear transfer to generate testable results lead us to research the application of viral infection in cultured placental tissue as a route to test the transcriptional regulation of oPL. Cotyledon tissue was collected from ewes, digested, and cell types separated on a percoll gradient. The cells were allowed to adhere to culture slides for infection with adenovirus or lentivirus. Several cell types (epithelial and stromal), including BNCs and syncycial plaques, were successfully transfected with this methodology. Figures 4.1 shows viral infected cells expressing GFP following 72 hours of culture. Both adenovirus (panels A and C) and lentivirus (panels B and D) could infect several cell types.

The viability of cells in culture is evidenced by the growth of cells during and following infection. Note that several cells are stained with DAPI that were not infected in panels C and D. These cells continued to grow following the removal of infection media, and subsequently are only stained with DAPI. The

expression of GFP was highest between 48 and 72 hr following infection, for both viral mediated infections.

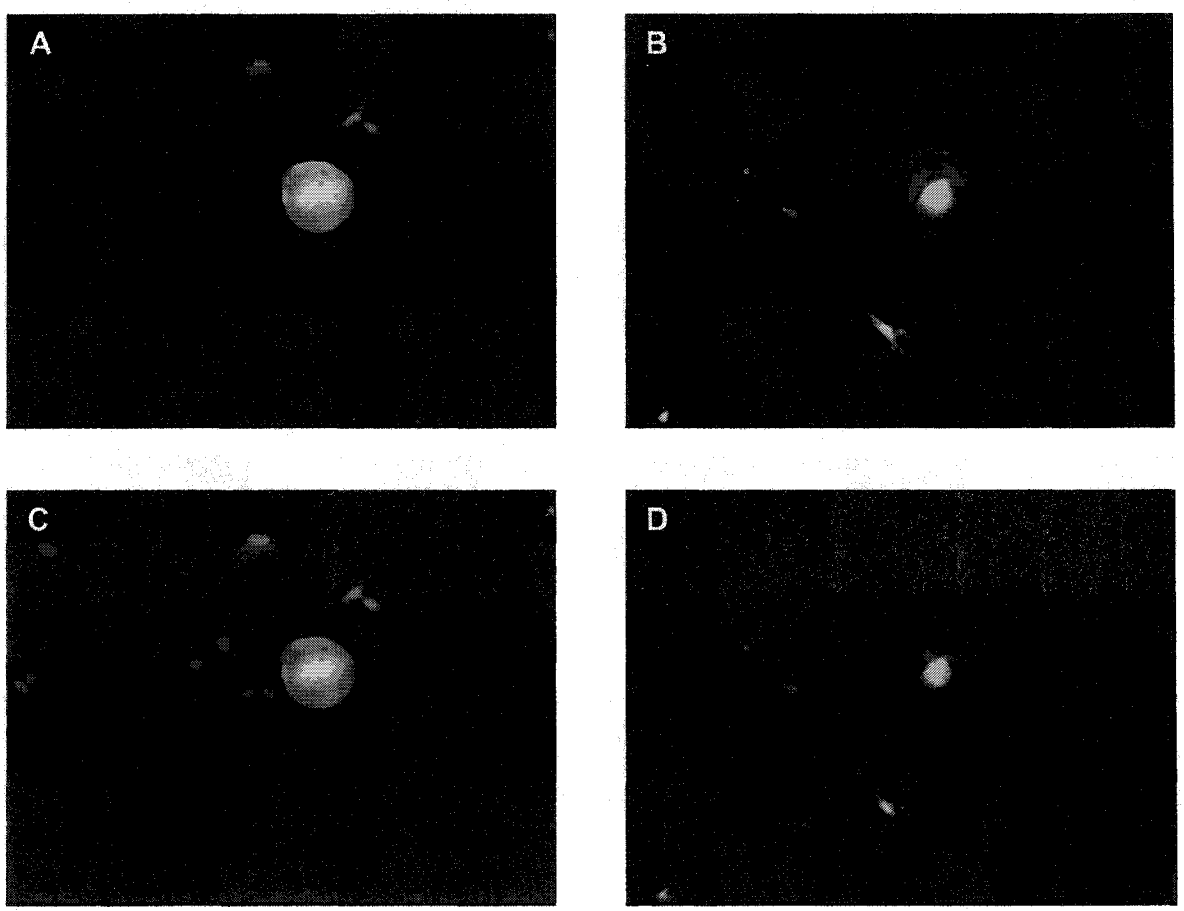

Figure 4.1: Viral infection of cultured placental cells. Panel A, GFP fluorescence of adenovirus BNC. Panel B, GFP fluorescence of lentivirus infected BNC. Panels C and D, overlay of DAPI stained nuclei for same cells in panels A and B respectively.

Discussion

Our goal was to use transgenic nuclear transfer to study the transcriptional regulation oPL. Although unsuccessful in attaining an answer to what portion of promoter sequence is necessary for BNC specific expression of oPL, several things were learned about this model of research. First the ability of different cell lines to generate a successful pregnancy is widely variable. The choice of cell line may be an important one, as it appears cell line and even clonal cell lines derived from the same fetus can impact blastocyst development (Kuhholzer et al., 2001). The day 47 cells were very stable in culture versus earlier gestational age fetal cell lines. Fetal cell lines from ~day 20 to 30 of gestation, were easily isolated, however the cells would differentiate and arrest their growth. Furthermore, the day 47 cell would remain a constant size during culture. As several of the older fetal cell lines (60 to 90 dGA) were cultured, they appeared to increase in size. The size of cells for nuclear transfer may not have an impact on success; however, the speed at which the smaller cells could be transferred to enucleated oocytes was higher for the smaller (47 dGA) cells. This gives a distinct advantage to the number of clones that could be generated for a given batch of recipient ewes.

Several clutches of oocytes were used to compare this cell line to others, and while the number of blastocysts derived through *in vitro* culture was not high (15.2%), the day 47 cells were more consistent in the rate of blastocyst formation. Of the pregnancies generated by this method of transgenic nuclear cloning prior to, and during this research, the day 47 cell line was the only cell

line to produce multiple healthy fetuses (multiple twins and one triplet) in a single pregnancy.

The reason for our inability to detect our EGFP reporter constructs is somewhat obscure. Several things could explain the lack of detectable expression of EGFP. First, the time of fixation for the tissue with paraformaldehyde may have blocked our ability to detect the EGFP. The tissue fixation overnight was similar to previous studies (Farin et al., 1989; Farin et al., 1990), where interferon-τ expression was detected in conceptus tissues. Furthermore, the same *in situ* hybridization protocol has been used successfully in this lab (Kappes et al., 1992). Therefore we can potentially rule out fixation as the problem in detection. Secondly, detection by immunohistochemistry, may not have been sensitive enough to localize EGFP. Even though the Vectastain Elite kit uses an avidin-biotin system to amplify the antigen signal, we were attempting to detect oPL promoter driven EGFP prior to maximal expression of oPL within the placenta (Handwerger et al., 1975; Handwerger et al., 1977; Kappes et al., 1992; Rueda et al., 1995). Consequently, there may not have been sufficient translated EGFP for detection.

Thirdly, the use of transgenic nuclear transfer as a method to generate tissue may have doomed our ability to generate appropriate tissue for detection of EGFP. Evidence in bovine clones suggests that our lack of successful pregnancies can be attributed to the limited ability of clones to establish pregnancy early in gestation (Hill et al., 2000). Hill et al reported that placental development of these clones was abnormal, and this contributed to loss of the

pregnancy (Hill et al., 2000). Placental insufficiency has also been implicated in loss of ovine nuclear transfer clones as well (De Sousa et al., 2001). If the placenta is not developing correctly in pregnancies resulting from nuclear transfer, we could have difficulty in detecting the EGFP reporter. Furthermore, it has been shown that clones generated from the same cell line are more variable in fetal and placental growth and development than half-siblings generated by artificial insemination (Lee et al., 2004). Most damaging to our methodology choice is the recent evidence that transcriptional regulation of particular genes including PL is altered in cloned pregnancies versus controls (Ravelich et al., 2004b; Ravelich et al., 2004a). Although we tried multiple routes to detect EGFP in the transgenic tissue, our choice to use nuclear cloning to generate the tissue may have predisposed us to failure.

Due to the lack of tangible results with the transgenic nuclear cloning project, we explored the use of viral mediated infection as a possible method to answer our questions about the oPL promoter. The culture of bovine trophoblast has been reported before (Shimada et al., 2001), and this cell line will spontaneously form BNCs that are positive for bPL production (Nakano et al., 2001; Nakano et al., 2002). Isolation and enrichment of ovine BNCs has been performed previously in this lab (Liang et al., 1999; Limesand et al., 2004; Limesand and Anthony, 2001), and using a similar protocol, we show here that BNC can be maintained in culture for successful viral infection (Figure 4.1). Several fractions of cells removed from the percoll gradient, contained BNCs; however, enriched fractions can be obtained for cell culture. Although successful

with infection by adenovirus and lentivirus, we did not optimize the conditions of either method. Further optimization may lead to higher infection efficiency and better expression of a reporter construct. Regardless, we have shown that both adenovirus and lentivirus can be used to infect placental cells, particularly BNC. This provides a method to further our pursuit of the transcriptional regulation of oPL and elucidate the promoter sequence necessary for cell specific production of oPL.

SUMMARY

While several proteins have been implicated in transcriptional regulation of PL in *in vitro* studies, little is known about the transcriptional regulation of oPL *in vivo*. The purpose of this research was to determine the promoter sequence necessary to give cell specific expression of oPL in BNCs. Ovine PL promoter deletion constructs (proximal 383-bp, 1616-bp and 4498-bp) were made to drive EGFP expression, and stably transfected into ovine fetal fibroblasts for nuclear cloning. Cloned embryos were transferred to synchronized recipient ewes, and successful pregnancy was determined by absence of estrus.

A 47 dGA cell line was chosen for nuclear cloning for advantages in cell culture stability before nuclear transfer and for appropriate growth, quality, and number of blastocysts derived from this cell line. Lines derived from earlier aged fetuses tended to differentiate and eventually arrest proliferation, therefore prohibiting their continued use. Cell lines from later gestational ages were stable in culture; however, the growth rate of embryos from the 60 dGA cell line was accelerated and the embryos would arrest at the morula stage. Furthermore, a

90 dGA cell line could generate blastocysts at a rate of ~10%, but the blastocysts were of poor quality and degenerative. While not statistically tested, these data indicate that choice of cell line for nuclear cloning is important to the success of embryo development.

Transgenic tissues were evaluated under fluorescence microscopy, where it was discovered that auto-fluorescence within placental tissue was going to make specific EGFP expression difficult to determine. Unsuccessful attempts were made to detect EGFP by immunohistochemistry and *in situ* hybridization; however, a positive control could be detected. The CMV promoter should yield expression of neomycin (positive control) within all cell types to compare against expected cell specific expression of the oPL promoter driven EGFP. Hybridization of the positive control was variable within and between the tested promoter constructs. The exact reason for the inability to detect EGFP is and the erratic detection of neomycin is unknown. Our fixation and immunohistochemistry and *in situ* hybridization techniques have been used successfully in previous research; therefore, we believe there was most likely a problem with the tissue. It has been reported that pregnancies derived from nuclear cloning have altered gene transcription (including oPL) and altered placental and fetal development.

Even if we had been able to detect transgenic EGFP expression, potentially altered regulation of oPL promoter may have lead to false conclusions about transcriptional regulation of oPL. With the lack of success in analyzing oPL transcriptional regulation by using transgenic nuclear cloning, we focused on

culture of placental cells *in vitro* and subsequent infection of the cells with adenovirus and lentivirus that carried CMV promoter driven GFP cassettes. Infection of multiple cell types including BNCs was successful with both adenovirus and lentivirus. Though we could not generate results with transgenic nuclear cloning to satisfy our initial goal, we have demonstrated that adenoviral and lentiviral mediated infection may be potential routes to explore the transcriptional regulation of oPL within BNCs.

CHAPTER V

EFFECTS OF EXOGENOUS GROWTH HORMONE ON PLACENTAL AND FETAL DEVELOPMENT

INTRODUCTION

In humans, growth hormone (GH) is part of a gene cluster that contains two copies of the GH gene and two copies of the placental lactogen (hPL) gene that produce functional proteins and one copy of hPL that is a pseudogene (Barsh et al., 1983; DeNoto et al., 1981; Hirt et al., 1987; Seeburg, 1982). The GH/PL gene cluster is located in a 66,500-bp region of chromosome 17 (Chen et al., 1989; George et al., 1981). All five genes are arranged sequentially (hGH-N, hPL-L, hPL-A, hGH-V and hPL-B in order) in the gene cluster, share a high sequence identity (91-99%), and are presumably the result of tandem duplications of the same gene (Barsh et al., 1983; Chen et al., 1989; Hirt et al., 1987), as speculated by others (Niall et al., 1971; Seeburg, 1982). Sheep also have a duplicate copy of the GH gene (Valinsky et al., 1990). While there are two genes for ovine growth hormone (oGH-1 and oGH-2), only oGH-2 has multiple alleles (oGH-2N and oGH-2Z (Gootwine et al., 1993; Valinsky et al., 1990). The sequence of the oGH-1 transcript was originally report by Orian et al. (Orian et al., 1988). The transcripts of the oGH-2N and -2Z alleles are more similar to the ovine GH sequence reported by Byrne et al. (Byrne et al., 1987).

The oGH-2 genes encode proteins similar to pituitary derived oGH-1 where only three amino acids are switched in the Z allele versus the N allele (Lacroix et al., 1996). Cleavage of a 26 amino acid secretion signal leaves a 191 amino acid mature peptide with a molecular weight of 22,000 (Byrne et al., 1987; Lacroix et al., 1996; Orian et al., 1988).

The expression of oGH-2 has been detected in the sheep placenta (Lacroix et al., 1996; Lacroix et al., 1999) and it has been inferred that both the N and Z alleles of the gene are expressed (Ofir and Gootwine, 1997). However, a distinct localization of oGH expression in the placenta is still lacking. The expression of oGH in the placenta is detected from approximately day 35 to 55 of gestation (Lacroix et al., 1996; Lacroix et al., 1999). Lacroix et al reported immunodetection of GH in maternal syncytial and stromal cells and the fetal trophoblast in day 40 to 45 placentomes (Lacroix et al., 1996). More recently, this group reported *in situ* hybridization of oGH in only the syncytium and trophoblast (Lacroix et al., 1999). Although both reports from the Lacroix group agree about detection of oGH within the placenta, the conclusions drawn about the localization of this expression are by no means definite. Furthermore, ongoing research in this lab has shown that oGH mRNA concentration in the caruncle is considerably higher than that seen in the cotyledon (Regnault and Anthony, unpublished data). Recently, it was found that placental oGH expression was decreased in a model of placental insufficiency resulting in placental and fetal growth restriction. This evidence lead us to hypothesize oGH may play a role in placental growth and development. Further research to

determine the true cellular origin of oGH in the placenta is needed in order to understand the role of oGH in the placenta.

Studies in sheep to elucidate the biological actions of placental growth hormone have produced little understanding of the role of growth hormone within the placenta. Administration of GH during elongation and early placentation has no effect on placental development (Spencer et al., 1999b). Only endometrial gland proliferation was stimulated and this effect was seen with concurrent interferon-τ administration (Spencer et al., 1999b). Wallace et al. reported an increase in placental weight due to GH infusion from 35-80 days of gestational age (dGA); however, this may have been due to increased fluid weight resulting from polyhydraminios instead of exogenous GH (Wallace et al., 2004). Additionally, the increase in placental weight reported by Wallace et al. was only seen in adolescent ewes maintained on a high dietary intake (Wallace et al., 2004). This feeding regimen for adolescent ewes is a model of growth restriction (Anthony et al., 2003; Wallace et al., 1997). Interestingly, in these overnourished adolescent ewes, serum GH is decreased, while serum IGF-I is increased (Wallace et al., 1997). This indicates a disconnection of the normal endocrine relationship of GH and IGF-I.

Administration of GH during mid-pregnancy (70-83 dGA) results in increased maternal serum non-esterified fatty acids, IGF-I and glucose, but no change in fetal growth (Jenkinson et al., 1999). Maternal infusion of GH at 98-111 days of gestation can increase fetal weight by 10% (Jenkinson et al., 1999); however, several reports saw no effect on fetal or placental weight with similar

temporal administration of GH (Currie et al., 1996; Harding et al., 1997; Stelwagen et al., 1994). Interestingly, all of the studies discussed above do not administer exogenous GH concurrent to growth hormone expression (~35 to 55 days of gestational age; dGA) within the placenta (Lacroix et al., 1996; Lacroix et al., 1999). It has been shown that administration of recombinant porcine GH (rpGH) to gilts during early to mid-gestation will increase fetal and placental weight (Sterle et al., 1995). Increases in fetal weight and length have also been reported for underfed gilts given rpGH during mid gestation (Gatford et al., 2000). It remains to be seen what the effects of GH administration will have in sheep, particularly if the GH administration is concurrent with the normal expression window of GH within the placenta. The purpose of the following studies was to further define location of oGH expression in the placenta and to determine the effects of exogenous recombinant bovine GH administration during normal window of placental oGH expression.

MATERIALS AND METHODS

cDNA Generation

In order to verify the origin of ovine growth hormone (oGH) expression within the placenta of sheep, placentomes were collected from day 45 post coitus sheep. Intact placentomes were excised, trimmed to 5 mm cross-sections, and fixed overnight in 4% paraformaldehyde. The fixed tissue was embedded in paraffin for sectioning. Complimentary DNA for ovine GH, PL, vimentin (marker of fibroblast cell lineage), cytokeratin 18 (marker of epithelial cell lineage), CD 18 protein (marker of leukocytes), and Pit-1 transcription factor were all obtained for

this analysis. See Table 5.1 for a summary of the primers, annealing temperatures and product size for the cDNA used for *in situ* hybridization analysis. The oGH cDNA used in hybridization was previously constructed (Orbus, 1999). The cDNA for cytokeratin 18 (1307-bp) was isolated from an ovine placental cDNA library previously (Limesand and Anthony, 2001). For oPit-I, oCD 18, and oVimentin cDNA, tcRNA from ovine pituitary (oPit-I), white blood cells (oCD 18), and caruncle (oVimentin) were used for reverse transcription.

Table 5.1: PCR primers, annealing temperatures and product sizes for *in situ* hybridization cDNAs.

cDNA	Forward Primer	Reverse Primer	Anneal	Product
oGH	gcccagcagaaatcagac	gggaggggtaacagcaga	59°C	406-bp
oVimentin	ctctccttccggagccagt	gttggtgcgggtggtcttg	64°C	369-bp
oCD 18	tggcagaaagcaacat	tcgcactcgcagaact	57°C	679-bp
oPit-I	tcattacggaaaccagtcatc	ttacaaaaccaaaccctcacc	59°C	618-bp

For the reverse transcriptase reaction, the SuperScript First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) was used following the manufacturer's protocol. For polymerase chain reaction (PCR), the reaction mix was 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 μM primer (forward and reverse) and 2 U of *Taq* Polymerase (Invitrogen, Carlsbad, CA, USA). The PCR products were generated by 40 cycles of 95°C 1

min to denature; 1 min to anneal; 72°C 1 min for extension. The PCR products for oVimentin, oCD 18, and oPit-I were cloned into PCR-Script Amp SK (Stratagene, Cedar Creek, TX, USA) for transformation, sequence validation, and subsequent propagation. The plasmid was harvested by alkaline lysis, and purification by centrifugation through a CsCl gradient (Liang et al., 1999). The plasmid preparations were sequenced by Macromolecular Resources (Colorado State University).

In situ Hybridization

To generate template for *in situ* hybridization, oGH (*Eco*RI), oPL (*Bam*HI and *Nde*I), oVimentin (*Eco*RI and *Not*I), oCytokeratin 18 (*Xho*I), oCD 18 (*Eco*RI and *Sac*I) and oPit-I (*Eco*RV and *Not*I) were digested from their respective plasmids and gel purified on 1% agarose gels. For a negative control, ~400-bp of non-coding sequence from PCR-Script Amp was digested out of intact plasmid with *Nsp*I and *Sac*II. Radiolabeling of cDNA for *in situ* hybridization was performed using 50-100ng of template in the DECAprime II random prime labeling kit (Ambion, Austin, TX, USA) and using radiolabeled cytosine tri-phosphate ($^{33}P\alpha$-dCTP) per manufacturer's instructions. Only probes with specific activities exceeding $5x10^8$ CPM/μg of DNA were used for *in situ* analysis. *In situ* hybridization was performed as described previously (Kappes et al., 1992).

Exogenous GH Administration

Thirty-two time-mated whiteface crossbred ewes were gestated to 35 dGA. At 35 dGA the ewes were randomly assigned to treatment groups for

subcutaneous injections of bGH (GH, n=16) or buffer control (CON, n=16). The GH group received 0.2 mg/kg of body weight of recombinant bovine growth hormone (rbGH; Monsanto, St. Louis, MO, USA) per day reconstituted in 100 mM NH_4HCO_3 buffer. The rbGH was given by two injections of 3 ml 12 hr apart. The CON group received two injections of 3 ml of 100 mM NH_4HCO_3 buffer. The injection regimen lasted for 20 days (35 to 55 dGA) to coincide with the normal expression window of oGH within the placenta (Lacroix et al., 1996; Lacroix et al., 1999). Prior to, and every 5 days during rbGH administration, blood samples were collected by jugular venipuncture to compare serum hormone concentrations of CON and GH groups.

Serum Analyses

A validated GH radioimmunoassay was used to determine serum GH levels (Hoefler and Hallford, 1987) for duplicate samples. Using NIDDK anti-oGH and NIDDK oGH, the intraassay coefficient of variation was 7.2% and recovery was 97% for 15 ng. A two-site immunoradiometric assay (IRMA; Diagnostic Systems Laboratories; Webster, TX, USA) was used to assay serum insulin-like growth factor I (IGF-I) as directed by the manufacturer. Intra-assay and interassay coefficients of variation were 3.43 and 3.39% for the 50 ng/ml control respectively and 2.29 and 2.27% for the 200 ng/ml control. To assay the serum progesterone concentrations, a progesterone enzyme immunoassay (EIA; Diagnostic Systems Laboratories) was used on extracted serum samples. For extraction, 500 μl of serum was diluted 10 fold with petroleum ether (Mallinckrodt Baker, Paris, KY, USA) and vortexed for 1 minute. The samples were frozen in

dry ice/methanol and the supernatant was collected. The samples were thawed and the extraction was repeated and the supernatant combined with the first extraction. The extracted samples were dried under nitrogen and then reconstituted with 500 μl of 1 x PBS with 1% BSA by incubation overnight at 4°C. The reconstituted samples were then assayed with the EIA in duplicate per manufacturer's recommendations. Using non-linear regression (Sigmaplot; SPSS Inc., Chicago, IL, USA), minimum (min), maximum (max), EC50, and Hillslope values were obtained. From these values, the concentrations of progesterone were predicted using the equation f=min+(max-min)/(1+(x/EC50)^Hillslope) where f is absorbance and x is ng/ml of progesterone. The intraassay and interassay coefficient of variation were 3.39 and 5.16% respectively for the 1 ng/ml control and 4.28 and 4.27% for the 7.5 ng/ml control.

Tissue Collection

Between days 54-56 of gestation, 8 CON and 8 GH ewes were euthanized (20mg/kg sodium pentobarbitol) and uterine, placental and fetal tissues were collected for analysis. Ewe weight, gravid uterine weight, placental weight, and fetal parameters (sex, weight, crown rump length, and liver weight) were all recorded. Using crown rump length (CRL) and fetal weight, the ponderal index (PI) for each fetus was calculated (PI=weight in grams/length in cm^3). Cotyledon and caruncle tissue from each placenta were manually separated and snap frozen in liquid nitrogen for later RNA analysis. The remaining ewes were gestated until 135 dGA to evaluate effects of rbGH administration near term.

Ewes were euthanized between 133 and 138 dGA with sodium pentobarbitol (20 mg/kg; Sigma, St. Louis, MO, USA) and the same data and tissue samples were collected as the 55 dGA groups. Additionally, empty uterine weight was measured for use in calculating fluid weight within the uterus. To calculate fluid weight, fetal, placental and empty uterine weights were subtracted from gravid uterine weight.

Total Cellular RNA Collection

Frozen caruncle and cotyledon tissues were crushed with mortar and pestle under liquid nitrogen to a fine powder. Total cellular RNA (tcRNA) was isolated as described before (Kappes et al., 1992; Warren et al., 1990b) An aliquot of the stock tcRNA samples was diluted to a concentration of 1 μg/μl ± 0.2 μg/μl for use as a working solution. The concentration of the working solutions was verified using the Ribogreen (Molecular Probes, Eugene, OR, USA) RNA quantification system. Measurements of fluorescence were taken using a Mithras LB 940 (Bertold Technologies, Oak Ridge, TN, USA). The samples were excited at 485 nm and emission was measured at 535 nm. The counting time was 0.1 seconds and the lamp energy was set at 10,000. The unknowns were compared against an RNA standard curve.

Reverse Transcriptase Real-time Polymerase Chain Reaction

For the reverse transcriptase reaction, 2 μg of tcRNA was used in the SuperScript First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). All samples of tcRNA for both caruncle and cotyledon were incubated at the same time with the same kit to avoid sample variation due to technician and/or kit

variation. The yield of cDNA from the RT reactions was quantified with the Oligreen (Molecular Probes, Eugene, OR, USA) ssDNA quantification kit. Samples were excited and measured with the Mithras LB 940 with the same settings as for the Ribogreen analysis. Following quantification, all samples were set to a cDNA concentration of 12.5 ng/μl for use in real-time polymerase chain reaction (PCR). The real-time PCR reaction mix was 12.5 μl of iQ SYBR Green Supermix (BIO-RAD), 200 nM primer (forward and reverse), 2μl of RT product (25 ng), and balanced to 25 μl total volume with sterile water with a neutral pH. For all real-time PCR reactions the first denaturing step was 95°C for 5 min and 45 seconds thereafter, the annealing time was 1 min with gene specific temperature (Table 5.2), and 1 minute of extension at 72°C. The reactions were run for 40 cycles, with a fluorescence measurement after each cycle, followed by a melting curve analysis initiated at 55°C and measuring fluorescence every 0.5°C to 95°C. For the real-time PCR analysis, cDNA for ovine IGF-I, IGF-II, and IGF binding proteins (BP) 1, 2, 3, and 4 and ribosomal protein S 15 (RPS15) were used as standard templates. The standard curve template concentrations spanned 100 pg/μl to 1 $x10^{-5}$ pg/μl at 10-fold increments. For each standard, 1μl of template was supplied to the reaction mix with the same primer concentration. Table 5.2 lists the primer sequences, annealing temperatures and product sizes generated by PCR. Primers were designed to span at least one intron of the mRNA sequence for each gene of interest and produce 130 to 350-bp of product. For each gene of interest the real-time PCR reactions were validated by electrophoresis of the PCR products to verify correct product size prior to

analysis. The standard reactions were run in duplicate and the unknown reactions were run in triplicate.

Table 5.2: PCR primers, annealing temperatures and product sizes for cDNA used in real-time PCR analysis.

cDNA	Forward Primer	Reverse Primer	Anneal	Product
IGF-I	tcgcatctctcttctatctggccctgt	acagtacatctccagcctcctcaga	62°C	238-bp
IGF-II	gaccgcggcttctacttcag	aagaacttgcccacggggtat	62°C	202-bp
IGF BP-1	tgatgaccgagtccagtgag	gtccagcgaagtctcacac	62°C	248-bp
IGF BP-2	caatggcgaggagcactctg	tggggatgtgtagggaatag	55°C	330-bp
IGF BP-3	ctcagagcacagacaccca	ggcatatttgagctccac	54°C	335-bp
IGF BP-4	tgtgcgtgtgtgttgatg	gagggagccaagatgagt	62°C	229-bp
RPS15	atcattctgcccgagatggtg	tgctttacgggcttgtaggtg	60°C	134-bp

Northern Hybridization Analysis

Radiolabeling of cDNA for was performed using 50-100 ng of template in the DECAprime II random prime labeling kit (Ambion, Austin, TX, USA) and using $^{32}P\alpha$-dCTP per manufacturer's instructions. Only cDNAs with specific activities exceeding $5x10^8$ CPM/μg of DNA were used for analysis. Caruncle tcRNA was hybridized with oGH cDNA, while cotyledon tissue was hybridized with oPL cDNA as described previously (Kappes et al., 1992). Following hybridization, the membranes were washed with 1 x SSC and 0.1% SDS for 15 min at room

temperature. Further washes in 0.2 x SSC and 0.1% SDS for 30 min at 42°C, 30 min at 60°C, and 30 min at 60°C to remove non-specific binding of the radiolabel. The membranes were exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA) overnight for oPL and 48 hours for oGH. The screens were scanned using a Storm Imager (Molecular Dynamics) to visualize hybridization. The membranes were then stripped with repeated washes of 200 ml of boiling stripping buffer (0.05 x SSC, 10mM EDTA and 0.1% SDS). Membranes were submerged and agitated for 15 min, then fresh stripping buffer was applied and agitation continued. The stripping continued until membranes were at background radiation levels as determined by handheld Geiger counter. The membranes were then blocked again with pre-hybridization buffer and hybridized with RPS 15 cDNA ($2x10^6$ CPM/ml) to serve as a normalization control. Following hybridization the membranes were exposed to a Phosphor screen overnight and scanned with the Storm Imager. Densitometric analysis of the Storm images for all northern hybridizations was completed with ImageQuant 5.0 (Molecular Dynamics). Densitometric values for oPL and oGH were divided by the corresponding RPS15 densitometric values for each sample.

Statistical Analysis

For the serum hormone analysis, means (CON and GH) for each sample day were separated by student's t-test. For northern and real-time PCR analyses normalized densitometric means for the CON and GH groups were separated by student's t-test. For all analyses, statistical significance was accepted at $P \leq 0.05$.

RESULTS

In order to localize the expression of oGH within the placenta, day 45 placentomes were embedded and sectioned at 6 μm. Using *in situ* hybridization, the sections were hybridized with cDNAs for oGH, oPL, oCytokeratin 18, and oVimentin. Analysis of tissue sections revealed a distinct population of cells at the maternal-fetal interface with positive hybridization of oGH. Figure 5.1 illustrates the hybridization of oGH in the placentomes sections. Positive hybridization of oPL was seen in BNCs within the fetal trophoblast. Figure 5.2 shows the light and dark phase images of oPL hybridization within the fetal trophoblast. The positive oGH expressing cells are not part of the maternal stroma or fetal trophoblast, and appear to be the remnants of maternal epithelium. The oGH positive cells were in random clusters at outer boundaries of maternal tissue.

To learn what cell type is expressing oGH, oCytokeratin 18 and oVimentin radiolabeled cDNAs were hybridized to day 45 placentome sections. Positive hybridization was seen in maternal and fetal stroma with oVimentin cDNA, as well as positive hybridization for oCytokeratin 18 in the maternal epithelium. Figures 5.3 and 5.4 represent light and dark phase images of oCytokeratin 18 and oVimentin hybridization. As seen in the oGH hybridization the epithelial derived cells at the maternal-fetal interface were not consistently present throughout the boundary of the maternal tissue (figure 5.3). The hybridization of oVimentin (figure 5.4) was detected throughout the maternal villous stroma, and to some extent in fetal villous stroma.

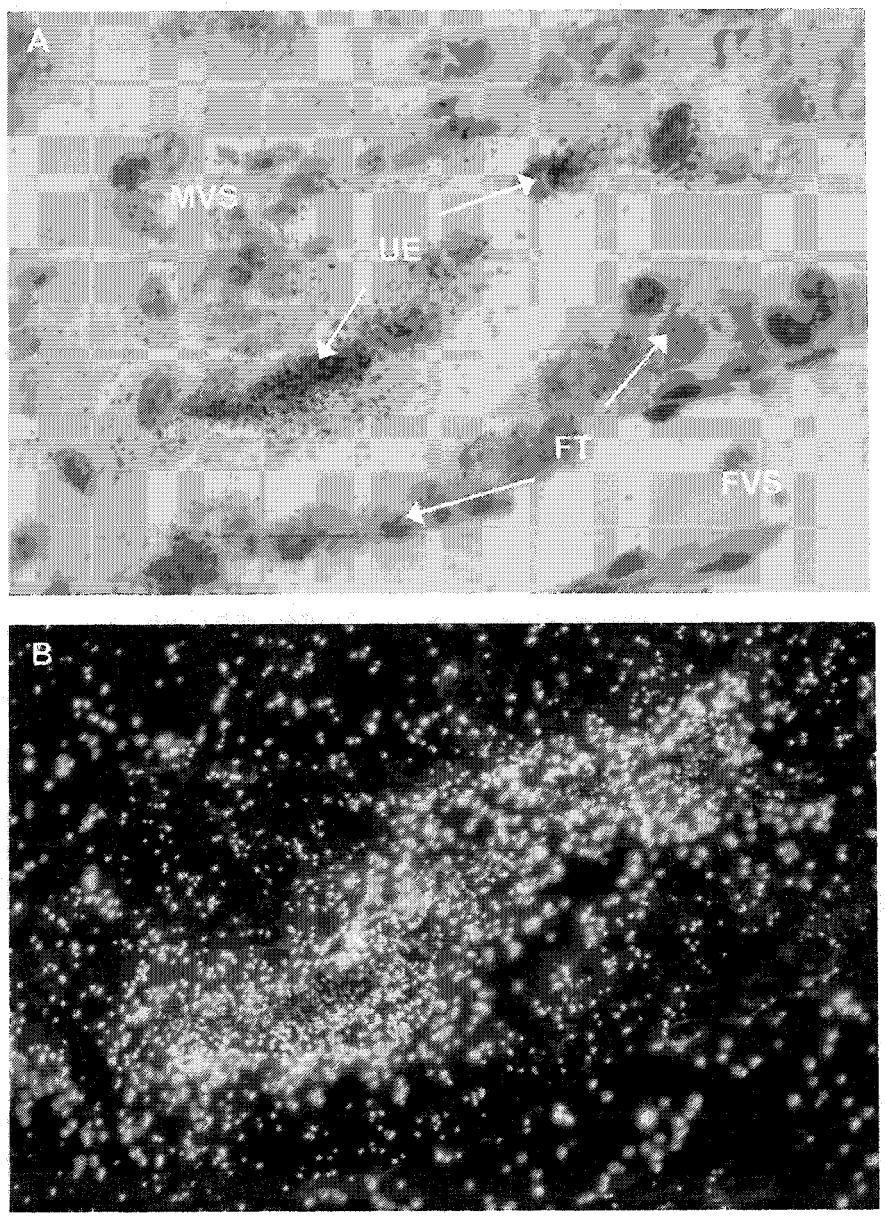

Figure 5.1: *In situ* hybridization with oGH cDNA. Panel A is a light field image of day 45 placentome. Panel B is the dark field of the same location. Positive hybridization is detected in UE. FT = fetal trophoblast; FVS = fetal villous stroma; MVS = maternal villous stroma; UE = uterine epithelium; Final magnification = 400x

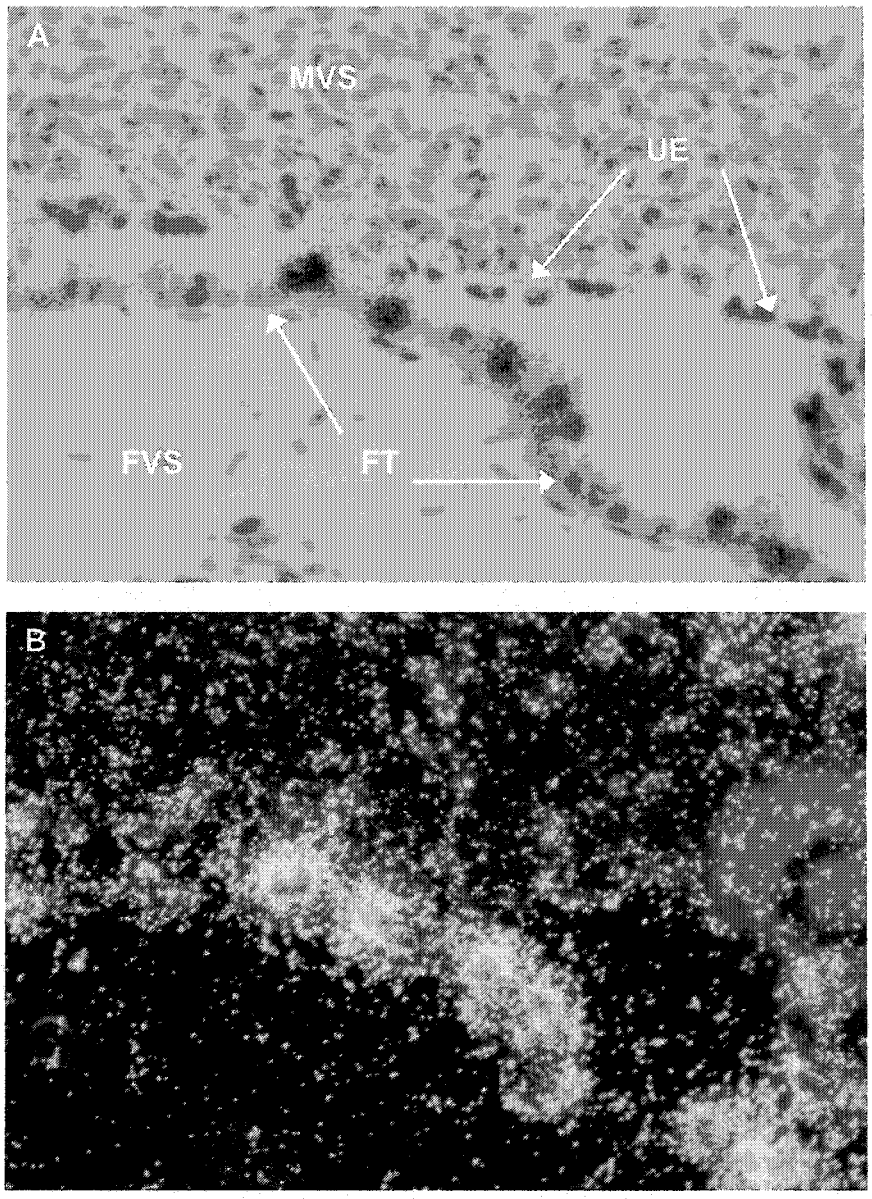

Figure 5.2: *In situ* hybridization with oPL cDNA. Panel A is a light field image of day 45 placentome. Panel B is the dark field of the same location. Positive hybridization detected in FT binucleate cells. FT = fetal trophoblast; FVS = fetal villous stroma; MVS = maternal villous stroma; UE = uterine epithelium; Final magnification = 200x

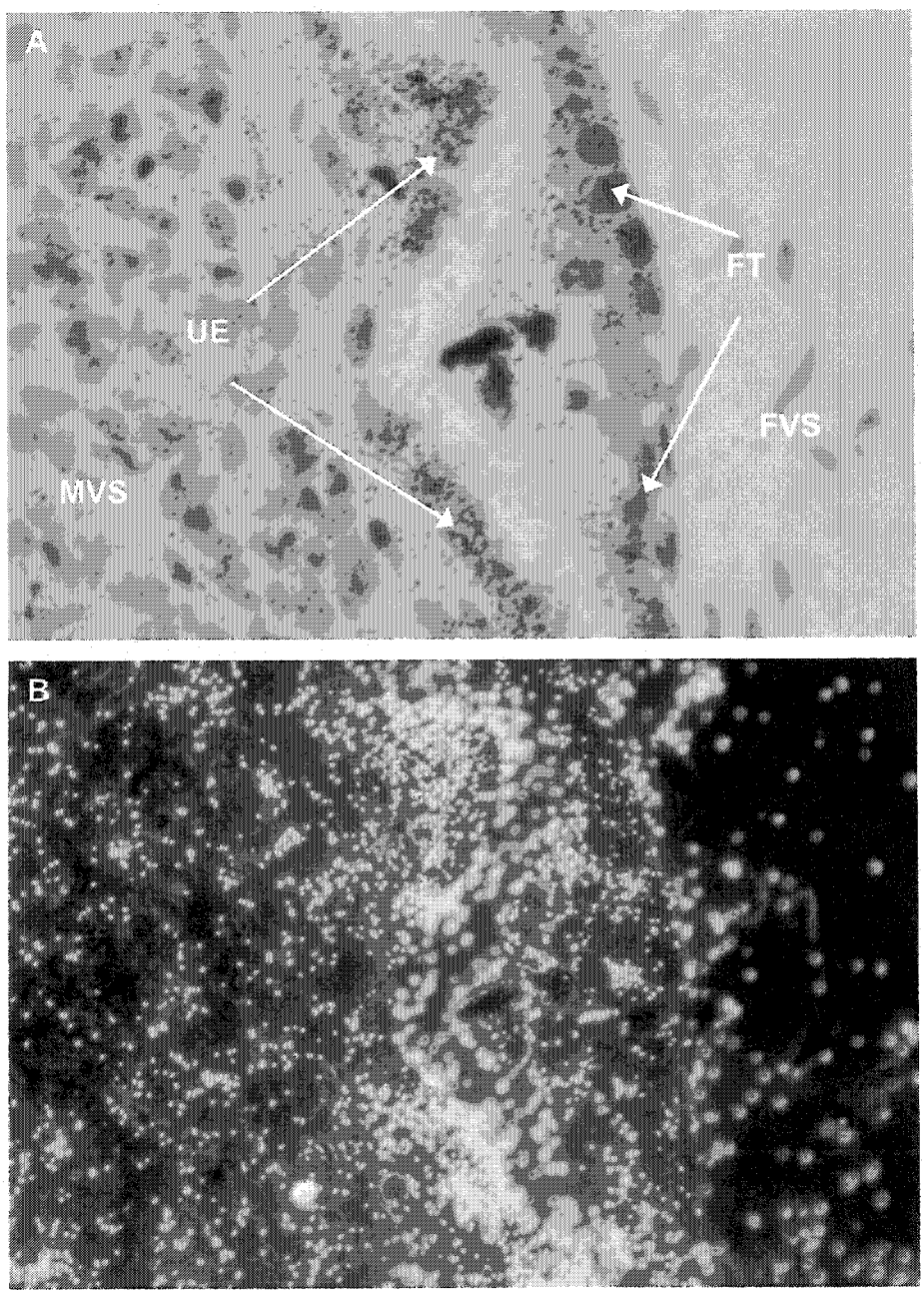

Figure 5.3: *In situ* hybridization with cytokeratin 18 cDNA. Panel A is a light field image of day 45 placentome. Panel B is the dark field of the same location. Positive hybridization is detected in UE. FT = fetal trophoblast; FVS = fetal villous stroma; MVS = maternal villous stroma; UE = uterine epithelium; Final magnification = 400x

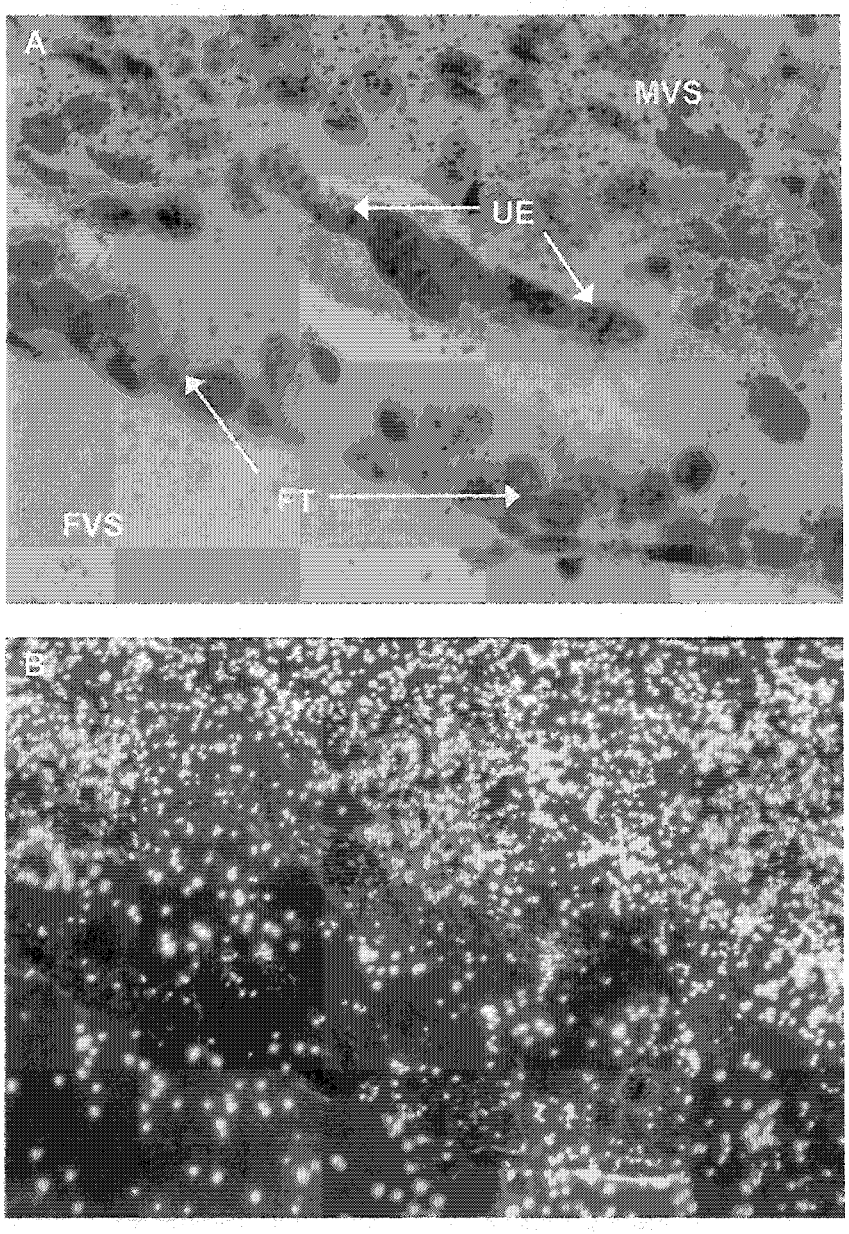

Figure 5.4: *In situ* hybridization with vimentin cDNA. Panel A is a light field image of day 45 placentome. Panel B is the dark field of the same location. Positive vimentin hybridization is detected in MVS. FT = fetal trophoblast; FVS = fetal villous stroma; MVS = maternal villous stroma; UE = uterine epithelium; Final magnification = 400x

Hybridization with an oPit-I cDNA revealed no cells present in the placentomes that were expressing Pit-I (data not shown). Additionally, hybridization of the day 45 placentomes with CD 18 radiolabeled cDNA, detected cells of leukocyte origin (data not shown). The CD 18 positive cells were deep in the endometrium and myometrium, far from the oGH positive cells within the maternal epithelium.

To determine the role of oGH in the placenta, exogenous rbGH was given to pregnant ewes from day 35 to 55 of gestation. The effects of GH were assessed at day 55 and day 135 of gestation. At necropsy, 12 of 16 ewes in the 55 dGA group and 11 of 16 ewes in the 135 dGA group were found to have twin pregnancies. In order to minimize the confounding effects of fetal number, the data presented are means from only the twin pregnancies. Our intent was to increase circulating GH concentrations ten-fold higher than normal. Figure 5.5 shows the results of serum GH concentrations in CON and GH ewes. The treated (GH) ewes had similar serum GH concentrations as controls at day 0 of the treatment period. The GH ewes had significantly ($P < 0.001$) higher concentrations of serum GH than CON ewes by day 5 of treatment and remained approximately 10 fold higher than the controls throughout the treatment period. The exogenous rbGH was biologically active in sheep as the treated ewes showed significant ($P < 0.001$) increases in serum IGF-I concentrations, as evidenced in Figure 5.6.

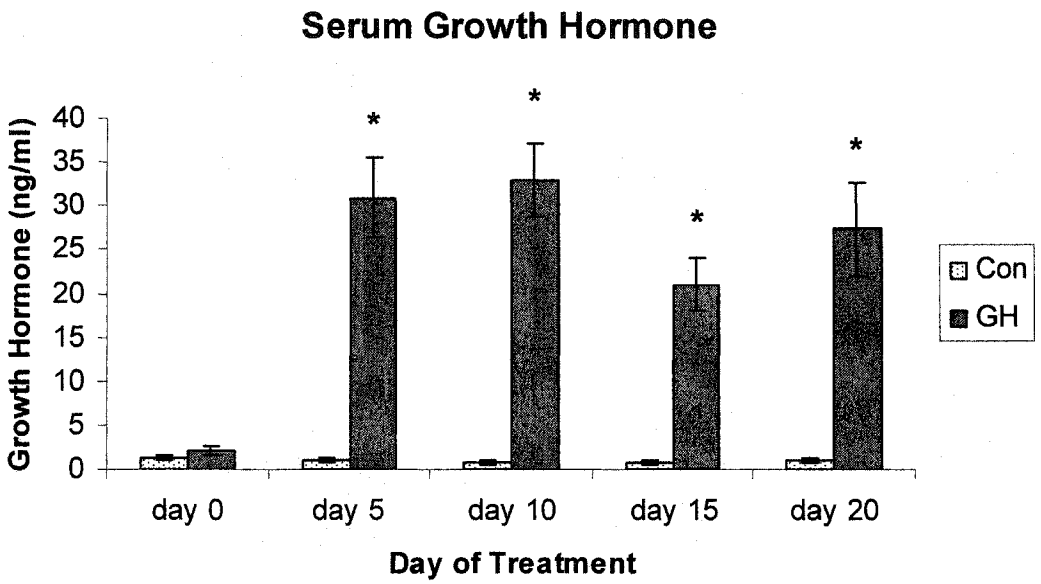

Figure 5.5: Serum growth hormone (GH) concentrations. Concentrations for control (Con) and GH treated (GH) ewes throughout the gestation treatment period (day 35-55) are presented as mean ± SEM with significant differences marked with an asterisk.

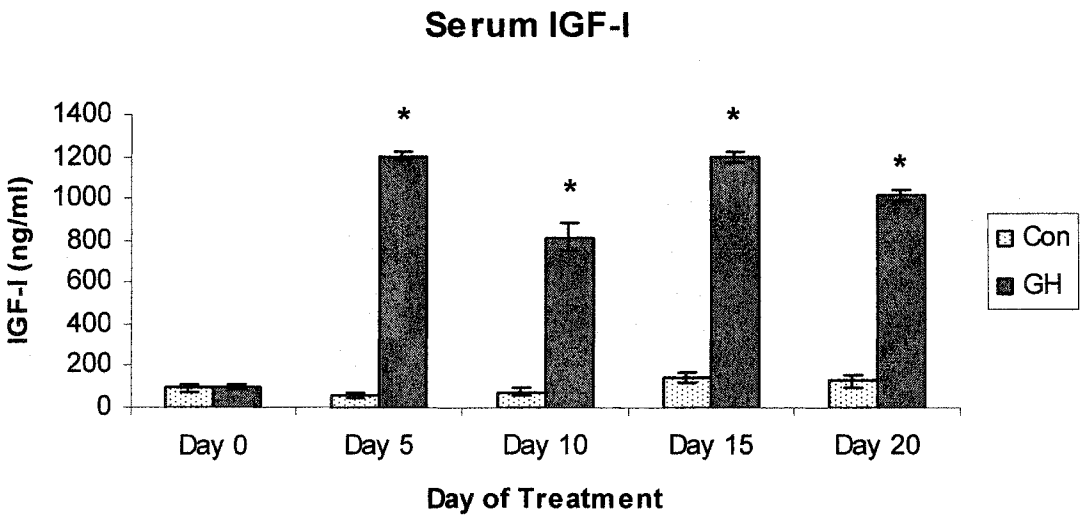

Figure 5.6: Serum IGF-I concentrations. Concentrations for control (Con) and GH treated (GH) ewes throughout the gestation treatment period (day 35-55) are presented as mean ± SEM with significant differences marked with an asterisk.

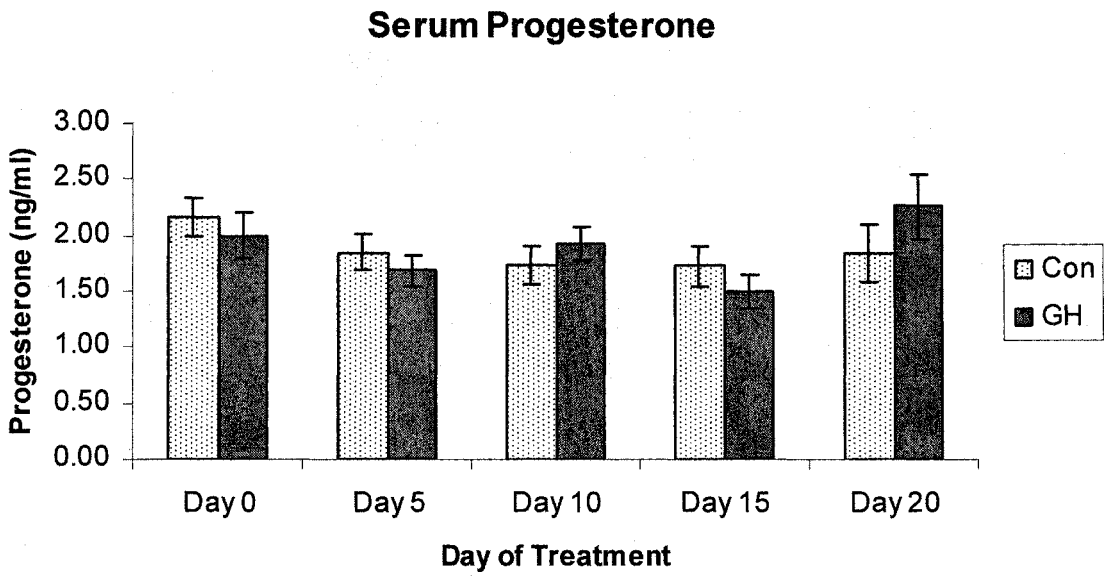

Figure 5.7: Serum progesterone concentrations. Concentrations for control (Con) and GH treated (GH) ewes throughout the gestation treatment period (day 35-55) are presented as mean ± SEM. There were no differences among Con and GH groups.

After placentation, the placenta becomes a major source of progesterone, which is released into the maternal blood stream. To indirectly assess the function of the placenta during the treatment period, serum progesterone concentrations were also determined. Figure 5.7 illustrates the mean progesterone concentrations of GH and CON ewes. No significant difference was detected between GH and CON ewes.

The physical data, for the ewes, fetuses and placenta collected at necropsy are summarized in tables 5.3 and 5.4. For the 55 dGA groups no differences were seen in uterine weight, placental weight, fetal weight, crown rump length (CRL), ponderal index (PI), liver weight or liver weight expressed by

kg of body weight (BW) between GH and CON groups. Similar results were obtained in the near term (135 dGA) ewes. No significant differences were seen between uterine, placental, calculated fluid, and fetal weights. Additionally, no significant differences were found in CRL, PI, liver weight, or liver weight/kg BW between treatments in the 135 dGA groups. There was no effect of fetal sex on the fetal parameters measured at day 55 and 135.

Table 5.3: Day 55 maternal and fetal physical data. Data for control (CON) and GH treated (GH) ewes are presented as mean ± SEM.

Treatment	Control	GH	P value
Days post coitus	55.14 ± 0.26	55 ± 0.32	0.734
Ewe weight (kg)	85.29 ± 2.31	82.82 ± 5.38	0.610
Uterine weight (g)	1773.57 ± 73.72	1934 ± 59.32	0.336
Placental weight (g)	693.26 ± 59.58	709.06 ± 90.75	0.882
Fetal weight (g)	32.23 ± 1.18	32.03 ± 1.28	0.910
Crown rump length (cm)	8.26 ± 0.16	8.48 ± 0.15	0.346
Ponderal index (g/cm^3)	0.057 ± 0.002	0.053 ± 0.001	0.075
Liver weight (g)	2.64 ± 0.15	2.48 ± 0.11	0.426
Liver weight/kg BW	81.62 ± 2.09	77.56 ± 1.98	0.190

To assess the local actions of exogenous GH, representative cotyledon and caruncle tissues were collected from each pregnancy from day 55. Total cellular RNA (tcRNA) was used in Northern hybridization analysis to detect

changes in cotyledon and caruncle expression of oPL and oGH respectively. For Northern analysis, the expression of RPS 15 was used for normalization. The concentration of RPS 15 was used for normalization due to changes by treatment in other housekeeping genes (actin and glyceraldehydes-3-phosphate dehydrogenase). As seen in figure 5.8, there was no effect of treatment on cotyledon expression of oPL. As seen with oPL in cotyledon, no change in caruncle oGH expression was detected for the GH group as compared to CON (Figure 5.9). Additionally, tcRNA was subjected to reverse transcriptase reaction to generate cDNA. Transcriptional changes in the tissues were detected by real-time PCR for IGF-I, IGF-II, IGF binding proteins (BP) -1, -2, -3, and -4. Each measured transcript was normalized by RPS 15 concentration as detected by real-time PCR. Figure 5.10 shows the comparison of relative expression of the selected transcripts of cotyledon tcRNA. The means are presented as gene of interest cycle threshold divided by RPS 15 cycle threshold (Ct/Ct). There was no significant difference in cotyledon expression of IGF-I, IGF-II or IGF BP-3 between treatments. Slight yet significant ($P < 0.05$) differences were detected for only IGF BP-1, -2, and -4. Expression of binding proteins 1 and 4 were increased, while IGF BP-2 was decreased in the GH treated fetal placenta. Figure 5.11 shows the comparison of relative expression of the selected transcripts within the caruncle. No significant differences were detected between treatments for caruncle IGF-I, IGF-II, and IGF BP-2. The expression of caruncular IGF BP-1 was significantly ($P < 0.05$) decreased, while, expression of IGF BP-3 and -4 were both significantly ($P < 0.05$) increased by GH treatment.

Table 5.4: Day 135 maternal and fetal physical data. Data for control (CON) and GH treated (GH) ewes are presented as mean ± SEM.

Treatment	CON	GH	P value
Days post coitus	136.00 ± 0.97	136.20 ± 0.80	0.880
Ewe weight (kg)	91.29 ± 4.78	97.27 ± 5.36	0.425
Uterine weight (kg)	13.80 ± 0.81	14.06 ± 0.66	0.816
Placental weight (kg)	1.45 ± 0.09	1.50 ± 0.11	0.748
Fluid weight (kg)	2.26 ± 0.39	2.21 ± 0.21	0.915
Fetal weight (kg)	4.45 ± 0.17	4.54 ± 0.13	0.655
Crown rump length (cm)	50.36 ± 0.79	51.70 ± 0.82	0.332
Ponderal index (g/cm^3)	0.035 ± 0.001	0.033 ± 0.001	0.325
Liver weight (g)	117.43 ± 6.36	125.20 ± 8.39	0.462
Liver weight/kg BW	26.33 ± 0.77	27.40 ± 1.31	0.473

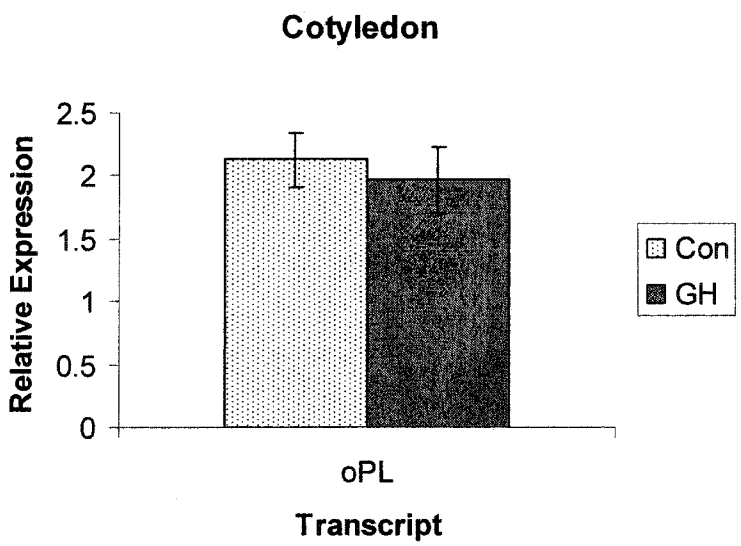

Figure 5.8: Relative expression of oPL in cotyledon. Densitometric values for the samples were normalized by densitometric values for RPS 15. Ten μg of tcRNA was loaded for Northern hybridization.

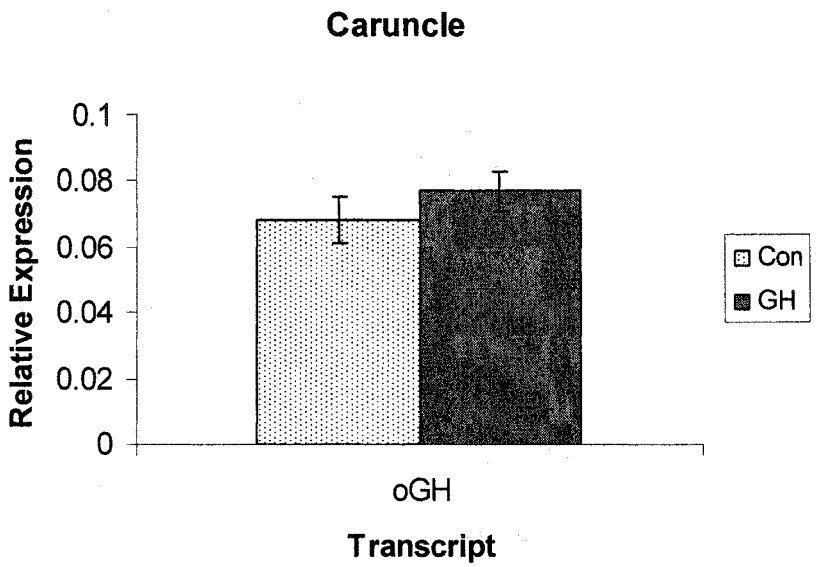

Figure 5.9: Relative expression of oGH in caruncle. Densitometric values for the samples were normalized by densitometric values for RPS 15. Ten μg of tcRNA was loaded for Northern hybridization.

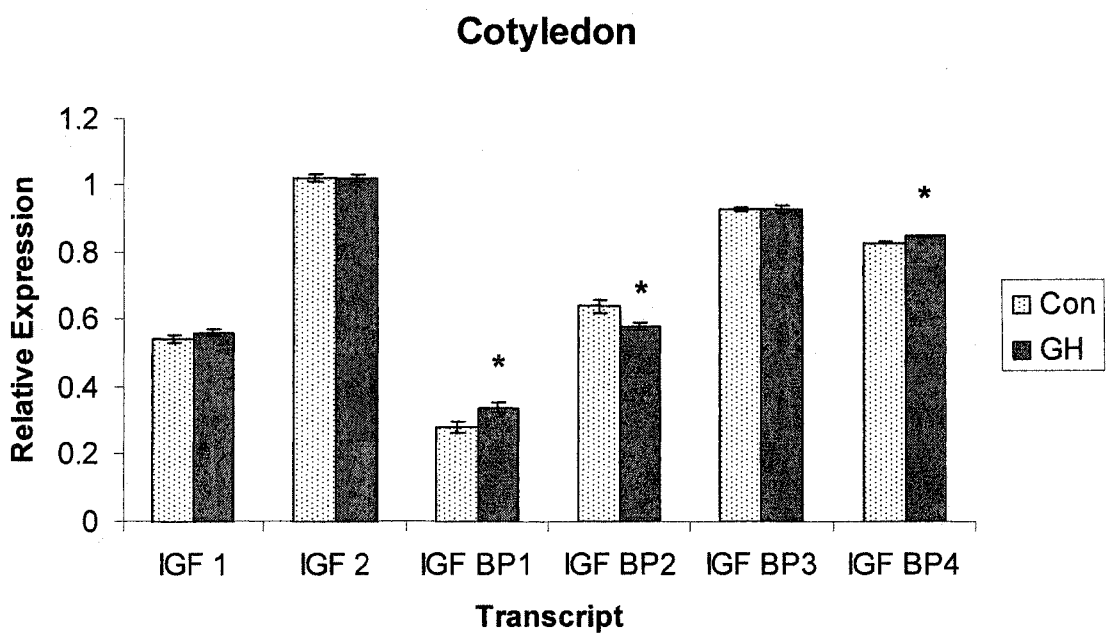

Figure 5.10: Relative expression of IGF-I, IGF-II, IGF BP-1, -2, -3, and -4 mRNA in cotyledon tissue. All transcripts were corrected (Ct/Ct) by RPS 15 mRNA. Data are expressed as mean ± SEM and significant differences ($P < 0.05$) are indicated by an asterisk.

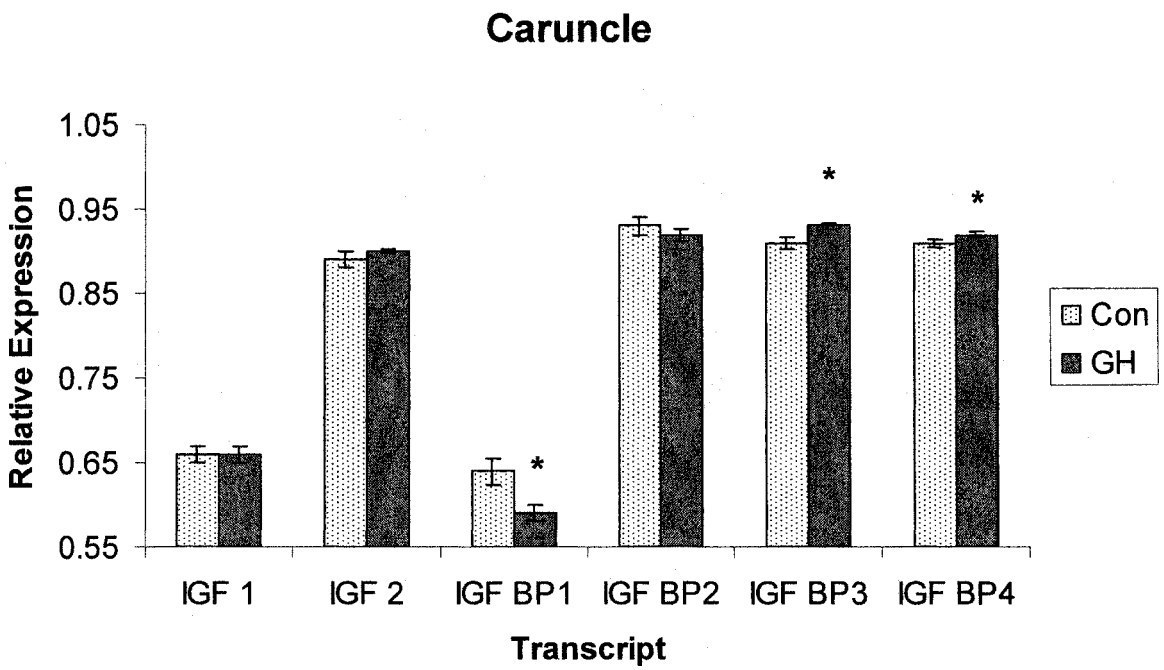

Figure 5.11: Relative expression of IGF-I, IGF-II, IGF BP-1, -2, -3, and -4 mRNA in caruncle tissue. All transcripts were corrected (Ct/Ct) with RPS 15 mRNA. Data are expressed as mean ± SEM and significant differences ($P < 0.05$) are indicated by an asterisk.

DISCUSSION

Growth hormone has been previously detected in the ovine placenta from day 35 to 55 of gestation; however, the cellular source of GH was not clearly defined (Lacroix et al., 1996; Lacroix et al., 1999). The first objective of this research was to definitively localize the expression of oGH in day 45 placentome tissue. These results of the *in situ* hybridization contrast with those reported by Lacroix et al. who reportedly detected oGH in maternal syncytium and stromal cells and fetal trophoblast. The use of *in situ* hybridization and visualization at high magnification reported here specifically detected hybridization of oGH radiolabel at the maternal fetal interface (Figure 5.1). These cells were not of

fetal trophoblast origin, as marked by oPL radiolabel (Figure 5.2). The cells that hybridized oGH radiolabel also showed positive hybridization for cytokeratin 18 (Figure 5.3) a marker of epithelial cells and not vimentin (5.4), a marker of fibroblast derived cells. Additionally, the oGH positive cells were negative for CD 18 hybridization precluding them from being leukocyte in origin (data not shown).

The results of the *in situ* hybridization analysis clearly indicate that oGH is expressed in maternal uterine epithelium and not detected in maternal villous stroma nor fetal trophoblast or villous stroma. Our detection of oGH in day 45 placental tissue coincides with the reported maximal expression of oGH (Lacroix et al., 1999). The pattern of oGH expression beginning at day 35, peaking at day 45 and decreasing to day 55 reported by Lacroix et al., is further supported by a much less robust detection of oGH in day 55 placentomes (Regnault and Anthony, unpublished data). While we do not understand the initiation of oGH in the placenta at approximately 35 dGA, the mechanism by which oGH expression is terminated may be the loss of the cells producing oGH. The loss of maternal epithelium within the caruncle due to syncytium formation is well documented, and by 55-60 dGA the epithelium is not detectable (Davies and Wimsatt, 1966; Stegeman, 1974). Therefore the cessation of oGH expression is likely not a change in transcriptional regulation, but the loss of oGH producing cells

Maternal growth hormone infusion late in gestation (day 98-111) has been shown to increase fetal growth in singleton pregnancies (Jenkinson et al., 1999); however, these results are not consistent within the same breed receiving the same oGH treatment (Currie et al., 1996). Wallace et al. initiated GH

administration to adolescent ewes at the appropriate time (day 35); however, the GH treatment continued to day 80 (Wallace et al., 2004), well past the reported cessation of oGH expression in the placenta (Lacroix et al., 1996; Lacroix et al., 1999). Although Wallace et al. reported an increase in placental weight in the over-nourished adolescent ewes with singletons, these ewes are predisposed to placental and fetal growth restriction due to the high plane of nutrition. The overnourished adolescent ewes exhibit an interesting change in GH/IGF-I endocrinology. These ewes have decreased maternal serum GH while at the same time have increased serum IGF-I (Wallace et al., 1997). This indicates a disconnection of the normal positive endocrinology of GH and IGF-I. Therefore, caution should be used when interpreting the results of maternal oGH infusion in this model. Maternal infusion of GH maternally may have resulted in increases in placental weight; however this may represent a resetting of the normal GH/IGF-I system thus providing a repartitioning of nutrients toward the pregnancy to normalize placental and fetal growth.

In our research, maternal GH treatment significantly ($P < 0.001$) raised maternal serum concentrations of GH and IGF-I ten-fold higher than CON ewes. by the 5^{th} day of treatment, and maintained the increase throughout treatment. The peak GH concentration was similar to that reported by Jenkinson et al., while the peak IGF-I concentration was similar to Stelwagen et al. (Jenkinson et al., 1999; Stelwagen et al., 1994). Although the ewes responded to GH treatment with increased maternal serum IGF-I, there was no concurrent increase in serum progesterone. Exogenous GH had no effect on uterine, placental, and fetal

weight or fetal organ weight measured at either day 55 or day 135 of gestation. These results support the conclusion that elevated maternal serum GH and IGF-I concentrations during the normal expression window of oGH have little impact on placental and fetal growth in ewes with twin pregnancies. This contrasts the results of exogenous GH administration in pigs, where GH administration increased fetal weight (Sterle et al., 1995). We specifically chose twin pregnancies to evaluate, as the multiple fetuses would put increased demand on the maternal system to provide sufficient nutrition to the uterus versus singletons. Furthermore the only normal sheep pregnancies to respond to maternal GH treatment were twin pregnancies (Jenkinson et al., 1999). The response seen by Jenkinson et al. more than likely is due to partitioning of maternal nutrients towards the pregnancy late in gestation, versus an alteration of placental development.

In an effort to gain insight into the potential effects of GH on gene expression within the placenta, we used northern hybridization and reverse transcriptase reaction and real-time PCR to quantify GH, oPL, IGF-I, IGF-II, and IGF binding proteins 1, 2, 3, and 4 in day 55 caruncle and cotyledon. Maternal GH treatment had no effect on GH expression in the caruncle tissue, nor did GH impact oPL expression in cotyledon tissue. In placental growth IGF-II plays a crucial role as evidenced by growth restriction in knockout and targeted disruption studies (Constancia et al., 2002; DeChiara et al., 1990). Had the exogenous GH significantly changed the IGF-II levels in the placenta, it would be expected that a change in placental growth would be evident. The role of IGF-I

may be as a less potent stimulator of proliferation, that has stimulatory effects on trophoblast differentiation and migration (Lacey et al., 2002; Maruo et al., 1995). With no gross changes in placental weight and fetal weight evident at necropsy, the lack of effect on these growth factors is not surprising.

In contrast, within the cotyledon, maternal GH treatment increased expression of IGF BP-1 and -4, while IGF BP 2 expression was decreased and IGF BP-3 was unchanged. Within the caruncle exogenous GH increased expression of IGF BP-3 and -4, while decreasing the expression of IGF BP-1. In the caruncle, IGF BP-2 was unaffected by maternal GH treatment. The decrease in caruncle expression of IGF BP-1 can be attributed to two possible mechanisms. First, IGF BP-1 is inversely regulated by insulin (Suikkari et al., 1989), and it has been shown previously that GH administration during gestation leads to hyperinsulinemia (Wallace et al., 2004). Given that we achieved significant stimulation of IGF-I similar to that reported by Wallace et al., one could reason that we generated a similar insulin response that reduced caruncle expression of IGF BP-1. Second, it has been shown that IGF-II can regulate IGF BP-1 expression at the maternal interface to control trophoblast invasion (Hamilton et al., 1998; Irwin et al., 2001). Although we did not measure maternal serum IGF-II concentrations, an increase of serum IGF-II due to GH treatment could also reduce the IGF BP-1 expression in the caruncle. The increase in caruncular IGF BP-3 agrees with reports that both GH and IGF-I are stimulatory to IGF BP-3 expression as reviewed by Clemmons (Clemmons, 1997). The increased expression of IGFBP-4 is not surprising, as it has been reported to be

a negative regulator of IGF-1 action in other tissues (Clemmons, 1997). Within the caruncle, IGF BP-4 may be upregulated to mitigate the effects of increased serum IGF-I.

Within the cotyledon, it is more difficult to interpret the changes in IGF BP-1, BP-2 and BP-4. Given the low amount of blood in a day 55 fetus, it is difficult to obtain serum analysis of GH, IGF-I, IGF-II and insulin in fetal circulation. Theses hormones, as discussed above, regulate the expression of IGF binding proteins and thus determination of their concentration would be necessary to give accurate interpretation of the transcriptional changes within the placenta. This is the first evidence of the transcriptional changes caused by GH administration during the normal placental oGH expression window within the placenta. While we did not alter the local expression of oGH in the placenta, this may be indicative of alternative regulation of oGH in the placenta versus the pituitary. Our evidence suggests that increased serum GH does not cause the normal negative feedback on GH expression in the placenta that is seen in the pituitary. Cloning and analysis of the complete promoter sequence for oGH 1 and oGH 2 may lead to discovery of differential regulation of the promoters. Heterologous studies with the human GH genes revealed that hGH-V lacked a proximal pituitary-specific protein (Pit-1) site that is one of two present in the hGH-N promoter (Nickel et al., 1991). This may indicate a tissue-specific transcriptional regulation for human GH in the placenta as compared to the pituitary. With the lack of placental response to maternal GH treatment, further research to

elucidate the role of placental oGH will likely necessitate a knockdown of oGH expression to gain insight into oGH function within the placenta.

SUMMARY

Our current understanding of growth factors regulating placental growth lacks clear definition. Ovine growth hormone was previously reported to be expressed in placental tissue for a brief (20 days) period during maximal placental growth and development. Previously in this lab we found that placental expression of oGH is decreased in a model of placental insufficiency that leads to fetal growth restriction. This lead us to hypothesize that oGH may play a role in early placental growth and development which impacts subsequent fetal growth. Our objectives here were to define the cellular source of oGH in the placenta, and study the impact of exogenous GH during the normal window (35-55 dGA) of placental expression.

The results of *in situ* hybridization indicate that oGH is expressed by uterine luminal epithelium and no tissues of fetal origin. The detection of oGH mRNA is reduced by day 55, likely due to loss of maternal epithelium as the maternal-fetal syncytium is formed. In the second study, the effects of maternal GH administration were measured at day 55 and 135 of gestation. For GH treated ewes, serum GH and IGF-I concentrations were increased approximately 10-fold ($P < 0.001$) by day 5 of treatment and the increase was maintained throughout the treatment period. Serum progesterone concentrations were unaffected by treatment. Uterine, uterine fluid, placental and fetal weights were unaffected by treatment for both day 55 and 135 groups. Fetal length, liver

weight, and liver weight per kg of body weight were not changed by maternal GH treatment. Using Northern hybridization, reverse transcriptase reaction, and real-time PCR, we measured transcriptional changes in caruncle and cotyledon tissue in the 55 dGA group. Maternal GH administration did not significantly alter GH (caruncle), or oPL (cotyledon) mRNA concentration as detected by Northern hybridization. In the cotyledon, IGF BP-1, and BP-4 were significantly ($P < 0.05$) increased, while IGF BP-2 was significantly decreased. The expression of IGF BP-3 was unaffected by treatment. Within the caruncle, IGF BP-1 was decreased, while IGF BP-3 and IGF BP-4 were increased, and IGF BP-2 was unchanged due to GH treatment.

In contrast to pigs, these data indicate that maternal serum concentrations of GH and IGF-I have no significant effect on placental and fetal growth. Although we did not impact placental or fetal growth, exogenous GH administration does impact the abundance of mRNA encoding IGF binding proteins within the placenta. Increase in caruncular IGF BP-3 is likely a response to increased maternal serum IGF-I. The increase in IGF BP-4 may also be due to increased IGF-I as IGF BP-4 is a negative regulator of IGF-I action. The decrease in IGF BP-1 is possibly the result of increased maternal insulin in response to GH treatment as insulin decreases IGF BP-1 transcription. Cotyledonary changes in transcription are difficult to interpret without knowing fetal serum GH, IGF-I, IGF-II and insulin concentrations. While we cannot explain the changes in transcription within the cotyledon, they are important in trying to explain the role of the IGF system at the maternal-fetal interface in

growth and differentiation. Although we now know the cellular source of oGH, further research is needed to refine the understanding of the role of oGH within the placenta.

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