THESIS

THE DEVELOPMENT OF OVINE PLACENTAL LACTOGEN DEFICIENT PREGNANCIES

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ABSTRACT

THE DEVELOPMENT OF OVINE PLACENTAL LACTOGEN DEFICIENT PREGNANCIES

Intrauterine growth restriction (IUGR) results in significant fetal and neonatal mortalities and morbidities. Additionally, infants surviving IUGR experience increased incidence of heart disease, diabetes, hypertension and stroke during adulthood. A major placental secretory product, placental lactogen (PL), is found at high levels in maternal and fetal circulation, and is significantly reduced in both human and sheep IUGR pregnancies. While the exact function of PL has not been defined for any species, it is thought to modulate the mobilization of maternal nutrients to the fetus. Recently, the development of lentiviral-mediated expression of short hairpin RNA (shRNA) within sheep conceptuses has provided a means of examining placental gene function in sheep. The objective of this research was to generate ovine PL (oPL) deficient sheep pregnancies using lentivirus targeting the degradation of oPL mRNA, in order to assess the function of PL during pregnancy. We hypothesize that oPL deficiency during pregnancy will lead to IUGR near term.

To test our hypothesis a preliminary study was conducted to evaluate the *in vivo* efficacy of lentiviral-oPL targeting vectors in the sheep placenta at 55 days gestational age (dGA). Efficiency of oPLmRNA degradation was first measured *in vitro* using twelve promoter-targeting sequence combinations, tested in three cell lines overexpressing oPL. Two oPL target sequences (tg2 and tg6) were used, as either shRNA or shRNAmiR (microRNA mimic) sequences. Subsequently, three lentiviral

constructs; hLL3.7 tg6 (human U6 promoter expressing oPL tg6 shRNA), hEF-1 tg2 (human elongation factor-1α promoter expressing oPL tg 2 shRNAmiR) and oPGK tg6 (ovine phosphoglycerate kinase-1 promoter expressing oPL tg 6 shRNAmiR), were selected to be tested *in vivo*. Day 9 blastocysts were harvested from naturally mated donor ewes, infected with one of the lentiviral constructs, and 2 to 3 blastocysts were surgically transferred to recipient ewes. At 55 dGA, uterine vein (UtV) blood and placental tissues were collected for analysis of oPL expression, and compared to naturally mated controls (NMC). Based on a 95% confidence interval created from UtV oPL concentrations in NMC pregnancies (n=4), 3 out of 4 hLL3.7 tg6 pregnancies, 3 out of 4 hEF tg2 pregnancies and 4 out of 4 oPGK tg6 pregnancies were classified as responder pregnancies. Compared to NMC pregnancies, UtV oPL concentrations were significantly reduced (P≤0.05) in responder pregnancies. While we hypothesized that oPL deficiency will result in IUGR near-term, at 55 dGA there were no differences in fetal weights.

To test our overall hypothesis, we generated 8 hEF-1-SC (human elongation factor-1α promoter expressing scrambled control shRNAmiR), 9 hEF-1 tg2, 7 hEF-1 tg6 (human elongation factor-1α promoter expressing oPL tg6 shRNAmiR) and 9 hLL3.7 tg6 singleton pregnancies that were harvested at 135 dGA. Based on two standard deviations below the mean placental weight of the hEF-1 SC pregnancies, 2 out of 7 hEF-1 tg6 pregnancies and 6 out of 9 hLL3.7 tg6 pregnancies were classified as tg6 responder pregnancies (tg6). Tg6 pregnancies resulted in significantly decreased (p≤0.05) oPL mRNA concentrations, placental weight and fetal body weight compared to the controls at 135 days of gestation. These data confirm the effect of lentiviral-oPL

targeting vectors and suggest that oPL plays a significant role in early placental development. Interestingly, we also observed that tg6 pregnancies had significantly increased ($P \leq 0.05$) placental efficiency relative to controls, which may function as a coping mechanism to maintain pregnancy in the face of oPL deficiency. Uterine artery to uterine vein glucose gradients were also increased ($p \leq 0.05$) in tg6 pregnancies compared to controls, which may be indicative of increased glucose uptake by the placenta in order to maintain function. Further analysis revealed that circulating fetal insulin tended to be decreased in the umbilical artery ($p \leq 0.10$) of tg6 fetuses relative to control fetuses, thus supporting a role for oPL in altering fetal insulin production. Finally, mRNA concentrations of insulin-like growth factors (IGF) -I and -II, and insulin-like growth factor binding proteins (IGFBP) -2 and -3 were significantly reduced ($P \leq 0.05$) in fetal liver tissue of tg6 fetuses compared to the controls. While this could be an indirect result of IUGR, oPL may well induce the expression of fetal insulin-like growth factors during *in utero* development. Based on these results, our hypothesis that oPL deficiency during gestation would result in intrauterine growth restriction appears correct. Surprisingly, it appears that oPL may have its greatest impact during early pregnancy when the placenta is developing, however the presence of adequate oPL is likely to be important throughout gestation for healthy fetal growth

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CHAPTER I: INTRODUCTION

The placenta is a multifaceted endocrine organ that nurtures healthy fetal growth through management of maternal-fetal nutrient exchange during pregnancy. The mature placental takes on several different morphologies depending on the species, but is commonly derived from a single layer of trophectoderm cells that surround the late stage embryo (blastocyst). During the early stages of pregnancy the trophectoderm cells form an intimate relationship with the maternal uterine endometrium through precisely timed cell signaling mechanisms, thus leading to the formation of a complex vascular and nutrient exchange system that is the mature placenta. The maternal and fetal contributions of the mature placenta form the chorionic villi, the primary exchange unit of the placenta. The chorionic villi house critical vasculature for nutrient exchange as well as placenta specific cell types that secrete a plethora of growth factors, protein hormones, steroids, cytokines and other biochemical factors that are vital for the development and maintenance of a healthy pregnancy. Proper placental establishment and function are critical for prenatal and postnatal health of the fetus. Functional placental insufficiency often leads to intrauterine growth restriction (IUGR), neonatal complications, and has been associated with the development of chronic adult onset disease. The creation of animal models to study placental function and consequences related to placental dysfunction will give a better understanding of the biological driving forces behind IUGR, and may lead to the development of effective tools for the management of IUGR in humans.

Placental lactogen (PL) is a placenta specific protein hormone abundantly secreted by the placenta throughout gestation, and is thought to play an important role

in modulating maternal metabolism to support fetal growth. PL is conserved across several mammalian species including humans, ruminants and rodents, and is derived from the growth hormone (GH)/prolactin (PRL) gene family. Since GH and PRL are well established protein hormones with an important role in growth, the close sequence homology of PL with GH and PRL suggests that PL may have a similar somatogenic function during pregnancy. Indirect evidence indicates a role for PL in maternal and fetal amino acid, carbohydrate and lipid metabolism through the stimulation of insulin-like growth factors. Additionally, the level of circulating PL during pregnancy has been directly associated with placental and fetal weights at birth. Below normal circulating levels of PL have been associated with compromised fetal growth and IUGR. Despite the discovery of PL more than 50 years ago, the exact biological function of PL during pregnancy has yet to be established largely due to the lack of an effective animal model. Similarities between pregnancy and the secretory pattern of PL in humans and sheep have made the sheep an extensively used model for studying PL during pregnancy. PL is secreted by the syncytiotrophoblast of the human placenta and comparable binucleate cells of the sheep placenta. Secretion of PL begins upon formation of the syncytiotrophoblast, or binucleate cells and increases until parturition. In the past, experiments conducted in the sheep to elucidate a role for PL have led to variable results with difficult interpretation. However, recent advancements in the development of lentiviral-meidated expression of short-hairpin RNA (shRNA) provide the means to generate a PL deficient sheep model.

This research is focused on creating ovine PL (oPL) deficient sheep pregnancies using lentiviral mediated shRNA, in order to establish a role for PL in supporting healthy

fetal growth. We hypothesize that oPL deficiency throughout gestation will result in IUGR. To test this hypothesis, a series of oPL targeting lentiviral vectors were constructed and their efficiency was tested *in-vitro* before conducting experiments *in vivo*. Subsequently, the *in vivo* efficiency of three different oPL targeting lentiviral vectors was tested at 55 days of gestation (dGA). After establishing the effectiveness of *in vivo* oPL targeting lentivirus, our goal was to determine the impact of oPL deficiency throughout gestation on near term fetal growth and development. Using three different oPL targeting lentiviral vectors, oPL deficient pregnancies were generated and harvested near term (135 dGA). As we anticipated, oPL deficiency throughout gestation resulted in IUGR. Interestingly, our results indicate that PL has an important function during early pregnancy when the placenta is developing. In addition oPL may influence fetal insulin production and insulin like growth factor expression. Overall, these studies provide a greater understanding for role of PL during pregnancy as well as useful insight towards mechanisms driving PL's action and the development and management of IUGR.

CHAPTER II: LITERATURE REVIEW

PLACENTATION

In 1937 Mossman broadly defined the placenta as an apposition of parental and fetal tissue for the purposes of physiological exchange' (Wooding & Burton, 2008). The placenta is, in fact, a complex, transient endocrine organ responsible for maximizing nutrient exchange and providing immune protection to fuel fetal growth during pregnancy. In mammals, the placenta is comprised of a fetal component derived from the chorion, amnion and allantois and a maternal component typically derived from the uterine endometrium (Senger, 2005). Together the fetal and maternal contributions form the primary exchange unit of the placenta, known as chorionic villi (Senger, 2005). The structure and distribution of chorionic villi among the placenta is extremely diverse among mammalian species, and has been classified based on anatomical appearance, histological structure (Grosser Classification), types of interdigitation, and maternal-fetal blood flow (Wooding & Burton, 2008; Benirshke, Beargen & Burton, 2012).

Anatomical Appearance

Classification based on anatomical appearance categorizes placentae into four different groups according to the macroscopic arrangement of chorionic villi in the mature placenta (Wooding & Burton, 2008). The first type is the diffuse placenta, which can be observed in pigs and horses. Chorionic villi of the diffuse placenta are evenly distributed across the entire chorion. In contrast, chorionic villi of cotyledonary, zonary and discoid placentas are concentrated to specific areas of the chorion. Cotyledonary

placentas are found in ruminant species such as the cow and sheep, and consist of very discrete exchange structures known as placentomes. Each placentome consists of the maternal caruncle derived from the uterine endometrium, and the fetal cotyledon at which chorionic villi are highly concentrated. The number and size of placentomes across the placenta varies according to species (Amoroso, 1952). Carnivorous species, such as canines and felines, have a zonary placenta in which the chorionic villi are isolated to an equatorial band around the chorion and near the middle of the conceptus (Wooding & Burton, 2008; Senger, 2005). The final classification is the discoid placenta found in higher primates and rodents. A disc shaped area of the chorion that is densely populated by chorionic villi and the primary site for metabolic exchange characterizes the discoid placenta. Classification of the placenta based on anatomical structure provides critical information pertaining to the various arrangements of the placenta; however, this system provides little information regarding the physiological function of each placental type.

Grosser Classification

In 1909 Otto Grosser established a placental classification system according to the type of tissue and number of layers separating maternal and fetal blood supplies (Amoroso, 1952.). Grosser classification is a widely accepted classification system, but has undergone modifications as our knowledge of placental structures has advanced. Grosser classification was originally thought to be more elusive to the functional exhange of nutrients and waste between mother and fetus, (Power, 2012; Senger, 2005; Wooding, 1992; Wooding & Burton, 2008) however, recent studies have

confirmed that histological structure of each placental type is not reflective of its functional efficiency (Enders & Carter, 2004). To date, the histological structure, or Grosser classification can be broken down into four different categories: epitheliochorial, synepitheliochorial, endotheliochorial, and hemochorial (Wooding, 1992; Wooding & Burton 2008).

The number of layers separating maternal and fetal blood supply is dictated by the degree of invasion by the fetal chorion into maternal endometrium. The epitheliochorial placenta is the least invasive placenta type in which the intact uterine epithelium is in direct contact with the epithelium of the fetal chorion (Wooding & Burton, 2008). In total, there are six layers of tissue separating maternal and fetal blood supplies: fetal capillary endothelium, fetal connective tissue, chorionic epithelium, endometrial epithelium, maternal connective tissue, and maternal capillary endothelium. Since there is distinct isolation between maternal and fetal tissues, the epitheliochorial placenta provides a greater degree of immunological protection in comparison to more invasive placental types. On the other hand, there is greater difficulty in the passage of materials across the interhemal barrier, which is overcome by indenting of the chorionic epithelium and endometrial epithelium near capillaries (Enders & Carter, 2004). Ruminant placentae are similar to the epitheliochorial placenta, but fusion between the fetal chorion and uterine epithelium results in the classification of ruminant placentae as the synepitheliochorial. Grosser originally classified the ruminant placenta as syndesmochorial based on the observation that uterine epithelium was eroded, and the fetal trophectoderm, or fetal chorion, was apposed to the uterine connective tissue (Wooding, 1992). More recent evidence shows that the uterine epithelium persists in a

modified state. Fetal chorionic binucleate cells, specific to ruminant species, migrate and fuse with the uterine epithelium to form a hybrid fetomaternal syncytium at the interhemal membrane (Wooding, 1992). In the mature ruminant placenta, fetomaternal syncytia account for a large area of the chorionic villi surface (Wooding, 1984). The third classification, endotheliochorial placenta, is defined by its intermediate invasiveness and erosion of the uterine epithelium and maternal connective tissue so that the fetal chorion is in direct apposition to the maternal capillary endothelium (Benirshke, Beargen & Burton, 2012). The maternal endothelium may prevent shedding of fetal cells into maternal circulation as a means of immunological protection. Still yet, there is tendency for maternal capillaries to remodel and grow into the fetal trophoblast layer (Enders & Carter, 2004). The endotheliochorial placenta is commonly found in carnivores (Bjorkman, 1970), as well as insectivores (Malassine & Leiser, 1984) and lower primates (Kaufmann, Bruns, Leiser, Luckhard & Winterhager, 1985). Lastly, the hemochorial placenta is the most invasive placental type that is characteristic of humans, higher primates and rodents (Enders, 1965; Ramsey, 1982). During hemochorial placentation the fetal trophoblasts cells invade through the maternal capillary endothelium resulting in pools of maternal blood that bathe fetal chorionic villi. The hemochorial placenta allows for the most direct route of oxygen exchange with maternal blood, as well as transport of maternal substrates such glucose and amino acids through transporters located on the surface of fetal trophoblast cells of the chorionic villi (Battaglia & Regnault, 2001; Cariappa, Heath-Monnig & Smith, 2003; Takata & Hirano, 1997). In contrast, direct exposure of maternal blood to fetal tissues poses a greater threat of immune response by the mother. While several speculations

have been made regarding the advantages and disadvantages of placental types under Grosser classification, it remains established that the histology of the placenta is not reflective of functional efficiency (Dantzer, Kaufmann & Luckhardt, 1988).

Maternal-Fetal Interdigitation

Placental transport efficiency may be attributed in large part to surface area occupied by chorionic villi. The type of interdigitation between maternal and fetal tissues greatly impacts the amount of surface area covered by the placenta, thus making it a noteworthy functional classification system. Recent evidence demonstrates a positive correlation between the degree of maternal-fetal interdigitation and fetal growth rates, suggesting that the interdigitation influences nutrient transfer rates (Capellini, 2012). Additionally, this relationship has been noted to foster shorter gestation lengths (Capellini, 2012). There are five known categories of maternal-fetal interdigitation with progressive levels of interdigitation beginning with folded type, and increasing in lamellar, trabecular, villous, and labyrinthine types respectively (Benirshke et al., 2012). Folded type interdigitation is often found in species with a diffuse placenta, such as the pig, to compensate for the poorly branched folds of the chorion that interdigitate with grooves in the uterine endometrium (Benirshke et al., 2012). The lamellar type has a slightly higher degree of interdigitation and is characterized by more intimate chorionic folds that complement folds of the maternal endometrium. Lamellar interdigitation is commonly observed in carnivores (Leiser & Kohler, 1984). Humans and ruminants share the villous type interdigitation represented by extensively branched tree-like structures, rather than chorionic folds. These villous structures extend into the

maternal endometrium (ruminants), or become bathed in maternal blood (humans) depending on the amount of invasion by the fetal chorion. Intermediate between lamellar and villous is trabecular interdigitation, which is defined by a combination of folding and branching of fetal chorion in conjunction with uterine endometrium. Finally, labyrinthine types are found in all rodents and are a complex network of maternal and fetal tissue with cavities of either maternal or fetal blood (Benirshke et al., 2012; Capellini, 2012).

Maternal-Fetal Blood Flow

Lastly, the arrangement of maternal and fetal vasculature is also of important functional relevance. The way in which fetal capillaries are organized relative to maternal blood supply determines the direction of blood flow and has impacts on the ability for exchange and diffusion across the placenta. The three basic types of vascular arrangement known to exist among mammalian species include countercurrent, multivillous and crosscurrent exchange types. While there are definitive definitions of each exchange type, the vascular system within any placenta is complex and may include a combination of different arrangements (Enders & Carter, 2004). Nonetheless, the interrelationship between maternal and fetal blood flow is directly related to fetal-placental weight ratios at term (Dantzer et al., 1988). The countercurrent exchange system orients maternal and fetal capillaries parallel to each other, but with blood flowing in opposite direction. Because blood flows in opposite directions, countercurrent exchange provides maximum transfer efficiency (Benirshke et al., 2012). As nutrients and waste are transferred between blood supplies, there is a more gradual change in

concentration gradient, thus making more productive use of the potential generated by the concentration gradient. Countercurrent exchange is the predominant arrangement type in horses, guinea pigs and rabbits (Rurak, 2001). In contrast, multivillous and crosscurrent exchange types have intermediate transfer efficiencies. Multivillous arrangement occurs when fetal blood flows in and out of “capillary loops” within branching villi that are consequently bathed by maternal blood or surrounded by large maternal vessels (Rurak, 2001). Chorionic villi bathed by maternal blood within the intervillous space of the human placenta are a prime example of multivillous arrangement. Multivillous arrangement has also been observed in the ruminant placenta (Dantzer et al., 1988). Finally, crosscurrent arrangement is characterized by the arrangement of fetal vessels, and direction of blood flow, at 90° angles to maternal blood flow and vasculature. This type of arrangement is dominant in the placenta of carnivores, some lower primates and the pig (Benirshke et al., 2012; Dantzer et al., 1988; Leiser & Kohler, 1984; Luckhardt, Kaufmann & Elger, 1985).

ANIMAL MODELS

In order to make advances in our knowledge of placental physiology, gene regulation within the placenta, and improve the prognosis for placental insufficiencies a relevant animal model is crucial. It has been accepted that in cases of insufficient placental function, for both humans and animals, offspring are at risk for impaired development leading to increased risk of heart disease, diabetes, hypertension and stroke later in life (Gluckman & Hanson, 2005). Therefore, detailed characterization of placentation across species is of great medical importance for selecting animal models

to study human pregnancy. Unfortunately, there is no perfect model for human pregnancy aside from humans themselves, which is not ethically viable in many circumstances. Furthermore, clinical trials focused on placenta function can be challenging largely due lack of patient consent. As such, animal models are invaluable tools for pregnancy research, as long as the appropriate model is utilized. Different animal models address certain aspects of human pregnancy and placentation better than others. A comprehensive understanding of the advantages and disadvantages of each animal model is critical to adequately assess an experimental hypothesis and optimizing experimental design. To date, the most common animal models for studying the human placenta include the mouse, sheep and non-human primate (Old World monkeys) (Carter, 2007).

Mouse

The mouse has been a convenient model due to its small size, short gestation length, and its classification as a hemochorial placenta. Some have argued that the close phylogenetic relationship between rodents and humans also makes them a beneficial model (Springer, Murphy, Eizirik, & O'Brien, 2005), however a high mutation rate and fragmentation within the mouse genome act against that relationship (Wu, 1985; Li et al., 2004). The fact that humans and mice both have a hemochorial placenta makes the mouse a favorable animal model, but there are still distinct differences between them that are important to consider. For instance, mice are litter bearing animals that deliver altricial young, where as humans typically give birth to a single, precocial offspring (Carter, 2007). Since mice have a short gestation length, they give

birth do undeveloped young and their offspring experience several developmental processes during postnatal life that are otherwise experienced in utero during human pregnancy (Carter, 2007). Furthermore, there is a lesser degree of trophoblast invasion in the mouse compared to humans (Redline & Lu, 1989). The mouse placenta has three trophoblast layers separating maternal and fetal blood supplies (hemotrichorial), whereas humans have a single syncytiotrophoblast layer (hemomonochorial). Nevertheless, studies have shown similarities between placental membranes, which favor similar transport mechanisms between humans and mice (Dilworth & Sibley, 2013). Alternatively, the endocrine function of the mouse placenta varies from that of the human. In humans progesterone, a steroid hormone required for the maintenance of pregnancy, is produced and secreted by the corpus luteum in response to human chorionic gonadotropin (hCG) during the first trimester, after which the trophoblast cells of the placenta secrete enough progesterone to meet the demands of pregnancy. In contrast, mice require corpus luteum derived progesterone throughout gestation (Carter, 2007). In addition the mouse and human placenta both secrete placental lactogen, however, multiple temporal isoforms exist in the mouse (Roberts & Anthony, 1994). Despite these differences, the genes regulating placental development are similar between the mouse and human (Rossant & Cross, 2001), making them a useful model for gene function and mutation studies related to placental physiology. Since transgenesis has become a routine procedure in mice, such studies are easily attainable in rodent models. In all, short gestation length, ease of transgenic studies and similarities between placenta types make the mouse a suitable model for the study of particular exchange mechanisms as well as placental development.

Sheep

The pregnant ewe offers several characteristics that make them a unique and beneficial model for human pregnancy. The synepitheliochorial placentation observed in the sheep is often considered a major deficit of the sheep model, however, there are many physiological similarities between placental function and fetal development in humans and sheep. To start, sheep often carry singleton and twin pregnancies for a longer gestational period, and give birth to precocial young of similar weight to newborn humans, which is a more accurate reproduction of human pregnancy than that of the mouse (Carter, 2007). Throughout gestation humans and sheep undergo several developmental processes during similar stages of gestation, and reach a comparable level of fetal maturity at parturition (Carter, 2003; Carter, 2007). In addition, placental vasculature within the placentomes (fetal cotyledon and maternal caruncle) of sheep resembles that of the discoid placenta in humans. At the start of pregnancy in both human and sheep, the conceptus survives in a hypoxic environment until fetal vasculature begins to develop and invade into the maternal tissue (Steven, 1975). In the sheep, the fetal cotyledon establishes villous tree like structures that branch and invade into crypts of the maternal caruncle, apposed to the maternal epithelium. These villi exist in three different forms: stem, intermediate and terminal villi, all of which are structurally similar to the chorionic villi of the human placenta (Leiser, Krebs, Ebert & Dantzer, 1997). These key similarities are followed up by commonalities between oxygen and nutrient transport. A number of studies have demonstrated that placental oxygen consumption, transfer and fetal oxygen consumption rates between the human and sheep are similar throughout pregnancy (Barry & Anthony, 2008). Likewise, the

placenta of human and sheep employ comparable mechanisms for glucose transport, the primary substrate for placental function and fetal growth (Barry & Anthony, 2008). While the above physiological traits of the sheep placenta are of great significance when choosing the appropriate animal model, the ability to use *in vivo* instrumentation during sheep pregnancy is the most valuable trait of the sheep model (Barry & Anthony 2008). Surgical techniques allowing the placement of catheters into maternal or fetal vasculature have resulted in a much more detailed understanding of placental and fetal physiology for sheep (Barry & Anthony, 2008; Carter, 2007). On the other hand, cost and labor demands of using sheep for large-scale research can be problematic for experimental design (Carter, 2007). Altogether, with the appropriate facilities and funding, sheep provide an excellent model for studying relevant developmental, metabolic and transport aspects of human pregnancy.

Non-Human Primates (Old World Monkeys)

Non-human primates are the closest evolutionary relative to the human, and deviate only slightly from humans in placental development and fetal growth. Old world monkeys, such as macaques and baboons have a hemochorial placenta with villous structures that appear morphologically identical to that of humans (Carter, 2007). The remodeling of spiral arteries in Old World monkeys is also indistinguishable from that of humans (Blankenship & Enders, 2003). Key differences to consider when using the Old World monkey as a human model are the invasive characteristics of cytotrophoblasts during early implantation in the Old World monkey (Carter, 2007; Enders, Lantz, & Schlafke, 1996). For instance a large part of the blastocyst remains in the uterine cavity

during implantation in the Old World monkey, and in humans the blastocyst invades just beneath the maternal endometrial epithelium (Carter, 2007). However, the interactions between the trophoblast and maternal endometrium in Old World monkeys and humans are similar (Carter, 2007). From a physiological standpoint, the non-human primate is an ideal model for human pregnancy; however, logistical and ethical concerns surrounding their use often outweigh the benefits. The level of intelligence and complex behavior patterns make their housing and maintenance costly. Additionally, it is a sensitive debate as to whether or not the housing of such an intelligent species in a research environment crosses any ethical lines.

As our knowledge of phylogenetic relationships expands, and our *in vivo* technologies improve, our access to more efficient, relevant animal models will advance the study of human pregnancy and disease. Until then, it is imperative to choose a relevant animal model that best represents the aspect of human pregnancy under study.

PLACENTAL ESTABLISHMENT AND FORMATION

The early stages of pregnancy during which the developing embryo implants into the maternal endometrium and placental formation is established, are critical time points of pregnancy. Complications during early pregnancy may lead to significant clinical consequences for both the mother and her offspring; such as preeclampsia, placental insufficiency and fetal growth restriction. Additionally, a large portion of pregnancy loss across species can be accounted for during early pregnancy. In humans, only 50-60% of all conceptions progress beyond 20 weeks gestation, and 75% of failed pregnancies are a consequence of failed implantation (Norwitz, Schust & Fisher, 2001). The steps

leading up to and during the process of implantation require precise coordination, regulated by molecular signaling and synchronous gene expression between the developing embryo and maternal uterine environment. To date, most of our knowledge regarding the molecular cues and gene regulation during early pregnancy has been extracted from studies conducted in rodents. These studies have laid the foundation upon which discoveries of early pregnancy regulation in alternative species have been made. Further identification of the key events and markers controlling successful implantation and placental formation, are important for the development of clinical treatments to mitigate the rate of early pregnancy loss and pregnancy disease.

Early Embryo Development

From the time of fertilization in mammals, the nascent embryo undergoes a phase of rapid cell division as it travels down the fallopian tubes in route towards the uterus as its final destination. During this time of rapid cell cycle division the embryo's primary source of nourishment is maternal oocyte derived mRNA and protein, until zygotic genome activation (Bettegowda, Lee & Smith, 2007; Guzeloglu-Kayisli, Basar & Arici, 2007). Zygotic genome activation typically occurs between the eight- and 16-cell stage of development (Bettegowda et al., 2007), when the maternal mRNA and proteins have been depleted and embryonic development shifts to complete control by the zygotic genome (Murchison et al., 2007; Tang *et al.* 2007). As the embryo advances to the morula stage, the totipotent cells located near the periphery of the morula undergo the first stages of differentiation towards the trophectoderm. The trophectoderm (TE) is a layer of cells lining the perimeter of the blastocyst that will ultimately form the placenta

and extraembryonic membranes (Kay, Nelson & Wang, 2011). Subsequent blastocyst formation results in the formation of the inner cell mass (ICM), consisting of pluripotent cells that will develop into the fetus, and the surrounding TE (Cross, Werb & Fisher, 1994; Guzeloglu-Kayisli et al., 2007; Kay, Nelson & Wang, 2011; Watson, 1992). The up regulation of Cdx2 marks the segregation of TE cell lineage and suppresses the expression of pluripotency genes Oct4 and Nanog that are otherwise expressed in the ICM (Johnson & McConnell, 2004). The mature, fully expanded blastocyst has a third cell lineage known as the primitive endoderm, described as the layer of cells lining the ICM and interfacing the amniotic cavity that are destined to develop into other extraembryonic membranes. Gata6 gene expression is characheristic of the cells comprising the primitive endoderm (Guzeloglu-Kayisili et al., 2007). Once the embryo has reached the late blastocyst stage, it is ready to hatch from the zona pelucida, a protective layer made of glycoproteins (Wassarman, 1988).

Implantation

After the mammalian embryo has hatched from the zona pelucida, the exposed trophectoderm is available to begin the process of implantation. There are four recognized types of implantation that occur in three general stages: apposition, adhesion and invasion (Hamilton, Mossman & Boyd 1972; Guzeloglu-Kayisili et al., 2007). First is centric implantation found in ungulates, lower primates and carnivores and is classified by embryo expansion prior to implantation and maintenance of the embryo in the center of the uterus (Steven, 1975). Eccentric implantation, found in rodents, is defined by blastocyst contact with a uterine crypt or recess (Steven, 1975).

Superficial implantation occurs in ruminants and pigs and involves only minor blastocyst invasion into the maternal endometrium following adhesion. Finally, interstitial implantation is the most invasive type of implantation classified by complete immersion of blastocyst within the uterine endometrium. Interstitial implantation is characteristic of humans, guinea pigs, hedgehogs and chimpanzees (Steven, 1975).

In order to condition the maternal endometrium for embryo receptivity, a period of decidualization is key. Decidualization is a phase of rapid proliferation and differentiation of uterine stromal cells. In most mammalian species, decidualization is stimulated upon implantation; however, humans, old world monkeys and great apes undergo a unique phase of dedicualization in preparation for implantation during the secretory phase of the menstrual cycle. In the case of non-pregnancy, the decidualized stromal tissue is shed during menstruation. Since species such as rodents and ruminants do not experience dedidualization until stimulated by blastocyst implantation, this eliminates the need for menstruation (Ramathal et al., 2010). Despite the difference in timing of decidualization, the regulation and overall function of the decidua is similar among mammalian species. Estrogen and progesterone are the primary steroidal regulators of decidualization. Increased estrogen primes the endometrium for decidualization by stimulating proliferation of the uterine stromal cells, while a subsequent rise in progesterone levels dictates their differentiation. The uterine stromal cells transform from a fibroblast-type cell to a large, polygonal, polyploid cell-type with unique biosynthetic and secretory functions (Ramathal et al., 2010). The decidualized endometrium secretes histotroph, a nutrient rich secretion comprised of growth factors and cytokines to support conceptus growth (Finn, 1977; Glasser et al., 1991), and is

largely populated by immune cells such as macrophages, T-lymphocytes and uterine natural killer cells (uNK) to mediate the maternal immune response to implantation (Kay et al., 2011). Further evidence also suggests a role for decidualized cells in the remodeling of maternal vasculature in support of the developing embryo (Ramathal et al., 2010). While implantation has been shown to progress in the absence of decidualization, the success of the pregnancy and establishment of adequate blood supply is significantly compromised (James, Carter & Chamley, 2012).

Apposition is the first stage of attachment during which the trophectoderm cells of the developing conceptus contact the luminal epithelium of the uterine wall (Guzeloglu-Kayisli, 2007; James et al., 2012; Kay et al., 2011). In humans and rodents, apposition occurs shortly after the blastocyst hatches from the zona pelucida. The sheep conceptus, on the other hand, hatches from the zona pelucida and goes through a period of rapid elongation between days 12 and 15 post-conception before the trophectoderm makes initial contact with the caruncular epithelium (Boshier, 1968). During this period of elongation the conceptus takes on a tubular and then filamentous form. In humans the TE cells nearest the ICM are considered the polar TE and also the site of contact between the TE and luminal epithelium (Hertig A, 1968). Similarly the TE nearest the ICM of the filamentous sheep conceptus is where apposition occurs. (Wintenberger-Torres & Flechon, 1974). In order for the conceptus to achieve a more stable adhesion, there is complex cross talk between the uterine epithelial cells and the trophectoderm that drive the expression of critical cell adhesion molecules, integrins and various glycoproteins. For example, L-selectin is an important adhesion molecule known to be present on the surface of trophectoderm cells in the mouse, sheep and

human blastocyst, and is thought to play a role in adhesion by binding oligosaccharide ligands on the surface of the receptive uterine luminal epithelium (James et al., 2012). In sheep, evidence suggests that L-selectin binding facilitates communication between the blastocyst and luminal epithelium (LE), leading to the activation of various integrins and further blastocyst adhesion (Spencer, 2004). A similar function for L-selectin has been observed in humans as well (James et al., 2012). MUC-1 is an additional factor shown to have an important role during the time of blastocyst adhesion and uterine receptivity. MUC-1 is an anti-adhesive glycoprotein on the LE (Brayman, Thathiah, & Carson, 2004; Cross & Fisher, 1994) that is down regulated in the mouse and sheep to allow for integrins on the surface of the TE to interact with their receptors on the LE (Burghardt et al. 2002; Carson et al. 2000). This differs from humans in that MUC-1 is only down regulated at the site of implantation, but upregulated elsewhere on the LE (Brayman et al. 2004; Carson et al. 2000). Sectreted Phosphoprotein 1 (SPP1) is also a noteworthy contributor to adhesion in humans, sheep and mice. SPP1 is an extracellular matrix (ECM), cytokine protein known to mediate cell-cell and cell-ECM communication, and is secreted as a part of uterine histotroph (Johnson et al., 2003). During implantation SPP1 functions to promote cell adhesion, spreading and migration (Spencer, 2004). To conclude, these are a few of many factors involved in blastocyst apposition and adhesion, which is a complex process requiring precise timing and communication between the uterine environment and the developing conceptus.

Trophoblast Differentiation

After apposition and adhesion, the differentiation of trophoblast cell lineages and invasion into the maternal endometrium leads to the formation of the mature placenta and lays the groundwork for efficacious maternal-fetal exchange. Abnormalities in trophoblast differentiation may lead to detrimental placental insufficiencies typically resulting from the lack of blood and nutrient supply from the mother to the fetus. Despite differences in the resulting placentation types between species, the overall nature of trophoblast cell lineages is comparable between all mammals.

In humans, cytotrophoblasts are the initial trophoblast lineage to arise from the trophectoderm upon blastocyst adhesion. The primary functions of cytotrophoblasts are to act as precursor cells to all other trophoblast lineages, and to provide a source of regeneration to those lineages throughout pregnancy (Cross et al., 1994; Fitzgerald, Poehlmann, Schleussner & Market, 2008; Knofler, 2010). In the mature placenta, cytotrophoblasts populate at the chorionic plate (Benirshke et al., 2012) and proliferate continuously to meet the demands of the growing fetus and corresponding placenta. As the blastocyst begins to invade the maternal endometrium, the most peripheral cytotrophoblasts differentiate and fuse to form the primary invasive trophoblast lineage known as the syncytiotrophoblast layer. The syncytiotrophoblast is a terminally differentiated, polyploid cell type derived from and sustained by the proliferation and fusion of underlying cytotrophoblasts (Benirshke et al., 2012). This multinucleated layer of tissue is the functional barrier between mother and fetus that actively absorbs and exchanges nutrients, and also has a wide array of endocrine functions (Malassine & Cronier, 2002). The mass of syncytiotrophoblast rapidly increases in size as it

continues to invade the maternal endometrium by disrupting apical junctional complexes and degrading the extracellular matrix (ECM) to make space for syncytiotrophoblast branching (Benirshke et al., 2012). Within a couple of days, the blastocyst and surrounding syncytiotrophoblast becomes completely emersed within the uterine endometrium, constituting interstitial implantation that is characteristic of human pregnancy (Hertig, Rock & Adams, 1956). Further growth of the syncytiotrophoblast results in the formation of a network of lacunae, or vacuoles, with the surrounding syncytiotrophoblast arranged as trabeculae (James et al., 2012; Benirshke, 2012; Kay et al., 2011). At this stage, cytotrophoblast rapidly proliferate and invade through the trabeculae to form cellular columns that extend through the syncytiotrophoblast until they meet the maternal endometrial tissue, creating the cytotrophoblastic shell (Benirshke et al., 2012). Cells of the cytotrophoblastic shell that populate at the interface of the endometrial tissue further differentiate into what is known as an extravillous trophoblast (EVT) cell. The differentiation and migration of the EVTs into the maternal endometrium is critical for remodeling of maternal spiral arteries to accommodate blood supply demands of the placenta (Benirshke et al., 2012). The EVTs invade the maternal endometrium, even as far as the myometrium, and adhere to the maternal endothelial cells lining the spiral arteries that supply blood to the uterus and eventually the placenta (Ferretti, Bruni, Dangles-Marie, Pecking & Bellet, 2007; Kaufmann & Castellucci, 1997). This cell-cell interaction leads to altered dilatory properties of the spiral arteries so that there is increased blood perfusion to the placenta and fetus throughout pregnancy (Pijnenborg, Bland, Robertson & Brosens 1983). Meanwhile the syncytiotrophoblast of the lacunae network grows and erodes maternal

sinusoids located within the endometrium (Hertig et al., 1956), thus forming the intervillous space (IVS) in which maternal blood bathes the fetal derived syncytiotrophoblast, establishing the fundamental nutrient and endocrine exchange site (Ferretti et al., 2007). As the IVS grows, cytotrophoblasts continue to proliferate and arrange into primary chorionic villi and eventually the mature villous tree as described earlier.

The trophoblast lineages of the sheep's synepitheliochorial placenta are significantly less invasive than those required to achieve interstitial implantation in humans. None the less, there are still noteworthy functional and morphological similarities between trophoblast lineages in humans and sheep. There are two primary trophoblast cell types derived from the trophectoderm of the sheep conceptus known as the mononucleate cell (MNC) and the binucleate cell (BNC) (Wimsatt, 1951). MNCs typically function in nutrient transport, while BNCs are majorly responsible for the endocrine/secretory functions of the placenta (Duello, Byatt & Bremel 1986; Myers and Reimers, 1988). Morphologically similar to an epithelial cell type (Igwebuike, 2006), MNCs are the most populous cell type lining the chorioallantois (Boshier & Holloway, 1977) and sustain proliferation throughout gestation, much like human cytotrophoblasts. Characteristic microvilli are located on the apical membranes of MNCs, and interact with similar microvilli structures of the uterine epithelial cells at the maternal-fetal interface (Bjorkman, 1969; Dent, 1973). As the fetal chorion invades the crypts of the maternal caruncle (Davis, Fisher & Schlafer, 2000) these MNC microvilli are essential for increasing surface area for absorption of nurtrients (Igwebuike, 2006). Similar to cytotrophoblast derived syncytiotrophoblast in humans, the MNCs also give rise to

polyploid BNCs. Unlike humans, the formation of binucleate cells begins prior to conceptus adhesion, around day 15 of gestation (Boshier, 1969). In addition, rather than the fusion of MNCs to generate BNCs, such as the differentiation of cytotrophoblasts to syncytiotrophoblast, BNCs arise by acytokinetic mitoses (Bjorkman, 1968; Wimsatt, 1951). BNCs initially reside amongst the MNCs in the trophectoderm in an intraepithelial position (Igwebuike, 2006; Wango, Wooding & Heap, 1990; Wooding, 1984). However, once the BNCs have been established, they acquire essential migratory properties that direct them towards the maternal-fetal interface (Igwebuike, 2006). The BNCs will travel through the MNCs without disrupting their tight junctions by extending cytoplasmic processes within the tight junctions (Wango et al., 1990). When the BNCs meet the uterine epithelial interface, they fuse with maternal epithelial cells, thus generating a maternal-fetal hybrid trinucleate cell (TNC) (Wooding, 1984; Wooding, 1992; Wango et al., 1990). Continued migration and fusion of BNCs with the uterine epithelium and previously established TNCs results in the formation of syncytial plaques (Wooding, 1984, 1992). These syncytial plaques represent modifications to the maternal epithelium that facilitate transport of maternal substrates as well as fetal secretory products (Wooding, 1984). The generation and migration of BNCs is constant throughout pregnancy in order to maintain and increase the area of syncytial plaques as fetal growth demands rise (Lawn, Chiquoine & Amoroso, 1969). The morphological and physiological characteristics of BNCs and syncytial plaques are analogous to that of the human syncytiotrophoblast and respective syncytium, thus contributing to the practicality of using the sheep as a model for human pregnancy.

Extraembryonic Membranes

The extraembryonic membranes of all vertebrates are derived from the ectoderm, endoderm and mesoderm germ layers of the embryo (Wooding, 2008), and are essential structures for the formation of a functional placenta (Steven 1975). The chorion, amnion and allantois constitute the extraembryonic membranes that develop as a result of the three germ layers. Chorioallantoic placentation is defined by the fusion of the chorion and allantois, and is the broadest classification of placentation under which eutherian mammals fall (Carter, 2012). The chorion is derived from the fusion of the trophectoderm with underlying mesoderm cells (Wooding, 2008) and represents the outermost extraembryonic membrane that contacts the maternal interface. The amnion is the innermost extraembryonic membrane. In species that have lost their polar trophectoderm, such as ruminants and carnivores, the chorion folds to produce the fluid-filled amniotic sac surrounding the embryo (Wooding, 2008). Alternatively, species that maintain a polar trophectoderm, such as humans, the amnion is derived from cavitation of the inner cell mass (Wooding, 2008). The allantois is a highly vascularized extraembryonic membrane that serves as a precursor to the umbilicus and is the primary source of fetal blood supply to the placenta (Arora & Papaioannou, 2012). The allantois arises as an extension of mesoderm that buds from the embryonic hind gut, and continues to grow outward towards the chorion (Arora & Papaioannou, 2012; Wooding, 2008). Once the allantois reaches the chorion and spreads along the surface of the chorion, there is fusion between the two membranes, thus generating the chorio-allantoic membrane (Arora & Papaioannou, 2012). The chorion portion develops into chorionic villi and the allantois provides essential fetal vasculature to these structures

(Watson, 2005). Development and maintenance of such extraembryonic structures is vital for adequate placental function, as well as protection and growth of the fetus.

ENDOCRINE FUNCTION OF THE PLACENTA

The placenta is an incredibly adaptive organ with endocrine function that is imperative to a healthy pregnancy. The endocrine actions of the placenta support the establishment and maintenance of pregnancy, adaptation of maternal metabolism, fetal growth, and parturition (Evain-Brion & Malassine, 2003). A wide array of protein and steroid hormones, growth factors, cytokines and other biochemical signals are synthesized and secreted by the placenta to achieve its specialized endocrine function (Chard & Grudzinskas, 1992; Talamantes & Ogren, 1988; Prager, Weber & Herman-Bonert, 1992). A large portion of placental secretory products are also products of other endocrine and non-endocrine organs in the body. For instance, progesterone secretion from the placenta in humans is required for the maintenance of pregnancy, but is also secreted by other reproductive tissues during times of non-pregnancy. Alternatively, there is a group of hormones that are considered true placental hormones because their presence can only be detected in maternal circulation during pregnancy and they originate only from placental tissue (Anthony, Pratt & Holland, 1995). The most extensively studied true placental hormones include chorionic gonadotropins (CG), trophoblast interferons (INF-τ), and placental lactogens (PLs) (Roberts & Anthony, 1994). Placental endocrine activity begins prior to invasion by the developing embryo when the trophectoderm cells secrete signals that are received as a maternal recognition of pregnancy. In humans, hCG is required for maternal recognition and

maintenance of pregnancy. hCG is a glycoprotein hormone synthesized and secreted by the syncytiotrophoblast, and acts on the corpus luteum to prolong progesterone secretion (Evion-Brion & Malassine, 2003). hCG levels decline after 8 weeks of gestation and are sustained at low levels until term (Roberts & Anthony, 1994). In ruminant species, such as sheep, INF-τ is the documented signal for maternal recognition of pregnancy. INF-τ is a type I interferon (Roberts, Cross & Leaman, 1990) secreted by the trophectoderm of the preimplantation conceptus in order to prevent regression of the corpus luteum and maintain progesterone production for pregnancy (Roberts & Anthony, 1994). INF-τ secretion is terminated shortly after adhesion by the elongated conceptus (~d21 in sheep) (Roberts et al., 1990). Unlike hCG and INF-τ, placental lactogens (PL's) arise from mature placental cells and have pronounced secretion throughout pregnancy in most species. The existence of placental lactogens has been confirmed in species such as primates, rodents and ruminants (Forsyth, 1984). During pregnancy the secretion of PL is reflective of placental growth (Grumbach, Kaplan, Sciarra & Burr, 1968; Spellacy, Buhi, Schram, Birk & McCreary, 1971), and is the most abundantly secreted hormone by the placenta. The exact biological role of PL has yet to be confirmed, however evidence suggests it may be a key player in placental and fetal growth through the modulation of maternal nutrient supplies.

PLACENTAL LACTOGEN

Background

Placental lactogens belong to the growth hormone (GH)/prolactin (PRL) gene family and are believed to have a significant role in modulating the mobilization of maternal nutrient supplies to support normal fetal growth during pregnancy. Placental lactogens have been isolated and purified in several species, including humans (Sciarra, Kaplan & Grumbach, 1963), goats (Currie, Card, Michel & Ignotz, 1990) sheep (Martal & Djiane, 1975), cattle (Murthy, Schellenberg & Friesen, 1982) and rodents (Robertson & Friesen, 1981). In general terms, primate PL's tend to be structurally more similar to GH, whereas non-primate PL's tend to be more similar to PRL (Anthony et al., 1995). In 1968 and 1971 Grumbach et al., and Spellacy et. al respectively, found a positive correlation linking circulating levels of maternal human PL (hPL), placental mass, and fetal weight, thus leading to further studies investigating the relationship between PL, maternal metabolism and fetal growth. Due to similarities among structure, and secretory patterns, the pregnant sheep has been an extensively used for examining the biological roles of PL in humans and other ruminant species. Despite several studies speculating the physiological role of PL, the exact biological function and factors regulating PL activity are still largely unidentified.

Placental Lactogen Structure

Placental lactogen is a protein hormone that is structurally similar to either GH or PRL depending on the species. The structural similarity of GH and PRL genes suggests that they have diverged from a common ancestral gene (Soares, 2004) as a

result of gene duplication approximately 350 million years ago (Miller & Eberhardt, 1983). PL is thought to have evolved after the divergence of GH and PRL and the separation of the main order mammals. This is supported by the fact that the structure of PL in humans and most primates closely resembles GH, while PL in rodents, ruminants and many other mammalian species is closer in structure to PRL (Soares, 2004; Roberts & Anthony, 1994).

The human PL genes are located within the human GH (hGH) gene cluster on chromosome 17 and share significant nucleotide and amino acid sequence homology with hGH (Hirt et al., 1987; Owerbach, Rutter, Martial, Baxter, & Shows, 1980; Roberts and Anthony, 1994). hPL shares 87% amino acid, and 93.5% nucleotide sequence homology with hGH but only 35% and 41% homology with hPRL respectively (Hirt et al., 1987; Owerbach et al., 1980; Shome & Parlow, 1977). There are three genes encoding hPL (hPL_1, hPL_3, hPL_4). hPL_3 and hPL_4 are transcriptionally active and encode proteins that are structurally and functionally identical, aside from a single amino acid within the signal sequence (position 24), where hPL_3 codes for an alanine residue and hPL_4 codes for a proline residue (Barrera-Saldafia, Seeburg, & Saunders, 1983). In contrast, hPL_1 is a non-functional pseudogene in which the resulting mRNA transcript is never fully processed (Chen et al., 1989; Hirt et al., 1987). hPL genes contain five exons and 4 introns (DeNoto, Moore & Goodman, 1981; Seeburg, 1982) that are transcribed and translated to yield a 191 amino-acid single polypeptide chain. The mature hPL protein is non-glycosylated with two disulfide bridges, and has an apparent molecular weight (M_r) of 22,000 (Li, 1972). Initially, hPL has an M_r of 25,000,

however cleavage of the signal sequence from the amino terminus yields the mature hPL (Barrera et al., 1983).

Despite the fact that ovine PL (oPL) is thought to have evolved from the PRL gene rather than GH, the oPL protein product has structural and functional characteristics more similar to that of hPL than other ruminant and rodent species (Liang, Limesand & Anthony, 1999). The oPL gene is located on chromosome 20 (Gootwine & Yossafi, 1998) and was found to span 11.2 kilobases (kb) (Liang, 1995). oPL shares 49% sequence homology with ovine PRL (oPRL) and only 28% with ovine GH (oGH) (Colosi et al., 1989; Warren, Liang, Krivi, Siegel & Anthony, 1990). In conservation with the GH/PRL gene family, the gene encoding oPL contains five exons and four introns (Liang et al., 1999). In 1999 Liang and colleagues characterized the structure of the oPL gene. They determined that exon 1 contains the 5' untranslated region (UTR) followed by the methionine start codon (AUG) and 17 amino acids of the leader sequence. Exon 2 encodes the remaining 19 amino acids of the leader sequence and 42 amino acid residues of the mature oPL protein. Exons 3,4 and 5 contain 35, 60 and 61 amino acid residues of the coding sequence respectively. Following the coding region is a translational stop codon, the 3' UTR and a polyadenylation signal. Like hPL, the mature oPL protein has an M_r of 22,000, is a single polypeptide chain that is non-glycosylated and contains two disulfide bridges (Warren et al, 1990). Additionally, oPL begins as a M_r 25,000 precursor polypeptide before the signal sequence is cleaved from the amino terminus (Warren et al, 1990). It should be noted that bovine PL (bPL) has 67% amino acid sequence identity with oPL, however there are multiple isoforms of the mature bPL protein that are glycosylated with

apparent molecular weights between 32,000-34,000 (Murthy et al., 1982; Arima & Bremel, 1983). The physiological consequence of these structural differences between ovine and bovine PL genes have yet to be determined.

Rodents are unique in that there is temporal expression of two different PL genes, PL-I and PL-II, both of which are structurally more similar to PRL than GH (Roberts & Anthony, 1994). PL-I is expressed by the trophoblast giant cells of the rodent placenta up until mid-gestation, at which point PL II expression becomes the predominant form of PL expressed by giant cells (Faria, Ogren, Talamantes, Linzer & Soares, 1991). PL-I has been described as a high molecular weight, N-linked glycoprotein in both the mouse (M_r 29,000-32,000, and M_r 36,500-42,000) (Colosi, Talamantes & Linzer, 1987) and rat (Mr 40,000-50,000) (Robertson, Gillespie & Friesen, 1982). After cleavage of the amino terminus signal sequence the mature proteins are 194 and 200 amino acid residues for mPL-I and rPL-I respectively (Colosi et al. 1987; Robertson et al. 1982). The mature rodent PL-II protein is structurally more similar to human and ovine PL proteins than PL-I. Mature PL-II in the rat and mouse is a 191 amino acid protein that is non-glycosylated with a M_r between 20,000-25,000 (Duckworth, Kirk & Friesen, 1986; Jackson, 1986). The temporal expression of two structurally distinct placental lactogen proteins throughout pregnancy in the rodent suggests that each protein also has a distinct biological role. Furthermore, the differences between rodent and human PL expression makes the rodent a less effective translational model for PL studies. For this reason rodent PL will not be a focus for discussion.

Transcriptional Regulation of Placental Lactogen

Since the expression of placental lactogen is limited to the syncytiotrophoblast of the human placenta and the binucleate cells of the ovine placenta, its regulation is unique within the GH/PRL gene family (Jiang & Eberhardt, 1994). Several early studies were conducted to describe the tissue specific transcriptional regulation of hPL genes, and were followed by similar studies to characterize the transcriptional regulation of oPL.

Studies conducted by Selvanyagam and collegues (1984) demonstrate hPL gene expression under the control of a promoter spanning a 500 base pair (bp) region 5' to the transcriptional start site (+1) of all hPL genes. The primary transcriptional start site, which accounts for 82-83% of all hPL transcripts is located approximately 30bp downstream from a consensus TATA box, and ~61bp upstream of the methionine translational start codon (Selvanyagam et al., 1984). Such arrangement of the TATA box suggests that it is involved in routine transcription initiation of hPL genes (Selvanyagam et al., 1984). An alternative transcriptional start site located further upstream of the +1 site contributes to 8% of all hPL transcripts, and is accompanied by several other transcriptional start sites that contribute only a minor portion of all hPL transcripts (Selvanyagam et al., 1984). Despite the essential role that the hPL promotor region plays in patterns of hPL gene expression, there is no evidence suggesting it modulates tissue specificity (Rogers, Sobnosky & Saunders, 1986). This is supported by the absence of a placenta specific transcription factor that binds to the hPL promotor region (Jiang & Eberhardt, 1994), such as the pituitary-specific factors that bind the hGH promoter (Bodner and Karin,1987; Bodner et al., 1988). In 1986 Rogers et al.

identified a 1kb enhancer region approximately 2kb downstream of the hPL_3 gene that influences tissue specificity. The enhancer was found to act in an orientation independent manner, positive to the transcriptional start site, and was able to act on heterologous promoters to stimulate transcription, all of which are defining traits of transcriptional enhancers (Rogers et al., 1986). While the enhancer was not proven to be completely tissue specific, it was shown to increase transcription efficiency in placental cell types several-fold more than that of pituitary cells (Rogers et al., 1986). Rogers originally reported that the hPL_3 enhancer was only located adjacent to the hPL_3 gene, with no evidence of a homologous enhancer region for any other hPL gene (Rogers et al., 1986); however, hPL_3 accounts for only 40% of hPL mRNA in placental tissue and hPL_4 makes up the remaining 60% of hPL mRNA (Barrera-Saldafia et al., 1983), which does not align with the reported enhancer activity. Clarification from a later study conducted by Chen et al., 1989 identified two homologous enhancer regions for hPL_1 and hPL_4 located approximately 2 kb from the 3' end of the respective hPL gene, the same relative location as the hPL_3 enhancer. Further studies localized the hPL enhancer to a 138bp region within the original enhancer region identified by Rogers et al. (Fitzpatrick, Walker & Saunders, 1990). The same study confirmed binding activity of placenta specific nuclear proteins to this unique hPL enhancer, culminating in highly efficient, tissue specific hPL gene expression (Fitzpatrick et al., 1990). In conjunction with the tissue specific enhancer region, there are also pituitary specific repressors located ~2kb upstream of the hPL genes to ensure suppression of placenta specific genes in the pituitary (Nachtigal, Nickel & Cattini, 1993).

In contrast to humans, sheep have only a single copy of the PL gene, but expression is still limited to placental tissue as it is in humans. The oPL transcriptional start site is located 91bp upstream from the AUG translational start codon (Liang et al., 1999), and tissue specific regulation of oPL gene expression has been mapped to a 4.5kb region of the 5' flanking sequence. More recently, a 383bp proximal promoter within this 4.5kb region has been identified as having trophoblast specific regulatory elements responsible for oPL gene transcription (Liang et al., 1999). Liang and colleagues used DNAse I protection assays and promoter deletion constructs to investigate the regulatory elements that may be responsible for the tissue specific enhancement of oPL gene transcription and discovered multiple *cis*-acting elements. Two sites within the proximal promoter containing the sequence GAGGAG (DR-1 sites) were found to be essential for maximal oPL expression. A similar sequence has been observed in the hPL promoter, and is the binding site for an SP1 transcription factor (Fitzpatrick et al., 1990). Transcription factors SP1 and SP3 were later confirmed to be the *trans*-acting elements interacting with the DR-1 sites of the oPL promoter (Jeckel, Limesand & Anthony, 2009). Oddly, a functional TATA consensus sequence was not identified within the oPL promoter. Conversely, DNase I protection observed within the minimal promoter (-124bp to +16bp) suggest the presence of an initiator element (Liang et al.,1999), which is necessary for the formation of the preinitiation complex and transcription in the absence of a TATA box (Aso, J.W. Conaway, & R.C. Conaway, 1994). In addition, an AP-2 binding element was also mapped to the minimal promoter of oPL as an additional tissue specific transactivator of oPL gene transcription. AP-2 typically requires interaction with alternative *cis*-acting elements to actively enhance

gene transcription (Liang et al., 1999). Localization of a GATA (Liang et al., 1999) as well as a Purα (Limesand, Jeckel & Anthony, 2004) cis-acting elements within the proximal promoter suggests that there may be interaction between the three cis-acting elements via trans-acting factors which confers oPL gene expression.

While the factors modulating PL gene transcription differ between humans and sheep, the structural similarities, patterns of gene expression throughout pregnancy and the proposed biological actions appear to be remarkably similar.

Mechanism of Action and Biological Role of Placental Lactogen

Since the discovery of placental lactogen more than 50 years ago, indirect evidence has suggested a role for PL in the modulation of maternal nutrient supplies to support healthy fetal growth. However, numerous studies regarding PL activity report contradictory findings due to inconsistent experimental design and the lack of an effective translational model; therefore, definitive conclusions for the exact biological role of PL have not been made.

Synthesis and Secretion

Placental lactogen is known to be synthesized by the syncytiotrophoblast of the human placenta and the binucleate cells of the sheep placenta, and is secreted into both maternal and fetal circulations (Gootwine, 2004). In humans production of hPL begins as soon as the syncytiotrophoblast has achieved full differentiation from cytotrophoblasts (Walker, Fitzpatrick, Barrera-Saldana & Resendez-Perez, 1991). hPL is packaged into secretory vesicles for exocytosis into maternal circulation at the apical

membrane of the chorionic epithelium (Caulfield, Duong & Rosenblatt). hPL mRNA has been localized specifically to the syncytiotrophoblast via *in situ* hybridization (Hoshina, 1982) and can be detected 5-10 days post implantation via immunohistochemistry (Beck, Gordon, Donald & Melvin,1969; Sciarra, Kaplan, Grumbach, 1963). As pregnancy progresses levels of hPL in maternal circulation increase relative to the mass of syncytiotrophoblast (Braunstein, Rasor, Engrail & Wade, 1980). hPL is first detected in maternal circulation during the third week postconception (Sciarra, Sherwood, Varwa & Lunberg, 1968) and will peak at 5-15 µg/ml (Walker et al., 1991) during the third trimester, making PL the most abundantly secreted hormone in primates (Walker et al., 1991). The route of hPL entry into fetal circulation is laregely unknown, and detection of hPL in fetal circulation is complicated by the fact that a human fetus cannot be catheterized. Nonetheless, measurements from intact human fetuses reveal hPL concentration of 5 ng/ml at 20 weeks of gestation and 20-30 ng/ml at birth (Hill, Freemark, Strain, Handwerger & Milner, 1988)

Placental lactogen production in sheep begins upon formation of binucleate cells (BNC) from mononucleate cells (Wooding & Burton, 2008; Wooding, 1992). It has been well established that oPL is packaged into membrane bound secretory vesicles within the BNCs and subsequent migration and fusion of BNCs with maternal epithelium is the primary mode of oPL transport into maternal circulation (Wooding & Burton, 2008). However, oPL transport to fetal circulation is not well defined (Byatt et al., 1992). oPL is not able to cross the placenta from maternal to fetal circulation (Grandis & Handwerger, 1983), and BNCs consistently migrate to the maternal side to release contents. Since the synthesis and packaging of BNC secretory granules occurs prior to BNC migration

(Wooding, Flint, Heap & Hobbs; 1981; Lee, Wooding & Brandon, 1986), there may be exocytosis of oPL into fetal circulation at the basal plate, however this is only speculation. The secretory pattern of oPL aligns closely to that of humans. In 1977, Martal and Djiane used radioimmunological methods to identify the presence of oPL in placental tissue around days 16-17 post-conception, which coincides with the formation of binucleate cells. By day 50 of gestation oPL is detectable in maternal circulation and steadily increases until parturition (days 131-141) (Chan, Robertson & Friesen, 1978). The mean maternal oPL serum concentrations in pregnancies of multiple fetuses is 232.4 ng/ml and 59.9 ng/ml in singleton pregnancies (Kappes, Warren, Pratt, Liang & Anthony, 1992). oPL in fetal circulation increases from day 60 until day 90 and plateaus until 135 days gestation (Kappes et al., 1992). Average fetal oPL serum concentrations are higher in pregnancies of multiple fetuses (25 ng/ml) compared to that of singleton pregnancies (20 ng/ml). Concentrations of oPL in fetal circulation do not follow the same pattern as maternal oPL likely due to the exponential increase in fetal blood volume throughout gestation (Kappes et al., 1992).

Maternal System

Since PL has obvious structural similarities to the GH/PRL gene family, it is also thought to have similar somatogenic properties in maternal circulation. Several studies have reported PL's involvement in the mobilization of free fatty acids (FFA), increased insulin resistance, increased glucose and nitrogen retention, as well as mammogenesis in the maternal system. In 1966, Grumbach et al. originally reported an increase in FFA of hypopituitary individuals treated with intramuscular injections of hPL. From this they

hypothesized that hPL may modulate the mobilization of maternal fat stores as source of energy for the mother in order to spare glucose and nitrogen for fetal energy. On the other hand, a 5 hour intravenous infusion of hPL into non-pregnant humans did not alter plasma FFA levels (Beck & Daughaday, 1967). A study conducted by Waters et al. 1985 reported a decrease in maternal FFA in oPL immunoneutralized pregnant ewes during late gestation. Interestingly, adipocytes isolated from rats in the fasted state displayed increased lipolysis in response to treatment with hPL, whereas adipocytes isolated from rats in the fed state experienced lipogenesis in the presence of hPL. Altogether, Grumbach's original hypothesis should not be refuted, however conditions such as stage of pregnancy and fed vs. fasted state may complicate PL's impact on maternal lipolysis.

It has been well established that a certain degree of maternal insulin resistance is associated with pregnancy, and reports suggest PL activity may promote insulin resistance. Pregnant women treated with insulin experience a smaller decline in corresponding serum glucose levels than that of non-pregnant women (Burt, 1956). Beck and Daughaday (1967) noted a comparable result when men and women treated with intravenous hPL responded with increased levels of circulating insulin without change in glucose metabolism. Additionally, the degree of insulin resistance throughout pregnancy increases temporally with the steady rise of hPL until term (Beck & Daughaday, 1967). The potential propogation of maternal insulin resistance by PL may coincide with increased glucose retention. Thordarson et al. (1987) reported that non-pregnant, non-lactating ewes had increased glucose and urea nitrogen without any change in insulin after treatment with partially purified PL for 36 hours. In addition,

increased oPL concentrations in pregnant ewes (mid to late gestation) induced by fasting lead to decreased glucose clearance rates compared to fed ewes; however, there was no difference when compared to non-pregnant ewes (Butler, Huyler, Grandis & Handwerger, 1987). In contrast, an experiment conducted on fasted pregnant and non-pregnant sheep treated with placental extracts containing oPL resulted in decreased maternal glucose and amino acid nitrogen, with a corresponding increase in maternal plasma insulin (Handwerger et al. 1976). Hypoglycemia induced pregnant ewes via intravenous insulin experienced decreased glucose as well as decreased oPL in maternal circulation (Brinsmead, Bancroft, Thorburn & Waters, 1981). In the same way, normal pregnant women who underwent glucose loading responded with decreased levels of hPL 30 minutes post treatment (Burt, Facog & Rhyne, 1970). Overall, inconsistencies in experimental design have complicated our interpretation of PL's role, nonetheless there is an apparent function for PL in modulating maternal metabolism.

Early studies conducted in pregnant, hypophysectomized rodents were the first to suggest that placental secretions, such as PL, may be responsible for mammary development and transient lactation (Forsyth, 1984). This was later supported by studies conducted in the sheep (Denamur & Martinet, 1961) and goat (Buttle, Cowie, Jones & Turvey, 1979), which include the temporal correlation between the rise of PL during pregnancy and the onset of mammary development (Martal and Dijiane, 1977; Hayden, Thomas, Smith & Forsyth, 1980). A number of studies support a mitogenic role for PL to promote mammary development. For instance, in 1978 McManus et al., and colleagues found that hPL administered to rats with benign human breast tumor

transplants had increased DNA synthesis within the ductal epithelium. Researchers also saw an increase in mammary DNA of nulliparous ewes treated with oPL after induced lactation (Kann, Delobelle-Beroide, Belair, Gertler & Djiane, 1999). Under conditions of decreased prolactin, placental lactogen may compensate by stimulating mammary epithelial cell differentiation (Buttle et al. 1979; Denamur & Martinet, 1961; Martal and Djiane, 1977). Thus, in addition to PL's important role in fetal development, its effect on mammogenesis may be imperative for healthy lactation and post-natal life.

Fetal System

Placental lactogen has been labeled the "growth hormone" of pregnancy due to its somatogenic properties within the fetal system. Much like the post-natal functions of growth hormone, an accumulation of data indicates that PL functions to promote muscle accretion, DNA synthesis, glycogen synthesis and IGF-I secretion in the fetus. This is supported by *in vitro* studies demonstrating increased amino acid uptake, DNA synthesis and IGF-I production in human fetal myoblasts, fibroblasts and hepatocytes after treatment with hPL (Handwerger & Freemark, 2000). In such studies, treatment with IGF-I antiserum diminished the effects of hPL treatment on DNA synthesis and amino acid transport studies, thus implying that PL may achieve its actions through the stimulation of fetal IGF-I secretion (Handwerger and Freemark, 2000). It was also found that hPL promoted DNA synthesis and insulin production in fetal pancreatic explants (Brelje et al., 1993). Fetal lambs given a chronic intravenous infusion of purified oPL during late gestation experienced increased IGF-I contentrations as well as in increase in hepatic glycogen storage (Schoknecht, Mcguire, Cohick, Currie & Bell, 1996). A

similar outcome was observed in rat and ovine hepatocytes treated with oPL (Freemark & Handwerger, 1985). In addition to PL's important anabolic role during advanced stages of fetal growth and development, PL has recently been associated with early embryonic development as well. In a study conducted by Seyoum and colleagues (1999), enhanced rat PL (rPL) in the rat visceral yolk sac stimulated DNA synthesis and yolk sac diameter. Furthermore, rPL increased DNA synthesis and crown-rump length in the cultured rat embryo. To conclude, there is an abundance of evidence that proposes a critical anabolic effect of PL on fetal metabolism, growth and development, however the exact route of action is still poorly understood.

Placental Lactogen Receptor Signaling

The lack of knowledge regarding the physiologic impact of PL is in part due to a poor understanding of PL's receptor activity and signaling cascade *in vivo.* A number of studies have documented PL's ability to bind the growth hormone receptor (GHR), prolactin receptor (PRL-R), and possibly a distinct PL receptor specific to fetal tissue; however, a definitive route for signal transduction has yet to be identified (Herman et al., 2000; Pratt, Kappes, Anthony, 1995). The GHR and PRL-R are members of the Jak/Stat superfamily, which are membrane bound receptor proteins that utilize tyrosine phosphorylation to initiate a signaling cascade (Gertler & Djiane, 2002; Rawlings, Rosler & Harrison, 2004). Because of the structural homology between PL, GH and PRL, it is probable that PL functions through a similar pathway. Human PL has been shown to bind to the GHR with low affinity (Kd 770 nM), and the prolactin receptor with an affinity of 0.046 nM (Handwerger & Freemark, 2000). It has also been proven to have binding

activity in the fetal liver, small intestine, adrenal, heart, lunch, pancreas, thymus, kidney, brain, testis and skeletal muscle (Handwerger & Freemark, 2000). A study conducted by Hill et al. (1988), analyzed the relative binding affinities of hPL, hGH and hPRL to fetal liver and skeletal muscle particulate cell membranes. In this study hPL bound to hepatic membranes with a stronger affinity than that of hGH and hPRL, and also exhibited distinct binding in the presence of hGH or hPL competition. Human PL was the only substrate with affinity for fetal skeletal muscle. A similar result was observed in sheep using fetal hepatic microsomes, in which a high affinity binding site was observed for oPL but no specific binding sites were identified for oGH or oPRL (Pratt, Kappes & Anthony, 1995). The Kd of the receptor was 122.1 +/- 8.2 pM, and the expression of the receptor (per mg of protein) remained constant throughout gestation (Pratt et al., 1995). A more recent experiment using gel filtration demonstrated that oPL also has the ability to heterodimerize the extracellular domains of the GHR and PRLR. Cells co-transfected with the GHR and PRLR exhibited increased oPL binding activity relative to single transfection with either GHR or PRLR (Herman et al., 2000). Despite PL's ability to bind both the GHR and PRLR, evidence suggests that PL likely functions through a unique PL receptor that has yet to be characterized. Further experimentation is necessary to purify and sequence the unidentified PL receptor.

INTRAUTERINE GROWTH RESTRICTION

Intrauterine growth restriction (IUGR) can be defined as a fetus that has failed to reach its growth potential in utero. IUGR is the second leading cause of perinatal mortality and complicates up to 6% of all pregnancies (Gagnon, 2003). In addition,

IUGR is associated with increased risk of stillbirth, neonatal death, cerebral palsy (Gagnon, 2003) and adult onset disease such as diabetes, hypertension, heart disease and stroke (Barker et al., 1989, 1990, 1993a, b). IUGR has been correlated to a number of environmental cues such as maternal malnutrition, substance abuse, diabetes, and multiple pregnancy, but abberations in placental function account for nearly 60% of IUGR cases in normally formed fetuses (Ghidini, 1996). Since the placenta is an incredibly adaptive endocrine organ that secretes a diverse group of growth factors and hormones, it is not surprising that alterations in a number of placental secretory products, such as placental lactogen, have been linked to IUGR (Regnault, Galan, Parker, & Anthony, 2002). Reduced PL has been associated with human and sheep IUGR pregnancies (Lea et al., 2007; Spellacy, Buhi & Birk, 1976). In fact, low concentrations of hPL on three different occasions during the last five weeks of pregnancy is correlated with a 71% risk of fetal distress, a 13% risk of perinatal mortality and a 57% risk of IUGR (Letchworth & Chard, 1972). Despite common links to IUGR, the exact biological driving forces have yet to be described, thus impeding our ability to confidently diagnose and treat IUGR pregnancies. Recent advancements in technology have allowed for more accurate assessment of growth restriction on an individual basis using fetal growth curves/rates and a series of ultrasound evaluations, however, IUGR is still commonly overlooked in low risk pregnancies (Figeuras & Gardosi, 2011). Since IUGR has only been documented in humans, it has been difficult to conduct the necessary studies to resolve IUGR. The creation of an animal model to accurately address the circumstances of IUGR in humans is critical. Several models for IUGR have been developed in the sheep, however these models employ techniques such as

maternal malnutrition, hyperthermia, carunclectomy, administration of glucocorticoids and uteroplacental embolization, all of which may have side effects that distort results (Anthony, Scheaffer, Wright & Regnault, 2003). Until a more accurate mode is developed to better understand biological principles of IUGR, it will continue to complicate pregnancies and post-natal life.

LENTIVIRAL GENE KNOCKDOWN

In the past, our inability to create sufficient gene knockdown models in the conceptus of large animals has deferred the identification of specific biological roles for PL during fetal growth. Fortunately, the recent discovery of microRNAs (miRNA) and development of short hairpin RNA (shRNA) methodologies has made targeted gene expression experiments attainable in large animals. MicroRNAs are small (20-23 nucleotides), endogenous non-coding RNA sequences, and are involved in gene regulation through translation inhibition or transcript degradation, depending upon target sequence homology (Bartel, 2004). To achieve their action, primary miRNAs are transcribed by RNA polymerase II or III cleaved by RNAse III Drosha enzyme, and exported out of the nucleus as a characteristic hairpin loop structure (pre-microRNA) (Winter, Jung, Keller, Gregory & Diederichs, 2009). In the cytoplasm, the loop is cleaved for degradation by RNase Dicer complexed with a double stranded RNA binding protein, to yield the mature hairpin structure (Winter et al., 2009). The functional miRNA is bound by Argonaute proteins containing RNase H activity and acts as a guide strand to complete the RNA induced silencing complex (RISC) that is the catalyst of miRNA gene regulation (Winter et al, 2009). In the case that the target sequence is not

completely complementary to the miRNA sequence, translational repression is likely to take place, otherwise the target transcript in cleaved for degradation by RISC (Anthony, Cantlon, Gates, Purcell & Clay, 2011; Winter et al., 2009). Small hairpin RNA for a particular gene of interest can now be introduced into cells via lentiviral infection and processed as endogenous miRNA for targeted gene repression. Lentiviral transgenesis is particularly useful for large animal models, such as the sheep and cow, which are notoriously difficult to genetically manipulate. For instance, lentiviral infection of the trophectoderm layer of hatched blastocysts allows for placenta-specific gene transgenesis and assessment of gene function (Anthony et al., 2011). Similarly lentiviral infection of zygotes has the potential to produce global gene knockdown in large animal species (Hofmann et al., 2003). shRNA for a particular gene of interest is designed using two inverted repeats that will compliment the mRNA of interest separated by a loop sequence and a poly(T) tail to terminate transcription (Paddison, Caudy, Bernstein, Hannon & Conklin, 2002; Anthony et al., 2011). The shRNA can then be incorporated into a lentiviral vector behind a polymerase II or III promoter. Once the lentivirus has infected the host cell the vector can stably integrate into the host genome to create long-term transgenesis (Anthony et al., 2011). This technique was utilized by Purcell et al., (2009) to target gene knockdown of proline-rich 15 (PRR15) during early conceptus development in the sheep. In this experiment uniform expression of a fluorescent marker in control groups and conceptus demise in PRR15 targeted knock down groups, validated the effectiveness of lentiviral mediated transgenesis in sheep. Using this approach, a more accurate role may be distinguished for placental lactogen as well as many other placental gene products.

SUMMARY

The placenta is an extraordinary endocrine organ that ties together maternal metabolism with fetal growth. Placental lactogen (PL), a protein hormone, is the most abundant secretory product of the placenta and appears to be a key player in the modulation of maternal nutrient supplies in support of fetal growth. PL is conserved across several mammalian species and was thought to have diverged from the GH and PRL genes approximately 350 million years ago. Due to its sequence and structural homology with either GH (primates) or PRL (ruminants and rodents), studies speculate that PL has similar somatogenic function to GH and PRL during pregnancy. PL is synthesized and secreted by the syncytiotrophoblast of the human placenta and the comparable binucleate cells of the sheep placenta beginning during the early stages of pregnancy. In both humans and sheep, secretion of PL is directly correlated to placental mass and fetal growth throughout pregnancy, further suggesting a cohesive relationship between the production of PL and the development to the feto-placental unit. In addition, indirect evidence indicates a role for PL in maternal and fetal amino acid, carbohydrate and lipid metabolism through the stimulation of insulin-like growth factors. Below normal circulating levels of PL have been associated with compromised fetal growth and IUGR. Even with an accumulation of data regarding the structure, secretion and potential functions of PL, the exact biological mechanism has yet to be characterized largely due to the lack of an effective animal model.

In terms of pregnancy, sheep are commonly used as a human model. Similarities between stages of fetal development, birth weights, gestation length, placentation, nutrient transport and the ability to use instrumentation are among the

benefits of using sheep as a model for human pregnancy. In the past, our inability to use transgenic techniques in ruminant species lead scientists to develop IUGR and placental lactogen deficient models using environmental stressors. Inconsistencies in the experimental design of such studies lead to variable results that are difficult to interpret. Recent advancements in lentiviral mediated shRNA gene knockdown techniques provide new opportunities to introduce transgenics to ruminant species such as the sheep.

In the following chapters, the generation of PL deficient pregnancies in sheep was accomplished using lentiviral mediated mRNA knockdown. Using quatitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), Western immunoblotting and radioimmunoassays the relative amount of PL gene knockdown was determined and subsequent physiologic responses were evaluated. Our ability to create PL knock down pregnancies in sheep allowed for more accurate assessment of PL's biological mechanisms during pregnancy and gave valuable insight towards the use of lenitviral genetic manipulation in future studies.

CHAPTER III: TESTING THE *IN VIVO* EFFICIENCY OF LENTIVIRAL-OVINE PLACENTAL LACTOGEN TARGETING VECTORS AT 55 DAYS OF GESTATION

INTRODUCTION

Intrauterine growth restriction is a leading cause of fetal and neonatal mortalities and morbidities. In addition, IUGR has been linked to increased risk of adult onset disease such as diabetes, hypertension, heart disease and stroke (Barker et al., 1989, 1990, 1993a,b). While the exact cause of IUGR is largely unknown, reduced PL has been associated with human and sheep IUGR pregnancies (Lea et al., 2007; Spellacy, Buhi & Birk, 1976). Even though PL was discovered more than 50 years ago, the exact contribution of this abundantly produced placental hormone to the development of a healthy pregnancy has yet to be identified. Original reports hypothesized that PL is an important modulator of maternal nutrient stores to support fetal growth (Grumbach et al., 1966). In the fetus, it is thought that PL plays an anabolic role through the repartitioning of nutrients and substrates for fetal use (Handwerger & Freemark, 2000). Even so, inadequacies in experimental design have complicated interpretations of PL's purpose. Since loss of function experiments often reveal crucial biological functions, the development of a PL deficient animal model would give useful insight towards PL's biological role during pregnancy. Recent advancements in microRNA (miRNA) and short-hairpin RNA (shRNA) technologies has made the development of gene knockdown models in large animal species more available. In 2009 Purcell and colleagues successfully knocked down the expression of proline-rich 15 (PRR15) *in vivo* using lentiviral mediated shRNA and inhibited conceptus elongation in the sheep.

Despite the apparent ability to knockdown the expression of genes such a PRR15 during early pregnancy in the sheep, the efficiency of mRNA degradation for highly expressed genes such as PL has yet to be assessed. Therefore, the purpose of this preliminary study was to test the *in vivo* efficacy of lentiviral-oPL targeting vectors in the sheep placenta at 55 days gestational age (dGA).

MATERIALS AND METHODS

All procedures conducted with animals were approved by the Colorado State University Institutional Animal Care and Use Committee.

Lentivirus Vector Construction

Lentiviral infection was used to stably integrate and express shRNA targeting oPL mRNA into the host cell. Twelve different lentiviral vectors were constructed (Table 3.1) using two different plasmids: pLentiLox3.7 (plasmid 11795; Addgene, Cambridge, MA) and pGIPZ (Thermo Fischer Scientific, Waltham, MA), and two different oPL targeting sequences: target 2 (tg2) and target 6 (tg6). The lentiviral vector pLentiLox3.7 (pLL3.7) originally contained the mouse RNA polymerase III U6 promoter, upstream of the multiple cloning sites for the introduction of shRNA cassettes, as well as the cytomegalovirus (CMV) promoter upstream of enhanced green fluorescent protein (EGFP). In order to introduce the human RNA polymerase III U6 promoter into the pLL3.7 and remove the mouse U6 promoter, the pLKO.1 vector (plasmid 10878; Addgene) which contains the desired human U6 promoter, was used as a template. Oligonucleotides containing oPL-targeting shRNA sequences (tg2 shRNA and tg6

shRNA) were designed with 5' *AgeI* and 3' *EcoRI* restriction sites for compatibility with pLKO.1 (Table 3.2)

Table 3.1 Lentiviral Vectors

Construct Name	Vector	Promoter	oPL Target
hLL3.7 tg6	pLentiLox 3.7	human U6	shRNA-tg6
hLL3.7 tg2	pLentiLox 3.7	human U6	shRNA-tg2
hEF-1 tg6	pGIPZ	human elongation factor-1α	shRNAmiR-tg6
hEF-1 tg2	pGIPZ	human elongation factor-1α	shRNAmiR-tg2
oPGK tg6	pGIPZ	ovine phosphoglycerate kinase-1	shRNAmiR-tg6
oPGK tg2	pGIPZ	ovine phosphoglycerate kinase-1	shRNAmiR-tg2
mPGK tg6	pGIPZ	mouse phosphoglycerate kinase-1	shRNAmiR-tg6
mPGK tg2	pGIPZ	mouse phosphoglycerate kinase-1	shRNAmiR-tg2
UbC tg6	pGIPZ	human ubiquitin C	shRNAmiR-tg6
UbC tg2	pGIPZ	human ubiquitin C	shRNAmiR-tg2
CMV tg 6	pGIPZ	cytomegalovirus	shRNAmiR-tg6
CMV tg 2	pGIPZ	cytomegalovirus	shRNAmiR-tg2

Table 3.2 oPL-targeting shRNA/shRNAmiR sequences

Oligonucleotide	Sequence (5'-3')
tg2 shRNA sense	CCGGAAGGTCATCAACTGCCACACTGTCGACAGTGTGGCAGTTGATGACCTTTTTTTG
tg2 shRNA anti-sense	AATTCAAAAAAAGGTCATCAACTGCCACACTGTCGACAGTGTGGCAGTTGATGACCTT
tg6 shRNA sense	CCGGAAGGCCAAAGTACTTGTAGACGTCGACGTCTACAAGTACTTTGGCCTTTTTTTG
tg6 shRNA anti-sense	AATTCAAAAAAAGGCCAAAGTACTTGTAGACGTCGACGTCTACAAGTACTTTGGCCTT
tg2 shRNAmiR sense	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGGTCATCAACTGCCACACTTTAGTGAA GCCACAGATGTAAAGTGTGGCAGTTGATGACCTTGCCTACTGCCTCGG
tg2 shRNAmiR anti-sense	AATTCCGAGGCAGTAGGCAAGGTCATCAACTGCCACACTTTACATCTGTGGCTTCACTAA AGTGTGGCAGTTGATGACCGCGCTCACTGTCAACAGCAATATACCTTC
tg6 shRNAmiR sense	AATTCCGAGGCAGTAGGCAAAGGACAAAGTACTTGTAGACGTACATCTGTGGCTTCACT ACGTCTACAAGTACTTTGGCCTGCGCTCACTGTCAACAGCAATATACCTTC
tg6 shRNAmiR anti-sense	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCAGGTACTTGTAGACGTAGTGAAGCCA CAGATGTACGTCTACAAGTACTTTGGCCTTTGCCTACTGCCTCGG

Oligonucleotides were ordered with 5' phosphates and PAGE purified (Integrated DNA Technologies, Coralville, Iowa). Oligonucleotides were resuspended in water to 60 pmol/µl, and one microliter of the sense oligonucleotide and one microliter of the anti-sense oligonucleotide were annealed in 48 µl of annealing buffer (100 mM potassium acetate; 30 mM HEPES-KOH, pH7.4; 2 mM magnesium acetate). The annealing mixture was incubated at 95ºC for 4 minutes, 70ºC for 10 minutes and 4ºC for 10

minutes. Annealed oligonucleotides were ligated into pLKO.1 digested with *AgeI*/*EcoRI* restriction enzymes and treated with calf intestinal phosphatase (New England Biolabs Inc., Ipswich, MA). The human U6 promoter and downstream oPL-targeting shRNA sequences within pLKO.1 were PCR amplified using a forward primer with a 5' *XbaI* restriction site (5'-TCTAGATTCACCGAGGGCCTATTTCCC-3') and a reverse primer containing a 3' *XhoI* restriction site (5'- GAATACTGCCATTTGTCTCGAGGTCG-3'). The resulting PCR amplicon was cloned into pPCR- Script Amp SK(+) vector (Stratagene, La Jolla, CA) and verified by sequencing (Colorado State University Proteomics and Metabolomics Facility, Fort Collins, CO). The human U6 promoter and oPL-targeting shRNA DNA fragment was digested from pPCR-Script Amp SK(+) using the *XbaI/XhoI* restriction enzymes. Subsequently, the DNA fragment was ligated into the pLL3.7 vector also digested with *XbaI/XhoI* and treated with calf intestinal phosphatase (New England Biolabs Inc). Insertion of the human U6 promoter and oPL-targeting shRNA sequence into pLL3.7 was verified by restriction digest to confirm the presence of the DNA fragment between *XbaI* and *XhoI* restriction sites, as well as the disappearance of an *AgeI* restriction site. In addition, sequencing was conducted to verify stability of the shRNA construct during cloning (Colorado State University Proteomics and Metabolomics Facility).

The lentiviral vector pGIPZ originally contained the cytomegalovirus promoter (CMV) upstream of the turbo green fluorescent protein (tGFP), puromycin and multiple cloning site for the introduction of microRNA mimic sequences (shRNAmiR). In addition to the CMV promoter, the pGIPZ vector was constructed to contain either the human elongation factor-1α (hEF-1), human ubiquitin C (UbC), mouse phosphoglycerate

kinase-1 (mPGK) or ovine phosphoglycerate kinase-1 (oPGK) promoters upstream of oPL tg2 or tg6 shRNAmiR sequences (Table 3.1). In order to create these constructs, oPL targeting shRNAmiR oligonucleotides (Table 3.2) were first inserted into the pGIPZ vector containing the CMV promoter. Oligonucleotides were designed with 5' *XhoI* and 3' *EcoRI* restriction sites. Oligonucleotides were ordered with 5' phosphates and PAGE purified (Integrated DNA Technologies). Oligonucleotides were resuspended in water to 60 pmol/µl, and one microliter of the sense oligonucleotide and one microliter of the anti-sense oligonucleotide were annealed in 48 µl of annealing buffer (100 mM potassium acetate; 30 mM HEPES-KOH, pH7.4; 2 mM magnesium acetate). The annealing mixture was incubated at 95ºC for 4 minutes, 70ºC for 10 minutes and 4ºC for 10 minutes. Annealed oligonucleotides were ligated into the pGIPZ digested with *XhoI/EcoRI* and treated with calf intestinal phosphatase (New England Biolabs Inc.,). Resulting positive clones were sequenced and analyzed for the correct orientation and shRNAmiR sequence. To introduce the different promoters into pGIPZ and remove the CMV promoter, a restriction site was engineered between the 3' end of the promoter and the transcription start site for tGFP. Within pGIPZ, the CMV promoter-tGFP cassette is flanked by *XbaI* and *BsrGI* restriction sites on the 5' and 3' ends, respectively. In addition, *BstBI* was identified as a unique restriction site between the 3' end of the promoter and the transcription start site for tGFP, and it was not found in the published sequences of the hEF-1, UbC, mPGK, and oPGK promoters. *XbaI* sites also are not present in the UbC, hEF-1, mPGK, promoters. Therefore, primers were designed to amplify the four promoters and include a 5' *XbaI* site in the forward primer and a 3' *BstBI* site on the reverse primer (Table 3.3).

Table 3.3 pGIPZ Promoter Primers

Primer	Forward (5'-3')	Reverse (5'-3')
UbC	TCTAGATATCGGATCTGGCCTCCG	TTCGAAGCTTCGTCTAACAAAAAAGCCAAAAAC
hEF-1	TCTAGACGCTCCGGTGCCCGTC	TTCGAAAGCTTCAGCTGTGTTCTGGCGGC
mPGK	TCTAGATTCGTCGACCTCGAAATTCTACC	TTCGAAAGATGGATGCAGGTCGAAAGG
oPGK	GCTAGCCTAAGCAACCTCTTTGTGCATCAGTTCATTC	TTCGAAGGCAGAGACTGAGACCGACTC
tGFP	TTCGAAACCATGGAGAGCGACGAGAG	GCTACTTGTACATTATTCTTCACCGGCATCTG

Since the oPGK promoter contains an *XbaI* site within its sequence, a forward primer was designed to have a 5' *NheI* site, which encodes the same 5' overhang sequence as *XbaI*. Templates used for the promoters include: pLenti6/UbC/V5-DEST (Addgene plasmid 17224) for UbC, pSico-EF1a-mCh-Puro (Addgene plasmid 31845) for EF-1, pSico-PGK-GFP (Addgene plasmid 12093) for mPGK, and sheep genomic DNA for the oPGK promoter. All PCR products were cloned into the pPCR- Script Amp SK(+) vector (Stratagene) and verified by sequencing using T3 and T7 primers (Colorado State University Proteomics and Metabolomics Facility). Similarly, tGFP was PCR amplified using a forward priming containing a 5' *BstBI* restriction site and reverse primer containing a 3' *BsrGI* restriction site (Table 3.3). The resulting PCR product was cloned into the pPCR- Script Amp SK(+) vector (Stratagene), and verified by sequencing using the T3 and T7 primers (Colorado State University Proteomics and Metabolomics Facility). Promoters and tGFP were digested from pPCR-Script Amp SK(+) using *XbaI* (or *NheI* for oPGK)*/BstBI* and *BstBI/BsrGI* respectively, and subsequently ligated into pGIPZ digested with *XbaI/BsrGI* and treated with calf intestinal phosphatase (New England Biolabs Inc.). Resulting positive clones were sequenced using forward (5'-GGAACGGCACTGGTCAACTTGG-3') and reverse (5'-CACACCGGCCTTATTCCAAGCG-3') sequencing primers and verified for the correct

orientation and sequence (Colorado State University Proteomics and Metabolomics Facility).

Lentivirus Generation

Lentiviral particles were generated in 293FT cells (Invitrogen) grown in high-glucose DMEM medium containing 10% fetal bovine serum at 37ºC and 5% CO_2. On the day of transfection, cells were grown to 60-70% confluency on a 150 mm tissue culture dish. For each 150 mm dish, a total of 80 ug of DNA was used for transfection: 50% oPL-targeting plasmid (40ug), 36% psPAX2 packaging plasmid (28.8 ug; Addgene) and 14% pMD2.G packaging plasmid (11.2 ug; Addgene). Plasmid DNA was vacuum concentrated using the Savant SpeedVac ISS 100 Centrifugal Evaporator (Thermo Scientific) and the resulting pellet resuspended in 1.8 mL sterile water and 200 ul of 2.5 M calcium chloride ($CaCl_2$). The DNA/$CaCl_2$ solution was added dropwise to 2 mL of a 2X Hepes buffered solution (HeBS; 140 mM NaCl, 25mM HEPES, 1.4 mM Na_2HPO_4, pH 7.1) in order to create a calcium phosphate precipitate. The precipate was incubated at room temperature for 20 minutes before being evenly distributed over the cells. After cells were incubated with the transfection mixture overnight, the medium was removed, cells were washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.3) and fresh complete medium was added. The lentiviral containing medium was collected at the end of 48 hours incubation with the 293FT cells and the supernatant was ultracentrifuged over a 20% sucrose cushion at 47,000xg for 2 hours at 4ºC. After ultracentrifugation, lentiviral pellets were re-suspended in either PBS (for cell culture) or chemical defined medium for late stage embryos (CDM-2) medium and

stored in aliquots at -80ºC. Frozen aliquots of concentrated lentivirus were thawed, initially diluted 1:40 and then 10-fold serially diluted using high-glucose DMEM supplemented with 10% FBS. Serial dilutions ranging 10^{-2} to 10^{-7} and 8 µg of polybrene (Sigma) per milliliter of media were added to either 12-well or 6-well tissue culture dishes of HEK 293 cells at 80% confluency. Cells were incubated with virus dilutions overnight, washed with PBS, and fresh complete medium was added. If titering pLL3.7 vector based lentivirus, the number of eGFP-positive cells within the last well containing fluorescent cells were counted 48 hours post infection. The titer was then calculated by dividing the number of live cells by the serial dilution factor from which the live cells were counted and multiplying this number by the initial dilution factor (40) used to dilute the original stock of virus. If titering pGIPZ vector based lentivirus, cells were infected with serial dilutions of virus in replicates of four, and put under puromycin selection (1 µg/ml media) 72 hours after infection. The titer was calculated between 5 and 7 days after the start of selection. To calculate the titer, the number of replicates of the last serial dilution containing live cells was input into the $TCID_{50}$ calculator (Hierholzer & Killington, 1996). The $TCID_{50}$ calculation was then multiplied by the initial dilution factor (40) used to dilute the original stock of virus in order to obtain a final concentration of virus.

Cell Culture, Transfection and Lentiviral Infection

ACH-3P, COS-7 and CHO cells were used to generate oPL overexpressing cell lines in order to test the knock-down efficiency of our oPL targeting lentiviral vectors. ACH-3P cells are a human first trimester trophoblast cell line derived from the fusion of

AC1-1 cells with primary first trimester trophoblasts (Hiden et al., 2007). COS-7 are an African Green Monkey kidney fibroblast cell line derived from the transformation of the CV-1 cell line with Simian Vacuolating Virus 40 (SV40) (Gluzman, 1981). CHO cells originated from the Chinese Hamster ovary through single cell cloning (Tijo & Puck, 1958). ACH-3P cells were grown in Ham's F-12 medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution containing 10,000 IU/mL penicillin, 10,000 ug/mL streptomycin and 25 ug/mL amphotericin. COS-7 and CHO cells were grown in high-glucose DMEM medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution containing 10,000 IU/mL penicillin, 10,000 ug/mL streptomycin and 25 ug/mL amphotericin. All cell lines were incubated at 37ºC and 5% CO_2.

The pcDNA3.1 vector (Invitrogen, Carlsbad, CA) was used to create stably transfected ACH-3P, COS-7 and CHO cell lines expressing the oPL gene. cDNA for the full oPL coding sequence was cloned and introduced into the pcDNA3.1 vector which contains a multiple cloning site for the insertion of cloned cDNA, as well as a neomycin (G418) resistance gene to allow for selection of stably transfected cells. Cell lines were stably transfected in three biological replicates on a 6-well dish, with SuperFect (10 µl/well; Quiagen, Valencia, CA) transfection reagent and 2 µg of pcDNA3.1 plasmid containing the oPL coding sequence according to the manufacturer's protocol. Transfected cells were selected with a final concentration of 400 µg/ml neomycin (Sigma). The amount of oPL mRNA in transfected cells was evaluated using quantitative real-time reverse transcriptase PCR (qRT-PCR), as described below.

The mRNA degradation efficiency of the twelve different lentiviral vectors (Table 3.1) was tested by generating lentiviral particles, as described below, and infecting oPL

overexpressing lines with each of the lentiviral vectors. ACH-3P, COS7 and CHO cells expressing oPL were infected in three biological replicates with lentivirus at a multiplicity of infection (MOI) of 100 viral particles per cell in 35-mm tissue culture dishes. At the time of infection, cells were also treated with 8µg of polybrene per milliliter of media (Sigma, St. Louis, MO). After overnight incubation, the medium containing virus and polybrene was aspirated, cells were washed in PBS and fresh complete medium was added. Cells were allowed to recover for 48 hours after treatment with virus. Cells infected with pGIPZ vector based lentivirus were selected with 1ug/mL of puromycin (Sigma). Cells infected with pLL3.7 vector based lentivirus were not put under antibiotic selection. The concentration of oPL mRNA in infected cell lines was measured by quantitative real-time reverse transcriptase PCR, as described below.

Blastocyst Collection and Transfer

Blastocyst collection and transfer were conducted based upon a previously described protocol (Purcell et al., 2009). Briefly, standing estrus in all ewes was determined using a vasectomized ram. Blastocyst donor and recipient ewes were synchronized by twice-daily i.m. injections of $PGF_{2\alpha}$ given four hours apart (10mg i.m per dose; Lutalyse, Pfizer, New York, NY). Forty-eight hours after synchronization, all donor ewes displaying standing estrus were bred by three different intact rams over a 24 hour time period. Late stage blastocysts were collected from donor ewes nine days after breeding. Donor ewes were fasted for approximately 12 hours prior to blastocyst collection. Donor ewes were euthanized using sodium pentobarbital (90 mg/kg, i.v.; Pentasol, Vibrac, Fort Worth, TX), a complete hysterectomy was performed and the

blastocysts were flushed from the uterus using DMEM/F-12 (1:1) medium supplemented with 0.25% BSA (Bovine Serum Albumin). Collected blastocysts were washed in Hepes buffered chemically defined medium for late stage embryos (HCDM-2; De La Torre-Shanchez, Garder, Preis & Seidel, 2006). To infect blastocysts with lentivirus, hatched blastocysts were incubated in 100ul drops overlaid with mineral oil. Each drop contained 100,000 TU/embryo of concentrated lentivirus, 80 ng of polybrene (8ng/µl; Sigma), and CDM-2. Drops containing blastocysts were cultured in 5% CO_2, 5% O_2 , and 90% N_2 at 37ºC for approximately 5 hours before being transferred. Blastocysts were washed in HCDM-2 immediately prior to transfer.

Recipient ewes that had displayed standing estrus behavior within 24 hours of the donor ewes were eligible to receive blastocysts. Recipient ewes were fasted for approximately 12 hours prior to blastocyst transfer. Recipient ewes were sedated using ketamine (12.5 mg/kg i.v.; Ketacine, Vetone, Boise ID) and diazepam (0.125 mg/kg, i.v.; Hospira, Lake Forest, IL), intubated with an endotracheal tube and maintained on 2L/min O_2 and 1%-2% isofluorane (Fluriso, Vetone) during the surgical embryo transfer. The transfer was performed using a fine, fire-polished glass pipette inserted through a puncture wound into the uterine horn ipsilateral to the corpus luteum (CL). One to three blastocysts were transferred into each recipient. Post-operative pain was managed by i.m. administration flunixin meglumine (100 mg i.m. per dose; Banamine, Merck, Whitehouse Station, NJ) immediately prior to surgery and again at 24, 48 and 72 hours after surgery. In addition amplicillin was administered (1g i.m. per dose; Polyflex, Boehringer Ingelheim Inc., St. Joseph, MO) immediately prior to surgery and again at 24

and 48 hours after surgery. After transfer, recipient ewes were given an ad libitum diet of alfalfa hay and monitored for 72 hours post-surgery.

Tissue Collection

For analysis of oPL gene knockdown, placental tissues were collected from mature crossbred ewes on Day 55 of gestation. Pregnant recipient ewes were sedated using ketamine (12.5 mg/kg i.v.; Ketacine, Vetone) and diazepam (0.125 mg/kg, i.v.; Hospira), intubated with an endotracheal tube and maintained on 2L/min O_2 and 1%-2% isofluorane (Fluriso, Vetone). Uterine vein blood was collected from right and left uterine horns of recipient ewes and allowed to coagulate at room temperature. All blood samples were centrifuged at 2,000xg for 10 minutes at 4ºC, serum was collected and stored at -80ºC until use. Recipient ewes were euthanized (90 mg/kg, i.v. sodium pentobarbital; Pentasol, Vibrac) and a fetectomy was performed. For all recovered fetuses: sex, body weight (FBW) and crown-rump-length (CRL), were recorded. Cotyledon tissue from approximately 15 placentomes nearest the umbilicus were excised, washed in PBS, stored in 50 mL conicals and snap frozen in liquid nitrogen. After being frozen, cotyledon tissue was pulverized using a mortar and pestel and stored at -80ºC until use. Pulverized placental tissue was analyzed for oPL mRNA by qRT-PCR and oPL protein by western immunoblotting.

RNA Isolation

Total cellular RNA was isolated from cells and day 55 cotyledon tissue using the RNeasy Mini Kit (Quiagen) according to the manufacturer's protocol. RNA

concentration was quantified using the Biotek Synergy 2 Microplate Reader (Biotek, Winooski, VT) and quality was measured by the 260/280 nm absorbance ratio. Samples were stored at -80ºC until use.

cDNA Synthesis and Quantitative Real-Time PCR

cDNA was synthesized from 1 ug of total cellular RNA by reverse transcription at 55ºC for 50 minutes using oligo(dT) primers (Superscript III Supermix; Invitrogen), according to the manufacturer's protocol. After cDNA synthesis, all cDNA samples, were treated with 5 units of RNase H (Thermo Fischer Scientific, Waltham, MA) at 37ºC for 20 minutes. To control for variance in efficiency of the reverse transcription reaction, cDNA was quantified using the Quant-iT OliGreen ssDNA Assay Kit (Invitrogen) according to the manufacturer's protocol and quality was measured according to the 260:280 nm absorbance ratio. An equal mass of cDNA was used for each sample in the qRT-PCR reaction.

Quantitative real time RT-PCR (qRT-PCR) was performed using the Lightcycler 480 (Roche Applied Science, Indianapolis, USA) and protocol as previously described (Purcell, et al., 2009). All primer sets for qRT-PCR were designed using Oligo software (Molecular Biology Insights, Cascade, CO) to amplify an intron-spanning product. Primer sequences and conditions for qRT-PCR are shown in Table 3.4. Prior to qRT-PCR, a PCR product for each gene was generated, cloned into PCR-Script (Agilent, Santa Clara, CA) and sequenced to verify amplification of the correct mRNA (Colorado State University Proteomics and Metabolomics Facility). In order to measure amplification efficiency and quantify the temporal expression of each gene, a standard

Table 3.4 PCR Primers, annealing temperature, and product sizes for cDNA used in real-time PCR Analysis

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Anneal (°C)	Product (bp)
oPL	ataaactccgaatccaaggtc	gttccttttgagtttgccag	58	177
oRPS15	atcattctgcccgagatggtg	tgctttacgggcttgtaggtg	58	124
oPol II	agtccaacatgctgacggacatga	agccaagtgccggtaattgacgta	60	332
oGAPDH	accactgtccactgccatcac	cctgcttcaccaccttcttga	60	268
hamGAPDH	ttctggcaaagtggaagttgt	ttgatgacaagctttccattc	55	137
hRPS15	ttccgcaagttcacctacc	cgggccggccatgctttacg	60	361

curve was generated from $1x10^{2}$ to $1x10^{-6}$ pg using a PCR product amplified from the sequenced plasmid for each gene. Amplification was detected using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 200nM of forward and reverse primer for each gene. Samples were amplified by 45 cycles of 95ºC for 1 min, 58ºC for 1 min, and 72ºC for 1 min. A melt-curve analysis was conducted by increasing the temperature 0.5ºC at 10-sec intervals from 55ºC to 100ºC. All standards were run in duplicate, and cDNA from each sample was run in triplicate. Product specificity was confirmed by observation of a single melt curve for each gene. The starting quantity (picograms) was normalized to the starting quantity (picograms) of the respective housekeeping genes. Housekeeping genes include: human ribosomal protein S15 (hRPS15), hamster GAPDH (hamGAPDH), ovine ribosomal protein S15 (oRPS15), ovine GAPDH (oGAPDH), and ovine RNA Polymerase II (oPol II).

Western Immunoblotting

Cellular protein from Day 55 cotyledon tissue was assessed using Western immunoblot analysis. To isolate total cellular protein, 0.1 g of tissue was lysed in 500 ul of Western lysis buffer (0.48 M Tris, pH 7.4; 10 mM EGTA, pH 8.6; 10 mM EDTA, pH 8;

0.1 mM PMSF; 0.1 mM AEBSF; 0.0015 mM Pepstatin A; EG4 0.0014 mM; 0.004 mM Bestatin; 0.002 mM Leupeptin; 0.00008 mM Aprotinin) and sonicated on ice. After sonication, samples were centrifuged at 14,000xg for 3 minutes and the supernatant collected for protein quantification. Protein concentration was measured using the Bradford assay method (Bradford, 1976) and the absorbance read at 595 nm using the Biotek Synergy 2 Microplate Reader (Biotek). A total of 25 ug of protein was electrophoresed through 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY) at a constant 200 V for one hour at 4ºC. Protein was then transferred to a 0.45 um pore nitrocellulose membrane at a constant 100 V for one hour at 4ºC. Membranes were blocked in 5% non-fat dry milk (NFDM)-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for one hour at room temperature to reduce non-specific binding, then incubated with primary antibody at 4ºC overnight. Membranes were washed in TBST, and then incubated with a horseradish peroxidase-conjugated secondary antibody for 3.5 hours at room temperature. To detect oPL a polyclonal antibody generated in the rabbit (1:15,000 dilution; α-oPL-54; Kappes et al., 1992) in conjunction with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; product no. sc-2004; Santa Cruz Biotechnology Inc., Dallas, TX) was used. As a loading control protein and housekeeping to normalize oPL, a polyclonal antibody to β-actin (1:1,000 dilution; product no. sc-47778; Santa Cruz Biotechnology Inc.) bound by a horseradish peroxidase-conjugated secondary antibody (1:5,000; product no. sc-2005; Santa Cruz Biotechnology Inc.) was used. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham, Pittsburgh, PA), and imaged

using the ChemiDoc XRS+ chemiluminescence system (BioRad). Densitometry calculations were performed using the Image Lab Software (BioRad).

Radioimmunoassay

The concentration of serum oPL was assessed by radioimmunoassay (RIA) as previously described (Kappes et al., 1992) with an intra-assay coefficient of variance of 3.3%.

Statistical Analysis

All data were analyzed using Student's *t*-test to compare differences between the NMC and responder pregnancies. A standard of $P \leq 0.05$ set as statistically significant.

RESULTS

In vitro analysis of oPL mRNA Degradation

Prior to testing oPL shRNA knockdown *in vivo*, the efficiency of oPL mRNA degradation by two different lentiviral vector types was tested *in vitro*: pLentiLox 3.7 containing the human U6 promoter (hLL3.7) and GIPZ. The hLL3.7 is considered a "first generation" vector, and uses the RNA polymerase III enzyme (Pol III) to initiate gene transcription at the human U6 promoter that drives the expression of a simple stem-loop shRNA structure. Unlike the hLL3.7 vector, "next generation" vectors, such as GIPZ, use the RNA polymerase II (Pol II) enzyme to initiate gene transcription, and drive the expression of a microRNA mimic shRNA (shRNAmiR).

The hLL3.7 and GIPZ vectors were constructed to contain one of six different promoters driving the expression of either oPL shRNA/shRNAmiR target 2 or target 6 (Table1.). In order to test the efficiency of each lentiviral construct, ACH-3P, COS-7 and CHO cell lines overexpressing oPL were established and subsequently infected with each of the lentiviral constructs. The degree of oPL mRNA degradation was determined using quantitative real-time RT-PCR (qRT-PCR) and the average percent oPL mRNA knockdown was calculated and compared to oPL overexpressing control lines (Table 3.5).

Table 3.5 Average percent oPL mRNA knockdown compared to oPL overexpressing control lines.

Construct	ACH-3P	COS-7	CHO
hLL3.7 tg6	43.49	99.90	88.66
hLL3.7 tg2	28.84	99.99	80.77
hEF-1 tg6	92.09	72.27	-27.02
hEF-1 tg2	96.58	99.88	99.92
oPGK tg6	95.91		99.91
oPGK tg2	95.61		59.31
mPGK tg6	98.53	99.99	97.58
mPGK tg2	90.29	99.97	98.61
UbC tg6	96.20	99.99	99.53
UbC tg2	36.05	99.99	
CMV tg6	64.72	35.94	7.92
CMV tg2	46.40	66.57	70.52

Since the hLL3.7 tg6 vector appeared to be the most efficient “first generation vector”, it was chosen to be tested *in vivo.* The hEF-1 tg2 vector was also chosen to test in vivo given that it exhibited a consistent degree of oPL mRNA knockdown across all cell types, and it would allow for comparison between oPL target 2 and oPL target 6. Finally, since the oPGK tg6 vector contains an intrinsic sheep promoter and stimulated

a greater degree of oPL mRNA knockdown in ACH-3P and CHO cells than its oPL target 2 counterpart, it was also chosen to test *in vivo*.

Day 55 Fetectomy and Fetal Measurements

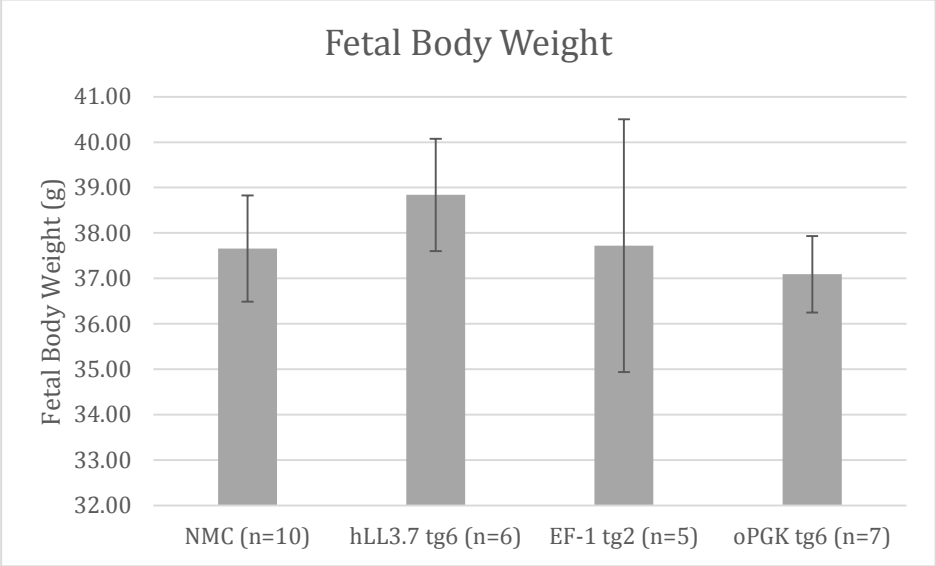
A total of 29 blastocysts were infected with lentivirus and transferred to 13 recipient ewes. Embryo transfer success rates are summarized in Table 3.6

Table 3.6 Summary of sheep embryo transfer

	Treatmet		
Parameter	**hLL3.7 tg6**	**hEF-1 tg2**	**oPGK tg6**
Recipient Ewes	4	5	4
Embryos Transferred	11	10	8
Embryos Recovered	8	7	7
Percentage Success	72.7	70.0	87.5
Total Embryos Transferred	29		
Total Embryos Recovered	22		
Total Percentage Success	75.9		

Fetal body weights and crown-rump lengths (CRL) were recorded and results are summarized in Figure 3.1. All lentivirus treated fetuses were compared to 10 fetuses recovered from 4 naturally mated control (NMC) ewes. There were no differences in fetal body weights or CRL between NMC and any of the treatment groups, however at 55 dGA we were not anticipating a growth restricted phenotype.

A.



B.

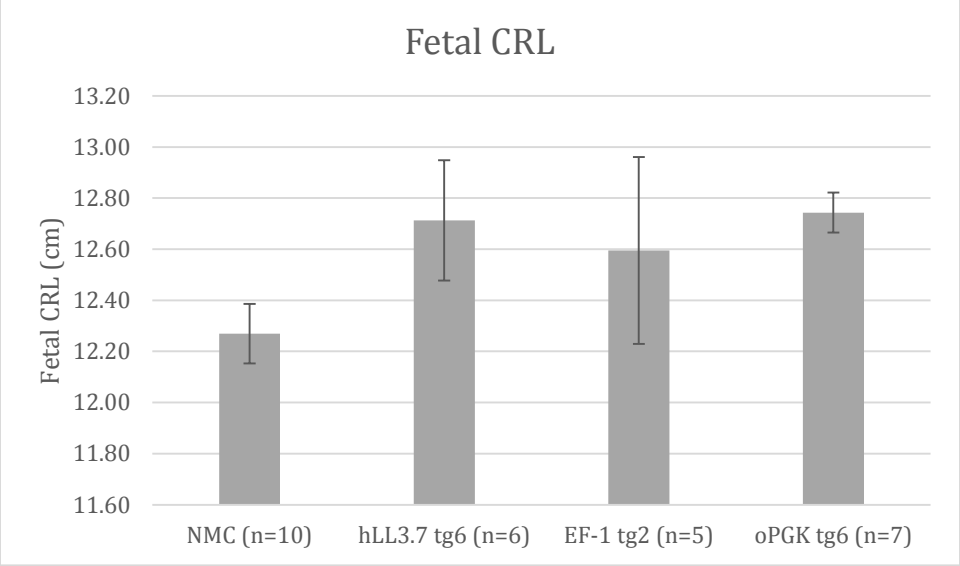


Figure 3.1 (A) Average fetal body weights for each treatment group. (B) Average crown-rump lengths for each treatment group. Treatment groups were compared to the NMC group with no significant differences.

Serum oPL Measurements

At 55 dGA uterine vein (UtV) blood samples were collected from right and left uterine horns for the analysis of circulating oPL concentrations. Based on a 95%

confidence interval calculated from the UtV oPL concentrations of the NMC (n=4), anything that fell below the lower limit (30.78 ng/ml) of the 95% confidence interval was considered a responder pregnancy. As a result, 3 out of 4 hLL3.7 tg6 pregnancies, 3 out of 4 hEF-1 tg2 pregnancies, and 4 out of 4 oPGK tg6 pregnancies were classified as responder pregnancies. This classification for responders was carried over for the analysis of qRT-PCR data, as well as western immunoblot data. Compared to NMC (36.59 ± 2.96 ng/ml), UtV oPL concentrations, 16.64 ± 3.21 ng/ml (hLL3.7 tg6), 12.83 ± 0.96 ng/ml (hEF-1 tg2) and 18.81 ± 2.46 ng/ml (oPGK tg6) were reduced (P≤0.05), representing 55%, 65% and 49% reduction in oPL secretion in these pregnancies, respectively (Figure 3.2).

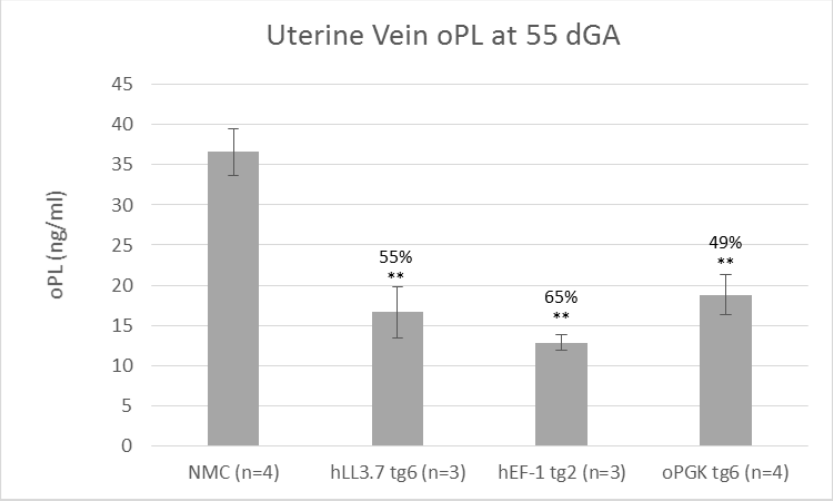


Figure 3.2 Effect of lentivirus targeting oPL mRNA on uterine vein oPL concentrations. Average oPL concentrations (ng/ml) for each treatment group were compared to NMC. ** indicates p≤0.05 when compared to NMC.

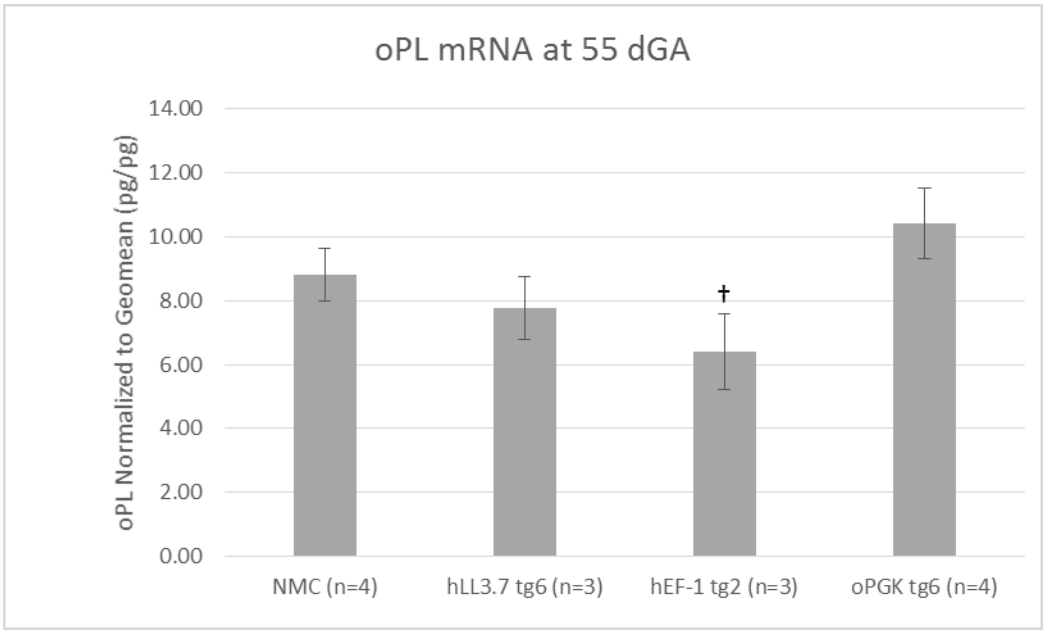
Concentration of oPL mRNA and Protein in Cotyledon Tissue

The concentration of oPL mRNA and protein was measured in cotyledon tissue at 55 dGA for the analysis of oPL gene expression. Quantitative real-time RT-PCR was

performed to determine the temporal expression of oPL mRNA in cotyledon tissue, and oPL mRNA concentrations were normalized to the geomean of all housekeeping genes (oRPS15, oGAPDH, oPol II). Compared to the NMC (8.83 ± 0.82 pg/pg), hLL3.7 tg6 responder pregnancies (7.78 ± 0.97 pg/pg), hEF-1 tg2 responder pregnancies (6.40 ± 1.17 pg/pg), and oPGK tg6 (10.42 ± 1.11 pg/pg) were not significantly different (Figure 3.3). However, hEF-1 tg2 responders showed a trend towards decreased mRNA ($P \leq 0.1$) and the hLL3.7 tg6 responders were numerically decreased, representing 28% and 12% oPL mRNA knock-down respectively, when compared to NMC.

The presence of oPL in 55 dGA cotyledon tissue was detected using western immunoblotting and densitometry measurements were calculated to compare responder pregnancies to NMC. The relative levels of oPL were calculated and normalized to β-actin. The hEF-1 tg2 responder pregnancies (0.66 ± 0.11) resulted in significantly reduced ($P \leq 0.05$) levels of oPL when compared NMC (1.29 ± 0.19), which is equivalent to a 49% oPL knock-down (Figure 3.3). Alternatively hLL3.7 tg6 responders (1.12 ± 0.25) and oPGK tg6 responders (0.96 ± 0.14) were not statistically significant (Figure 3.3) when compared to NMC. Despite increased concentrations of oPL mRNA in oPGK tg6 responders compared to NMC, the levels of oPL in cotyledon tissue were only numerically decreased relative to the NMC pregnancies.

A.



B.

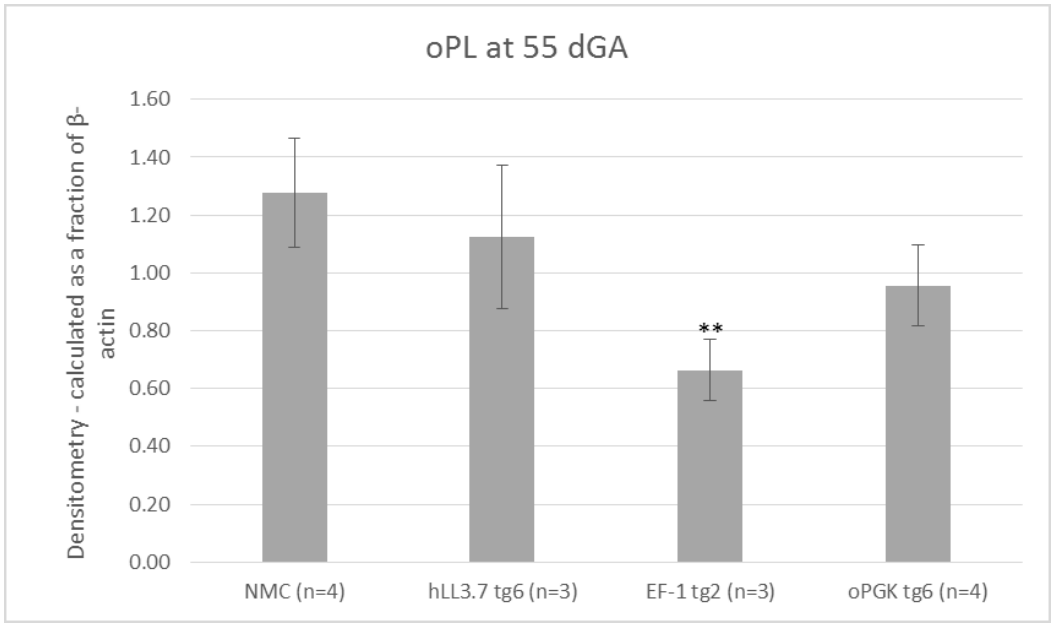


Figure 3.3 Effect of lentivirus targeting oPL mRNA on oPL mRNA and protein levels in 55 dGA cotyledon tissue. (A) Average oPL mRNA (pg/pg) for each treatment group measured by quantitative real-time RT-PCR and normalized to the geomean. † indicates P≤0.10 when compared to NMC. (B) Average oPL for each treatment group measured by western immunoblotting and normalized to β-actin. ** indicates P≤0.05 when compared to NMC.

DISCUSSION

A series of *in vitro* studies were conducted to test the mRNA degradation efficiency of twelve different oPL targeting lentiviral constructs in three different mammalian cell lines over expressing oPL. Consequently, three different lentiviral constructs were chosen to test *in vivo*: (1) the pLL3.7 vector base containing the human U6 promoter driving the expression of oPL targeting sequence 6 as shRNA (hLL3.7 tg6), (2) the pGIPZ vector base containing the human elongation factor-1α promoter driving the expression of oPL targeting sequence 2 as an shRNAmiR (microRNA mimic) (hEF-1 tg2), and (3) the pGIPZ vector base containing the ovine phosphoglycerate kinase promoter driving the expression of oPL targeting sequence 6 as an shRNAmiR (oPGK tg6). The hLL3.7 vector uses the RNA polymerase III (Pol III) enzyme to initiate gene transcription at the human U6 promoter, and drive the expression of a simple stem-loop shRNA structure. Pol III is a constitutively active replicase enzyme involved in cellular replication as well as other areas of DNA metabolism (Kelman and O'donnell, 1995). Alternatively, the GIPZ base vector uses the RNA polymerase II (Pol II) enzyme to initiate transcription and drive the expression of the shRNAmiR. The Pol II enzyme is present in eukaryotic cells and catalyzes the transcription of DNA into mRNAs, small nuclear RNAs (snRNA) and endogenous microRNAs (miRNA) (Sims, Mandal & Reinberg, 2004). Additionally, the shRNAmiR of GIPZ was designed to have the 5' and 3' flanking sequences of the human mir30 endogenous miRNA which is believed to provide more consistent and effective targeting of mRNA (Silva et al., 2005).

At 55 dGA serum concentrations of oPL from uterine vein blood were reduced in lentiviral treated pregnancies, suggesting that lentivirus targeting the degradation of oPL

mRNA reduces the secretion of oPL into maternal circulation during early pregnancy. However, the corresponding effect on oPL mRNA and protein levels in placental tissue was not as significant. The complexity of these results may be reflective of experimental design. The inability to control for uniform lentiviral infection and viral genome integration within the host trophectoderm cells is one of the major challenges facing *in vivo* lentiviral experiments. For instance, the number of viruses that infect each cell may vary, thus leading to a certain degree of mosaicism of the shRNA transgene expression among the binucleate cells of the placenta. In this study, both non-expanded and fully expanded blastocysts were infected with virus. Conceivably, there is less surface area for infection of the non-expanded blastocysts, which may further enhance the variability of infected cells. It was also noted that under circumstances where blastocysts were mechanically hatched, there was a lesser degree of oPL knock down and decreased pregnancy success rates. In future studies, infected blastocysts should be naturally hatched and expanded at the time of virus infection. In addition, the location within the host genome at which the viral DNA is integrated may impact the degree of transgene expression within a particular cell. This reinforces the concept of mosaicism, and may also explain the variation in oPL expresson observed between mRNA and protein in cotyledon tissue. Furthermore, day 9 blastocysts were incubated in 100 ul drops of CDM-2 in groups of up to 3 blastocysts with 100,000 TU of concentrated virus per blastocyst. This method of blastocyst culture may increase the variability of lentiviral infection and integration between blastocysts, and therefore skew the results. In future studies, single blastocysts should be cultured in individual drops of media with 100,000 TU of concentrated virus for more consistent

infection/integration rates between blastocysts. In this study only a portion of the placenta closest to the umbilicus was sampled and whole placental weight was not recorded since we were not anticipating an effect on placental weight. However, sampling from the whole placenta and record of placental weight may have provided useful supporting evidence to confirm the the efficiency of *in vivo* oPL targeting vectors and potentially reduce variability caused by mosaicism. Finally, IUGR is common among twin and triplet pregnancies in nature, thus performing double and triple blastocyst transfers complicates data interpretation. Since live fetuses were recovered from 75.9% of embryos transferred, performing single blastocyst transfers may provide less variation within treatment groups. Adjusting for such inconsistencies in experimental design would likely improve the degree of oPL knock down and lead to more reliable results. In conclusion, the use of lentivirus targeting oPL mRNA degradation is a useful approach to generating oPL deficient sheep pregnancies.

SUMMARY

Intrauterine growth restriction (IUGR) is a leading cause of fetal and neonatal mortalities and morbidities, and has also been linked to chronic adult onset disease. While the biologic driving forces of IUGR remain unidentified, placental lactogen (PL) is consistently reduced in IUGR pregnancies and may be an important regulator of healthy fetal growth. Placental lactogen is a protein hormone secreted in abundance by the placentae of several species, including humans and sheep, however its exact biological function during pregnancy has yet to be determined since its discovery more than 50 years ago. The failure to identify PL's function may be traced back to the absence of an

appropriate PL deficient animal model. Recent advancements in microRNA (miRNA) and short-hairpin RNA (shRNA) gene manipulation techniques have created the opportunity to develop a PL deficient model in the sheep. In this preliminary study, the in vivo efficiency of three lentiviral-oPL targeting vectors was assessed at 55 days of gestation (dGA). Despite inconsistency in mRNA and protein data from cotyledon tissue, the effect of lentivirus targeting oPL mRNA lead to significantly decreased oPL concentrations in maternal circulation. After reevaluating experimental design, these data suggest that *in vivo* lentiviral mediated gene knock-down techniques provides the opportunity to develop an effective PL deficient ovine model.

CHAPTER IV: THE IMPACT OF OVINE PLACENTAL LACTOGEN DEFICIENCY ON NEAR-TERM FETAL GROWTH AND DEVELOPMENT

INTRODUCTION

Intrauterine growth restriction (IUGR) complicates up to 6% of all human pregnancies and occurs when a fetus has failed to reach its growth potential (Gagnon, 2003). IUGR is the second leading cause of perinatal mortality and has been linked to increased risk of adult onset disease such as diabetes, hypertension, heart disease and stroke (Barker et al., 1989, 1990, 1993 a,b; Gagnon, 2003;). While several environmental cues such as maternal malnutrition, substance abuse, diabetes and multiple pregnancy have been associated with the development of IUGR, abberations in placental function account for nearly 60% of IUGR cases in normally formed fetuses (Ghidini, 1996). Additionally, in pregnancies complicated by IUGR, placental lactogen (PL) concentrations are reduced in maternal circulation for both humans and sheep (Lea et al., 2007; Spellacy et al.,1976). PL is a placenta specific protein hormone that belongs to the growth hormone/prolactin gene family, and is the most abundant secretory product of the placenta. The syncytiotrophoblasts of the human placenta and the binucleate cells of the sheep placenta are the sites of PL synthesis and secretion into maternal and fetal circulations (Gootwine, 2004). The production of PL begins during the earliest stages of placental development, and continues throughout gestation. (Walker et al., 1991; Wooding, 2008).

Since its discovery more than 50 years ago, there has only been indirect evidence suggesting a role for placental lactogen in the modulation of maternal and fetal

amino acid, carbohydrate and lipid metabolism to support healthy fetal growth. Maternal PL is reported to be involved in the mobilization of free fatty acids, increased insulin resistance and increased glucose and nitrogen retention (Beck & Daughaday, 1967; Brinsmead et al., 1981; Burt et al., 1970; Butler et al., 1987; Grumbach et al., 1966; Handwerger et al.1976; Thordarson et al., 1987; Waters et al., 1985). Fetal PL is believed have an anabolic role possibly by eliciting the actions of insulin-like growth factors (Schoknecht *et* al., 1996). However, inconsistencies in experimental design have led to variable results regarding PL's function, thus making a precise role for PL difficult to define. In order to determine the exact biological role for PL during pregnancy, the development of a PL deficient animal model is critical.

To date, a PL deficient model has not been achieved in any species. While gene knockout models in rodents have been available for several decades, rodents uniquely express two structurally distinct forms of PL during pregnancy (Robertson & Friesen, 1981), which may not be readily translatable to primates. Furthermore, the lack of gene knockout techniques in large animal species has inhibited the development of a PL deficient model. Fortunately, recent experiments conducted by Purcell et al. (2009) demonstrated successful knock down of proline-rich 15 (PRR15) expression *in vivo* using lentiviral mediated shRNA and inhibited conceptus elongation in the sheep. This evidence suggests that lentiviral mediated shRNA may be an effective method for the development of a PL deficient sheep model. Therefore, the objective of this study was to knock down ovine PL (oPL) expression *in vivo* using lentiviral mediated shRNA in order to determine the impact of ovine PL (oPL) deficiency during pregnancy on near

term (135 days of gestation) fetal growth. We hypothesized that oPL deficiency could result in intrauterine growth restriction of fetal lambs.

MATERIALS AND METHODS

All procedures conducted with animals were approved by the Colorado State University Institutional Animal Care and Use Committee.

Lentivirus Vector Construction

Lentiviral infection was used to stably integrate and express shRNA targeting oPL mRNA into the host cell. Four different lentiviral vectors were constructed (Table 4.1) using two different plasmids: pLentiLox3.7 (plasmid 11795; Addgene, Cambridge, MA) and pGIPZ (Thermo Fisher Scientific, Waltham, MA), two different oPL targeting sequences: target 2 (tg2) and target 6 (tg6), and a scrambed control sequence (SC).

Table 4.1 Lentiviral Vectors

Construct Name	Vector	Promoter	oPL Target
hEF-1 SC	pGIPZ	human elongation factor-1α	shRNAmiR-SC
hEF-1 tg2	pGIPZ	human elongation factor-1α	shRNAmiR-tg2
hEF-1 tg6	pGIPZ	human elongation factor-1α	shRNAmiR-tg6
hLL3.7 tg6	pLentiLox 3.7	human U6	shRNA-tg6

The lentiviral vector pLentiLox3.7 (pLL3.7) originally contained the mouse RNA polymerase III U6 promoter, upstream of the multiple cloning site for the introduction of shRNA cassettes, as well as the cytomegalovirus (CMV) promoter upstream of enhanced green fluorescent protein (EGFP). In order to introduce the human RNA polymerase III U6 promoter into the pLL3.7 and remove the mouse U6 promoter, the pLKO.1 vector (plasmid 10878; Addgene), which contains the human U6 promoter, was

used as a template. Oligonucleotides containing the oPL tg6 shRNA sequence were designed with 5' *AgeI* and 3' *EcoRI* restriction sites for compatibility with pLKO.1 (Table 4.2). Oligonucleotides were ordered with 5' phosphates and PAGE purified (Integrated DNA Technologies, Coralville, Iowa). Oligonucleotides were resuspended in water to 60 pmol/µl and one microliter of the sense oligonucleotide and one microliter of the anti-sense oligonucleotide were annealed in 48 µl of annealing buffer (100 mM potassium acetate; 30 mM HEPES-KOH, pH7.4; 2 mM magnesium acetate). The annealing mixture was incubated at 95ºC for 4 minutes, 70ºC for 10 minutes and 4ºC for 10 minutes. Annealed oligonucleotides were ligated into pLKO.1 digested with *AgeI/EcoRI* restriction enzymes and treated with calf intestinal phosphatase (New England Biolabs Inc., Ipswich, MA).

Table 4.2 oPL-targeting shRNA/shRNAmiR sequences

Oligonucleotide	Sequence (5'-3')
tg6 shRNA sense	CCGGAAGGCCAAAGTACTTGTAGACGTCGACGTCTACAAGTACTTTGGCCTTTTTTTG
tg6 shRNA anti-sense	AATTCAAAAAAAGGCCAAAGTACTTGTAGACGTCGACGTCTACAAGTACTTTGGCCTT
tg2 shRNAmiR sense	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGGTCATCAACTGCCACACTTTAGTGAA GCCACAGATGTAAAGTGTGGCAGTTGATGACCTTGCCTACTGCCTCGG
tg2 shRNAmiR anti-sense	AATTCCGAGGCAGTAGGCAAGGTCATCAACTGCCACACTTTACATCTGTGGCTTCACTAA AGTGTGGCAGTTGATGACCGCGCTCACTGTCAACAGCAATATACCTTC
tg6 shRNAmiR sense	AATTCCGAGGCAGTAGGCAAAGGACAAAGTACTTGTAGACGTACATCTGTGGCTTCACT ACGTCTACAAGTACTTTGGCCTGCGCTCACTGTCAACAGCAATATACCTTC
tg6 shRNAmiR anti-sense	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCAGGTACTTGTAGACGTAGTGAAGCCA CAGATGTACGTCTACAAGTACTTTGGCCTTTGCCTACTGCCTCGG

The human U6 promoter and downstream oPL tg6 shRNA sequence within pLKO.1 was PCR amplified using a forward primer with a 5' *XbaI* restriction site(5'-TCTAGATTCACCGAGGGCCTATTTCCC-3') and a reverse primer containing a 3' *XhoI* restriction site (5'- GAATACTGCCATTTGTCTCGAGGTCG-3'). The resulting PCR amplicon was cloned into the pPCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA)

and verified by sequencing (Colorado State University Proteomics and Metabolomics Facility, Fort Collins, CO). The human U6 promoter and oPL tg6 shRNA DNA fragment was digested from pPCR-Script Amp SK(+) using *XbaI/XhoI* restriction enzymes. Subsequently, the DNA fragment was ligated into the pLL3.7 vector also digested with *XbaI/XhoI* and treated with calf intestinal phosphatase (New England Biolabs Inc). Insertion of the human U6 promoter and oPL tg6 shRNA sequence into pLL3.7 was verified by restriction digestion to confirm the presence of the DNA fragment between *XbaI* and *XhoI* restriction sites, as well as the disappearance of an *AgeI* restriction site. Sequencing was also conducted to verify stability of the shRNA construct during cloning (Colorado State University Proteomics and Metabolomics Facility).

The lentiviral vector pGIPZ originally contained the cytomegalovirus promoter (CMV) upstream of the turbo green fluorescent protein (tGFP), puromycin resistance gene and a multiple cloning site for the introduction of microRNA mimic sequences (shRNAmiR). The pGIPZ vector was reconstructed to contain the human Elongation factor-1α (hEF-1α) promoter upstream of oPL tg2, tg6 or SC shRNAmiR sequences (Table 4.1). In order to create the oPL targeting constructs, shRNAmiR oligonucleotides (Table 4.2) were first inserted into the pGIPZ vector containing the CMV promoter. Oligonucleotides were designed with 5' *XhoI* and 3' *EcoRI* restriction sites. Oligonucleotides were ordered with 5'phosphates and PAGE purified (Integrated DNA Technologies). Oligonucleotides were resuspended in water to 60 pmol/µl, and one microliter of the sense oligonucleotide and one microliter of the anti-sense oligonucleotide were annealed in 48 µl of annealing buffer (100 mM potassium acetate; 30 mM HEPES-KOH, pH7.4; 2 mM magnesium acetate). The annealing mixture was

incubated at 95ºC for 4 minutes, 70ºC for 10 minutes and 4ºC for 10 minutes. Annealed oligonucleotides were ligated into the pGIPZ vector digested with *XhoI*/*EcoRI* and treated with calf intestinal phosphatase (New England Biolabs Inc., Ipswich, MA). Resulting clones were sequenced and analyzed for the correct orientation and shRNAmiR sequence. To introduce the hEF-1α promoter into pGIPZ and remove the CMV promoter, a restriction site was engineered between the 3' end of the promoter and the transcription start site for tGFP. Within pGIPZ, the CMV promoter-tGFP cassette is flanked by *XbaI* and *BsrGI* restriction sites on the 5' and 3' ends, respectively. In addition, *BstBI* was identified as a unique restriction site between the 3' end of the promoter and the transcription start site for tGFP, and was not found in the published sequence of the hEF-1α promoter. The *XbaI* site is also not present in the hEF-1α promoter. Therefore, primers were designed to amplify the hEF-1α promoter and include a 5' *XbaI* site in the forward primer (5'-TCTAGACGCTCCGGTGCCCGTC-3') and a 3' *BstBI* site in the reverse primer (5'-TTCGAAAGCTTCAGCTGTGTTCTGGCGGC3'). The pSico-EF1a-mCh-Puro plasmid (plasmid 31845; Addgene) was used as a template for the hEF-1α promoter. The PCR product was cloned into the pPCR- Script Amp SK(+) vector (Stratagene) and verified by sequencing using T3 and T7 primers (Colorado State University Proteomics and Metabolomics Facility). Similarly, tGFP was PCR amplified using a forward primer containing a 5' *BstBI* restriction site (5'-TTCGAAACCATGGAGAGCGACGAGAG-3') and reverse primer containing a 3' *BsrGI* restriction site (5'-GCTACTTGTACATTATTCTTCACCGGCATCTG-3'). The resulting PCR product was cloned into the pPCR- Script Amp SK(+) vector (Stratagene), and verified by

sequencing using the T3 and T7 primers (Colorado State University Proteomics and Metabolomics Facility). The hEF-1α promoter and tGFP were digested from pPCR-Script Amp SK(+) using *XbaI/BstBI* and *BstBI/BsrGI* respectively, and subsequently ligated into pGIPZ digested with *XbaI/BsrGI* and treated with calf intestinal phosphatase (New England Biolabs Inc.). Resulting positive clones were sequenced using forward (5'- GGAACGGCACTGGTCAACTTGG-3') and reverse (5'-CACACCGGCCTTATTCCAAGCG-3') sequencing primers and verified for the correct orientation and sequence (Colorado State University Proteomics and Metabolomics Facility). Since a non-silencing pGIPZ vector (Thermo Fisher Scientific) was available containing a scrambed control shRNAmiR sequence downstream from the original CMV promoter, the CMV promoter was removed and replaced with the hEF-1α promoter according to the procedure described above.

Lentivirus Generation

Lentiviral particles were generated in 293FT cells (Invitrogen) grown in high-glucose DMEM medium containing 10% fetal bovine serum at 37ºC and 5% CO_2. On the day of transfection, cells were grown to 60-70% confluency on a 150 mm tissue culture dish. For each 150 mm dish, 8.82 ug of oPL-targeting plasmid, 6.66 ug of psPAX2 (Addgene) packaging plasmid and 2.70 ug of pMD2.G (Addgene) packaging plasmid DNA was used for transfection. The plasmid DNA mixture was brought to a total volume of 675 ul using serum-free, antibiotic-free high-glucose DMEM. The PolyFect transfection reagent (180 ul; Quiagen), was added to the plasmid DNA mixture and incubated for 10 minutes at room temperature to allow for complex formation.

Immediately prior to treating cells with the transfection mixture, medium was gently aspirated from cells and replaced with 18 mL of fresh, complete medium. After complex formation, 885 ul of PolyFect transfection mixture was added to each 150 mm dish of 293FT cells drop-wise for even distribution. Transfection medium was replaced with fresh, complete medium after 4-6 hours of incubation at 37ºC and 5% CO_2. The lentiviral containing medium was collected at the end of 48 hours incubation with the 293FT cells and the supernatant was ultracentrifuged over a 20% sucrose cushion at 47,000xg for 2 hours at 4ºC. After ultracentrifugation, lentiviral pellets were re-suspended in chemically defined medium for late stage embryos (CDM-2; De La Torre-Sanchez et al., 2006) and stored in aliquots at -80ºC. Frozen aliquots of concentrated lentivirus were thawed, initially diluted 1:40 and then 10-fold serially diluted using high-glucose DMEM supplemented with 10% FBS. Serial dilutions ranging 10^{-2} to 10^{-7} and 8 µg of polybrene (Sigma) per milliliter of media were added to either 12-well or 6-well tissue culture dishes of HEK 293 cells at 80% confluency. Cells were incubated with virus dilutions overnight, washed with PBS, and fresh complete medium was added. If titering pLL3.7 vector based lentivirus, the number of eGFP-positive cells within the last well containing fluorescent cells were counted 48 hours post infection. The titer was then calculated by dividing the number of live cells by the serial dilution factor from which the live cells were counted and multiplying this number by the initial dilution factor (40) used to dilute the original stock of virus. If titering pGIPZ vector based lentivirus, cells were infected with serial dilutions of virus in replicates of four, and put under antibiotic selection (puromycin, 1µg/ml) 72 hours after infection. The titer was calculated between 5 and 7 days after the start of selection. To calculate the titer, the

number of replicates of the last serial dilution containing live cells was input into the TCID50 calculator (Hierholzer & Killington, 1996). The TCID50 calculation was then multiplied by the initial dilution factor (40) used to dilute the original stock of virus in order to obtain a final concentration of virus.

Blastocyst Collection and Transfer

Blastocyst collection and transfer were conducted based upon a previously described protocol (Purcell et al., 2009). Briefly, standing estrus in all ewes was determined using a vasectomized ram. Blastocyst donor and recipient ewes were synchronized by twice-daily i.m. injections of PGF2α given four hours apart (10mg i.m per dose; Lutalyse, Pfizer, New York, NY). Forty-eight hours after synchronization, all donor ewes displaying standing estrus were bred by three different intact rams over a 24 hour time period. Late stage blastocysts were collected from donor ewes nine days after breeding. Donor ewes were fasted for approximately 16 hours prior to blastocyst collection. Donor ewes were euthanized using sodium pentobarbital (90 mg/kg, i.v.; Pentasol, Vibrac, Fort Worth, TX), a complete hysterectomy was performed and the blastocysts were flushed from the uterus using DMEM/F-12 (1:1) medium supplemented with 0.25% BSA (Bovine Serum Albumin). Collected blastocysts were washed in Hepes buffered chemically defined medium for late stage embryos (HCDM-2; De La Torre-Shanchez, Garder, Preis & Seidel, 2006). To infect blastocysts with lentivirus, single, hatched blastocysts were incubated in 100ul drops overlaid with mineral oil. Each drop contained 100,000 TU of concentrated lentivirus, 80 ng of polybrene (8ng/µl; Sigma), and CDM-2. Drops containing blastocysts were cultured in 5% CO2 , 5% O2 , and 90%

N2 at 37ºC for approximately 5 hours before being transferred. Blastocysts were washed in HCDM-2 immediately prior to transfer.

Recipient ewes that had displayed standing estrus behavior within 24 hours of the donor ewes were eligible to receive blastocysts. Recipient ewes were fasted for approximately 16 hours prior to blastocyst transfer. Recipient ewes were sedated using ketamine (12.5 mg/kg i.v.; Ketacine, Vetone, Boise ID) and diazepam (0.125 mg/kg, i.v.; Hospira, Lake Forest, IL), intubated with an endotracheal tube and maintained on 2L/min O2 and 1%-2% isofluorane (Fluriso, Vetone) during the surgical blastocyst transfer. The transfer was performed using a fine, fire-polished glass pipette inserted through a puncture wound into the uterine horn ipsilateral to the corpus luteum (CL). A single blastocyst was transferred into each recipient. A total of 49 blastocysts were transferred to recipient ewes, including: 13 hEF-1 SC (SC), 11 hEF-1 tg2, 10 hEF-1 tg6 and 15 hLL3.7 tg6 infected blastocysts. Post-operative pain was managed by i.m. administration flunixin meglumine (100 mg i.m. per dose; Banamine, Merck, Whitehouse Station, NJ) immediately prior to surgery and again at 24, 48 and 72 hours after surgery. In addition amplicillin was administered (1g i.m. per dose; Polyflex, Boehringer Ingelheim Inc., St. Joseph, MO) immediately prior to surgery and again at 24 and 48 hours after surgery. After transfer, recipient ewes were given an ad libitum diet of alfalfa hay and monitored for 72 hours post-surgery.

Tissue Collection

For analysis of oPL gene knockdown, terminal surgeries were conducted on mature crossbred ewes at 135 days of gestation (dGA) and tissues were collected. A

total of 33 pregnancies were harvested at 135 days of gestation, including: 8 hEF-1 SC (SC), 9 hEF-1 tg2, 7 hEF-1 tg6 and 9 hLL3.7 tg6 pregnancies. Pregnant recipient ewes were food and water restricted for 16 hours prior to surgery. Pregnant recipient ewes were sedated using ketamine (12.5 mg/kg, i.v.; Ketacine, Vetone) and diazepam (0.125 mg/kg, i.v.; Hospira), intubated with an endotracheal tube, and maintained on 2L/min O_2 and 1%-2% isofluorane (Fluriso, Vetone). Fetal blood was collected from the umbilical artery and vein, and maternal blood was collected from the uterine artery and vein ipsilateral to the fetus. The fetus was then euthanized using sodium pentobarbital by intravenous administration through the umbilical vein (90mg/kg; Pentasol, Vibrac), and removed. The recipient ewe was also euthanized using sodium pentobarbital (90 mg/kg, i.v.; Pentasol, Vibrac). All blood samples were allowed to coagulate and then centrifuged at 2,000xg for 10 minutes at 4ºC. Serum was collected and stored at -80ºC until use. Sex, body weight (FBW) and crown-rump-length (CRL) were recorded for the recovered fetus. The fetal liver was harvested, recorded for weight, and stored in a 50 mL conical that was snap frozen in liquid nitrogen. A portion of the maternal liver was also harvested and stored in a 50 mL conical that was snap frozen in liquid nitrogen. After a complete hysterectomy was performed, all placentomes were excised, washed in PBS and recorded for placental weight and total placentome number. Placentomes were then classified based on morphology as either type A/B or type C/D placentomes and the total number of each placentome type was recorded. Type A placentomes are concave in shape and the maternal caruncle surrounds a small portion of the fetal cotyledon tissue (Vatnick et al., 1991). In contrast, type D placentomes are convex in shape and the fetal cotyledon tissue surrounds the maternal caruncle tissue (Vatnick et

al., 1991). Type B and C placentomes are intermediate in morphology (Vatnick et al., 1991). Three placentomes from each pregnancy were used to fix 3 mm sagittal sections from the center of each placentome in 4% paraformaldehyde (PFA) overnight at 4ºC. Placentome sections were then stored in 70% ethanol at 4ºC until use. Fetal cotyledons were separated from maternal caruncles, and the cotyledon tissue was stored in 50 mL conicals that were snap frozen in liquid nitrogen. After being frozen, tissue samples were pulverized using a mortar and pestel. Pulverized tissue was kept frozen at -80ºC until use.

RNA Isolation

Total cellular RNA was isolated from 135 dGA fetal cotyledon, fetal liver and maternal liver tissue using the RNeasy Mini Kit (Quiagen) according to the manufacturer's protocol. RNA concentration was quantified using the Biotek Synergy 2 Microplate Reader (Biotek, Winooski, VT) and quality was measured by the 260/280 nm absorbance ratio. Samples were stored at -80ºC until use.

cDNA Synthesis and Quantitative Real-Time PCR

cDNA was synthesized from 1ug of total cellular RNA by reverse transcription at 55ºC for 50 minutes using iScript Reverse Transcription Supermix for RT-qPCR (BioRad) according to the manufacturer's protocol. After cDNA synthesis, all cDNA samples, were treated with 5 units of RNase H (Thermo Fischer Scientific, Waltham, MA) at 37ºC for 20 minutes. To control for variance in efficiency of the reverse transcription reaction, cDNA was quantified using the Quant-iT OliGreen ssDNA Assay

Kit (Invitrogen) according to the manufacturer's protocol and quality was measured according to the 260:280 nm absorbance ratio. An equal mass of cDNA (20 ng) was used for each sample in the qRT-PCR reaction.

Quantitative real time RT-PCR (qRT-PCR) was performed using the Lightcycler 480 (Roche Applied Science, Indianapolis, USA) and protocol as previously described (Purcell, et al., 2009). All primer sets for qRT-PCR were designed using Oligo software (Molecular Biology Insights, Cascade, CO) to amplify an intron-spanning product. Primer sequences and PCR conditions are summarized in Table 4.3.

Table 4.3 PCR primers, annealing temperatures, and product sizes for cDNA used in qRT-PCR

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Anneal (°C)	Product (bp)
oPL	ataaactccgaatccaaggtc	gttccttttgagtttgccag	58	177
oRPS15	atcattctgcccgagatggtg	tgctttacgggcttgtaggtg	58	124
oPol II	agtccaacatgctgacggacatga	agccaagtgccggtaattgacgta	60	332
oGAPDH	accactgtccactgccatcac	cctgcttcaccaccttcttga	60	268
oIGF-I	tcgcatctctcttctatctggccctgt	acagtacatctccagcctcctcaga	62	238
oIGF-II	gaccgcggcttctacttcag	aagaacttgcccacggggtat	62	202
oIGFBP-1	tgatgaccgagtccagtgag	gtccagcgaagtctcacac	62	247
oIGFBP-2	caatggcgaggagcactctg	tggggatgtgtagggaatag	55	330
oIGFBP-3	ctcagagcacagacaccca	ggcatatttgagctccac	54	335

Prior to qRT-PCR, a PCR product for each gene was generated, cloned into PCR-Script (Agilent, Santa Clara, CA) and sequenced to verify amplification of the correct mRNA (Colorado State University Proteomics and Metabolomics Facility). In order to measure amplification efficiency and quantify the temporal expression of each gene, a standard curve was generated from $1x10^{2}$ to $1x10^{-6}$ pg using a PCR product amplified from the sequenced plasmid for each gene. Amplification was detected using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 200nM of forward and reverse primer for each gene. Samples were amplified by 45 cycles of 95ºC for 1 min, 60ºC for

1 min, and 72ºC for 1 min. A melt-curve analysis was conducted by increasing the temperature 0.5ºC at 10-sec intervals from 55ºC to 100ºC. All standards were run in duplicate, and cDNA from each sample was run in triplicate. Product specificity was confirmed by observation of a single melt curve for each gene. The starting quantity (picograms, pg) was normalized by dividing the starting quantity of the gene of interest by the geomean of starting quantities (pg) for the housekeeping genes. Housekeeping genes include: ovine ribosomal protein S15 (oRPS15), ovine GAPDH (oGAPDH), and ovine RNA Polymerase II (oPol II).

Western Immunoblotting

Cellular protein from 135 dGA cotyledon tissue was assessed using Western immunoblot analysis. To isolate total cellular protein, 0.1 g of tissue was lysed in 500 ul of Western lysis buffer (0.48 M Tris, pH 7.4; 10 mM EGTA, pH 8.6; 10 mM EDTA, pH 8; 0.1 mM PMSF; 0.1 mM AEBSF; 0.0015 mM Pepstatin A; EG4 0.0014 mM; 0.004 mM Bestatin; 0.002 mM Leupeptin; 0.00008 mM Aprotinin) and sonicated on ice. After sonication, samples were centrifuged at 14,000xg for 3 minutes and the supernatant collected for protein quantification. Protein concentration was measured using the Bradford assay method (Bradford, 1976) and the absorbance read at 595 nm using the Biotek Synergy 2 Microplate Reader (Biotek). A total of 10 ug of protein was electrophoresed through 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY) at a constant 200 V for one hour at 4ºC. Protein was then transferred to a 0.45 um pore nitrocellulose membrane at a constant 60 V for five hours at 4ºC. Membranes were blocked in 5% non-fat dry milk (NFDM)-TBST (50 mM Tris, 150 mM NaCl, 0.05%

Tween 20, pH 7.6) for one hour at room temperature to reduce non-specific binding, then incubated with primary antibody at 4ºC overnight. Membranes were washed in TBST, and then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. To detect oPL a polyclonal antibody generated in the rabbit (1:25,000 dilution; α-oPL-54; Kappes et al., 1992) in conjunction with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; product no. sc-2004; Santa Cruz Biotechnology Inc., Dallas, TX) was used. As a loading control protein and housekeeping to normalize oPL, a polyclonal antibody to β-actin (1:250 dilution; product no. sc-47778; Santa Cruz Biotechnology Inc.) bound by a horseradish peroxidase-conjugated secondary antibody (1:5,000; product no. sc-2005; Santa Cruz Biotechnology Inc.) was used. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham, Pittsburgh, PA), and imaged using the ChemiDoc XRS+ chemiluminescence system (BioRad). Densitometry calculations were performed using the Image Lab Software (BioRad). To correct for technical error between western immunoblots, a common sample was included in each western immunoblot. Densitometry measurements were then adjusted proportional to the average densitometry measurement of the common sample.

Radioimmunoassay

The concentration of serum oPL was assessed by radioimmunoassay (RIA) as previously described (Kappes et al., 1992) with an intra-assay coefficient of variance of 5.5%. The concentration of insulin in maternal and fetal circulation was measured

according to the procedure described by Camacho, Benavidez and Hallford (2012) with a 4.3% intra-assay coefficient of variance.

Serum Glucose and Lactate Measurements

Maternal and fetal serum glucose and lactate concentrations were measured using the YSI 2700 SELECT Biochemistry Analyzer (YSI Inc., Yellow Springs, OH) according to the manufacturer's protocol. The following enzymatic membranes and standard solutions were used to detect the presence of glucose and lactate: glucose (dextrose) membrane kit (model # 2365; YSI Inc.), lactate membrane kit (model # 2329; YSI Inc.), dextrose/lactate standard (2.5g/L destrose, 0.5 g/L lactate; model # 2777; YSI Inc.), buffer concentrate kit (model # 2357; YSI Inc.). Samples were thawed and allowed to adjust to room temperature before being measured in duplicates using 25 ul of serum per read. A calibration was performed after every fifth measurement and accuracy was confirmed using a standard control sample.

Statistical Analysis

All data were analyzed using SAS software (SAS Institute, Cary, NC). All data were subjected to analysis of variance using the PROC GLM procedure, with treatment, gender and sire as dependent variables and all interactions considered. There were no treatment by gender or treatment by sire interactions that were confounding to the overall statistical analysis. This was followed by Dunnett's *t*-test where all three treatment groups were compared to the scrambled control (SC) pregnancies, with gender and sire as dependent variables. This analysis revealed parameters where

gender and sire effects were expected, such as placental weight, fetal weight, fetal crown-rump length and fetal liver weight. Subsequently, analysis of variance was conducted using the PROC GLM procedure with gender and sire as dependent variables, to compare SC pregnancies to tg6 shRNA/shRNAmmiR pregnancies with placental weights that fell below two standard deviations of the mean placental weight for scrambled controls. Such tg6 shRNA/shRNAmiR pregnancies were defined as Tg6 responders (tg6), and were evaluated for all parameters. An n of 8 tg6 were compared to an n of 8 SC pregnancies. Statistical significance was set at P≤0.05, and statistical tendency was set at P≤10. NS indicates no statistical difference

RESULTS

Day 135 Fetectomy and Fetal and Placental Measurements

A total of 49 day 9 blastocysts were infected with lentivirus and surgically transferred as single blastocyst transfers to recipient ewes. Of the 49 single blastocyst transfers, 33 resulting pregnancies were harvested near term (135 dGA), which was equal to a 67.3% blastocyst transfer success rate (Table 4.4).

Table 4.4 Blastocyst Transfer Success

Treatment Group	Embryos Transferred	Returned to Estrus	Positive Ultrasounds	Near Term Pregnancies	% Success
SC	13	5	8	8	61.5
hEF-1 tg2	11	2	9	9	81.8
hEF-1 tg6	10	3	7	7	70.0
hLL3.7 tg6	15	5	10	9	60.0
Total	**49**	**15**	**34**	**33**	**67.3**

Within each treatment group, there was an n of 8 hEF-1 SC (SC), 9 hEF-1 tg2, 7 hEF-1 tg6 and 9 hLL3.7 tg6 near term pregnancies. For each pregnancy fetal body weight

(FBW), and placental weight (PW) was recorded and summarized in Figure 4.1. The average PW for SC pregnancies was 0.781 ± 0.029 kg, with 0.612 kg being two standard deviations below the average. Compared to the SC pregnancies the average placental weight for all hEF-1 tg2 pregnancies was 0.751 ± 0.074 kg, all hEF-1 tg6 pregnancies was 0.600 ± 0.087 kg ($p \leq 0.10$), and all hLL3.7 tg6 pregnancies was 0.526 ± 0.071 kg ($p \leq 0.05$), representing 3.8%, 23.1% and 32.6% change in placental weight, respectively. The average FBW for SC pregnancies was 4.904 ± 0.114 kg, with 4.26 kg being two standard deviations below the average. Compared to SC pregnancies the average FBW for all hEF-1 tg2 pregnancies was 4.432 ± 0.193 kg, all hEF-1 tg 6 pregnancies was 4.218 ± 0.377 kg ($p \leq 0.10$), and all hLL3.7 tg6 was 3.954 ± 0.375 ($p \leq 0.05$), representing 9.6%, 14.0%, and 19.4% change in FBW respectively. While the objective was to create oPL deficient pregnancies, placental and fetal development was only assessed at 135 dGA, and the events that occurred throughout gestation can only be speculated. Accordingly, as apparent from Figure 4.1, there was variability in PW and FBW of oPL targeted pregnancies. Since a greater degree of growth restriction was observed in PW compared to FBW, we chose to evaluate pregnancies in which the placental weight fell below two standard deviations of the mean placental weight of SC pregnancies. While there was one hEF-1 tg2 pregnancy that met this criteria, overall the hEF-1 tg2 lentivirus did not appear to be as effective as the hEF-1 tg6 and hLL3.7 tg6 lentivirus based off of PW, FBW and oPL mRNA concentrations (Figure 4.2).

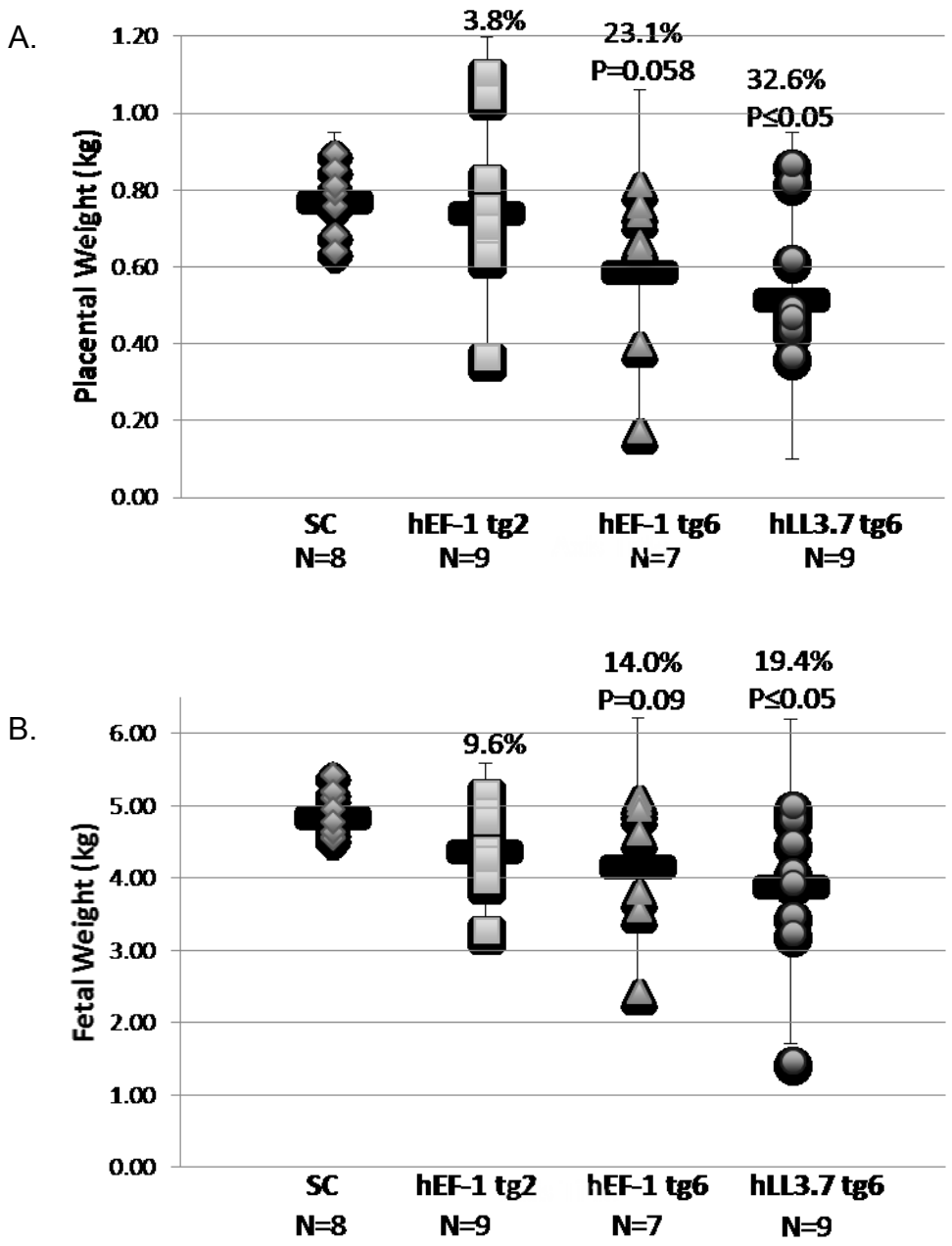


Figure 4.1 (A) Scattergram of placental weight distribution between treatment groups. Horizontal, bold bars represent mean placental weight and vertical bars represent two standard deviations. Percentages represent average percent growth restriction relative to scrambled controls (SC). The p-value represents statistical analysis compared to SC, and N represents the number of singleton pregnancies (B) Scattergram of fetal body weight distribution between treatment groups. Horizontal, bold bars represent mean fetal body weight and vertical bars represent two standard deviations. Percentages represent average percent growth restriction relative to SC. The p-value represents statistical analysis compared to SC. N represents the number of singleton pregnancies.

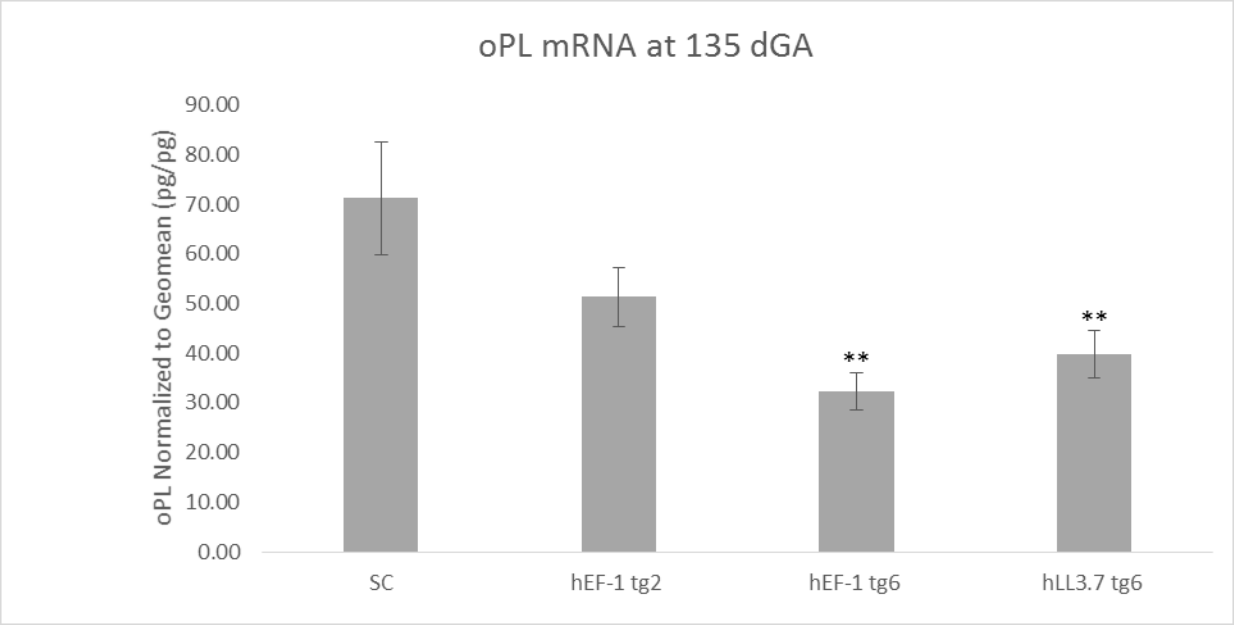


Figure 4.2 Concentration of oPL mRNA in 135 dGA placental tissue. Quantitative real-time RT-PCR for oPL normalized to the geomean. ** indicates P≤0.05 when compared to SC.

Since the inefficiency of hEF-1 tg2 lentivirus relative to hEF-1 tg6 and hLL3.7 tg6 may be related to the different oPL targeting sequences, pregnancies treated with the tg6 oPL targeting sequence that had placental weights two standard deviations below the mean PW for the SC group were defined as tg6 responders (tg6). As a result, 2 out of 7 hEF-1 tg6 pregnancies and 6 out of 9 hLL3.7 tg6 were considered tg6 pregnancies. The remainder of our analysis focused on comparing tg6 (n=8) to the SC (n=8) pregnancies.

The PW of tg6 pregnancies (0.374±0.042 kg) was significantly reduced (p≤0.05) compared to the PW of SC pregnancies (0.781±0.030 kg) (Figure 4.3), representing a 52% placental growth restriction. Similarly, the FBW of tg6 pregnancies (3.346±0.346 kg) was significantly reduced (p≤0.05) compared to that of SC pregnancies (4.904±0.114 kg) (Figure 4.3), representing a 32% fetal growth restriction.

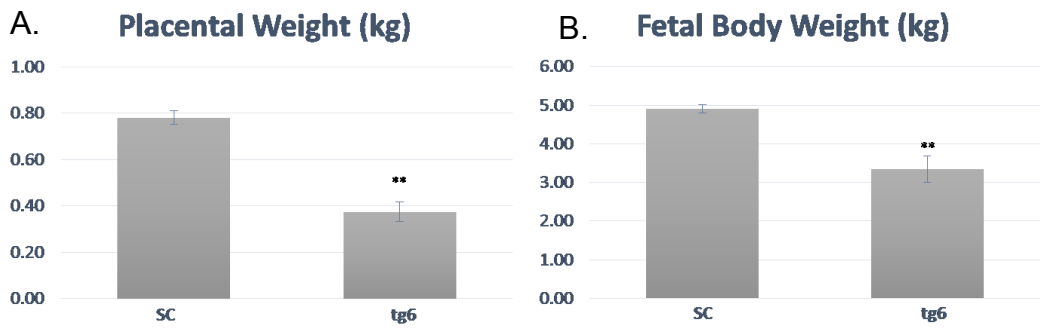


Figure 4.3 (A) Average placental weight of tg 6 versus SC. ** indicates P≤0.05 when compared to SC. (B) Average fetal body weight of tg 6 and SC. ** indicates P≤0.05 when compared to SC.

In addition, placentome number, placentome morphology and placental efficiency (FBW/PW) were evaluated (Table 4.5). The tg6 pregnancies tended to have a decreased total number of placentomes (p≤0.10), as well as a decreased number of type C/D placentomes compared to SC (p≤0.10). The tg6 pregnancies also had significantly increased placental efficiency (p≤0.05) relative to the SC group.

Table 4.5 Placentomes and Placental Efficiency

Parameter	SC	tg6	p-value
Placentome Number	68.5±3.8	56.6±6.1	p≤0.10
Type A/B Placentomes	42.9±9.5	48.9±8.9	NS
Type C/D Placentomes	24.4±7.6	7.8±3.7	p≤0.10
Placental Efficiency (FBW/PW)	6.3±0.2	9.2±0.8	p≤0.05

Fetal crown-rum length (CRL), fetal liver weight, and ponderal index were also recorded and compared between tg6 and SC pregnancies (Table 4.6). The CRL and fetal liver weights of tg6 pregnancies were significantly decreased (p≤0.05) compared to SC. On the other hand, no change was observed in ponderal index (Table 4.6)

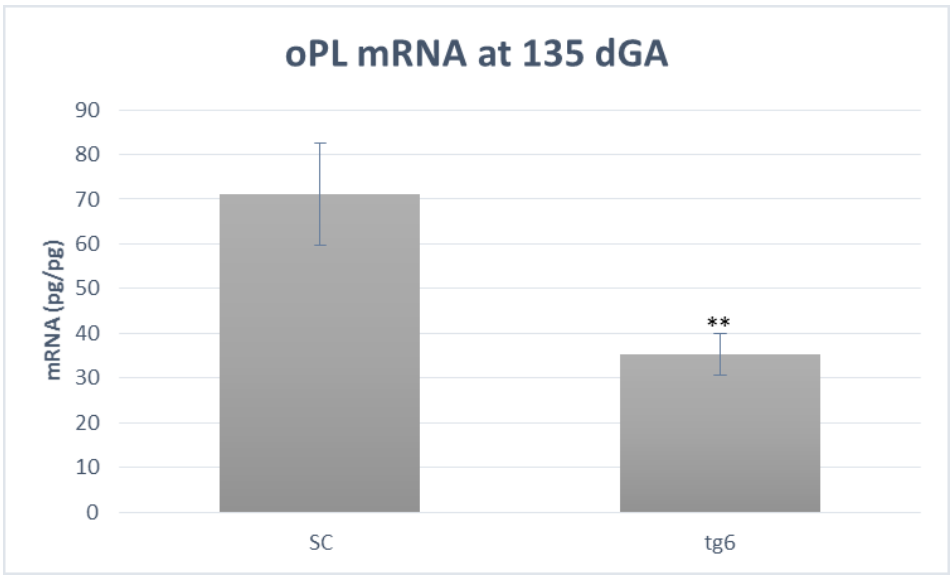
Table 4.6 Fetal Measurements

Parameter	SC	tg6	p-value
Fetal CRL (cm)	51.31±0.57	44.00±1.43	p≤0.05
Fetal Liver Weight (kg)	0.165±0.008	0.098±0.013	p≤0.05
Ponderal Index (kg/cm^3)	3.31±0.03	3.36±0.05	NS

oPL Expression in Day 135 Placental Tissue

The concentrations of oPL mRNA and protein were measured in cotyledon tissue at 135 dGA for analysis of oPL gene expression. Quantitative real-time RT-PCR was performed to determine the expression of oPL mRNA in cotyledon tissue. Compared to the SC group (71.22 ± 11.40 pg/pg), we observed significantly decreased (p≤0.05) oPL mRNA concentrations in the cotyledon tissue of the tg6 group (35.35 ± 4.68 pg/pg) (Figure 4.4), which is equal to a 50% reduction. The presence of oPL in 135 dGA cotyledon tissue was detected using western immunoblotting and densitometry measurements were calculated to compare tg6 to SC. β-actin was used as a loading control to verify equal loading for all samples and. The tg6 group (1.76±0.12 optical density units) displayed reduced (P≤0.10) levels of oPL compared to the SC group (3.37±0.93 optical density units), which was equivalent to a 48% change in oPL concentration (Figure 4.4).

A.



B.

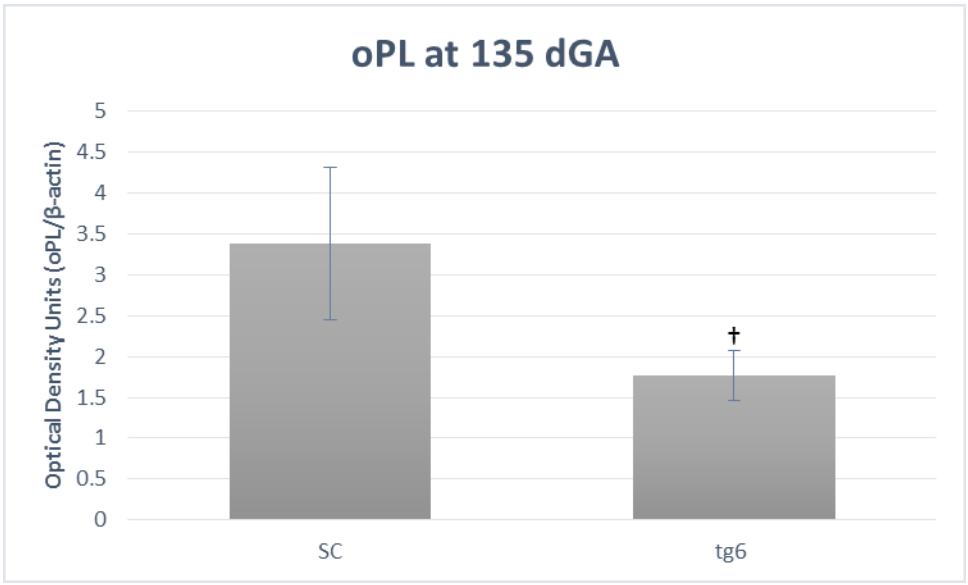


Figure 4.4 Effect of tg6 lentivirus on oPL mRNA and protein in 135 dGA cotyledon tissue. (A) Average oPL mRNA (pg/pg) for each treatment group measured by quantitatve real-time RT-PCR and normalized to the geomean. ** indicates P≤0.05 when compared to SC. (B) Average oPL for each treatment group measured by western immunoblotting and normalized to β-actin. † indicates P≤0.10 when compared to SC.

Day 135 Serum Measurements

On day 135 of gestation fetal blood samples were collected from the umbilical artery and umbilical vein, and maternal blood samples were collected from the uterine

artery and uterine vein ipsilateral to the fetus. Samples were collected at a single point in time during terminal fetectomy surgery and after approximately 16 hours of fasting. Circulating concentrations of oPL were measured in uterine vein and umbilical vein serum samples (Table 4.6). There were no statistical differences in maternal and fetal circulating oPL; however, there were 24% and 12% reductions, respectively, in tg6 relative to SC pregnancies. Likewise, we did not observe any statistical differences in insulin and glucose concentrations in maternal circulation between tg6 and SC pregnancies. However, the uterine artery to uterine vein glucose gradient was significantly increased ($p \leq 0.05$), and the uterine artery insulin:glucose ratio tended to be increased ($p \leq 0.10$) in tg6 pregnancies compared to SC. Concentrations of insulin and glucose in fetal circulation were not statistically different with the exception that umbilical artery insulin tended to be decreased ($p \leq 0.10$) in tg6 pregnancies compares to SC (Table 4.6).

Table 4.6 Maternal and Fetal Serum Measurements

Parameter	SC	tg6	p-value
Utr. Vein oPL (ng/ml)	797.43±84.34	609.02±102.72	NS
Umb. Vein oPL (ng/ml)	45.64±3.74	40.00±5.77	NS
Utr. Artery Insulin (ng/ml)	0.404±0.080	0.944±0.313	NS
Umb. Artery Insulin (ng/ml)	0.564±0.126	0.286±0.065	p≤0.10
Utr. Artery Glucose (mmol/L)	3.389±0.562	3.701±0.545	NS
Utr. Vein Glucose (mmol/L)	3.063±0.505	3.342±0.556	NS
Utr. Artery-Vein Glucose (mmol/L)	0.233±0.035	0.359±0.034	p≤0.05
Utr. Artery Insulin:Glucose	0.120±0.017	0.237±0.058	p≤0.10
Umb. Artery Glucose (mmol/L)	0.797±0.310	0.532±0.210	NS
Umb. Vein Glucose (mmol/L)	0.968±0.333	0.767±0.221	NS
Umb. Vein-Artery Glucose (mmol/L)	0.171±0.039	0.236±0.066	NS
Utr. Artery-Umb. Vein Glucose (mmol/L)	2.426±0.201	2.933±0.385	NS
Umb. Artery Insulin:Glucose	1.212±0.275	1.773±0.776	NS

Insulin-like Growth Factors in Maternal and Fetal Liver

Since oPL has been proposed to achieve its actions through insulin-like growth factors, the mRNA concentrations of insulin-like growth factors and their binding proteins were measured in cotyledon tissue at 135 dGA. Quantitative real-time RT-PCR was performed to determine the temporal expression of insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II), as well as insulin-like growth factor binding proteins 1 (IGFBP-1), 2 (IGFBP-2) and 3 (IGFBP-3) in both maternal and fetal liver tissue. There were no significant differences in the concentration of IGF-I, IGF-II, or IGFBP-1, 2, and 3 in the liver tissue of the tg6 mothers compared to SC (Table 4.7).

Table 4.7 Maternal Liver Insulin-like Growth Factor mRNA

Maternal Liver			
Parameter	**Scrambled Controls**	**tg6 Responders**	**p-value**
IGF-I mRNA (pg/pg)	0.035±0.012	0.041±0.033	NS
IGF-II mRNA (pg/pg)	0.085±0.008	0.091±0.020	NS
IGFBP-1 mRNA (pg/pg)	0.582±0.168	0.671±0.101	NS
IGFBP-2 mRNA (pg/pg)	0.187±0.100	0.208±0.072	NS
IGFBP-3 mRNA (pg/pg)	0.077±0.023	0.079±0.013	NS

On the other hand, IGF-I and IGF-II mRNA concentrations in liver tissue of tg6 fetuses were significantly decreased ($p \leq 0.05$) by 83% and 71% respectively compared to SC (Table 4.8). Additionally, compared to the SC group, IGFBP-2 and IGFBP-3 mRNA concentrations in tg6 fetal livers were also significantly reduced (Table 4.8). IGFBP-1 mRNA concentrations were not statistically different between fetal liver of tg6 and SC.

Table 4.8 Fetal Liver Insulin-like Growth Factor mRNA

Fetal Liver			
Parameter	**Scrambled Controls**	**tg6 Responders**	**p-value**
IGF-I mRNA (pg/pg)	0.044±0.007	0.008±0.002	P≤0.05
IGF-II mRNA (pg/pg)	0.489±0.077	0.142±0.035	P≤0.05
IGFBP-1 mRNA (pg/pg)	0.720±0.157	0.637±0.306	NS
IGFBP-2 mRNA (pg/pg)	0.181±0.044	0.048±0.027	P≤0.05
IGFBP-3 mRNA (pg/pg)	0.089±0.014	0.017±0.006	P≤0.05

DISCUSSION

Grumbach et al. (1968) and Spellacy et al. (1971), made the first observations that circulating levels of maternal human PL (hPL) were positively correlated with placental mass and fetal body weight. This relationship has since led to a series of experiments investigating a role for PL in modulating maternal metabolism and promoting fetal growth. Unfortunately, numerous studies regarding the activity of PL during pregnancy report contradictory findings due to inconsistent experimental design and the lack of an effective translational model. The aim of this study was to develop a PL deficient sheep model to further investigate a precise biological role for PL during pregnancy. To address this, ovine PL (oPL) deficient pregnancies were generated using lentiviral mediated shRNA targeting oPL mRNA, and assessed for the impact on placental and fetal development near term. Data presented here confirm earlier reports linking PL to placental weight and fetal body weight, and provide additional evidence illustrating a role for PL in early pregnancy development and fetal insulin like growth factor (IGF) expression.

Pregnancies impacted by oPL deficiency (tg6 pregnancies) led to substantially decreased placental weight and fetal body weights at 135 dGA. Surprisingly, we

observed a greater impact on placental weight (52% reduction) than fetal body weight (32% reduction) in tg6 pregnancies. The overall function of the placenta is to accommodate fetal growth; therefore, if placental development is inhibited, then fetal growth will also be repressed. Accordingly, if oPL expression was reduced during early pregnancy, this could have interfered with placental development, and consequently fetal development, suggesting a direct action for PL in early placental development. Additionally, despite significantly decreased fetal body weights, crown-rump lengths and liver weights of fetuses harvested from tg6 pregnancies, the ponderal index of these fetuses did not differ from that of the control fetuses. Ponderal index is a useful indicator of body proportionality, and a method for distinguishing between symmetric and asymmetric growth restriction (Landmann et al., 2006). Similar ponderal index between growth restricted fetuses and control fetuses indicates that the growth restriction experienced by fetuses harvested from tg6 pregnancies was symmetric. Since symmetric growth restriction typically results from an insult encountered early *in utero* (Landmann et al., 2006), this provides additional evidence that reduced PL early in pregnancy may impede placental development. A study conducted by Spencer et al. (1999) also reported a potential role for placental lactogen during early pregnancy development, in which PL may regulate glandular epithelium proliferation and differentiation in preparation for placentation. If PL has the ability to stimulate proliferation and differentiation of the maternal epithelium, it could have a similar function during the development of the placenta, however this is only speculation and further experiments are necessary to confirm the function of PL during early placental growth and development.

Significant decreases in placental mass and fetal size were accompanied by a 50% decrease in oPL mRNA and a 48% decrease in oPL in 135 dGA placental tissue of tg6 pregnancies relative to the controls. These data imply that lentiviral mediated shRNA targeting the degradation of oPL mRNA leads to decreased oPL expression in placental tissue. Previous studies in sheep have demonstrated that decreased placental mass and fetal growth restriction are associated with decreased maternal circulating concentrations of oPL as well as the expression of oPL in placental tissue (Lea et al., 2007). Based on this relationship, a decrease in serum oPL concentrations proportional to the 52% and 50% reductions in placental mass and oPL mRNA, respectively, would be expected in tg6 pregnancies. However, serum concentrations of oPL were variable, and there were no significant changes in maternal or fetal serum oPL concentrations between tg6 and SC pregnancies. Previously, measurements of placental lactogen in ruminants have proven to be highly variable and can undergo short term fluctuations (Bauer et al, 1995; Butler et al., 1987). For instance, a study in which pregnant ewes and their fetuses were chronically catheterized from day 110 of gestation until term reported that oPL in maternal plasma showed no circadian rhythm, and that plasma oPL has the ability to vary up to two-fold in an hour (Taylor et al., 1980). In the same study, it was also reported that surgery elevated concentrations of oPL in maternal and fetal plasma for up to 5 days follow the operation. Given this evidence, measuring serum concentrations of oPL from blood samples collected at a single point in time under the influence of anesthesia, as was conducted in this study, may not accurately represent the overall secretion of oPL into circulation. Additionally, a number of studies have also demonstrated nutritional regulation of circulating PL

(Bauer et al., 1995; Butler et al., 1987; Oliver et al., 2005). In particular, fetuses of pregnant ewes that were fed a high plane of nutrition and underwent a short term fast experienced markedly elevated concentrations of plasma oPL during starvation (Oliver et al, 1992). Since pregnant ewes in the current study underwent a short-term fast prior to terminal surgery, this may have altered the rate of oPL secretion into maternal and fetal circulation, thus leading to skewed measurements of plasma oPL. To obtain a more accurate assessment of circulating oPL, blood samples should be collected after chronic catheterization and instrumentation of pregnant ewes, under non-anesthetized/non-stressed and steady-state conditions.

During pregnancy maternal metabolism undergoes major alterations to maintain an adequate supply of nutrients to support fetal growth. Previous studies have indicated that PL may be involved in maternal glucose retention and the mobilization of maternal carbohydrates (Handwerger and Freemark, 2000). Buter et al. (1987), noted that increased oPL concentrations induced by fasting in pregnant ewes at mid- to late gestation led to reduced glucose clearance rates compared to fed ewes. In addition, non-pregnant, non-lactating ewes treated with partially purified PL experienced increased blood glucose concentrations (Thordarson et al.; 1987). These studies illustrate that PL may modulate the availability of glucose to support pregnancy. In this study, serum samples collected at a single point in time on day 135 of gestation were evaluated for the impact of oPL deficiency on insulin and glucose metabolism in both maternal and fetal systems. While we did not observe any changes in the concentrations of maternal insulin or glucose, a significant increase in the uterine artery to uterine vein glucose gradient was revealed for tg6 pregnancies compared to controls.

Conversely, no changes were observed in the uterine artery to umbilical vein glucose gradient between tg6 pregnancies and controls. Together these data infer that glucose uptake by the placenta of tg6 pregnancies was increased in comparison to control pregnancies. Since, the placenta of tg6 pregnancies were significantly growth restricted, increased glucose uptake may serve as a compensatory mechanism in order to maintain adequate placental function for fetal survival. Furthermore, these data also coincide with a 32% increase in placental efficiency observed in tg6 pregnancies compared to controls. Even though tg6 pregnancies resulted in placental and fetal growth restriction, they were able to achieve greater fetal body weights per mass of placenta compared to control pregnancies, potentially through increased glucose uptake and utilization. In order to verify alterations in glucose uptake by the placenta in the case of oPL deficiency, chronic catheterization and instrumentation would be needed to monitor blood flow and utero-placental uptake and utilization of nutrients.

Fetal growth restriction, or IUGR is commonly associated with fetal hypoinsulinemia in parallel with fetal hypoglycemia (Langer et al., 1986; Limesand et al., 2007). While circulating fetal insulin was decreased by 49% in tg6 pregnancies compared to the controls, a corresponding decrease in circulating concentrations of glucose was not observed. These results may signify increased insulin sensitivity in fetuses of oPL deficient pregnancies, since increased insulin sensitivity was also reported for sheep IUGR fetuses as an adaptation to maintain normal rates of fetal glucose metabolism (Limesand et al., 2007). However, these results could also denote an effect of oPL deficiency on beta-cell function during development of the fetal pancreas. A study conducted using human fetal pancreatic explants revealed

significantly increased insulin and IGF-1 content and release in pancreatic tissue treated with human PL (hPL) in the presence of glucose (Swenne et al., 1987). This proposes that PL may modulate beta-cell function and contribute to fetal pancreatic development (Swenne et al., 1987). In order to meet the metabolic demands of pregnancy, increased beta-cell proliferation in the maternal pancreas has also been associated with the activity of placental lactogen (Sorensen & Brelje, 1997). Overall, these data raise the possibility that PL has an important function in early fetal development, and may help set metabolic standards for continued fetal growth. Nonetheless, additional experiments are required to elucidate the exact biological role of PL on fetal insulin metabolism and pancreatic function.

PL is believed to have an important anabolic function in the fetus, possibly through the stimulation of insulin-like growth factors (IGF) (Handwerger and Freemark, 2000; Schoknecht et al., 1996). To address the relationship between PL and IGFs, the expression of IGFs and insulin-like growth factor binding proteins (IGFBP) was measured in maternal and fetal liver tissue. While no significant changes were noted in IGF and IGFBP expression in maternal liver tissue, fetal liver from tg6 pregnancies revealed 83% and 71% reductions in IGF-I and IGF-II mRNA, respectively, compared to control fetuses. This was accompanied by significant reductions in fetal liver IGFBP-2 and IGFBP-3 mRNA concentrations of tg6 pregnancies compared to controls. This data supports results of earlier studies in which PL stimulated fetal IGF secretion *in vitro* and *in vivo. In vitro* studies demonstrate increased IGF-I production in human fetal myoblasts and fibroblasts treated with hPL, which was diminished after treatment with IGF-I antiserum (Hill et al., 1985). Furthermore, fetal lambs given a chronic intravenous

infusion of purified oPL during late gestation experienced increased IGF-I concentrations (Schoknecht et al., 1996). Insulin-like growth factors and their associated binding proteins are critical for conceptus cell proliferation, differentiation and metabolism (De Vrijer et al., 2006; Fowden, 2003; Jones & Clemmons, 1995). Similarly IGFs have been noted to be altered in relation to the development of intrauterine growth restriction (De Vriver et al., 2006). While the results collected in this study could be an indirect result of IUGR, oPL may well induce the expression of the insulin-like growth factors, supporting earlier reports (Hill et al., 1985; Schoknecht et al., 1996). Additionally, if oPL was significantly reduced during early gestation, leading to a lack of fetal IGF production/secretion, then fetal liver development may have been permanently disrupted. Supporting evidence shows that fetal IGF-I and IGF-II gene expression occurs from the earliest preimplantation stages (Watson et al., 1994), and can be affected by the endocrine environment *in utero* (Fowden, 1995). Furthermore, the 'Barker hypothesis' states that adverse influences that occur during intrauterine life can lead to permanent alterations in physiology and metabolism, which increase the risk for adult onset disease (De Boo & Harding, 2006; Barker & Osmond,1986). Since data collected here was for the purpose of assessing the impacts of oPL deficiency near term, in order to gain a better understanding of the importance for PL in fetal development, additional studies should be conducted focused on oPL deficiency during early pregnancy.

Placental lactogen is an abundantly secreted placenta specific hormone and has a number of postulated somatogenic functions, however the exact biological mechanisms behind PL's activity are largely unknown. Previously, the lack of an

appropriate PL deficient animal model has inhibited our ability to clearly define PL's function. In this study, oPL deficient sheep pregnancies were generated using lentiviral mediated shRNA targeting oPL mRNA in order to shed light on the role of PL in promoting fetal growth. While the results of oPL deficiency support an important role for PL in the modulation of placental and fetal growth, additional experiments are necessary to specify the exact biological mechanism by which PL achieves its actions.

SUMMARY

Intrauterine growth restriction (IUGR) is a leading cause of neonatal mortality and morbidity. Additionally, it has been well established that infants surviving IUGR are at increased risk for adult onset disease. Placental lactogen (PL) is a placenta specific secretory product found at high levels in maternal and fetal circulation throughout gestation and is significantly reduced in human and sheep IUGR pregnancies. While PL is thought to have important somatogenic functions that promote maternal adaptations to pregnancy and support fetal growth, the exact biological function of PL has not been defined for any species. Therefore, the objective of this study was to knock down ovine PL (oPL) expression *in vivo* using lentiviral mediated shRNA in order to determine the impact of ovine PL (oPL) deficiency on near term (135 days of gestation) placental and fetal growth. We hypothesized that oPL deficiency could result in intrauterine growth restriction of fetal lambs. Three different lentiviral oPL-targeting constructs; hEF-1 tg2 (human elongation factor-1α promoter expressing oPL tg2 shRNAmiR), hEF-1 tg6 (human elongation factor-1α promoter expressing oPL tg6 shRNAmiR), and hLL3.7 tg6 (human U6 promoter expressing oPL tg6 shRNA) were used to generate oPL deficient pregnancies that were compared to pregnancies

generated with an hEF-1 SC lentiviral control construct (human elongation factor-1α promoter expressing scrambled control shRNAmiR). Day 9 blastocysts were collected from naturally mated donor ewes, infected with one of the lentiviral constructs, and single blastocysts were surgically transferred to synchronized recipient ewes. At 135 days of gestation (dGA) 8 hEF-1 SC, 9 hEF-1 tg2, 7 hEF-1 tg6 and 9 hLL3.7 tg6 singleton pregnancies were harvested and assessed for the impact of oPL deficiency. Based on two standard deviations below the mean placental weight of the hEF-1 SC pregnancies, 2 out of 7 hEF-1 tg6 pregnancies and 6 out of 9 hLL3.7 tg6 pregnancies were classified as tg 6 responder pregnancies (tg6). Tg6 pregnancies resulted in significantly decreased oPL mRNA, placental weight and fetal body weight ($p \leq 0.05$) at 135 dGA compared to control pregnancies. Interestingly, we also observed that tg6 pregnancies had significantly increased ($p \leq 0.05$) placental efficiency and uterine glucose uptake relative to controls, which may be indicative of coping mechanisms to maintain pregnancy in the face of oPL deficiency. Further analysis revealed a 49% ($p \leq 0.10$) decrease in circulating fetal insulin in tg6 fetuses compared to control fetuses, thus supporting a role for oPL in altering fetal insulin secretion. Finally, mRNA concentrations for insulin-like growth factors (IGF) –I and –II, and insulin-like growth factor binding proteins (IGFBP) -2 and -3 were significantly reduced ($p \leq 0.05$) in fetal liver tissue of tg6 pregnancies compared to controls. While this could be an indirect result of IUGR, oPL may well induce the expression of fetal IGFs and their respective binding proteins. These data give insightful evidence for PL's role during pregnancy, however the precise biological actions of PL are still unclear. Additional studies are necessary to elucidate the mechanisms by which PL achieves its actions. Nonetheless,

based on these results our hypothesis that oPL deficiency during gestation would result in IUGR appears correct. Surprisingly, it appears that oPL could have its greatest impact during early pregnancy when the placenta is developing.

CHAPTER V: GENERAL DISCUSSION

The placenta is a complex, transient endocrine organ responsible for mediating exchange between mother and fetus in order to support fetal development. Aberrations in placental function commonly lead to a condition known as intrauterine growth restriction (IUGR), in which as fetus has failed to reach its growth potential. Life threatening neonatal complications can arise from pregnancies complicated by IUGR. In addition, infants that survive IUGR have been associated with increased incidence of chronic adult on-set disease. Placental lactogen (PL) is a major placental secretory product found at high levels in maternal and fetal circulation, but is significantly reduced in both human and sheep IUGR pregnancies. While the exact biological function of PL is largely unknown, it is thought to modulate maternal and fetal carbohydrate, amino acid and lipid metabolism. Our inability to define PL's function is in part due to the fact that a PL deficient animal model has not been achieved in any species. However, the recent development of lentirvral-mediated expression of short hairpin RNA (shRNA) within sheep conceptuses has provided a means of examining placental gene function in sheep. In this study, ovine (oPL) deficient sheep pregnancies were generated using lentiviral-mediated shRNA targeting the degradation of oPL mRNA, in order to assess the importance of PL during pregnancy.

Pregnancies impacted by oPL deficiency lead to substantially decreased placental weight, fetal body weight and oPL expression at 135 dGA. Interestingly, we also observed that oPL deficient pregnancies appeared to have significantly inceased uteroplacental glucose uptake and utilization in conjunction with increased placental

efficiency compared to control pregnancies. Together, these may function as a coping mechanisms to maintain placental function and sustain fetal growth. Further analysis revealed that oPL deficienct pregnancies experienced decreased circulating concentrations of fetal insulin, thus supporting a role for PL in modulating fetal insulin secretion. Finally, oPL deficient pregnancies demonstrated significant reductions in insulin-like growth factor (IGF) –I and –II, and insulin-like growth factor binding proteins (IGFBP) -2 and -3 mRNA concentrations, which may point to direct stimulation of the IGF axis by PL. Altogether, these results indicate the PL may have an important function in early placental and fetal development, however further studies are necessary to elucidate the exact biological actions of PL.

Since this study demonstrated significant reductions in IGF expression in the fetal liver of oPL deficient pregnancies, futher studies should be conducted to address the effect of oPL deficiency on placental IGF expression. In addition to supporting fetal growth, insulin-like growth factors have also been implicated in promoting placental development. Insulin-like growth factors in fetal circulation are primarily derived from the fetal liver, but the placenta is also a source of IGF production and secretion during pregnancy (De Vriver et al., 2006). In fact, IGF production begins during the early preimplantation stages of pregnancy, and may have an important autocrine function to support placental development (Reynolds et al., 1997; Watson et al., 1994). In future experiments, the impact of oPL deficiency on IGF expression in the placenta will be evaluated using quantitative real-time PCR (qRT-PCR) to measure mRNA concentrations for IGFs and their respective binding proteins in placental tissue.

PL is believed to have an important function in maternal and fetal glucose metabolism. Using qRT-PCR, the expression of gluconeogenic enzymes in maternal and fetal liver may be analyzed to identify modifications in the rate of gluconeogenesis, and reveal alterations in maternal and/or fetal glucose metabolism between normal and oPL deficient pregnancies. Since this study also exhibits evidence for increased glucose uptake and utilization by the placenta of oPL deficient pregnancies, GLUT1 and GLUT3 expression in the placenta will be assessed. Transplacental glucose transport is a facilitated diffusion process mediated by GLUT1 and GLUT3 transporters (Langdown & Sugden, 2001). Therefore, qRT-PCR will be conducted to measure mRNA concentrations of GLUT1 and GLUT3 expression in response to decreased oPL.

While this study demonstrates important implications of oPL deficiency on near-term (135 dGA) fetal growth and development, the events occurring during early gestation in response to oPL deficiency can only be speculated. Follow-up studies should be conducted to closely monitor circulating concentrations of oPL, nutrient utilization and placental and fetal development during early, mid- and late gestation. At 55 dGA the placenta is fully established and undergoing maximum growth. During this early stage of gestation, chronic catheterization of the maternal femoral artery and uterine vein along with blood flow probes for both uterine arteries over a 10 day period would allow for the assessment of glucose and oxygen uptake, as well as serum concentrations of oPL and insulin under steady-state, non-anethetized conditions. In addition, the whole placenta and fetal tissues should be collected to evaluate any changes in the expression of metabolic or angiogenic factors that may lead to abberations in placental or fetal development in response to oPL deficiency at 55 dGA.

Similar experiments may be conducted at mid-gestation (90 dGA) in order to track enhanced or changing pathways as a result of oPL deficiency. Finally, oPL deficient pregnancies should be assessed near-term (125-135 dGA) using full instrumentation to monitor nutrient transport across the placenta, blood flow, and circulating oPL and insulin concentrations. Catheters placed in the uterine artery, uterine vein, umbilical vein and fetal abdominal aorta would allow for metabolic measurements of the uteroplacental unit and fetus simultaneously (Barry & Anthony, 2009). Similarly, such instrumentation also allows for close monitoring of circulating oPL, insulin and IGF concentrations. At 135 dGA, additional focus should be placed on individual fetal tissues to further evaluate the consequences of oPL deficiency on fetal metabolism. Altogether, these follow-up studies would provide additional evidence supporting a role for placental lactogen in early placental development and fetal growth, as well as the impacts of PL throughout gestation. Furthermore, a detailed understanding of the downstream effects of oPL deficiency may lead to the identification of the biological mechanisms behind PL activity and the development of IUGR.

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