DISSERTATION

# CHARACTERIZATION OF PERIATTACHMENT FACTOR

Submitted by:

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In partial fulfillment of the requirements

For the degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERATION PREPARED UNDER OUR SUPERVISION BY SCOTT PURCELL ENTITLED CHARACTERIZATION OF PERATTACHMENT FACTOR BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF PERIATTACHMENT FACTOR

The purpose of these studies was to characterize expression of periattachment factor (PF) mRNA in the developing sheep conceptus, localize PF in the sheep conceptus, determine if degrading PF mRNA in the sheep conceptus was detrimental to development, and characterize PF in human tissues and cells. Sheep conceptuses were collected on d 11, 13, 15, 16, 17, 21, and 30 post-mating. oPF mRNA exhibited a quadratic expression pattern ($P<0.05$). No oPF mRNA was detected in d 11 conceptuses. From d 13, oPF mRNA increased to a peak at d 16 before declining. Peak expression of oPF mRNA in the conceptus occurs during rapid elongation and initial attachment of the conceptus to the endometrium. Immunolocalization of PF in a d 15 conceptus showed predominantly nuclear staining in trophectoderm and trophendoderm. Next, embryos collected from superovulated ewes on d 8 post-mating were infected with either a lentivirus expressing a short-hairpin (sh) RNA designed to target PF mRNA for degradation, a lentivirus expressing a shRNA containing 3 mismatched nucleotides, or a control lentivirus expressing no shRNA. Following infection, blastocysts were transferred into recipient ewes and collected back on d 15 of gestation. While 94 and 88% of the control and mismatched shRNA-treated conceptuses elongated by d 15, none of the embryos treated with the lentivirus expressing shRNA against PF mRNA elongated, and most died. This indicates that oPF is required for conceptus elongation and survival. Human PF mRNA was detected in human carcinomas and in the first trimester cytotrophoblast cell line, hTR8. Immunohistochemistry showed PF in the

nuclei of carcinomas and in first and second trimester cytotrophoblasts. PF was also present in the invading cytotrophoblast columns. In an in vitro invasion assay of first trimester cytotrophoblasts, hPF mRNA increased from 0, 3, to 12 h as invasion occurred. To further characterize PF in human cells, a lentiviral construct expressing an shRNA targeting the hPF mRNA sequence was developed that resulted in 63% mRNA reduction in BeWo cells.

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# CHAPTER I

## INTRODUCTION

Successful establishment and maintenance of pregnancy requires negotiation through critical developmental windows such as fertilization, conceptus elongation, maternal recognition of pregnancy, and apposition and implantation. Embryo mortality during these early periods of development is high and occurs in all mammalian species. Pregnancy loss can have large economic impacts in livestock production and can be emotionally challenging for couples hoping to conceive. Perhaps even more important in the context of human reproduction are abnormalities in early embryo development and implantation that lead to complications of pregnancy such as preeclampsia or intrauterine growth restriction, that can in turn cause death or health problems with either the baby or the mother. Furthermore, there is increasing focus on the developmental origins of adult disease: the idea that perturbations during early fetal, or even embryo development can have adverse health effects later in life and into adulthood. A greater understanding of placental function could provide physicians with the ability to better treat pregnant mothers with complications. The global challenge of providing enough food for a growing world population also makes reproductive biology a very important field for research. More efficient reproduction and less embryo loss in livestock species generates more meat and milk, and the protein they provide, more economically. The understanding of early reproductive events could also lead to more options for contraception, another important aspect of human and companion animal reproduction.

In ungulate species, including cattle and sheep, the preimplantation conceptus goes through a period of rapid trophoblast elongation prior to attaching to the uterus. By

contrast, in humans, other primates, as well as rodents, the embryo begins to implant into the uterus while still a spherical blastocyst, soon after hatching from the zona pellucida. After attachment, the placental structure diverges across species. Although the gross anatomy of the placenta varies, placental function is similar among species. It provides a 'life support' system for the developing fetus and performs most of the physiological functions that the developing fetus is unable to do. The functional unit of the placenta that is involved in blood, nutrient, and gas exchange, the chorionic villus, is very similar across species, and similar molecules regulate its function. Comparative physiology is of great value in the field of reproductive biology due to the inherent difficultly and ethical issues of doing research in humans or other primates. This, combined with the high degree of homology at the gene level across all mammalian species allows model systems to be used and valid comparisons to be made to other species.

The gene that is the focus of these studies was first identified as G90 in the gastrointestinal tract of the mouse. Later this same gene was independently identified by our laboratory in trophoblast tissue during critical periods of conceptus development in cattle and called periattachment factor (PF). Subsequently, this gene has been found in a variety of mammalian species and is well conserved at both the DNA and protein level, indicative of a similar function in all these species. While much research today is done at the molecular level, the studies contained herein provide information on both the cellular and molecular aspects of early placental development, as well as a direct physiological consequence when RNA interference was used to target PF mRNA for degradation in ovine blastocysts. Due to the importance of this period of early conceptus development in both livestock and humans, and the many unanswered biological questions about this

period, the study of genes critical for developmental success remains a major focus of research in reproductive biology.

# CHAPTER II

# REVIEW OF LITERATURE

## PREIMPLANTATION EMBRYO DEVELOPMENT

Embryo development from the time of fertilization up to the blastocyst stage, although not identical, is relatively similar and well conserved across most mammalian species. This is especially true when compared to post blastocyst-stage development. Following fertilization, the zygote goes through stages of mitotic cell division called cleavage divisions in which the daughter cells, called blastomeres become smaller (1). These cleavage divisions are asynchronous (2). Until the blastocyst stage the embryo is encased in the acellular glycoprotein shell, the zona pellucida. Eventually a ball of blastomeres forms resulting in a morula stage embryo, usually around the 16-32 cell stage in ruminants (2, 3). Tight junctions form in the outer layer of the morula, with gap junctions forming between the cells of the inner layer (4). This occurs on d 3-4 in the ewe and d 4 in the human. The morula compacts, corresponding with the maternal to zygote transition in ruminants that marks transcription and translation from the entire genome instead of only from the stored maternal mRNA transcripts. This occurs around the 4-8 cell stage in humans, and 16-32 cell stage in ruminants (1). The outer layer of the cells in the morula pump sodium ions to the inner layer, creating a fluid-filled blastoceole cavity (4). At the blastocyst stage the embryo has differentiated into two distinct cell types, the inner cell mass (ICM), or embryonic disc, which will give rise to the fetus, amnion, and allantois, and the trophoblast, composed of the trophectoderm layer and inner endoderm layer that encircle the entire embryo, which will give rise to the chorion of the placenta (2). This first differentiation event is in part controlled by the

transcription factor Oct4, which must be down-regulated for differentiation of the trophoblast to occur (5). As well as down regulation of Oct4, the up-regulation of transcription factors Sox2 and Cdx2 are also involved in early trophoblast regulation (5). One of these, Sox2 is required for production of fibroblast growth factor 4 (FGF4), that is required for trophectoderm proliferation (6). As the blastocyst grows in size due to the increase in blastocoele fluid, proteolytic enzymes are secreted by trophoblast cells and/or the uterus that aid in degradation of the zona pellucida to facilitate hatching (2, 7). Following the hatching of the zona pellucida (d 7-8 in the ewe; d 5-6 in human), ruminant and primate embryo development diverges significantly. The human and rodent blastocyst retains its spherical shape as it adheres and attaches to the uterine endometrium. The ruminant on the other hand, goes through a rapid elongation process that will be discussed in more detail.

The preimplantation period varies greatly between species; ranging from 4 days post fertilization in the mouse to 20-25 days post fertilization in the cow (1, 8). All ungulate embryos go through a rapid elongation phase prior to attachment to the endometrium (9). This elongation process does not occur in primates, rodents, or horses. Interestingly, in some ungulates that display embryo diapause such as roe deer, the elongation process can be delayed up to 5 months, during which time the blastocyst retains a spherical shape (10). The mechanisms regulating blastocyst elongation in ungulates are largely unknown, partly because of the difficulty in the in vitro culture of elongated conceptuses (9, 11). Similarly, trophoblastic vesicles dissected from an elongated conceptus can expand, but do not elongate under in vitro conditions (2). Elongation in the sheep conceptus begins around d 11-13 post-conception (3, 9, 12).

Prior to this time, ovine conceptuses retain a spherical shape, similar to an expanded blastocyst. Others have reported the beginning of elongation as early as d 10 (13). The conceptus continues to increase in length up to d 16-17; also at this time the beginning of a close apposition of the conceptus to the uterus is occurring (14, 15). At its maximum length, the elongated ovine conceptus can be greater than 10 cm in length, resembling a long, thin filament (7, 16, 17). Even more dramatic increases in conceptus elongation are observed in the pig; which can elongate from a 10 mm sphere to a 150 mm filament in 2-3 h (18). Both the onset and rate of elongation are extremely variable within the same species (2). Others have also reported a wide range of variation in the stage of development at any single day post conception in the sheep (19). At around the time elongation has completed, attachment to the uterus begins. It has been shown that on d 16 in sheep, the conceptus begins to adhere to the endometrium and that elongation has stopped (7, 16).

The increase in size of ungulate conceptuses corresponds to an increase in cell number and protein synthesis (16, 20, 21). However, Geisert et al. (18) have suggested that elongation occurs primarily through cellular remodeling and migration, not through hyperplasia. In that study there was an increase in DNA and RNA content from d 10 to 16 in porcine conceptuses; however, this was a linear increase, with no surge occurring at the time of elongation and no increase in mitotic index at the time of elongation. More recent studies have suggested hyperplasia is involved in porcine conceptus elongation (22). In ovine trophoblasts others have observed an increase in lipid content during elongation, and death and disintegration of some trophoblast cells (23). Secretions from the uterine environment appear to be critical for elongation to occur. Elongation does not

occur in vitro, blastocysts must be transferred to the uterus and elongation does not occur in sheep lacking uterine glands (24, 25). Additionally, elongation is not dependent on the inner cell mass, as trophoblastic vesicles are able to elongate in the ovine uterus (24).

Many previous studies have identified global gene expression changes during the elongation period of ungulate conceptus growth (16, 26, 27, 28). However, most of these studies identify previously known genes, and none provided evidence of critical function during elongation by using a knockdown or knockout approach. Furthermore, it is estimated that the expression of 10,000 genes is necessary for successful pre-implantation and early fetal development (29). Therefore, only a handful of the identified genes will be discussed here. In an effort to study genes that may be involved in sheep conceptus elongation Cammas et al. (16) compared gene changes in pre-elongated, elongated, and implanting ovine trophoblasts using microarrays. Of particular interest is TMSB10, a $\beta$-thymosin that sequesters actin and causes a stronger ability of cells to spread and adhere when overexpressed. Expression of this gene was increased during conceptus elongation in sheep and pigs (16, 30). In the pig, IL-1$\beta$ increased during elongation in the conceptus, but was unchanged in the uterus during the same time period (26). Interestingly, IL-1$\beta$ decreased after elongation had stopped. Besides these, expression of a number of other genes that have a role in mitosis, signaling, and cell to cell communication and attachment have been identified by analysis global gene changes. The epidermal growth factor (EGF) gene family has significant effects on growth of mammalian embryos (31). The EGF receptor, as well as its ligands, EGF and transforming growth factor-$\alpha$ (TGF- $\alpha$) have been identified in elongating bovine embryos (d 13 to 16) and bovine endometrium; however, no change in expression was

observed over this time period (31). Another gene family affecting embryo growth, the transforming growth factor $\beta$ (TGF-$\beta$) family was investigated in elongating ovine conceptuses. The mRNA for TGF-$\beta$1 and TGF-$\beta$2 isoforms increased from d 13 to 27 of gestation (32). Immunolocalization of these two isoforms showed an increase in staining from d 13 to d 16 in both trophectoderm and trophendoderm (32). Although expressed during the time period of elongation, an established role of any of these genes during that process has not been established.

During elongation, the extraembryonic membranes form in most domestic species, while in primates and rodents, the extraembryonic membranes do not form until after implantation (4, 7). Importantly, these membranes must form prior to attachment to the endometrium in ruminant species (4). At the time elongation begins, the cells of the trophoblast portion of the blastocyst make up three quarters of the total cell number in the embryo (2). As the blastocyst grows, the endoderm originates from the inner cell mass and elongates with the trophectoderm layer (2, 7, 12). The endoderm migrates downward from the inner cell mass, forming an epithelial sheet lining the blastocoele and eventually connecting to itself to form the yolk sac (12). The endoderm layer further differentiates into visceral endoderm (VE) located under the ICM, and mural or parietal endoderm (PE) which surrounding the rest of the blastocoele (12). The VE is an area of cell proliferation, while the PE is made of large flat cells that migrate with the trophectoderm during elongation (12). In cattle and sheep, the layer of trophectoderm covering the inner cell mass, termed Rauber's layer, eventually disintegrates as the blastocyst elongates, exposing some cells of the inner cell mass to the maternal milieu (2). The mesoderm also forms during the preimplantation period between the

trophectoderm and endoderm layers on d 14 to 16 in cattle (2). The mesoderm and trophectoderm fuse to form the chorion, which is the placental membrane that forms an attachment to the uterus. The chorion eventually fuses over the dorsal portion of the inner cell mass to form the amnionic sack. While the amnion is forming, the allantois forms from the primitive hindgut of the embryo, which collects waste from the embryo. The allantois eventually fuses to the chorion, forming the chorioallantois (33). A similar sequence of events occurs in most mammalian species; however, the timing of initiation of these events varies.

The preimplantation period is also the time of maternal recognition of pregnancy in both ruminants and primates during which the embryo must secrete factors that signal its presence in the uterus and prevent luteolysis. In a normally cycling animal, if fertilization of the ovulated oocyte and embryo development does not occur, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is released from the uterus (ruminants) or ovary (humans) to cause luteolysis of the corpus luteum (CL) on the ovary (34). However, if pregnancy is to be maintained progesterone must continue to be produced from the CL of the ovary during early gestation in both ruminants and humans (34, 35). In cows progesterone from the CL must be produced throughout gestation; however, in the human and ewe, the placenta takes over as the primary site of progesterone production by the $9^{th}$ week of gestation, and d 55 of gestation, respectively (36, 37). During the elongation phase the ruminant embryo secretes the pregnancy recognition signal, interferon-tau from (IFN-$\tau$) the trophectoderm cells (38). First identified as ovine trophoblast protein-1 (oTP-1), IFN-$\tau$ is a type I interferon that prevents luteolysis, resulting in maternal recognition of pregnancy in ruminants (34, 39). Importantly, the ruminant embryo must elongate for

IFN-$\tau$ secretion to occur (40, 41). Interferon-$\tau$ is a 172 amino acid, 18,000 MW protein that is present in the uterus from d 13-21 after ovulation (35, 38). It acts by preventing the formation of oxytocin receptors in the uterus, thereby preventing the pulsatile release of $PGF_{2\alpha}$ and luteolysis (42). In the human, the pregnancy recognition signal is the glycoprotein hormone human chorionic gonadotropin (hCG), which is secreted from trophoblast cells beginning on d 7-9 after ovulation and peaks at 12 weeks (4, 36). It functions to prolong the lifespan of the CL during early pregnancy, and has a role in cytotrophoblast function (36). Interestingly, the transcription factor Ets2 is a regulator for both IFN$\tau$ genes (43) and for hCG $\alpha$ and $\beta$ subunits (44, 45) and is required for placenta development of the mouse (46).

ADHESION AND ATTACHMENT TO THE ENDOMETRIUM

The first step in the formation of placenta is for the trophectoderm layer of the blastocyst to attach to the luminal epithelium of the endometrium. The anatomy of the endometrium is similar across species, and the time period for uterine receptivity for embryo attachment is relatively short. The mucosa and submucosa layer of the uterus make up the endometrium (4). The endometrium contains a monolayer of pseudostratified epithelium separated from the stroma by a basal lamina, known as the luminal epithelial. The epithelium contains microvilli and ciliated cells. The glandular epithelium lining the uterine glands located in the submucosa, opens into the endometrial lumen is responsible for secretion of products into the endometrium (7). The secretion of materials from the uterine glands into the lumen is important for survival and function of the perimplantation embryo (4). The mixture of enzymes, growth factors, cytokines, lymphokines, hormones, proteins, and other substances secreted into the uterus has been

termed the 'histotroph' (7, 47). To investigate the effects of histotroph on embryo development, the uterine gland knockout ewe (UGKO) model was generated by continuous exposure of lambs to progesterone from birth to postnatal d 56, which ablates the glandular epithelium and reduces luminal epithelium surface area (48). In this model, recurrent pregnancy loss occurs due to a lack of conceptus elongation (25). On d 14 after mating conceptuses developing in a UGKO ewe were either severely retarded or not present.

In ruminants, specialized nonglandular, highly vascularized structures called caruncles are present throughout the surface of the endometrium; these serve as the primary sites of placental attachment to the uterus in these species (4). Initial attachment of the cotyledon of the placenta to the caruncle in sheep takes place at about d 16, and a firm attachment is established on d 30 (7). During this time, the caruncles become folded and depressed on their surface, allowing attachment of the cotyledons. In humans and rodents, a modification of the endometrial stromal cells, uterine glands and vessels and uterine immune cells occurs, called decidualization (49). This process occurs during the normal menstrual cycle, but is more extensive during pregnancy and involves glycogen and lipid accumulation in the endometrial cells (36). Decidualization alters the expression of metalloproteinases (MMP), cytokines, surface integrins, and histocompatibility molecules (49). Although decidualization does not occur in ruminants, similar changes in the molecules, proteins, and lipids expressed on the endometrium occur, allowing for attachment to take place (7). Another possible role of the decidual reaction during pregnancy in the human is to control the invasiveness of the invading cytotrophoblasts and control migration extravillous cytotrophoblasts (50).

The process of attachment is highly complex in all species and involves the interaction of many molecules on both the maternal and fetal side. In sheep, the filamentous conceptus on d 14-16 is immobilized in the uterus and begins a close association with the luminal epithelium (7). The trophectoderm layer in most domestic species is covered in microvilli (2). The apical microvilli covering the surface of the trophectoderm are reduced at the time of attachment allowing for closer apposition (7, 51). The apposition first occurs near the inner cell mass before spreading to the polar ends of the trophectoderm (7). Importantly, although the caruncles are the primary sites of attachment, and the sites of nutrient exchange through the chorionic villi, attachments occur in the intercaruncular areas of the endometrium as well through microvilli (7).

Many of the interactions that take place during attachment are common to different mammalian species. In all mammals, progesterone is important during the establishment and maintenance of pregnancy. Progesterone receptors (PR) are expressed on the endometrial epithelium during the early luteal phase in all mammals (34). The loss of PR in the luminal epithelium occurs immediately prior to implantation due to continuous exposure to progesterone in both sheep and primates (34, 52). In the sheep, PR are present in the stroma of the uterus throughout pregnancy (34). Specific molecules determined to be important during the attachment process in both the sheep and human are MUC1, glycosylated cell adhesion molecule 1 (GlyCAM-1), integrins, and osteopontin (OPN) (7, 53). The transmembrane protein MUC1 covers the epithelial surface of the endometrium resulting in a thick mucin coating and may be down-regulated in ruminants to allow attachment; however, in humans it has been shown that MUC1 may only be down-regulated at specific sites of blastocyst implantation (49).

Integrins are heterodimeric transmembrane glycoproteins that bind to extracellular matrix ligands and function in adhesion, cell migration, proliferation, and differentiation (7, 54). Integrins are expressed on the both the trophoblast and endometrial surfaces in ruminants and humans. The endometrium of women expresses different integrin subunits dependent on the day of the menstrual cycle and alterations in expression has been linked to infertility (55) However, in sheep integrin subunits are expressed constitutively and do not change due to pregnancy or presence of a conceptus (7). Similar to integrins, OPN is a glycoprotein that binds integrin heterodimers and is expressed in the endometrium during the perimplantation period in sheep and humans, and OPN receptors have been identified on the ovine and human trophoblast and luminal epithelium (7, 55, 56). Osteopontin binding to integrin subunits has been suggested to promote cell adhesion, spreading, and migration (7). Galectin-15 is a secreted lectin protein identified in the sheep endometrium that appears to increase during the elongation phase of embryo development and is abundant at the time of attachment (17). This molecule also increased proliferation of ovine trophectoderm cells in vitro. Although other galectins are present in the human, galectin-15 has not been identified in the human endometrium. Adhesion molecules identified in the human placenta include VE-cadherin, VCAM-1 and PECAM-1 (53). As discussed, there are many commonalities in anatomy and in the expression of specific adhesion molecules in the process of adhesion and attachment to the endometrium between the ruminant and human placenta. However, after this initial attachment takes place, another major change in physiology occurs between the ruminant and the human. While the ruminant placenta forms permanent attachments to the endometrium, there remain a number of cell layers between the fetal and maternal

vasculature. The human placenta, invades into the endometrium much further, eroding away more layers of maternal tissue. The structure and development of the ruminant and human placenta will be discussed in more detail.

## THE HUMAN PLACENTA

Eutherian mammals, those with a placenta, have a reproductive advantage due to the ability to protect the developing fetus within the maternal environment during development. The placenta functions in transport, metabolism, protective, and endocrine functions. Due to their recent evolution, there is a great degree of variation in the structure of the placenta in eutherian mammals; however, the function of the placenta remains similar across species. Normal placental development is critical for successful gestation to term and birth of a healthy infant. In humans, implantation begins earlier in development than in ruminants, when the blastocyst invades into the uterine epithelium immediately following attachment to the endometrium. The human placenta is classified as hemochorial, consisting of three layers between the maternal and fetal vasculature: the syncytiotrophoblast, maternal stroma, and fetoplacental vascular endothelium (57); and in some areas the syncytiotrophoblast is bathed directly in maternal blood (36). The functional unit of the human placenta is the chorionic villous tree, which develops in three stages (57). Finger-like outgrowths of trophoblast termed primary villi begin to develop on d 13 post-conception and are made up exclusively of trophoblast cells. On d 21 post-conception, mesenchymal cells from the embryo invade the primary villi and are then termed secondary villi. Within days of this event, blood vessels form within the secondary villi, and they are now termed mature tertiary villi. Most maternal-fetal exchange occurs in the terminal regions of the villi. The multinucleated

syncytiotrophoblast forms the outer epithelial layer of the chorionic villi and is in direct apposition to the maternal environment (58). Spaces within the syncytial layer form, called lacunae that coalesce to form the intervillous space that fills with maternal blood (57). The tips of the villi project into the intervillous space, and from here the invasive cytotrophoblast columns form (36). The intervillous space is lined with the multinucleated syncytiotrophoblast. Expansion of the villous tree occurs throughout pregnancy (49). The formation of the syncytiotrophoblast and cytotrophoblast will be discussed in more detail.

Immediately after attachment there is rapid proliferation of the trophoblast cells of the blastocyst that retain a stem cell population that diverges into two trophoblast cell lineages; the villous, or multinucleated syncytiotrophoblast and the extravillous, or invasive cytotrophoblast (36, 53). The syncytiotrophoblast is non-proliferating (49). It grows from incorporation of new cytotrophoblast cells into the epithelial layer of the chorionic villous (36, 49). The multinucleated syncytiotrophoblast is the site of nutrient and gas exchange, as well as the site of production of hormones and growth factors such as placental lactogen, chorionic gonadotropin, and placental growth hormone (36, 49, 53).

On d 13-14 of pregnancy, cytotrophoblasts penetrate through the syncytiotrophoblast to form columns of invasive cytotrophoblasts (36). These cytotrophoblast columns may exist in a proliferative and undifferentiated state (59). These columns form the anchoring villi (36). From the tips of these columns, subpopulations of extravillous cytotrophoblasts invade further, reaching over a third of the way into the myometrium. The extravillous cytotrophoblasts either become

interstitial and attach the placenta to the uterus, or endovascular and invade the maternal uterine spiral arteries (53). The extravillous endovascular cytotrophoblasts express endothelial cell adhesion molecules, enabling them to attach to the uterine spiral arteries (53). The endovascular extravillous cytotrophoblasts displace the endothelial cells of the spiral arteries and degrade the muscle and elastic lining, thereby transforming them into vessels of low resistance and high capacitance (53). The maternal spiral arteries are initially plugged by the endovascular cytotrophoblast cells and placental development in the first trimester takes place in a hypoxic environment. By 10-12 weeks of pregnancy blood flow increases, and oxygen tension rises as the spiral arteries are remodeled and widened (36). The hypoxic environment of the first trimester placenta appears to be important for cytotrophoblast proliferation and differentiation (60). Remodeling of the spiral arteries is necessary for transformation of the vessels to low resistance, high capacitance vessels enabling efficient blood supply and exchange of nutrients, gases, and wastes between the fetus and maternal environment. The interstitial extravillous cytotrophoblasts increase the circumference of the placental attachment and subsequent recruitment of more maternal arteries (36). Additionally, some of these interstitial cytotrophoblasts become multinucleated and form placental bed giant cells, regarded as the terminal differentiation step in the extravillous cytotrophoblast pathway (36).

Although these invasive cytotrophoblasts aggressively invade the uterus, there is tight regulation in normal development (61). During first trimester placental development, there is a balance between the processes of proliferation, migration, differentiation, and apoptosis. In the mouse this process of trophoblast differentiation is controlled by the transcription factors Mash2, Id2, E-factor, and Sta13; however, little is

known about the function of these transcription factors in the human (62). The AP-2 and Sp transcription factor families function in syncytiotrophoblast fusion; and Hash-2 and Id-2 maintain cytotrophoblast cells in a proliferating state (49). Differentiation is also controlled by other hormones, growth factors, cytokines, and $O_2$ concentrations (49). For example, prior to the $10^{th}$ week of gestation, low $O_2$ concentrations stimulate trophoblast cell proliferation, but inhibit differentiation into the syncytiotrophoblast and invasive cytotrophoblast cell types (49). Apoptosis also plays a role in both normal and pathological human placental development and has been reviewed by others (58, 62). The invasive property of the human placenta has resulted in many comparisons between cytotrophoblast cells and cancer cells, and some cytotrophoblasts are in fact cancerous in the case of choriocarinomas (64, 65).

The process of invasion into the endometrium is one of the major differences in placental development between species. Invasion begins with the multinucleated syncytiotrophoblast invading through the epithelium, followed by the villous cytotrophoblasts which invade further. Invasion of the trophoblast in the human is regulated by a variety of molecules including: cell adhesion molecules, growth factors, cytokines, hormones, inflammatory factors, integrins, and extracellular degrading matrix proteinases (49, 66). In both the ruminant and the human, the complement of MMPs that degrade the extracellular matrix, and tissue inhibitors of MMPs are necessary for successful implantation (16, 36). Although ovine trophoblasts do not invade the endometrium as extensively as is seen in the human, they do express MMP-2 and MMP-9 (67). Similarly, MMP-9 is associated with the invasive cytotrophoblasts of the human placenta (68).

Abnormalities in placental development and formation early on can lead to embryonic death, which is when most pregnancy loss occurs. These abnormalities have been studied more extensively in human pregnancy than in ruminants. Perturbations in the first trimester that don't result in mortality can have consequences later in the third trimester such as preeclampsia, intrauterine growth restriction (IUGR), placenta abruption, or placental accrete (33). Many of these pathologies appear to result from abnormalities in cytotrophoblast invasion. Preeclampsia affects 2-3% of all pregnant women and is characterized by pregnancy induced hypertension, maternal edema, and proteinuria. In severe cases eclampsia, or seizures occur (53, 69). In preeclamptic pregnancies, shallow trophoblast invasion, or a reduced number of trophoblasts invading the uterus is commonly observed (53). Expression of molecules necessary for degradation of the extracellular matrix such as MMP-9 and uPA may be altered in preeclampsia (69). Intrauterine growth restriction is diagnosed not by low birthweight, but by a failure of infants to reach their genetic growth potential in utero, determined by prenatal screening (70). Many times the cause of IUGR is placental insufficiency. Up to 8% of all pregnancies are affected by IUGR, and many placental structural abnormalities have been associated with IUGR (71). Invasion of the cytotrophoblasts may be limited in IUGR, similar to what is observed in preeclampsia and alterations in structure and function of the chorionic villous is also observed in IUGR (57, 72). Conversely, placenta accrete and choriocarcinomas are associated with excessive cytotrophoblast invasion (49). For the above-mentioned reasons, the study of factors involved in trophoblast differentiation and invasion is a major focus of human pregnancy research.

For obvious reasons, studies of human placental development in vivo are very difficult; therefore animal models are usually employed. The placenta of some non-human primate species are very homologous to the human placenta in physiology and anatomy; however, cost and ethical concerns have limited their use in studying placental development. As an experimental model for placental development, mice and rats are quite commonly used as well. Their small size, low cost of maintenance, similarity to the human placenta based on maternal/fetal blood separation, and most importantly the ability to perform genetic manipulations, make them a desirable model for some circumstances. However, other traits make them less desirable as a model. They are a litter-bearing species, are too small to catheterize for repeated sampling, fetal development is not complete until after parturition, and they have a very rapid implantation and apposition, with attachment and invasion occurring within 6 h (73). Due to the extremely short apposition and implantation time period, they may not be good models for early embryo development (73).

THE RUMINANT PLACENTA

Sheep have been used extensively as a model for placental development. One method of placental classification is by distribution of the functional unit of the placenta, the chorionic villi. As opposed to the human placenta which has a single point of attachment made of many chorionic villi, the ruminant placenta is classified as cotyledonary and has many distinct points of attachment, with the placentomes made of a maternal (caruncle) and fetal (cotyledon) component (33). The fetal cotylendon is made up of the chorionic villi. Across the placental surface, the sheep contains 90-100 cotyledons and the cow 70-120 (4). The horse and pig have many more, diffuse points of

attachments, hence having a diffuse placenta. The cow, sheep, and pig all exhibit rapid embryo elongation, allowing a large surface area for the points of attachment to occur in order to compensate for the less invasive implantation. Between the cotyledons of the ruminant placenta is the interplacentomal region, with simple apposition between the trophoblast and endometrium where histotroph transfer occurs (74). By the 4$^{th}$ week of gestation in sheep there is interdigitation between maternal and fetal tissues (75). The cotyledons form villi that branch and elongate into the caruncles on the endometrial surface. The placentomes function to exchange nutrients and metabolites. Microvilli are present on the surface of the trophoblast cells in both regions. In the interplacentomal regions, papillae also develop and penetrate into the uterine gland openings (76).

Based on the number of layers between maternal and fetal vasculature, the ruminant placenta is generally designated as synepithelialchorial. It contains six layers between the fetal and maternal circulation: the chorionic or fetal capillary endothelium, fetal connective tissue, fetal chorionic epithelium, maternal epithelium, maternal connective tissue, and maternal capillary epithelium. The fetal chorionic epithelium and maternal epithelium fuse in some locations, making five layers, that will be discussed further below. The ruminant placenta contains two types of specialized cells on the trophectoderm epithelium; the mononuclear trophoblast cells, and the binucleate trophoblast cells, sometimes called trophoblast giant cells (33). Binucleate cells form from the mononuclear cells by acytokinetic mitosis and have similar features across ruminant species (77). Mononuclear cells are epithelial cuboidal or columnar cells that have microvilli on their surface, are involved in most nutrient exchange, and produce the pregnancy recognition molecule, IFN$\tau$ (33).

In ruminants, binucleate giant cells from the trophoblast migrate through tight junctions in adjacent mononuclear trophoblast cells where they fuse with columnar cells of the endometrial luminal epithelium into multinucleated syncytial plaques, which results in the ruminant placenta being classified as synepithelialchorial (33, 77). In cows and deer these are eventually displaced by mononuclear cells, but in sheep and goats they are present throughout gestation (33). This migration of binuleate cells into the endomtrium may aid in diffusion of nutrients by reducing the number of layers between maternal and fetal circulation. The binucleate cells can make up 20% of the fetal placenta. These syncytial plaques cover the caruncles by d 24 of pregnancy in the sheep. Similarly to the syncytiotrophoblast cells of the human placenta, the binucleate cells are endocrine cells that produce progesterone, estradiol 17$\beta$, and placental lactogen and the syncytium they form is essential for placental function (7, 33, 78). While true implantation into the uterus does not occur in ruminant species, implantation in ruminants can be defined as the process that results in attachment of the conceptus to the maternal endometrium, leading to the establishment of placental structures (79).

Although sheep and humans differ in gross anatomy of the placenta, at the level of materno-fetal interdigitation, both exhibit a multi-villous structure, wherein the chorionic villi fit into maternal endometrial crypts, creating a similar materno-fetal blood flow relationship for exchange of nutrients, hormones, oxygen, etc (70). In both ruminants and humans, these villi can be divided into stem, intermediate, and terminal villi (80). Additionally, the structure of the fetal placental blood vessels of sheep and humans is similar (70, 80). In the case of studying IUGR, sheep have proved useful because growth restriction can be induced in by a variety of relatively simple methods

(71). Furthermore, the large size of sheep enables catheterization and repeated sampling from both the maternal and fetal side of the placenta throughout gestation under different experimental conditions (70). As discussed, development of the placenta in ungulate species involves a rapid elongation of the trophoblast and a slower process of attachment as compared to humans or rodents; making these species good models for studying mechanisms of adhesion and attachment as well (73).

PREGNANCY LOSS

Loss of pregnancy can occur at any time during development; however, these losses usually occur after fertilization, but before fetal development begins. Fertilization rates in beef cattle are estimated at close to 90%, while conception rates average 60%, indicating that embryo mortality as the major cause of reproductive failure in this species (81). The reproductive performance of high producing dairy cattle is even lower, with conception rates around 40%, and a recent estimate of 28% embryo loss between d 19 and 26 of pregnancy (82, 83). Embryo losses between d 8 and 16 in cattle have been estimated close to 30% (84). In all domestic ruminants embryo mortality is most prevalent during the elongation phase of embryonic development, just prior to implantation, while later losses during the fetal period are only estimated at 5% (85, 86). Similarly in the pig, the majority of embryo loss occurs during the elongation phase (87).

In humans, embryo mortality rates are also high, and infertility is a problem for a large number of people. A recent survey conducted by the Centers for Disease Control indicated that 12% of women of child bearing age in the United States have received infertility service and that 7% of married women of reproductive age reported they had not used contraception for 12 months without becoming pregnant (88). It has been

estimated that 50% of in vitro-produced human embryos are retarded or do not develop to the blastocyst stage, and that less than 20% of transferred embryos develop into a baby (89, 90). More recently, the pregnancy rate following transfer of in vitro-produced embryos was estimated at only 28% (88). Because of this inefficiency, multiple embryos are often transferred, creating additional problems. It was recently estimated that 34.2% of all live births resulting from in vitro embryos were multiple-infant births. These cause increased risk of morbidity and mortality to the mother and infant (88, 91). In vivo, up to 60% of all embryo loss in humans occurs within two weeks of fertilization (92).

For an embryo to survive and establish itself in the uterus, it must avoid rejection by the maternal immune system and secrete factors that signal its presence and enable the maintenance of the CL during the first few weeks of pregnancy. This is especially important during the "critical period" (d 15 to 17 in cattle) during which the majority of embryo mortality occurs (93). The predominant factor determined to be important in ruminants is IFN-$\tau$ and in humans, hCG. However, many other factors, including growth factors, cell-adhesion molecules, cytokines, extracellular matrix metalloproteinases, hormones, and transcription factors are also produced by the conceptus, enabling it to establish itself and grow in the uterus (71). Furthermore, IFN-$\tau$ isoforms are only expressed in ruminant trophoblast as hCG is only produced by the human trophoblast (39).

Due to the high rates of pregnancy loss early in gestation in both livestock species and in humans, this time period of development has been a major focus of research. Furthermore, alterations or insults early in pregnancy can possibly have adverse health effects later in life (94, 95). Proper development of the placenta is necessary for fetal

growth and function. Any improvement in reproductive efficiency in livestock species would have a large economic impact. Likewise, preventing the loss of pregnancy in humans, and importantly, research into a better understanding of the complications of pregnancy would have a positive impact on human health.

PRIOR RESEARCH ON PERIATTACHMENT FACTOR

The gene that is the focus of these studies (periattachment factor, or PF) was first identified in trophoblast tissue through the use of mRNA differential display in elongating bovine embryos (96). Northern hybridization indicated a 10.6-fold greater mRNA concentration of PF in d 17.5 than d 15.5 embryos. However, no PF mRNA was detected by Northern hybridization in d 30 or d 36 bovine conceptuses, and in a panel of seven adult bovine tissues PF mRNA was detected only faintly in the kidney (96). The time period of PF expression corresponds with critical events during gestation, such as maternal recognition of pregnancy and the beginning of a close apposition of the embryonic trophoblast to the uterine endometrium. The narrow time period of expression at such a critical stage in development and the lack of expression observed in adult tissues led to further interest and research on PF.

In-silico analysis identified homologues of PF in the human, baboon, chimpanzee, mouse, dog, and rat genomes. A number of cDNAs were also identified, including those in the human placenta, colon, kidney, and stomach, as well as the mouse lung, neonatal eye, mammary gland, and brain. Two of these, the human colon and mouse mammary gland were derived from carcinomas. A mouse homologue to PF, named G90 is expressed in the small intestine, and to a lesser extent in the large intestine, kidney, and testis (97). Expression in the small intestine occurred in the villous epithelium (97).

Expression of G90 was also identified during mouse embryonic development in the brain, inner, and middle ear, olfactory epithelium, vomeronasal organ, nasopharynx, pituitary gland and epiglottis (98). In summary, many of the homologues of PF appear to be present in rapidly growing tissues or in tissues with expanding or growing epithelium.

The inferred amino acid sequence of bPF identified by Glover and Seidel (96) was submitted to various protein analysis sites. These have identified four protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites, and a nuclear targeting sequence; however, no RNA or DNA binding motif, or signal peptide sequence was identified. Interestingly, all six phosphorylation sites as well as the nuclear targeting sequence are identical between the cow and human sequence. Because of these indicators, a potential hypothesis is that PF functions as a coactivator or corepressor of transcription. Once phosphorylated, PF may migrate to the nucleus, or if already there become active in the nucleus and act on other transcription factors or with other coactivators or corepressors in the cell. Several transcription factors have been identified in the mouse that function as regulators of trophoblast differentiation and invasion (62). Others have identified transcription factors that function during specific times during conceptus development and trophoblast differentiation and proliferation (5). It may be that PF is a coregulator of one or more of these factors.

To begin testing this hypothesis a series of experiments were conducted at Colorado State University in the Anthony laboratory (99). An oPF cDNA was generated that encompassed the entire ORF and inserted into a mammalian expression vector. The vector contained a His and V5 tag, both of which can be used for immunoblot analysis. This was then used to stabily transfect Cos-7 and CHO-1 cell lines and used to isolate

nuclear and cytoplasmic fractions. Immunodetection indicated that PF was located in the nucleus; however, the fusion protein was also located in the cytoplasm (99). Other experiments were performed in the Anthony laboratory using Southern and Northern hybridization of bPF.

Southern hybridization of radiolabeled bPF cDNA to genomic DNA indicated that the PF gene was in the human, dog, cow, pig, rabbit, yeast, and horse genomes (99). Northern hybridizations of bPF cDNA to RNA were also performed on a variety of tissues and species to determine expression of the PF mRNA. Northern hybridization in sheep tissue indicated that PF was expressed in the d 13 and 15 conceptus, and faintly in the kidney and lung, but was absent from a variety of other adult sheep tissues. In the bovine, PF mRNA was detected in a d 21 conceptus, but not in a d 28 conceptus. The d 28 conceptus contained both fetal and trophoblast tissues, and when the trophoblast was dissected away and analyzed separately, PF mRNA was detected (99). The reason for this difference was likely due to the equal amount of RNA used for each hybridization reaction. Therefore, it is likely that the amount of PF RNA was a small percentage of the d 28 conceptus, while it would have been a greater percentage of the total RNA in trophoblast tissue alone. In the rat, PF mRNA was not detected in any adult tissue tested. The lack of detection in rodent tissues by Northern hybridization was likely due to the high stringency of the hybridization reaction and mismatches between the bovine and rodent mRNA sequences; the presence of PF in rodents had been previously reported (97). In the equine, PF was detected in the d 14, d 16, d 20, and d 30 conceptus; however, it was not detected in the d 30 fetus (99). Although detected during similar time periods as the bovine, there are major differences between the equine and ruminant

embryo and conceptus. Unlike ruminants, IFN-$\tau$ is not secreted from the equine conceptus to serve as a pregnancy signal. In fact, little is known about the process of maternal recognition of pregnancy in the mare, although embryo migration between days 8 to 17 and prostaglandin $E_2$ may play a role (100). Second, the equine conceptus does not elongate as it does in the ovine or bovine, but does grow rapidly, retaining a large spherical shape until around d 16, usually termed an embryonic vesicle (101). These Northern hybridization results show that PF is expressed in the developing conceptus in a variety of species, including non-ruminants, while it is absent in the majority of adult tissues tested, and supports the hypothesis that it may be important during conceptus development.

In summary, PF was first identified by RNA differential display at a greater concentration on d 17.5 than d 15.5 of pregnancy in the cow, corresponding in time with the greatest amount of embryo mortality, when the bovine embryo is rapidly elongating and initiating attachment to the uterine endometrium (96). Northern hybridization verified its expression in the conceptus of the sheep, and in a non-ruminant, the horse (99). Furthermore, the Northern hybridization results indicate it may be predominantly of trophoblast origin. In silico data indicates it is present in the human placenta as well. Due to the wide range of reproductive strategies between species, comparative physiology is especially useful in the field of reproductive biology (95). The studies described in this dissertation involve ruminant, as well as human cells and tissues. Little is known about the mechanisms regulating ruminant conceptus elongation or human cytotrophoblast proliferation and differentiation or invasion (7, 11, 61). Although there are differences in the structure and development of the placenta in these species, many of

the molecules regulating placental development are conserved across species, and the study of both will lead to greater understanding of the function of PF.

# CHAPTER III

# CHARACTERIZATION OF PERIATTACHMENT FACTOR IN THE OVINE CONCEPTUS

## INTRODUCTION

All ungulate embryos go through a rapid elongation phase prior to implantation, and in domestic ruminants embryo mortality is most prevalent during this elongation phase, while later losses during the fetal period are estimated at about 5% (85, 86). Furthermore, little is known about the cellular mechanisms involved in conceptus elongation in ruminants (12). For a ruminant embryo to survive and establish itself in the uterus, it must avoid rejection by the maternal immune system as well as elongate and secrete factors that enable maintenance of the CL. This is especially important during the "critical period" (d 13 to 15 in sheep and d 15 to 17 in cattle) during which the majority of embryo mortality occurs (88). The predominant regulatory factor during this critical period in ruminants is interferon-tau (IFN-$\tau$). First identified as ovine trophoblast protein-1 (oTP-1), IFN-$\tau$ prevents luteolysis, allowing for maternal recognition of pregnancy in ruminants (7, 39). However, many other factors, including growth factors, cell-adhesion molecules, cytokines, extracellular matrix metalloproteinases, hormones, and transcription factors are also produced by the conceptus, enabling it to establish itself and grow in the uterus (71).

Periattachment factor (PF) is a gene first described in the bovine conceptus at greater concentrations on d 17.5 than d 15.5 of pregnancy (96). Further screening of bPF mRNA resulted in detection in a d 25 cDNA library, but no detection in d 30 to 36 bovine trophoblast tissues by Northern hybridization. Sequence analysis of PF indicated an open

reading frame encoding a 126 amino acid protein. The inferred amino acid sequence predicted four putative protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites, as well as a nuclear targeting sequence (96). Further work confirmed the presence of PF mRNA in the ovine conceptus on d 15, and faintly in the adult sheep lung and kidney (99). In cattle, PF mRNA was detected in d 21 and 28 trophobast. In the horse, PF mRNA was detected in d 14 and 16 conceptuses, and in equine trophoblast tissue on d 20 and 30, but not the equine fetus on d 30 (99). These initial data generated through Northern hybridization analysis show expression of PF during an interesting time period of conceptus development in multiple species; however, the exact timing of expression throughout conceptus growth and elongation is unknown, nor is the cellular localization or function of this gene.

A common method for beginning to elucidate the function of a novel gene is through reverse genetics and the creation of a knockout animal, either through homologous recombination of embryonic stem (ES) cells, or pronuclear injection of plasmids into zygotes. However, this approach is difficult and inefficient in species other than mice (102). First described in the worm *Caenorhabditis elegans*, RNA interference (RNAi) is an evolutionarily conserved mechanism by which double-stranded RNA (dsRNA) activates sequence-specific gene silencing (103, 104). The process of RNAi occurs in a two-step mechanism. When dsRNA is introduced into a cell it is cleaved into 21 to 23 nucleotide (nt) long short interfering RNA (siRNA) by an RNase III family dimeric nuclease called Dicer. The siRNA then binds with the RNA-induced silencing complex (RISC), which then recognizes and degrades the target mRNA transcript by endonucleolytic cleavage (104). In mammalian cells, dsRNA can be presented in many

forms; however, those longer than 30 nucleotides lead to interferon-mediated antiviral responses and non-specific down regulation of gene expression. For this reason, either the injection of synthetic siRNAs of 21-22 nucleotides long that mimic the products of Dicer, or vectors that express a short hairpin RNA (shRNA) often are used to induce gene silencing. Short-hairpin RNAs are effective in gene silencing in a variety of mammalian cell types (105). Because they are cleaved by Dicer in the cell, it is thought that shRNAs may be more efficient in targeting genes for silencing than in vitro produced siRNA (105).

The use of lentiviruses, a type of retrovirus, to create transgenic or knockout animals with relatively high efficiency has been reported in mice and other species, particularly when compared to other methods of generating transgenic animals (102). Lentiviruses are a member of the *retrovidridae* family of dsRNA viruses that reverse transcribe into DNA once in the cell's cytoplasm, and then integrate into the host cells genome where their own RNAs are transcribed (106). Recently, Okada et al. (107) showed that lentiviral transduction of mouse blastocysts was not deleterious to blastocyst survival and resulted in trophoblast-specific transgenesis in all transduced embryos.

The aim of these studies were to determine the precise timing of PF mRNA expression during sheep conceptus development, to localize PF in the conceptus at the time of peak mRNA expression, and finally to use RNAi to study the development of the ovine conceptus treated to greatly reduce mRNA for PF.

## MATERIALS AND METHODS

### *Conceptus tissue collection*

All procedures with animals were approved by the Colorado State University Institutional Animal Care and Use Committee. For analysis of oPF mRNA concentration during early gestation and localization of PF, conceptuses were collected from mature crossbred ewes on d 11, 13, 15, 16, 17, 21, and 30 of gestation (n = 4 to 6 conceptuses/day). For collection of d 11 and d 13 conceptus tissue, ewes were superovulated by twice-daily i.m. injections for four days of FSH (30, 30, 20, 20, 10, 10, 10, 10 mg of Folltropin, Bioniche, Belleville, ON, Canada) beginning on d 7 of the estrous cycle. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse, Pfizer, New York, NY) was administered in two doses (5 mg, i.m. per dose) given 4 h apart beginning at the time of the $6^{th}$ FSH injection. Conceptuses on d 15 through 30 of gestation were collected from ewes synchronized by two injections (10 mg, i.m.) of $PGF_{2\alpha}$ given 14 d apart. After observation of standing estrus with a vasectomized ram, all ewes were bred by one of two intact rams. Pregnant ewes were sedated using sodium pentobarbital (20 mg/kg, i.v.), a complete hysterectomy was performed, and the ewes were euthanized (90 mg/kg sodium pentobarbital, i.v). Conceptuses were flushed from the uterus using Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham (DMEM/F12; Sigma, St. Louis, MO) supplemented with 0.25% Fraction V BSA (Sigma) warmed to 38°C. All recovered conceptuses were placed into 1.5 ml eppendorf tubes and briefly centrifuged before removing any liquid and then frozen at -80°C until tcRNA isolation. For d 21 and 30 conceptuses, the fetus was separated from the trophoblast before freezing.

*Total cellular RNA isolation and quantitative real-time PCR*

Total cellular RNA was isolated from individual d 11 - 30 ovine conceptuses and d 21 and 30 fetuses using RNeasy kits (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. For each day of gestation, tcRNA was isolated from four to six conceptuses. Quantity and integrity of RNA was confirmed by the absorbance ratio at 260:280 nm and by electrophoresis on a 1.2% formaldehyde denaturing gel and visualizing the 18S and 28S ribosomal subunits. Samples were stored at -80°C until use. Complementary DNA was generated from approximately 2μg tcRNA by reverse transcription (RT) at 55°C for 50 min using oligo dT primers (Superscript III, Invitrogen, Carlsbad, CA). A control RT reaction was performed in the absence of any RNA. Each cDNA sample was treated with 5 U RNase H (New England Biolabs, Ipswich, MA) for 20 min at 37°C. To control for variances in efficiency of the RT reaction, all cDNA was quantified by spectrophotometry based on absorbance ratio at 260:280 nm, and an equal amount of cDNA was used for each sample in the quantitative RT-PCR (qRT-PCR) reaction.

Primers for oPF were designed to amplify an intron-spanning 500 bp product based on the bPF cDNA sequence (96). The oPF primers used were: forward, 5' - TAG CTG GAC TGC AGC GAT TT - 3' and reverse, 5' - GAC ACT GGG GTG CTG ATT CT - 3'. The primers and Taqman probe sequences for oIFN-$\tau$, provided by Dr. Alan Ealy at the University of Florida, are able to detect all isoforms of oIFN-$\tau$ (108). Both oPF and oIFN-$\tau$ mRNA were normalized to oGAPDH mRNA levels after determining no differences existed in oGAPDH mRNA concentrations between any day of development (Fisher's LSD; $P > 0.10$). The oGAPDH forward primer, 5' - GAT TGT CAG CAA

TGC CTC CT - 3' and oGAPDH reverse primer, 5' - GGT CAT AAG TCC CTC CAC GA - 3'amplified a 94 bp product. Before qRT-PCR was performed, all primers were tested by amplification of a PCR product that was cloned into pPCR-Script Amp SK (+) vector (Stratagene, LaJolla, CA) and sequenced (Colorado State University Macromolecular Resources) to verify identity. An annealing temperature of 60°C was used for all genes.

For quantitative qRT-PCR analysis, a standard curve was created for each gene from 1 x $10^2$ to 1 x $10^{-6}$ pg using a PCR product generated from the sequenced plasmid. Amplification was detected using iQ SYBR Green Supermix for oPF and oGAPDH and iQ Supermix for oIFN-$\tau$ on a Biorad iCycler (Biorad, Hercules, CA) with the addition of 200 nM forward and reverse primers for each gene. Due to the high GC content of the oPF transcript (67%), betaine (N, N, N- trimethyl glycine; Sigma) at 1M final concentration and *Pyrococcus furiosus* (*Pfu*) polymerase (*Pfu* Turbo Hotstart, Stratagene) at 0.15 U were added per 25 µl qRT-PCR reaction. Samples were amplified by 45 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For all genes except oIFN-$\tau$, a melt curve analysis was conducted by increasing the temperature 0.5°C at 10 sec intervals from 55°C to 100°C. All standards were run in duplicate, and cDNA from each conceptus was run in triplicate. Product specificity was confirmed by observation of a single melt curve for each gene and by electrophoresis and visualization of all qRT-PCR products on a 2% agarose gel. Two control reactions were run for each gene, one containing all components except cDNA and one containing the RT control cDNA. The starting quantity (pg) of the gene of interest was normalized to the starting quantity (pg) of GAPDH.

*Recombinant oPF production*

Primers were designed to amplify the entire open reading frame (ORF) of oPF. The primer sequences were: forward: 5'- GCA CAC GTG ACT TCG ACA AA – 3', and reverse 5' – AAT GGG AAG AGA GTC CTG GAC -3'. Ovine PF was cloned into pENTR™/TEV/D-TOPO (Invitrogen), which contains a Tobacco Etch Virus (TEV) protease site on the 5' end of the PF cDNA, and then transferred to the Glutathione S-transferase (GST) fusion vector, pDEST™15 (Invitrogen), by site-specific recombination using LR clonase™II (Invitrogen). The resulting vector, pDEST/GST-TEV-oPF was transformed into chemically competent BL21(D3) bacteria by standard techniques. A culture of BL21(D3) bacteria was grown in LB-ampicillin (50μg/ml) at 37°C until the culture grew to an $OD_{260}$ of 0.5. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1mM) was added, and the culture was allowed to incubate an additional 5 h. The bacteria were pelleted by centrifugation at 750 *g* for 10 min. The bacterial pellet was lysed with 10 ml of B-PER (Pierce, Rockford, IL) and then centrifuged at 27,000 *g* for 15 min at 4°C. The supernatant from the centrifuged bacterial lysate was allowed to flow through a glutathione affinity column. The glutathione affinity column was washed with Pierce Wash Buffer 1, followed Wash Buffer 2 (Pierce 78200). A "cleavage mixture" of 2.5 ml Wash Buffer 2, 25μl AcTEV protease (Invitrogen), and 22 μl 0.1 M dithiothreitol (DTT) was mixed and added to the glutathione affinity column and the column was placed at 4°C overnight. The following day, the column was placed at room temperature for 30 min and then washed with 2.5 ml of Wash Buffer 2 two times, collecting the column eluate. The eluate was dialyzed against 1L of ammonium acetate buffer (50 mM, pH 5.5) for 2 h at 4°C. A chromatography column containing S-Sepharose was equilibrated with

30 ml of ammonium acetate start buffer (50 mM, pH 5.5). The dialyzed protein sample was then added to the S-Sepharose column and allowed to run through. The column was washed with start buffer, followed by successive 10 ml fractions of elution buffer (50 mM ammonium acetate, pH 5.5) containing increasing concentrations of NaCl (300 mM, 325 mM, 350 mM). Fractions were collected upon addition of the first 10 ml of elution buffer. The 280 nm absorbance of each fraction was determined, and an absorbance curve was plotted. Two absorbance peaks were observed: one in the first 5 fractions and another larger peak in fractions 9-16. The samples of the second absorbance peak were pooled and concentrated by processing in Centricon YM-10 centrifugal filter units (Millipore, Billerica, MA), at 5,000 *g* for 1 h. A concentrated sample was electrophoresed on a polyacrylamide gel which was then coomassie stained. Only a single band at the expected molecular weight was observed on the coomassie stained gel. A sample of the recombinant oPF (roPF) was also sequenced (Colorado State University Macromolecular Resources) to confirm its identity.

The recombinant protein was then sent to Covance Immuno Technologies (Denver, PA) for polyclonal antibody production. Two New Zealand White rabbits were injected with 125 μg of roPF with Freund's Incomplete Adjuvant at 21 d intervals repeated six times. Pre-immune serum was collected from each rabbit prior to any injections with roPF, and anti-sera were collected following the fifth and sixth injections. The anti-sera (CSU-αoPF-146) and pre-immune sera (CSU-oPF PB) collected from the rabbits were used for immunohistochemistry.

*Immunohistochemistry*

Day 15 conceptuses recovered from pregnant ewes were fixed in 4% paraformaldehyde for 1 h and then placed into 70% ethanol overnight at 4°C before paraffin embedding. Six μm sections were cut and placed onto Superfrost/Plus slides, (Fisher Scientific, Waltham, MA) and dried overnight. Slides were then deparaffinized, and rehydrated through a graded ethanol series (100%, 95%, 70%, 50%). An antigen retrieval step was performed by incubating slides in a 10 mM sodium citrate, 0.05% Tween 20 (Sigma) solution for 30 min at 85°C. Sections were then bathed in 3% hydrogen peroxide for 1 h to quench any endogenous peroxidase activity. Staining was performed using the Vectastain Elite ABC kit according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA). The antisera and pre-immune serum were diluted in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH 7.3) supplemented with 1.5% (v/v) normal goat serum at a 1:10,000 dilution. Peroxidase activity was visualized using 4% 3,3'diaminobenzidine solution for 90 sec before washing in water and dehydrating through a graded ethanol series. For morphological comparisons, a serial section was also stained with hematoxylin and eosin using standard procedures.

*Construction of lentiviral vectors*

Lentiviral infection of embryos was used to stably integrate and express short hairpin RNA (shRNA) targeting the oPF mRNA sequence. The lentiviral vector pLL3.7 (109) containing the RNA polymerase III promoter U6, upstream of the multiple cloning site for introduction of shRNA cassettes, as well as a cytomegalovirus promoter upstream of enhanced green fluorescent protein (EGFP) was used. The U6 promoter is an efficient

promoter for shRNAs produced by a lentiviral vector (110). Both of these cassettes integrate into the genome, enabling continual production of shRNA as well as EGFP to confirm integration. The shRNA target sequences were selected by submitting the full length oPF cDNA sequence to online target selection tools at both Ambion and Dharmacon. Target sequences were selected based upon their predicted efficacy, as represented by a "score" returned by the online selection tools. Oligonucleotides were designed to contain (from 5' to 3') a 5'Xho overhang, oPF target anti-sense sequence, a TTCAAGAGA loop sequence, oPF target sense sequence, 6 thymidine residues for PolIII termination, and a blunt 3' end. Oligonucleotides were ordered with 5' phosphates and then PAGE purified (Invitrogen). Oligonucleotides were diluted in water to 60pmol/ul. One μl each of complementary oligonucleotides was annealed in 48ul of annealing buffer (100mM potassium acetate, 30mM HEPES-KOH pH7.4, and 2mM Magnesium acetate). The annealing mixture was incubated at 95°C for 4 min, 70°C for 10 min, and then cooled slowly to 4°C by placing the 70°C heat block holding the annealing mixture in a styrofoam cooler in a 4°C refrigerator. The annealed oligonucleotides were ligated into pLL3.7 that was digested with XhoI/HpaI, and treated with 10 U calf intestinal phosphatase (New England Biolabs, Ipswich, MA)

Lentiviral particles were generated in 293FT cells (Invitrogen) grown in high glucose DMEM medium supplemented with 10% fetal calf serum in an incubator at 37°C and 5% $CO_2$. One day prior to transfection, 10 cm dishes of 293 FT cells were passaged in order to be 50-75% confluent and contain approximately 2 x $10^6$ cells on the day of transfection. For each 10 cm dish, serum-free DMEM was mixed with one of the pLL3.7 lentiviral constructs (3.92 μg), as well as the packaging vectors pRΔ8.74 (2.96μg;

gag/pol elements) and pMD2.G (1.12 μg; env elements) in a total volume of 300 μl. The transfection agent Polyfect (80 μl Qiagen, Valencia, CA) was added, as well as 1 mL of complete DMEM medium. This transfection mixture was added to 293FT cells with 7 mL complete DMEM medium. After overnight incubation, the medium was then aspirated off and discarded and fresh medium was added. The medium was then collected at the end of 24 and 48 h incubation with the 293FT cells. Following the guidelines of the protocol at Didier Trono's laboratory (http://tronolab.epfl.ch/), we ultracentrifuged lentivirus supernatant over a 30% sucrose cushion at 47,000g for 2 h at 16°C. The resulting pellets were then resuspended in Hepes-buffered chemical defined medium for late state embryos (HCDM-2; 111), aliquoted, and stored at- 80°C for future use. Frozen aliquots of concentrated virus were thawed and 10-fold serially diluted. Serial dilutions of virus were added to 6-well plates of HEK293 cells at ~90% confluency. The number of GFP-expressing cells within each well were counted, and the titer was calculated based upon the volume of the initial aliquot present in the well from which GFP-expressing cells were counted.

Prior to in vivo studies with transfected ovine embryos, three shRNA sequences were tested for their efficacy in degrading oPF mRNA in vitro using the psiCHECK vector (Promega, Madison, WI). In the psiCHECK vector the oPF ORF is inserted into the multiple cloning region downstream (3') of the *Renilla* luciferase gene stop codon. This vector was cotransfected into 293T and Cos-7 cells along with the pLL3.7 vector containing various shRNA cassettes. If a specific shRNA combines with RISC to target the endogenous mRNA (oPF), the RNAi process will be initiated and the fused *Renilla*/oPF mRNA will be degraded, thus decreasing the luciferase signal. If the shRNA

does not work, the *Renilla* mRNA will be translated into protein and the signal will be detected. One shRNA sequence (pLL3.7-shRNA3) produced 80% knockdown of luciferase expression and was chosen for the in vivo studies. An empty vector expressing no shRNA, but producing EGFP was used as a control (pLL3.7). As a second control, a lentiviral vector containing a 3 bp mismatch to oPF mRNA (pLL3.7-MM) was also generated. The shRNA produced by this vector is homologus to the human PF mRNA sequence. This vector was tested on the human BeWo cell line and produced 63% knockdown of hPF mRNA.

*Embryo collection and transfer*

Embryo donor ewes were superovulated by twice-daily i.m. injections of FSH for four consecutive days (48, 48, 36, 36, 24, 24, 20, 20 mg; Folltropin; Bioniche). At the time of the 5$^{th}$ FSH injection, $PGF_{2\alpha}$ was administered in two doses (5 mg, i.m. per dose) given 4 h apart. At the time of the 6$^{th}$ FSH injection, PMSG (150 U, s.c.) was administered, and GnRH (50 μg, i.m. OvaCyst; Vedco; St. Joseph, MO) was administered at the time of the 8$^{th}$ FSH injection. Recipient ewes were given two doses of $PGF_{2\alpha}$ (5 mg, i.m. per dose) 4 h apart beginning at the time the donor ewes received the 4$^{th}$ FSH injection. Standing estrus in all ewes was observed by using a vasectomized ram. After observation of standing estrus, all donor ewes were bred by one of two intact rams. For embryo collection, pregnant ewes were sedated using sodium pentobarbital (20 mg/kg, i.v.), a complete hysterectomy was performed, and the ewes were euthanized (90 mg/kg sodium pentobarbital, i.v.). Conceptuses were flushed from the uterus using modified Dulbecco's phosphate-buffered saline (m-PBS; 112) supplemented with 0.1% w/v Fraction V BSA (Sigma) warmed to 38°C.

Collected embryos were washed in HCDM-2. Because viruses are unable to cross the zona pellucida, embryos were recovered on d 8, a time when most are hatched. Recovered embryos that had not already hatched out of the zona pellucida in vivo were mechanically hatched at this time with a microblade or 26 ga needle. For virus infection, three blastocysts were incubated in 100 µl drops overlaid with mineral oil. Each drop contained 60 µl chemical defined medium for late state embryos (CDM-2; 111) 30 µl concentrated lentivirus (1 x $10^7$/mL), and 10 µl polybrene (8ng/mL, Sigma). Drops containing embryos were cultured in 5% $CO_2$ and 5% $O_2$ at 37°C for approximately 6 h prior to transfer. Embryos to be transferred were recovered in the morning and transferred the same day in the afternoon. Immediately prior to transfer, embryos were washed in HCDM-2 three times. When possible, embryos from any individual donor ewe were divided among multiple treatments. Only recipient ewes that had come into estrus within 24 h of the donor ewes received embryos.

Transfer was performed using a glass pipette inserted through a puncture wound into the uterine horn ipsilateral to the CL. Three embryos were transferred into each recipient. Each recipient was given flunixin meglumine immediately prior to surgery and again 12 h post-surgery (75 mg i.m., VedaGesic, Vedco). Recipient ewes were sedated using sodium pentobarbital (20 mg/kg, i.v.). An endotracheal tube was inserted, and recipients were maintained on 1 L/min $O_2$ and 20% isoflurane (Altane, Minrad Inc., Bethlehem, PA) during the transfer procedure. Following transfer, recipient ewes were maintained on an ad-libitum diet and monitored daily for 7 d after transfer. On d 7 after transfer (d 15 of gestation) the recipient ewes were euthanized as described previously

and conceptuses were recovered. Total cellular RNA from lentiviral-treated conceptuses was isolated and cDNA generated as previously described.

Recovered embryos were analyzed for EGFP expression to insure lentiviral infection had occurred using the following EGFP primers: forward 5' - TCT TCT TCA AGG ACG ACG GCA ACT - 3' and reverse 5' – TGT GGC GGA TCT TGA AGT TCA CCT – 3'. With the same cDNA from lentiviral-infected embryos that was used for qRT-PCR analysis, EGFP was amplified by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. A portion of the PCR product was electrophoresed on 2% agarose gel and a portion of the PCR product was sequenced (CSU Macromolecular Resources) to verify specificity.

### *Statistical analysis*

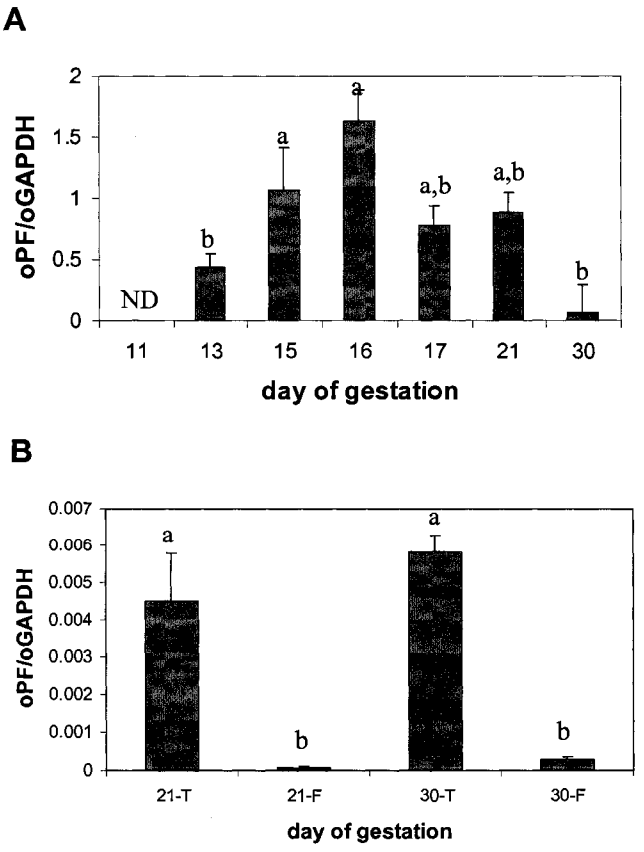
All data were analyzed using SAS software (SAS Institute, Cary, NC). For all qRT-PCR data, oGAPDH normalized oPF and oIFN$\tau$ mRNA concentrations were subjected to ANOVA using the PROC GLM procedure, with regression contrasts performed to further characterize the expression pattern. Comparisons between days of gestation, tissue types, and lentiviral constructs were made using Tukey's hsd. Due to heterogeneity of variance, qRT-PCR data for oPF mRNA across different days of gestation and data for lentiviral-treated embryos were log-transformed.

## RESULTS

### *Conceptus qRT-PCR during early gestation*

Previously, we detected PF mRNA in the developing conceptus of the cow, sheep, and horse by Northern hybridization (99). Quantitative RT-PCR (qRT-PCR) was performed in sheep to more accurately define the temporal pattern of expression during

early conceptus development. Although the housekeeping gene, oGAPDH mRNA was detected in d 11 conceptuses, no oPF mRNA was detected at this stage of gestation. This observation was based on the lack of an amplification curve in the qRT-PCR reaction, as well as the lack of a band after electrophoresis of the qRT-PCR products on a 2% agarose gel. Periattachment factor mRNA exhibited a quadratic expression pattern ($P < 0.05$) from d 13 to d 30 of gestation, with peak mRNA levels detected on d 16 (Fig. 1A). Expression of oPF mRNA on d 15 and 16 was significantly greater than on d 13 or 30 ($P < 0.05$). A comparison of oPF mRNA concentration between the fetal and trophoblast tissue of d 21 and 30 conceptuses was also performed. oPF mRNA concentrations were greater ($P < 0.05$) in trophoblast tissue than fetal tissue on d 21 and 30 (Fig. 1B). There were no significant differences in oPF mRNA concentrations between d 21 and 30 for either the trophoblast or fetal tissue.



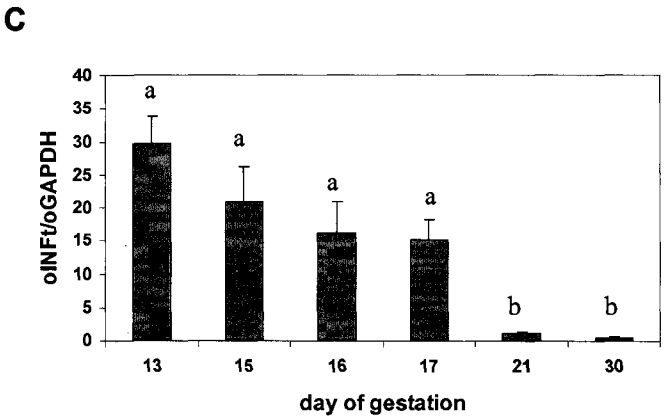



**Figure 1** Quantitative RT-PCR of oPF (**A, B**) and oIFN-$\tau$(**C**) mRNA normalized to oGAPDH mRNA in ovine conceptuses. Data represent lsmeans ± SEM. a,b; bars with a different letter ($P$< 0.05). (**A**) oPF mRNA exhibited a quadratic expression pattern ($P$ < 0.05). ND: No oPF amplification detected at this day. (**B**) oPF mRNA concentrations in ovine trophoblasts (T) and fetuses (F). (**C**) oIFN-$\tau$ mRNA exhibited a linear expression pattern ($P$ < 0.01).

Because little is known about oPF or its function, we wanted to compare the mRNA concentrations of oPF to those of a gene of known importance during early pregnancy, oIFN-$\tau$. From d 13 to d 30, oIFN-$\tau$ mRNA exhibited a linear decrease in expression ($P$ < 0.01; Fig. 1C). The concentrations of oIFN-$\tau$ mRNA on d 21 and 30 were lower than any other day of gestation studied ($P$ < 0.05).

*Immunohistochemistry*

The peak concentration of oPF mRNA as detected by qRT-PCR was on d 15 and 16 of gestation. For this reason, d 15 conceptus tissue was fixed and paraffin embedded in preparation for immunohistochemistry. Immunohistochemistry using an oPF-specific antibody on d 15 conceptus tissue showed staining throughout the length of the conceptus (Fig. 2A). Staining appeared to be predominantly nuclear, with some lighter staining in the cytoplasm (Fig. 2B). Periattachment factor was detected in the trophectoderm, as

well as the trophendoderm of the conceptus. No staining was detected in sections stained with pre-immune serum (Fig. 2C and 2D)

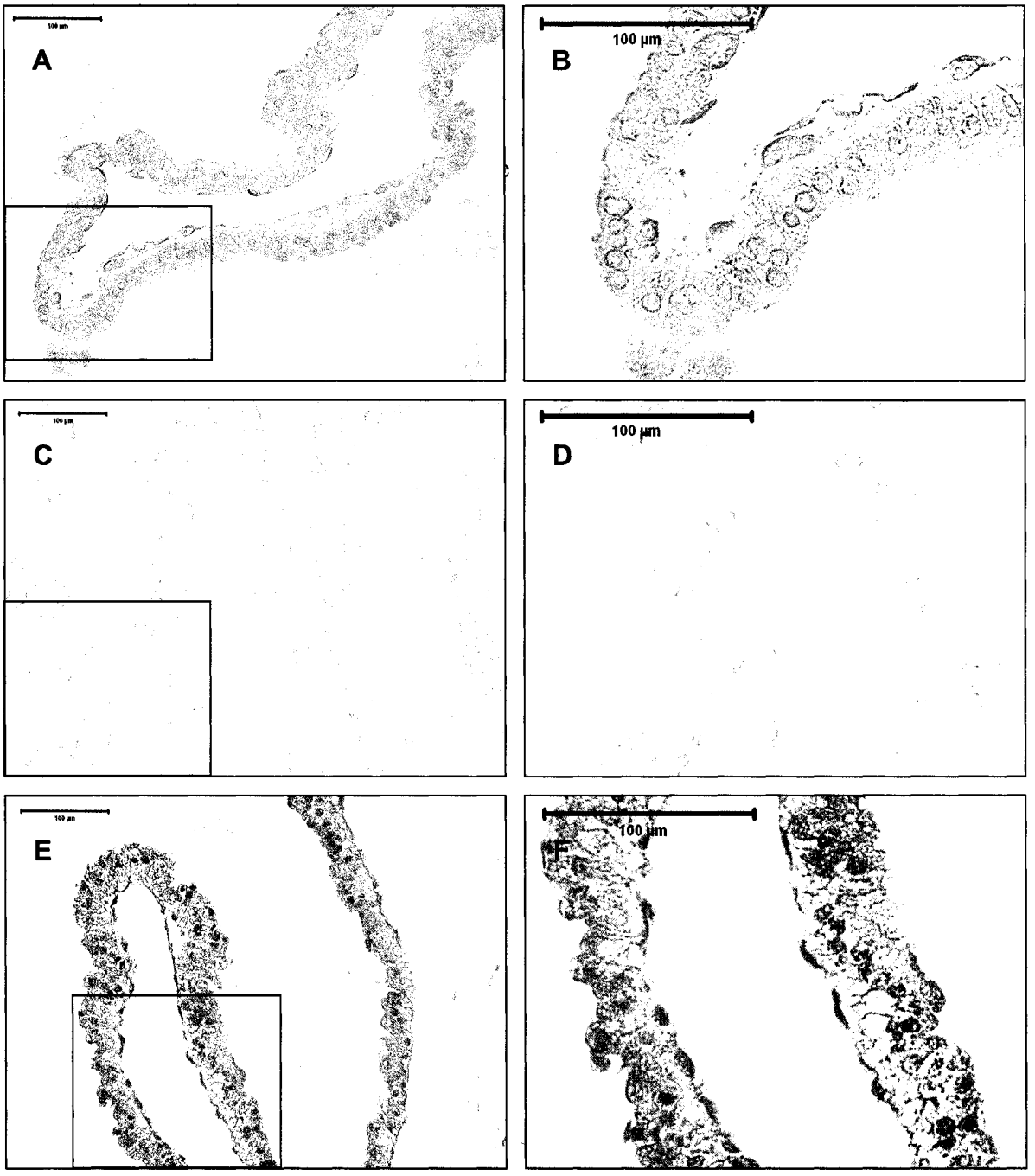



**Figure 2** Immunohistochemistry for oPF in serial sections of a paraffin-embedded d 15 ovine conceptus. **A**. Conceptus stained using rabbit anti-oPF antibody. **B**. Magnification of a portion of A. **C**. Staining with pre-immune serum from the same rabbit used to generate an antibody against oPF. **D**. Magnification of a portion of C. **E**. Hematoxylin and eosin staining. **F**. Magnification of a portion of E.

*Lentiviral infection and embryo transfer*

A total of 63 lentiviral-infected embryos were transferred into 21 recipient ewes. A summary of the results from each treatment is in Table 1. While 93.8% of the empty vector (pLL3.7) and 88.2% mismatched shRNA (pLL3.7-MM) -treated conceptuses had elongated by d 15 of gestation, 0% of the knockdown vector (pLL3.7-shRNA3) treated conceptuses had elongated by this point. Only four of the 21 embryos transferred were recovered back for the pLL3.7-shRNA3 treatment, indicating a high degree of embryo mortality in this treatment. Transfer of pLL3.7 and pLL3.7-MM treated embryos resulted in 66.7% and 94.4% recovery rates, respectively.

| | Treatment | | |
|---|---|---|---|
| | **pLL3.7** | **pLL3.7-MM** | **pLL3.7-shRNA3** |
| **Recipient ewes** | 8 | 6 | 7 |
| **Embryos transferred** | 24 | 18 | 21 |
| **Embryos recovered** | 16 | 17 | 4* |
| **Percent elongated embryos[a]** | 93.8% | 88.2% | 0.0%** |

**Table 1.** Summary of sheep embryo transfer results. [a] Percent elongated embryos of those recovered. Differs from other values within the same row (*P<0.05; **P<0.01)

At the time of collection, a subset of each treatment was assessed for EGFP expression by fluorescence microscopy. A portion of the conceptus was dissected off and placed in 70% ethanol and then placed on a glass slide. A glass coverslip was placed over the conceptus, and fluorescence was assessed using filters at 480nm excitation and 535 nm emission. A non-treated d 15 conceptus was used as a control. No EGFP expression was observed in the untreated sample (Fig 3E), while all lentiviral treated samples showed EGFP fluorescence (Fig 3F,G, and H). All conceptus samples to be used for qRT-PCR were analyzed for EGFP transgene expression by PCR. All samples indicated that insertion of the lentiviral vector DNA had occurred (Fig 3I).

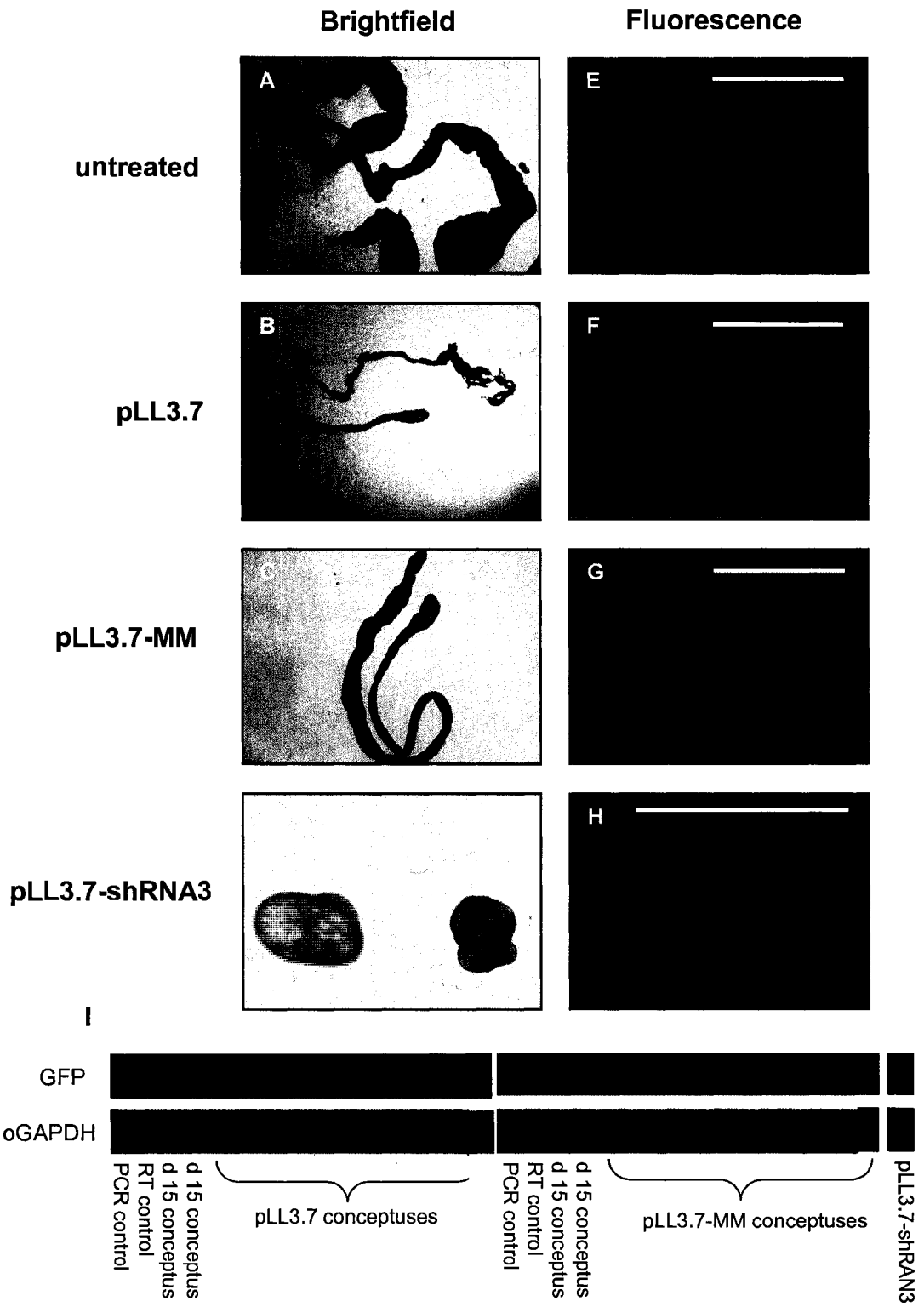



**Figure 3**. Brightfield (A-D) and fluorescence (E-H) microscopy of d 15 conceptuses treated with one of three lentiviral constructs and an untreated control. Scale bar represents ≈ 1mm. I. PCR for GFP and GAPDH was performed on cDNA from 12 pLL3.7 conceptuses, 12 pLL3.7-MM conceptuses, a pool of 2 pLL3.7-shRNA3, and 2 untreated control conceptuses. The PCR product was run out on a 2% agarose gel and visualized under UV light. No GFP was detected in the PCR control, RT control, or untreated d 15 control conceptuses. GAPDH was present in all conceptuses samples.

For qRT-PCR analysis, 12 pLL3.7, 12 pLL3.7-MM, and 2 pLL3.7-shRNA3 conceptuses were used. There was no difference in either oPF mRNA or oIFN$\tau$ mRNA between pLL3.7 and pLL3.7-MM treated conceptuses (Fig 4A and B). For conceptuses treated with pLL3.7-shRNA-3, oPF mRNA was undetectable and oIFN$\tau$ mRNA was reduced ($P<0.05$)

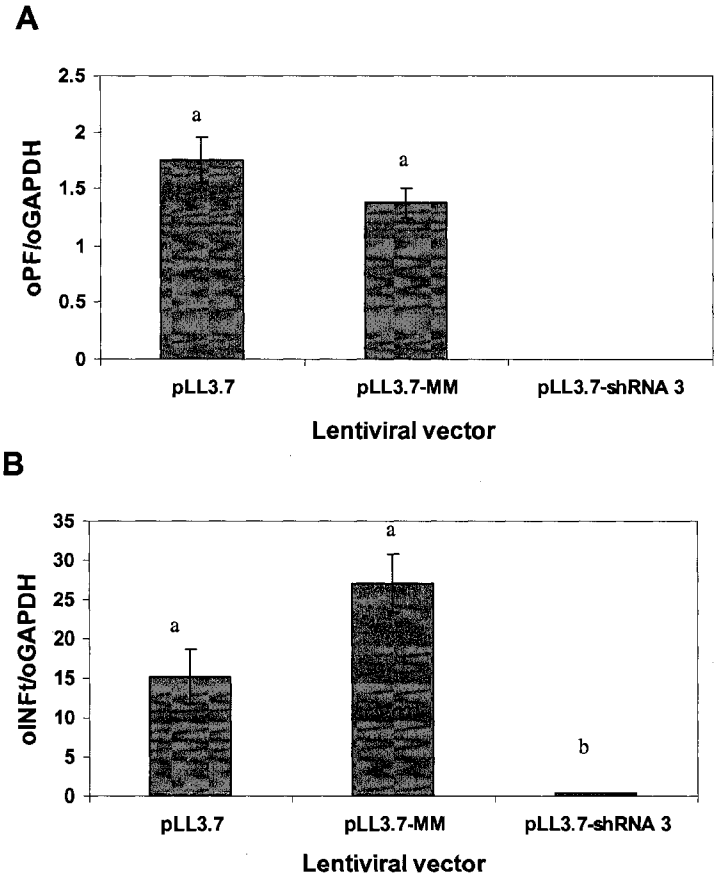



**Figure 4**. Quantitative RT-PCR analysis of oPF (A) and oIFN$\tau$ (B) mRNA in d 15 conceptuses treated with one of three lentiviral constructs (pLL3.7, pLL3.7-MM, or pLL3.7-shRNA3). Data represent lsmeans ± SEM. a, b bars with a different letter ($P < 0.05$)

## DISCUSSION

Quantitative RT-PCR was used to precisely determine the timing of oPF mRNA expression in the sheep conceptus during development from d 11 to d 30 of gestation (Fig. 1A). The time point when oPF mRNA was first detected (d 13) coincides in time

with when the ovine conceptus first begins a period of rapid elongation (3, 12). Prior to d 13, ovine conceptuses retain a spherical shape. In this study conceptuses recovered on d 11 of gestation were indeed spherical, and no oPF mRNA was detectable at this time, while the housekeeping gene, oGAPDH mRNA was present. It is difficult to speculate at this time what initiates PF mRNA transcription; although progesterone is elevated on d 13 in sheep, and progesterone response elements upstream of genes are known transcription regulators it is an unlikely candidate because progesterone is similarly high for a week prior to do 13. Furthermore, PF decreases from d 17 to 30 of gestation, a time when progesterone would remain elevated in the pregnant ewe. After oPF mRNA was first detected on d 13, oPF mRNA exhibited a quadratic pattern of expression, with peak mRNA concentration detected at d 16 of gestation before declining on d 17 through d 30. The increase in oPF mRNA to d 16 correlates well with previous studies that reported an increase in embryo length up to d 15 and the beginning of a close apposition of the conceptus to the uterus (14, 15). On d 16 in sheep, the conceptus begins to adhere to the endometrium, and elongation has stopped (7, 16). Although there are larger variances in oPF mRNA concentration on d 16, this could be due to an inherent variation in ovine conceptus size on any single day post conception (23). The decline in oPF mRNA from d 17 to d 30 roughly agrees with Northern hybridization results obtained in the cow (96, 99). In the cow, PF mRNA was undetectable from d 30 to 36, but was detectable at d 21 and 28, indicating a decrease in expression in this species as well. It is interesting to note the abrupt decrease in PF mRNA concentrations in ovine trophoblasts occurs within days following the burst of expansion (Fig. 1A). Initial Northern hybridization results indicated PF mRNA may be trophoblast specific (99). However, using qRT-PCR we

were able to detect PF mRNA in the sheep fetus, although at a much lower concentration than what was observed in the trophoblast (Fig. 1B). Furthermore, although PF mRNA was greater in trophoblast than fetal tissue on d 21 and 30, these days represent a significant decrease following the peak PF mRNA concentration on d 16 (Fig. 1A). Based on these observations, it appears likely that PF may have a functional role in the trophoblast, and not the fetus during conceptus expansion.

Interferon-tau is the pregnancy recognition signal in ruminants, secreted from d 10 to 21 in the sheep (113). For this reason we also measured oIFN-$\tau$ mRNA during conceptus development along with oPF mRNA. The linear decrease in oIFN-$\tau$ mRNA from d 13 to d 30 (Fig 1C) agrees with previous reports that described a decrease oIFN-$\tau$ mRNA from d 14 to d 22 of pregnancy in the sheep using dot blots (114), and a peak of oIFN-$\tau$ transcription on d 13 (115). Ovine IFN-$\tau$ binds to the IFNAR1 and IFNAR2 receptors used by all Type I interferons, and these receptors are present on most mammalian cells, and are components of signal transduction pathways that induce IFN-$\tau$ stimulated genes (ISGs; 35, 116). Due to the increase in oIFN-$\tau$ just prior to oPF mRNA expression it could be that signaling by oIFN-$\tau$ or an ISG could induce oPF transcription. However, one such ISG that has been investigated during early pregnancy in sheep, IFN-stimulated gene 15-kDa protein (ISG15) has not been reported to be present in the trophoblast; however, it is possible that other signaling pathways or ISG could act on the trophoblast and induce oPF expression. Because PF mRNA has been detected in the trophoblast of non-ruminant species such as the horse (99) and human, neither of which express IFN-$\tau$ as a pregnancy recognition signal, it is unlikely that IFN-$\tau$ and PF are

interacting with each other. Thus, oPF likely functions in a separate, but important capacity during this time period of conceptus development.

Recombinant oPF was used to generate polyclonal antibodies against PF in the rabbit. Immunohistochemistry of a d 15 ovine conceptus (Fig. 2) showed predominantly nuclear staining, confirming initial data generated by amino acid sequence analysis that reported a putative nuclear targeting sequence (96). Some staining was apparent in the cytoplasm; however, it is unknown how long PF remains in the cytoplasm after translation. One possible explanation is that a PKC phosphorylation event may be necessary to activate PF to translocate to the nucleus. While we hypothesized that oPF staining would be located to the trophectoderm only, it was also apparent in the trophendoderm. The cells of the trophendoderm develop from the ICM and migrate beneath the trophectoderm, further differentiating into the visceral endoderm (VE) beneath the ICM and the parietal endoderm (PE) surround the rest of the blastocoele cavity (12). This PE layer of cells also elongates in ruminant embryos, along with the trophectoderm (1, 7, 12). In sheep the PE cells stretch, and take on a multinucleated syncytial morphology, but retain the morphological characteristics of polarized epithelium (12). A multinucleated PE has also been observed in elongating bovine and porcine conceptuses (12, 18). The PE cells have epithelial cell characteristics such as desmosome-associated tonofilaments, structural polarization, and microvilli (2). The morphology of the PE could be the result of cellular adaptation to manage the rapid elongation of the trophoblast (12). Therefore, it is not surprising that oPF is found in both the trophectoderm and trophendoderm of elongating conceptuses if it is involved in transcriptionally regulating trophoblast outgrowth and migration. Furthermore, qRT-

PCR that compared oPF mRNA in trophoblast to fetus on d 21 and 30 indicated that PF mRNA is present in fetal tissues as well (Fig. 2B), indicating it is not exclusively trophoblast derived.

To begin to elucidate the function of PF during early conceptus development, we used a lentiviral vector that expresses an shRNA directed against the PF mRNA sequence. Knockdown of PF mRNA resulted in embryo mortality by d 15 of pregnancy, or a complete block of conceptus elongation, while a large majority of control embryos elongated (88.2 to 93.8%) and survived (66.7 to 94.4%; Table 1). Under normal in vivo conditions, PF mRNA was undetectable prior to initiation of elongation, and then increased as the conceptus elongates beginning on d 13 (Fig 1A). Conceptuses treated with pLL3.7-shRNA3 that were recovered had similar morphology to an unelongated d 11-12 conceptus, and no oPF mRNA was detected in these conceptuses (Fig 3D and 4A). Although peak expression of PF mRNA coincided with the time of trophoblast attachment to the uterus in sheep (Fig 1A), PF appears to have a functional role earlier than this time, as the process of elongation begins on d 11-12 and RNAi of PF mRNA prevented elongation (3, 9). The mRNA expression pattern of PF and the dramatic results observed following knockdown of PF mRNA suggest that PF likely is playing a critical role in the elongation process of the ruminant conceptus, but not in the attachment process. The phenomenon of conceptus elongation prior to implantation takes place in all ungulate species; however, the mechanisms controlling this process are largely unknown (9, 11).

Previous studies have identified global gene expression changes during the elongation period of conceptus growth (16, 26, 27, 28). However, most of these studies

identified previously known genes, and none provided evidence of critical function using a knockdown or knockout approach. One study of global gene expression changes in sheep during this time period showed that prior to elongation (d 12), protein trafficking genes were highly expressed, while during elongation (d 14) and attachment (d 15) signaling genes were more highly expressed (16). Suppressive subtractive hybridization was used to identify a highly upregulated novel gene during the elongation process in porcine conceptuses (26). However, sequence alignment between this gene (GenBank Accession Number AC009682) and PF showed no sequence similarity, and no further study on the function of this novel gene has been reported. Gene families that are known to influence growth, such as EGF as its ligands, EGF and TGF- $\alpha$, and the TGF-$\beta$ were identified as highly expressed during elongation in ovine and bovine conceptuses (31, 32). However, this expression did not closely resemble that of PF (Fig 1A) as there was no change in expression of EGF family members from d 13 to 16 in bovine conceptuses or endometrium, and a linear increase in TGF-$\beta$ from d 13 to 27 of gestation in sheep. Interestingly, immunolocalization of TGF-$\beta$1 and TGF-$\beta$2 isoforms showed that staining in both trophectoderm and trophendoderm increased from d 13 to 16 in ovine conceptuses, and staining was observed in d 23 and 30 chorion and allantois (32). Our data also show PF in both trophectoderm and trophendoderm on d 15; however PF was not immunolocalized on other days. In contrast to most genes studied during conceptus elongation, PF mRNA does not continue to increase throughout development, but peaks on d 16 before declining markedly through d 30 in trophoblast tissue. Day 16 corresponds to the beginning of attachment of the trophoblast to the uterus (14, 15). Perhaps physical attachment to the endometrium, and signaling through integrins or ECM

proteins functions to reduce transcription of PF mRNA in the ovine trophoblast at this time.

The rapid increase in conceptus size corresponds to an increase in cell number and protein synthesis (16, 20, 21). In the porcine conceptus, it has been suggested elongation occurs through cellular remodeling and not through hyperplasia, while others have suggested elongation in this species occurs through an increase in cell number as well (18, 22). It seems likely that a combination of increased cell number as well as cell remodeling causes elongation. Whether PF plays a role in one or both of these processes is unclear. Through the use of the uterine gland knockout (UGKO) ewe, it has been shown that the 'histotroph' secretions from the uterus of sheep are required for elongation to occur (25). The complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, proteins, and other substances in the histotroph could be involved in regulating PF expression, as PF is also required for conceptus elongation to occur in sheep. Furthermore, our qRT-PCR data (Fig 1B) and previous studies on PF (99) have show that it is predominantly of trophobast origin. Interestingly, previous studies have reported that the inner cell mass is not required for conceptus elongation in the sheep, as dissection of trophoblastic vesicles lacking the embryonic disc were able to elongate when placed into the uterus of recipient ewes (24). Therefore, PF may be interacting with another trophoblast specific protein to cause elongation.

As controls, we used a lentiviral vector expressing no shRNA (pLL3.7) and a vector expressing a shRNA with a 3 bp mismatch to the ovine PF mRNA sequence (pLL3.7-shRNA3). Others have reported that mismatches in an shRNA sequence can abolish the silencing effect (105). Importantly, we have shown that the pLL3.7-MM is

able to induce gene silencing of human PF mRNA, where it is not mismatched to the endogenous sequence. Therefore, the pLL3.7-MM is a control vector that is able to produce an shRNA that is recognized by Dicer and can be complexed to the RISC machinery. In our study, the elongation and survival rate of pLL3.7-MM-treated embryos was not different from those treated with the empty, pLL3.7 vector. The survival rate for pLL3.7 and pLL3.7-MM treatments, as assessed by total number of embryos recovered at d 15 was 66.7 to 94.4%, respectively (Table 1). This is well within the normal range (60 to 90%) reported for transfer of non-frozen blastocyst stage embryos in sheep (117, 118, 119). Although the recovery rate for pLL3.7-MM embryos was numerically greater than that of pLL3.7 embryos, there was a slightly reduced percentage of embryos recovered from this treatment that were elongated (88.2 vs. 93.8%, respectively), and these unelongated conceptuses would likely not have continued on to produce viable offspring (Table 1). Therefore, if embryo survival is calculated as the percentage of elongated embryos recovered on d 15, the rates for pLL3.7 and pLL3.7-MM treated embryos are more similar, at 62.5 and 83.3%, respectively. These survival rates also agree with previous studies using lentiviral vector transduction of embryos at various stages in mice, rats, pigs, and cows, that have all resulted in a high rate of embryo viability (>70%; 102). High rates of transgene integration (70 to 100%), and expression (64 to 100%), using lentiviral based vectors has been reported in pigs and cows (120, 121, 122, 123). These studies were done by injection into the perivitteline space of the zygote or unfertilized oocyte. Therefore, only one cell was available for transduction. In our study, we incubated blastocyst-stage embryos with the lentivirus, exposing the entire population of trophoblast cells to the lentivirus. Blastocyst stage ovine embryo contain

55 to 140 trophoblast cells (13, 124). Therefore, even based on a low rate of integration and expression, this likely produces knockdown of PF mRNA efficiently. Indeed, all conceptuses in this study observed by microscopy, or by PCR analysis showed that EGFP expression had occurred (Fig. 3). The number of actual integration sites for the lentiviral vector expression constructs in our experiment is unknown; however, it has been reported that only one copy of an shRNA transgene is sufficient to provide body-wide gene silencing in a mouse (125).

Another method of post-transcriptional gene silencing is through morpholino antisense oligonucleotides (MAO). Gene silencing occurs through binding of the MAO to mRNA and preventing its translation to functional protein through steric hindrance (126). Recently, Dunlap et al. (127) injected MAO directly into the uterus of sheep on d 8 of pregnancy to prevent translation of endogenous Jaagsiekte sheep retrovirus (enJSRV), which is produced by sheep trophoblast. This led to retarded trophoblast growth during elongation and inhibited binuceate cell formation on d 16 (127). We attempted to use MAO against PF mRNA in d 8 sheep blastocysts recovered after superovulation. In these preliminary experiments we transferred 24 anti-sense MAO-treated blastocysts into 8 recipients and 16 mismatch MAO-treated blastocysts into 6 recipients, resulting in 45 and 70% elongated embryos recovered back on d 15, respectively. This trend agrees with the results we subsequently observed in the lentiviral-treated embryos. This method of gene disruption was abandoned however, due to the observation that incubation with the MAO for 6 h in culture severely compromised embryo quality prior to transfer, regardless of the specific MAO treatment, concerns over dilution of the MAO in the uterus following the many cell divisions between transfer and

recovery on d 15, and low pregnancy rates of recipients receiving mismatch MAO-treated blastocysts. All of these issues were avoided with the use of lentiviral vectors expressing shRNAs against PF mRNA.

A particular issue in the case of investigating trophoblast specific genes is that the effect of degradation of a specific gene is confounded by possibly having an effect on the ICM, or fetus, as well as the trophoblast, or placenta. To address this issue Okada et al. (107) showed hatched blastocysts could be incubated with a lentiviral vector expressing an shRNA to produce trophoblast specific gene knockdown. The approach results in even distribution of shRNA expression in the placenta. We have used a similar approach in ovine embryos to successfully degrade PF mRNA and show that PF is required for successful ovine conceptus elongation.

## SUMMARY

Periattachment factor (PF) mRNA was first identified in trophoblast tissue in the bovine conceptus at markedly greater concentrations on d 17.5 than d 15.5 of gestation. Previous work in our laboratory showed PF mRNA to be present in both ruminant and non-ruminant species that display variations in the process of early embryo and placental development. The goal of these studies was to characterize expression of periattachment factor (PF) mRNA in the developing sheep conceptus, localize PF in the sheep conceptus, and determine if degrading PF mRNA in the sheep conceptus is detrimental to development. Sheep conceptuses were collected on d 11, 13, 15, 16, 17, 21, and 30 post-mating. oPF mRNA exhibited a quadratic expression pattern ($P<0.05$). No oPF mRNA was detected in d 11 conceptuses. From d 13, oPF mRNA increased to a peak at d 16 before declining. Peak expression of oPF mRNA in the conceptus occurs during rapid

elongation and initial attachment of the conceptus to the endometrium. A comparison of trophoblast and fetal tissues on d 21 and 30 identified the trophoblast as the primary source of oPF mRNA on both days. Immunolocalization of PF in a d 15 conceptus showed predominantly nuclear staining in trophectoderm and trophendoderm. Embryos collected from superovulated ewes on d 8 post-mating were infected with either a lentivirus expressing a short-hairpin (sh) RNA designed to target PF mRNA for degradation, a lentivirus expressing a shRNA containing 3 mismatched nucleotides, or a control lentivirus expressing no shRNA. Following infection, blastocysts were transferred into recipient ewes and collected back on d 15 of gestation. While 94 and 88% of the control and mismatched shRNA-treated conceptuses elongated by d 15, none of the embryos treated with the lentivirus expressing shRNA against PF mRNA elongated, and most died. This indicates oPF is required for conceptus elongation and survival.

# CHAPTER IV

# PERIATTACHMENT FACTOR IN HUMAN TISSUES AND CELL LINES

## INTRODUCTION

The human placenta differs from that of ruminants in gross morphology, the type of implantation, and the degree of invasiveness into the uterus. However, both require that specific trophoblast cells migrate and change morphology. In sheep, the trophoblast of the blastocyst elongates rapidly and expands prior to the attachment period. After attachment in the human, the trophoblast differentiates into the multinucleated syncytiotrophoblast, and the invasive cytotrophoblast forms columns that invade into the endometrium during the implantation process. The functional units of both the ruminant and human placenta, the chorionic villi, have a similar structure, and gas and nutrient exchange from maternal to fetal tissue is similar between the sheep and human (70).

Abnormalities or alterations in the formation of the placental villi in the human can lead to various pathologic conditions, such as preeclampsia and intrauterine growth restriction (IUGR). In human pregnancies, IUGR is a major cause of infant mortality and morbidity (128). There can be multiple complications arising from IUGR pregnancies, and many of these are associated with abnormalities in placental structure and function, particularly abnormalities in the chorionic villous tree (57, 71). One such complication that arises is a decrease in villous number, diameter, and surface area (129, 130). This can ultimately reduce blood flow and oxygen delivery to the placenta. In preeclampsia shallow or abnormal cytotrophoblast invasion is often observed (53). The location of PF in the human placenta has not been identified; however, PF mRNA or PF homologues

have been identified in placental tissues and other growing epithelial cell types, indicating that it may have a functional role in early placental development.

Periattachment factor is expressed during conceptus elongation and attachment to the uterus in sheep (Chapter III). Knockdown of PF mRNA in sheep resulted in a complete block of elongation or embryo mortality. In-silico analysis has identified a number of cDNA homologs to PF in a variety of tissues, including the human placenta, colon, kidney, and stomach, as well as the mouse lung, neonatal eye, mammary gland, and brain (97). Two of these, the human colon and mouse mammary gland were derived from carcinomas. In the human PF is located on chromosome 7. The National Center for Biotechnology Information (NCBI) database now identifies PF as PRR15 (GenBank Accession Number 222171). Many of the homologs of PF appear to be present in rapidly growing tissues or in tissues with expanding or growing epithelium. The high degree of homology at both the mRNA level and the protein level between the ruminant and human may indicate similar function.

The study of human placental development in vivo is inherently difficult. Therefore, cell lines and model systems are typically employed to understand the function of particular genes or molecules in placental development. To investigate the function of PF in the human, we used both primary cytotrophobasts, human placenta sections, cell lines derived from placental tissues, as well as human carcinoma cell lines. Although preliminary in nature, these experiments may provide some insight into the function of PF and give more focused direction to future research.

## MATERIALS AND METHODS

### *Human placental samples*

Total cellular RNA from first trimester (7 to 15 weeks gestation) human cytotrophoblasts was obtained from Dr. Virginia Winn at University of Colorado Health Sciences Center. The cytotrophoblast cells had been plated on an extracellular matrix causing them to differentiate into an invasive phenotype as previously described (131). Briefly, cytotrophoblast cells from anchoring villi were isolated and plated on plastic dishes coated with an extracellular matrix (Matrigel, BD Biosciences, San Jose, CA). These cells aggregate and begin to invade the extracellular matrix. The explants were cultured in serum-free DMEM supplemented with 4.5 g/L glucose, 2% Nutridoma (Boehringer Mannheim, Mannheim, Germany), penicillin/streptomycin (1000 U/mL penicillin G, 1000 μg/mL streptomycin sulfate), 1mM sodium pyruvate, 10 mM Hepes, and 50 μg/mL gentamycin sulfate on Matrigel coated plastic at 37°C in 5% $CO_2$ from 0 up to 36 h. In addition, one sample was subjected to hypoxic (2% $O_2$) vs. normoxic (20% $O_2$) conditions for 18 h. The tcRNA from these samples was reverse transcribed into cDNA using oligo dT primers (Superscript III, Invitrogen) as described for ovine cDNA generation in Chapter III.

Term human placental samples from normal (n = 16) and intrauterine growth restricted (IUGR; n = 11) pregnancies were obtained from Dr. Marcin Jozwik. Total cellular RNA was isolated by guanidine thiocyanate/CsCl extraction (132). Quantity and integrity of RNA was confirmed by the absorbance ratio at 260:280 nm and by

electrophoresis on a 1.2% formaldehyde denaturing gel and visualizing the 18S and 28S ribosomal subunits. Samples were stored at -80°C until use.

Total cellular RNA was reverse transcribed into cDNA as described above for cytotrophoblast samples.

*Cell culture*

The following carcinoma cell lines were used to analyze hPF mRNA expression.

| Cell line | Source | Media |
|---|---|---|
| BeWo | placental choriocarcinoma | F12-K (Mediatech 10-025-CV) |
| NCI-H1299 | non-small cell lung cancer | DMEM (Mediatech 15-017-CM) |
| Hep G2 | hepatocellular carcinoma | DMEM (Mediatech 15-017-CM) |
| MDA MB | mammary ductal carcinoma | DMEM (Mediatech 15-017-CM) |
| PC3 | prostate carcinoma | RPMI 1640 (Sigma R6504) |
| SK Mel | skin melanoma | DMEM (Mediatech 15-017-CM) |

Table 2. Cell lines used to study expression of hPF mRNA

Cells were grown at 37°C in 5% $CO_2$ in air. All media were supplemented with 10% FBS (Gemini Bioproducts, West Sacramento, CA) and antibiotic/antimycotic (1000 U/mL penicillin G, 1000 µg/mL streptomycin sulfate, 2.5 µg/mL amphotericin B; Gibco, Carlsbad, CA). In addition, cells grown in DMEM were also supplemented with 2mM L-glutamine and non-essential amino acids (Mediatech 25-025-CI; Herndon, VA). In addition to the carcinomas listed above, the hTR8 first trimester cytotrophoblast cell line was also used to examine the role of PF in the human. This cell line has been transfected with the pSV3neo plasmid which contains an SV40 Tag and neomycin phosphotransferase gene, conferring resistance to G-418 sulphate. In culture, these cells

can form multinucleated cells, secrete hCG, and stain positive for cytokeratin 7, a marker of cytotrophoblast phenotype (133). The hTR8 cells were cultured in RPMI 1640 (Sigma) supplemented with 10% FBS and antibiotic/antimycotic (1000 U/mL penicillin G, 1000 μg/mL streptomycin sulfate, and 2.5 μg/mL amphotericin B; Gibco) on Matrigel or plastic in 5 or 20% $O_2$ for 12 or 24 h. For all cell lines, RNA was harvested using the RNeasy kit and cDNA prepared as previously described.

*Quantitative RT-PCR*

For analysis of hPF mRNA expression, primers were designed to amplify a 228 bp intron-spanning product. The hPF primers used were: forward 5' – CCA GAA GCC TGA TCT CTC CA – 3' and reverse 5' – CCC TTT CTC CAC GTG GTC T – 3'. For all qRT-PCR analysis, hPF mRNA concentrations were normalized to hS15 mRNA concentrations. The hS15 primers amplified a 361 bp product and were: forward 5' – TTC CGC AAG TTC ACC TAC C – 3' and reverse 5' – CGG GCC GGC CAT GCT TTA CG – 3'. The PCR product generated by each primer set was cloned into pPCR-Script Amp SK (+) vector (Stratagene) and sequenced (Colorado State University Macromolecular Resources) to verify identity. Annealing temperatures were 61°C for hPF and 60°C for hS15. Similarly to oPF mRNA, hPF mRNA exhibited a high GC content. Therefore, betaine at 1M final concentration and *Pfu* at 0.15 U were added per 25 μl qRT-PCR reaction in all hPF samples. Quantitative RT-PCR was performed using SYBR green (Biorad) as described in Chapter III.

*Immunohistochemistry*

First and second trimester human cytotrophoblast samples that had previously been paraffin-embedded were used to immunolocalize hPF using the α-oPF antibody

described in Chapter III. Six μm sections were cut, and deparaffinization, dehydration, and staining was performed as described in Chapter III. For immunolocalization of hPF in various cell lines, cells were grown on chamber slides for 24-48 h prior to staining. The cells were then washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH 7.3) and fixed with 4% paraformaldehyde for 5 min followed by two more washing in PBS for 3 min each. Cells were then stained as described in Chapter III. The primary antibody was used at a 10,000:1 dilution. Either pre-immune serum, or a preabsorbed antibody was used as a control. For preadsorption, the primary antibody was diluted 10,000:1 in 10 mL PBS and coincubated with 2μg roPF for 6 h at room temperature.

*Lentivirus knockdown in human cells*

The BeWo cell line was used to test the efficacy of lentiviral knockdown of hPF mRNA. Four lentiviral constructs targeting the hPF sequence (h1 to h 4) as well the lentiviral construct used to knockdown oPF mRNA in ovine embryos, pLL3.7-shRNA3 (o3) were generated as described in Chapter III. The pLL3.7-shRNA3 contains a 3 bp mismatch to the hPF mRNA sequence. Lentiviral particles were generated in 293FT cells and added to BeWo cells in a 1:1 ratio with F12-K medium. The BeWo cells were treated with one of the four human shRNA constructs (h1, h2, h3, h4), the shRNA construct targeting the ovine sequence (o3), or no lentiviral construct (C). After 48 h of treatment, tcRNA was harvested from the cells using the RNeasy mini kit (Qiagen), and cDNA was generated as previously described. This was repeated three times, with a new virus being produced in 293FT cells each time. qRT-PCR was performed for hPF mRNA normalized to hS15 mRNA.

*Statistical analysis*

For all qRT-PCR data, concentrations of hPF mRNA were normalized to hS15 and analyzed by ANOVA using SAS software. For hTR8 cells comparisons of the main effects of Matrigel coating, oxygen content, and time, and all two-way interactions were analyzed by ANOVA. Comparisons between IUGR and normal term placenta were made using a paired t-test. Differences in hPF mRNA concentrations in BeWo cells treated with various lentivirus constructs were compared using Tukey's hsd.

## RESULTS

*hPF in carcinomas*

Periattachment factor mRNA was present in all the carcinoma cell lines screened by qRT-PCR. The highest abundance of hPF mRNA was observed in the HepG2, BeWo, and PC3 cell lines. These are derived from carcinomas of the liver, placenta, and prostate, respectively.

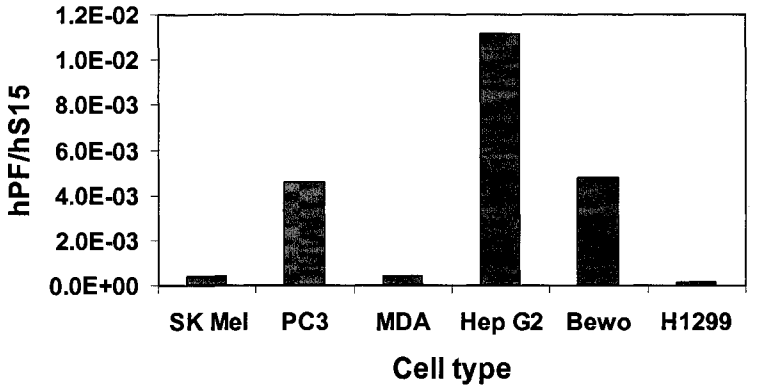



**Figure 5** Quantitative RT-PCR of hPF mRNA normalized to hS15 mRNA in various carcinomas.

*hPF in hTR8 cells*

hPF mRNA was detected in hTR8 cells in all treatment groups; however, there were no significant differences in hPF mRNA expression between any of the treatments or two-way interactions (Fig 6).

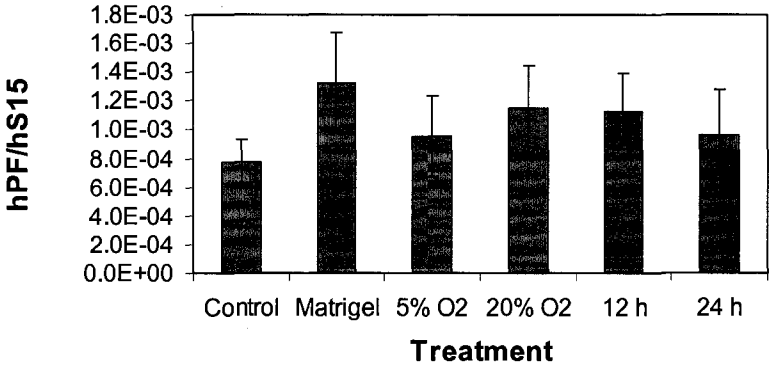



**Figure 6** Quantitative RT-PCR of hPF mRNA normalized to hS15 mRNA in hTR8 cells. Cells were treated with a 2x2x2 factorial with plate coating, oxygen content, and time as the main effects. Bars represent the means ± SEM

*hPF in human cytotrophoblasts and term placenta*

First trimester human cytotrophoblasts were cultured on Matrigel-coated dishes. In all four subsets, hPF mRNA increased as invasion occurred (Fig 7). In one subset (Fig 7D), hPF mRNA was decreased under 18 h of hypoxic (2% $O_2$) conditions relative to normoxic (20% $O_2$) conditions.

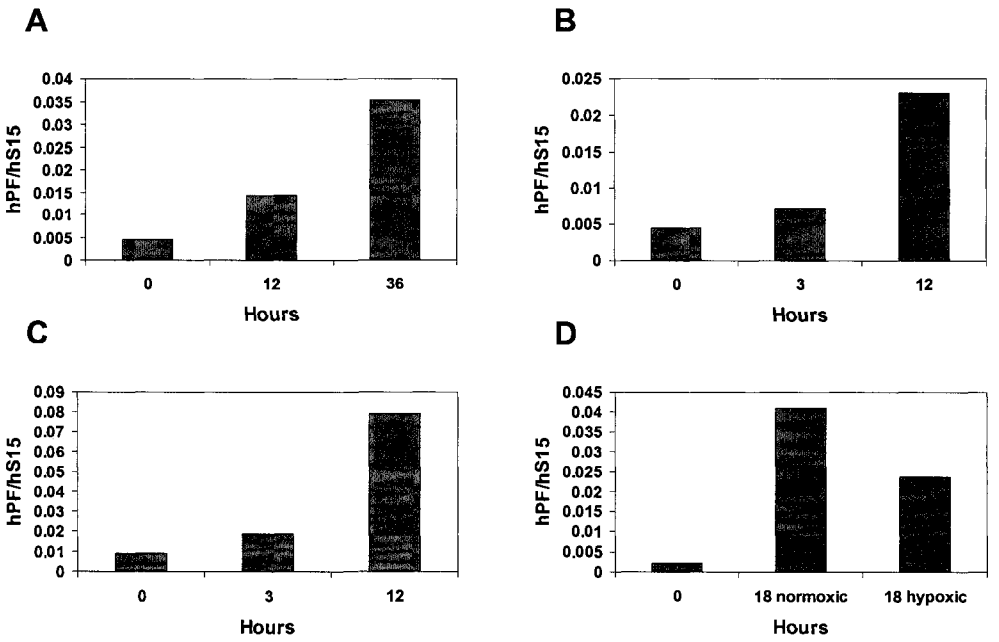



**Figure 7** Quantitative RT-PCR of hPF mRNA normalized to hS15 mRNA in 1st trimester human cytotrophoblast cells. A,B,C,D represent individual cytotrophoblast explants. Hours of invasion are represented on the Y-axis.

Tissue was also analyzed from human placental samples obtained at term from normal and IUGR pregnancies. There was no difference in hPF mRNA concentrations between IUGR and normal pregnancies (Fig. 8)

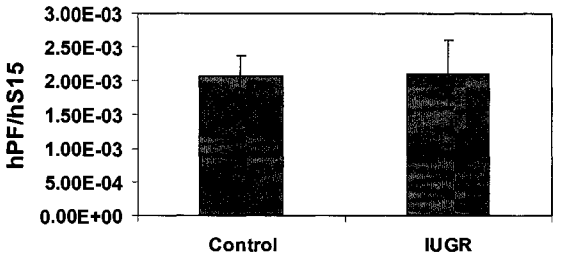



**Figure 8** Quantitative RT-PCR of hPF mRNA normalized to hS15 mRNA in control and growth restricted (IUGR) term human placenta. Bars are lsmeans ± SEM.

*Immunohistochemistry*

Periattachment factor immunolocalized to the nuclei of both first and second trimester human cytotrophoblast sections (Fig. 9), with some light staining present in the cytoplasm. Periattachment factor staining appeared more predominant in the cytotrophoblast cells (CTB) than the syncytiotrophoblast cells (S) of the villi. (Fig 9A). Staining was present in the invading extravillous cytotrophoblast (EVT) columns in the first trimester (Fig 9A). No PF was observed in sections that had been stained with α-PF that had been preadsorbed with roPF (Fig 9C, 9D). Staining of a variety of carcinoma cell lines also localized PF in the nuclei of these cells (Fig 10). Representative samples of BeWo, HepG2, and SK Mel cells are shown below.

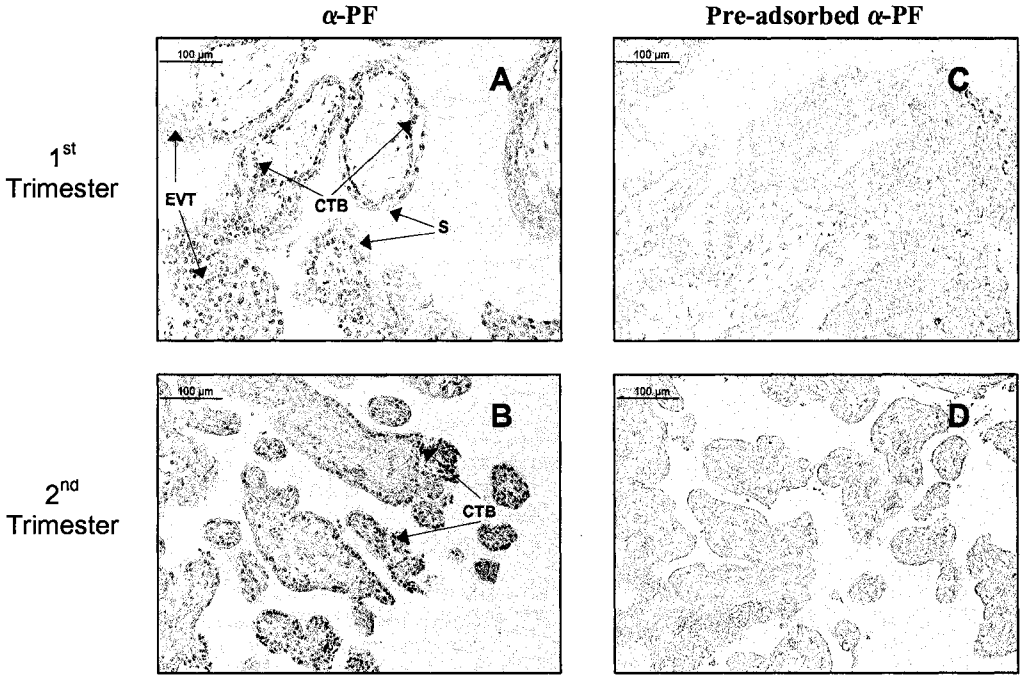



**Figure 9** Immunolocalization of PF in the 1st and 2nd trimester human placenta. Samples were stained with a 1:10,000 dilution of rabbit α-PF antibody (A,B). Control sections were stained with a 1:10,000 dilution of rabbit α-PF antibody preadsorbed with 1 ug of r-oPF. Sections show cytotrophoblasts (CTB), syncytiotrophoblasts (S), and extravillous cytotrophoblasts (EVT).

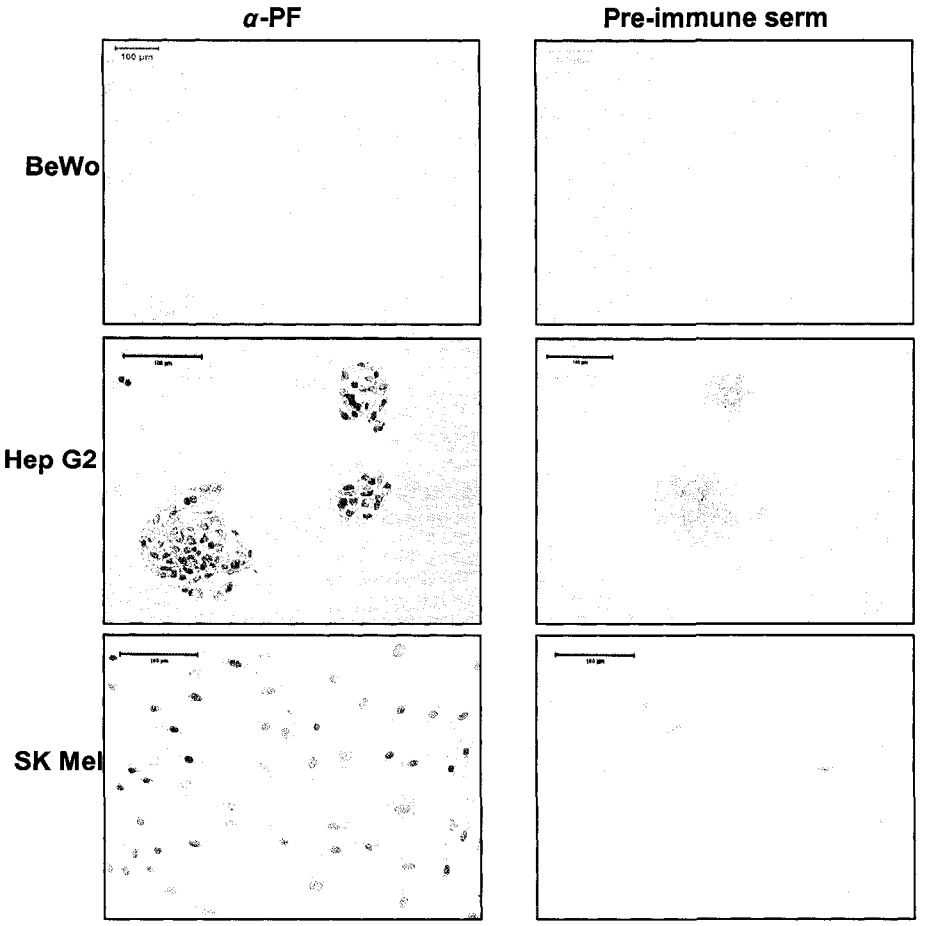



**Figure 10** Immunolocalization of PF in BeWo, HepG2, and SK Mel cell lines. Samples were stained with a 1:10,000 dilution of rabbit *α*-PF antibody.

*Lentivirus knockdown in human cells*

Lentiviral constructs targeting the human PF mRNA sequence were tested on BeWo cells in a 48 h culture. One construct, h2, resulted in 63% knockdown in hPF mRNA compared to controls (Fig 11). This was a significant decrease in mRNA concentrations from both control-treated and lentiviral vector o3-treated cells.

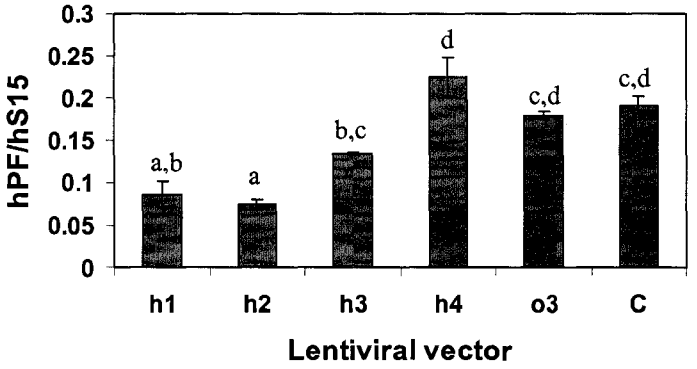



**Figure 11** Quantitative RT-PCR of hPF mRNA in BeWo cells treated with lentiviral vectors expressing shRNA against hPF mRNA (h1-h4) or against oPF mRNA (o3) and an untreated control. Data are lsmeans ± SEM. a,b,c,d Columns with different letters differ ($P < 0.05$)

## DISCUSSION

Expression of hPF mRNA and localization of PF in human tissues and cells were studied. The trophoblast of the human placenta diverges into two major lineages: the multinucleated syncytiotrophoblast or the invasive cytotrophoblast (36, 53). The syncytiotrophoblast forms the outer epithelial layer of the functional unit of the placenta, the chorionic villi (33, 58). The syncytiotrophoblast layer grows from incorporation of new cytotrophoblast cells into the epithelial layer of the chorionic villous (36, 49). The extravillous invasive cytotrophoblast columns form early in pregnancy; and all are responsible for both anchoring of the placenta to the uterus, and for invading and remodeling the maternal spiral arteries into low resistance, high capacitance vessels, enabling them to meet the requirements for increased blood as pregnancy progresses (53). Periattachment factor was immunolocalized to the nuclei of extravillous cytotrophoblast columns of the first trimester human placenta and in the cytotrophoblasts of first and

second trimester placenta (Fig 9). Extravillous cytotrophoblast columns were not present in the sections of second trimester placenta available for staining. Staining appeared to be more prominent in the cytotrophoblast cells than the multinucleated syncytiotrophoblast cells in villi (Fig 9A). This could be because the syncytiotrophoblast layer is made of non-proliferating cells, and studies of PF in sheep indicate that its expression increases during a time of rapid cell proliferation (49). It is unknown if PF is present in the binucleate cells of the ovine trophoblast, or if it is present in the syncytial plaques these cells form with endometrial epithelium. The predominantly nuclear staining observed in first and second trimester human placenta and in carcinoma cell lines agrees with results seen in the ovine trophoblast (Chapter III).

The expression of PF mRNA in the human cytotrophoblast increased as these cells differentiated into invasive cytotrophoblasts (Fig 7). Plating first trimester cytotrophoblasts onto an extracellular matrix causes them to differentiate into the invasive phenotype (131). In a previous study, when first trimester cytotrophoblasts were plated onto Matrigel-coated dishes, they migrated and formed aggregates within 8 h. These aggregates then formed processes and began to invade into the extracellular matrix (131). Associated with the change to an invasive phenotype was an increase in staining for human placental lactogen and expression of various MMPs (131). Interestingly, second trimester or term placental samples formed aggregates but did not invade the extracellular matrix (131). Although only four samples were available for the study of invasion, the pattern of increased PF mRNA expression was the same in each sample (Fig 7). Immunolocalization shows that PF is present in the extravillous invasive columns (Fig 9). These results indicate PF may play a role in development of the invasive

cytotrophoblast phenotype. Ovine trophoblasts do not invade the endometrium as extensively as is seen in the human; however, they do express MMP-2 and MMP-9; and MMP-9 is associated with the invasive cytotrophoblasts of the human placenta (67, 68). In the term human placenta, PF mRNA was present, but there were no differences in expression of hPF mRNA between normal and IUGR human pregnancies (Fig 8). There are many other common molecules expressed in both the ruminant and human during this early part of pregnancy; perhaps PF plays a role in transcription of one or more of these.

Quantitative RT-PCR detected hPF mRNA in the hTR8 cell line under all conditions tested. Expression was not significantly affected by treatment. Differentiation of cytotrophoblasts to an invasive phenotype is controlled by $O_2$ concentrations, as well as hormones, growth factors, and cytokines (49). Prior to the $10^{th}$ week of gestation, low $O_2$ concentrations stimulate trophoblast cell proliferation, but inhibit differentiation into the syncytiotrophoblast and invasive cytotrophoblast cell types (49). There was a numerical increase in hPF mRNA concentration when hTR8 cells were grown in 20% $O_2$.(Fig 6). Expression of hPF mRNA was numerically, but not statistically increased in hTR8 cells grown on Matrigel (Fig 6). Similarly, our results from cytotrophoblasts plated onto Matrigel show an increase in hPF mRNA as these cells invade over time (Fig 7). Although these data are preliminary, they suggest PF may be involved in development of the invasive cytotrophoblast phenotype in the human placenta. Experiments in hTR8 cells were repeated twice; perhaps further replicates in these cells and cytotrophoblast samples would result in statistical differences.

The function of PF in the human placenta is unknown at this time; however, defective placentation and invasion of the cytotrophoblast columns is the source of

several pregnancy associated disorders, such as preeclampsia or placenta accrete. During preeclampsia, the trophoblast columns may exhibit shallow invasion, fewer trophoblast cell numbers, or failure to remodel maternal arteries (72). Several transcription factors that function as regulators of trophoblast differentiation and invasion have been identified in the mouse (62). The transcription factors that function in the human are largely unknown (53). However, there is some homology in transcription factors important for trophoblast development in both the sheep and human. For example, the transcription factor Ets2 is a regulator for both IFN$\tau$ genes (34) and hCG $\alpha$ and $\beta$ subunits (44, 45) and is required for placental development of the mouse (46). The inferred amino acid sequence of PF shows no DNA or RNA binding motif, so therefore cannot act as a transcription factor per se; however, due to its localization in the nucleus, PF could be involved as a coactivator or corepressor of transcription. The predicted amino acid sequence of PF has four putative protein kinase C phosphorylation sites. It is known that the prostaglandin $PGE_2$ plays a role in human blastocyst implantation and is abundantly produced by the decidua. Interestingly, a recent paper reported that $PGE_2$, signaling through G-protein coupled receptors $G_{\alpha q}$ and $G_{\alpha s}$ can stimulate cytotrophoblast migration in first trimester explants, as well as in the hTR8 cell line (134, 135). The $G_{\alpha q}$ receptor for $PGE_2$, known as EP1 can stimulate the PKC pathway, and therefore possibly have an effect on the function of PF. However, others have reported contradictory results in the hTR8 cell line under slightly different culture conditions (136). Further studies are necessary to determine the role of PF in the human placenta.

Periattachment factor mRNA was detected in all of the carcinoma cell lines screened. Its concentration was greatest in the PC3, BeWo, and HepG2 cell lines, derived from cancers from the prostate, placenta, and liver, respectively (Fig 5). The PC3 cell line is an adenocarcinoma of the prostate isolated from a metastasis to bone, and does not require androgens for survival or growth (137, 138). The BeWo cell line was derived from a choriocarcinoma that had metastasized to the brain, and had been previously maintained in the cheek pouch of a hamster for 8 years (139). This cell line which secretes hCG, was the first endocrine secreting cell line established. The HepG2 cell line was established from a heterogeneous population of human liver biopsies initially grown on a feeder layer of irradiated mouse cells (140). Its properties indicate that it is a hepatoblastoma, from a patient less than 40 months old (141). Immunolocalization of PF in various carcinoma cell lines indicated predominantly nuclear staining (Fig 10). This agrees with the predicted nuclear localization signal in the amino acid sequence, as well as the staining we observed in d 15 sheep conceptus and in the human cytotrophoblast. Overall, staining appeared to be more markedly nuclear in the carcinoma cell lines than in the d 15 conceptus.

These carcinomas represent a wide range of original tissue types and functions. However, certain characteristics of the carcinomas may shed light on the function of PF. In the very basic sense, all are rapidly dividing, proliferating types of epithelial cells. This agrees with the data we observed in the ovine conceptus and human cytotrophoblast. The expression of PF mRNA in the sheep conceptus increased rapidly as this tissue was growing rapidly, and decreased coincident with the decrease in rapid growth of the conceptus. In the human cytotrophoblast, hPF mRNA increased as these cells invaded in

an in vitro assay. Additionally, the cytotrophoblast columns in the human exist in a proliferative and undifferentiated state similar to a cancer cell (59). Numerous groups have studied the similarities between the invasive trophoblast cells of the human placenta and cancer cells. The trophoblast cells, as well as carcinomas both show high cell proliferation, lack of contact inhibition, cell migration and invasion, as well as a lack of detection by the maternal immune system (64, 65). The similar behavior of trophoblast and cancer cells likely results from similar genes and proteins that play roles in their proliferation, migration, and invasion (65). A review of the some of the analogous factors between trophoblast and cancer cells, such as proto-oncogenes, growth factors, cell-surface receptors, enzymes, enzyme receptors and inhibitors, and various hormones and peptides was conducted by Ferretti et al. (65). In the context of our experiments, except for a carcinoma of the placenta, PF mRNA was greatest in liver and prostate. The proto-oncogene *c-fms,* the growth factors IGF-1 and 2, the growth factor receptor CSF1R, and the hormone activin A are all expressed by both cytotrophoblasts and carcinomas of the liver, while the receptors for insulin and granulocyte-macrophage colony stimulating growth factor, the hormones LIF-R and urocortin, and prostate specific antigen are all expressed by both cytotrophoblasts and carcinomas of the prostate (65). During human implantation, the blastocyst first attaches to the uterus where invasion of cytotrophoblast within the endometrium occurs, followed by deeper penetration of the cytotrophoblast through the decidua and into the maternal spiral arteries and the myometrium. These same invasive processes also are important in cancer cells as well. Specifically, both trophoblast and cancer cells change their complement of the hetero-dimeric transmembrane glycoproteins, integrins, during migration and invasion into different

tissues (64). Integrins appear to function in early trophoblast development in both human and ruminant species (7). The invasion into the uterus by the cytotrophoblasts results from attachment to the basement membrane followed by penetration by enzymes that degrade the extracellular matrix and similar enzymes and mechanisms involved during metastasis of cancer cells (142). In particular, matrix metalloprotease-9 (MMP-9) and the serine protease uPA are important for invasion of process in both cytotrophoblasts and matastatic cancers (64, 65). Another feature common to both trophoblast and cancer cells during the invasion process is an increase in vascularization. A subset of cytotrophoblasts invade and remodel the maternal spiral arteries to increase blood flow, while in cancers proliferation of capillary endothelial cells is increased by tumor-secreted proteins (64). Both normal trophoblast cells and malignant tumors must escape detection by the immune system to survive and grow. However, trophoblast cells do not express major histocompatibility complex (MHC) class I or II molecules, while MHC I is down regulated in 40-90% of human tumors (65, 143). Even in ruminant trophoblasts, MHC levels are low (2). Telomerase activity, that may allow proliferation of cells to continue by protecting the telomere ends of chromosomes, is upregulated in most cancers (144) Some studies have shown elevated telomerase activity in both the human placenta (145) as well as a loss of telomerase activity as trophoblast cells differentiate (146).

The major difference in the invasion observed in placental development and that of cancerous growth is that trophoblast growth is controlled spatially and temporally, whereas tumor growth is not. Trophoblast cells differentiate to an invasive phenotype, but eventually stop this process. For example, interstitial cytotrophoblast cells become multinucleated and form placental bed giant cells, regarded as the terminal differentiation

step in the extravillous cytotrophoblast pathway (36). This control of trophoblast invasion and proliferation is important, and dysregulation can lead to abnormal pregnancy conditions. Excessive invasion of the trophoblast into the uterus can lead to a condition known as placenta accrete; uncontrolled proliferation can lead to gestational trophoblast disease (GTD) such as a choriocarcinoma (the source of BeWo cells); inadequate invasion can lead to preeclampsia; and IUGR is also associated with abnormal placental structure (64). Comparing and contrasting the properties of carcinomas and the placenta may help provide more insight into the function of PF.

For studying the function of PF in any human tissue, the ability to knockdown its mRNA expression and observe any changes would be extremely useful. To this end we developed a lentiviral vector that expresses shRNA against the hPF mRNA sequence that was capable of providing 63% knockdown of hPF mRNA relative to controls. Importantly, we also showed that a vector producing an shRNA with a mismatch to the hPF mRNA sequence did not reduce hPF mRNA concentrations and can be used as an effective control as was done in our previous experiments with ovine embryos. Our percentage of knockdown may be improved with longer time in culture, and with more concentrated virus. In this experiment the virus was cultured with cells for only 48 h. It is likely that with more time, a greater reduction in hPF mRNA would occur. Although we have already shown that knockdown of PF in the sheep leads to inhibition of conceptus elongation or embryo mortality, mechanistic studies of the transcriptional regulation of PF, as well as other factors that PF may interact with, will be easier to conduct in the context of human cells or tissue due to the availability of reagents and validated protocols. Furthermore, the use of lentiviral transduced RNAi in human

placental cell lines, such as hTR8 may help determine if PF plays a role in migration or invasion in human placental development.

## SUMMARY

The purpose of these studies was to characterize PF in human cells and tissues. Immunolocalization showed PF predominantly in the nuclei of first and second trimester cytotrophoblasts, and in the extravillous invading cytotrophoblast columns in the first trimester. In an in vitro invasion assay of first trimester cytotrophoblasts, hPF mRNA increased from 0, 3, to 12 h as invasion occurred. In the term human placenta, there was no difference in hPF mRNA between growth-restricted and control pregnancies. Human PF mRNA was detected in all human carcinomas tested and in the first trimester cytotrophoblast cell line, hTR8. Expression of hPF mRNA was greatest in cell lines derived from cancers of the prostate, liver, and placenta. Immunohistochemistry showed PF in the nuclei of carcinomas. To further characterize PF in human cells, a lentiviral construct expressing an shRNA targeting the hPF mRNA sequence was developed that resulted in 63% mRNA reduction in BeWo cells. BeWo cells treated with a lentiviral construct that expressed an shRNA with nucleotide mismatches to the endogenous hPF mRNA sequence showed no difference in hPF mRNA expression compared to untreated BeWo cells. Although preliminary in nature, these results show PF is present in the early human placenta and carcinoma cell lines. These two cell types show many similarities in function, and PF could play a role in both of them.

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