Assessing gene function in the ruminant placenta

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The placenta provides the means for nutrient transfer from the mother to the fetus, waste transfer from the fetus to the mother, protection of the fetus from the maternal immune system, and is an active endocrine organ. While many placental functions have been defined and investigated, assessing the function of specific genes expressed by the placenta has been problematic, since classical ablation-replacement methods are not feasible with the placenta. The pregnant sheep has been a long-standing animal model for assessing *in vivo* physiology during pregnancy, since surgical placement of indwelling catheters into both maternal and fetal vasculature has allowed the assessment of placental nutrient transfer and utilization, as well as placental hormone secretion, under unanesthetized-unstressed steady state sampling conditions. However, in ruminants the lack of well-characterized trophoblast cell lines and the inefficiency of creating transgenic pregnancies in ruminants have inhibited our ability to assess specific gene function. Recently, sheep and cattle primary trophoblast cell lines have been reported, and may further our ability to investigate trophoblast function and transcriptional regulation of genes expressed by the placenta. Furthermore, viral infection of the trophoectoderm layer of hatched blastocysts, as a means for placenta-specific transgenesis, holds considerable potential to assess gene function in the ruminant placenta. This approach has been used successfully to "knockdown" gene expression in the developing sheep conceptus, and has the potential for gain-of-function experiments as well. While this technology is still being developed, it may provide an efficient approach to assess specific gene function in the ruminant placenta.

Introduction

The placenta is a multifaceted organ that plays critical roles in maintaining and protecting the developing fetus. These roles include transferring nutrients from the mother to the fetus and waste secretion from the fetus to the mother, acting as a barrier for the fetus against pathogens and the maternal immune system, and serving as an active endocrine organ. As the "vehicle" for amino acid, fatty acid, glucose and oxygen transfer to the fetus, the placenta utilizes a variety of transport mechanisms (Regnault *et al.* 2005; Jones *et al.* 2007). Nutrient supply to the fetus is not only dependent on available transport mechanisms, but also on the amount of placental substrate and hormone metabolism. At mid-gestation, the majority of substrate taken up by the gravid uterus is consumed by the placenta (Bell *et al.* 1986) but the proportion of

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substrate utilized by the placenta declines as gestation advances, with more transferred to the fetus. A portion of the nutrients utilized by the placenta serves to fuel the placenta as an active endocrine organ. The placenta is capable of secreting a plethora of hormones, growth factors and cytokines, many of which are also produced by other endocrine or non-endocrine organs (Talamantes & Ogren 1988). Some of these are true placental hormones since they are not produced by other organs and have known or inferred functions during pregnancy. Included in this category are the conceptus interferons, chorionic gonadotropins, and members of the growth hormone (GH)/prolactin (PRL) gene family (Roberts & Anthony 1994). While we know a great deal about the function of the placenta, we still know little about the specific function of individual genes expressed within the placenta.

Assessing specific gene function in the placenta has been difficult, since classical ablation-replacement strategies are not feasible with the placenta. The ability to surgically place and maintain catheters in both the maternal and fetal circulation of pregnant sheep (Meschia *et al.* 1965; Battaglia *et al.* 1968), allowing non-stressed repetitive sampling, has provided considerable insight into placental function in ruminants. However, the lack of ruminant-specific trophoblast cell lines or efficient means to alter specific gene expression within the placenta has often precluded the direct testing of gene function. Non-ruminant trophoblast-derived cell lines have been useful when examining ruminant gene regulation, but the recent availability of cattle (Talbot *et al.* 2000; Shimada *et al.* 2001) and sheep (Farmer *et al.* 2008) cell lines may allow a more robust assessment of ruminant gene regulation and function *in vitro*. Furthermore, recent *in vivo* loss-of-function experiments have been reported in sheep, where either antisense morpholino oligonucleotides were introduced into the uterine lumen (Dunlap *et al.* 2006), or blastocysts were infected with lentivirus expressing a short-hairpin RNA construct before embryo transfer (Purcell *et al.* 2009). Use of these newer experimental approaches may well provide the mechanism by which to directly assess placental gene function in ruminants.

Formation and structure of the ruminant placenta

In contrast to primates and rodents, the ruminant conceptus does not undergo invasive implantation. Rather the blastocyst, comprised of the inner cell mass (ICM) and the blastocoele cavity surrounded by a single layer of trophectoderm, elongates into a filamentous conceptus (Guillomot 1995). Extraembryonic primitive endoderm develops from the ICM and migrates beneath the trophectoderm, differentiating into visceral endoderm (beneath the ICM) and parietal endoderm (PE) beneath the trophectoderm (Flechon *et al.* 2007). As the conceptus elongates, the PE elongates with the trophectoderm (Flechon *et al.* 2007), taking on a multinucleated syncytial morphology but retaining the characteristics of polarized epithelium. By 15 days post coitus (dpc) in sheep, and 19-20 dpc in cattle, formal apposition occurs between the trophectoderm and uterine luminal epithelium (Guillomot 1995), followed a day later by adhesion along the feto-maternal interface. The process of apposition and adhesion coincides with expansion of the allantois from the hind-gut, providing fusion with the chorion and vascularization in the areas of maternal caruncle projections (Stegeman 1974). Interdigitation of the chorionic epithelium with the uterine epithelium located in the non-glandular caruncles provides for the initial formation of individual placentomes (Boshier 1969), comprised of the fetal cotyledon and maternal caruncle. As pregnancy progresses, growth of the fetomaternal interface leads to an apposing network of fetal and maternal villi. The fetal cotyledonary villous tree, when mature, is comprised of stem, intermediate and terminal villi (Leiser *et al.* 1997), within which are stem arteries and veins, intermediate arterioles and venules, and terminal capillaries, respectively.

Coincident with the time of conceptus apposition is the appearance of chorionic binucleate cells (BNC; Guillomot *et al.* 1981) within the trophectoderm layer. The BNC result from consecutive nuclear divisions of trophectoderm uninucleate cells (UNC), without cytokinesis following the second division (Wooding 1992), and comprise 15-20% of the trophectoderm. Essentially throughout gestation, 15-20% of the BNC are migrating through the tight junctions formed by the UNC (Wooding 1983). Fusion of the BNC with uterine epithelial cells form trinucleate cells, and continued fusion of BNC with the initial trinucleate cells result in the formation of syncytial plaques, comprised of 20-25 nuclei, linked by tight junctions (Wooding 1984; Wooding 1992). At mid-gestation in sheep and goats the fetomaternal interface is totally comprised of syncytium (Wooding 1984; Wooding 1992). By contrast, in cattle and deer, the uterine epithelium is reestablished, such that continued BNC migration and fusion results in transient trinucleate cells (Wooding & Wathes 1980). The formation, migration and fusion of BNC not only serves in the development and formation of the expanding fetal villi and the fetomaternal interface, but they also serve in the synthesis and delivery of placental hormones, such as placental lactogen (PL; Milosavljevic *et al.* 1989; Kappes *et al.* 1992) to the maternal circulation.

This brief description of ruminant placenta development and structure is meant to provide an appreciation of the complexity of the ruminant placenta. If one just considers the villous tree of the fetal cotyledon, it is comprised of UNC, BNC, villous mesenchyme, vascular endothelial and smooth muscle cells, and the proportion of each within a single cotyledon changes as pregnancy progresses (Kappes *et al.* 1992). Furthermore, there is the fetomaternal syncytium, and when separating the placentome into its cotyledon and caruncle components, residual tissue from either the maternal or fetal components contaminate the other (Bridger *et al.* 2007). Consequently, the recent use of microarray-based gene profiling methods to assess placental gene expression (Everts *et al.* 2008) in cattle may be confounded by the multiple cell types present in the ruminant placenta. Subsequent quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) can be used to validate the changes in mRNA concentration (Aston *et al.* 2009), but the combination of subsequent qRT-PCR and *in situ* hybridization (Ushizawa *et al.* 2007) likely gives a clearer interpretation of the microarray results. However, important changes in mRNA concentrations of "lowly"-expressed genes might be overlooked or muted by the relative composition of the tissue used for RNA isolation. Use of laser-capture microdissection to harvest the cell population of interest (e.g., trophoblast layer) may enhance the validity of the results obtained from gene profiling experiments.

In vitro assessment of placental gene function

For years, *in vitro* investigation into ruminant placental gene regulation and function was hampered by the lack of ruminant-specific cell lines. Often human or rat choriocarinoma cell lines were used to investigate the transcriptitional regulation of sheep and cattle genes expressed in the placenta (e.g., Liang *et al.* 1999; Ezashi *et al.* 2001; Limesand *et al.* 2004). To provide relevance to the ruminant, we also utilized nuclear extracts from isolated sheep BNC in DNase footprinting (Liang *et al.* 1999), electrophoretic mobility shift assays (Limesand *et al.* 2004) and Southwestern analysis (Jeckel *et al.* 2009), to verify the results obtained with choriocarcinoma cell lines. In our experience (Liang *et al.* 1999; Limesand *et al.* 2004; Jeckel *et al.* 2009) BeWo cells (human choriocarcinoma cell line) have served as a useful model system for studying the transcriptional regulation of sheep PL. Recently, Bridger *et al.* (2007) reported the establishment of trophoblast cultures from cattle placentomes, and subsequently they reported the culture of BNCs for 90 days in culture (Landim *et al.* 2007) and proliferation of bovine trophoblast

cell line F3 in response to epidermal growth factor (Hambruch *et al.* 2010). During early passages (P2) of the F3 cells, the BNC expressed PL, but in later passages (P18) PL was no longer detectable (Hambruch *et al.* 2010). As yet, there are no reports of successful transfection or other genetic manipulations of these cells. With the ovine trophoblast cells that we harvested to isolate nuclear protein (Liang *et al.* 1999; Limesand *et al.* 2004), part of these cells were placed in culture and passaged, and attempts were made to transfect these cells by a variety of techniques (e.g., lipid-mediated transfection, electroporation, $CaPO_4$ precipitation) without success. Our only success in transforming these cells was when we infected them with an adenoviral construct (Figure 1) or lentiviral construct, both of which expressed enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter. This approach was successful in obtaining EGFP expression in BNCs (Figure 1), but since the number of BNC diminished considerably with each successive passage, we have not pursued this approach further. It may however, be a useful approach to examine transcriptional regulation of genes expressed by BNC.

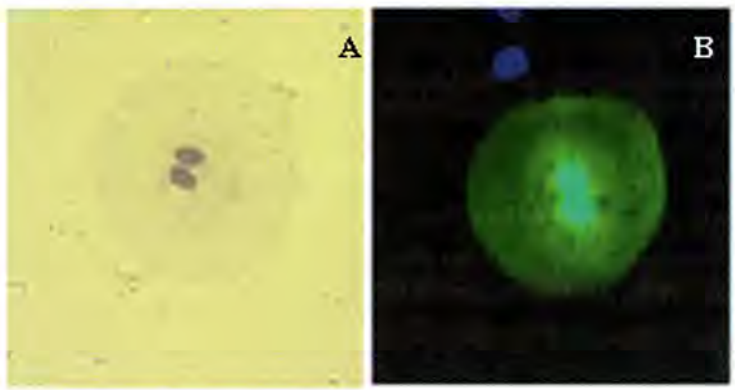

Fig. 1. Binucleate cell (BNC) expression of enhanced green fluorescent protein (EGFP). BNC were isolated from 100 dpc sheep placenta, placed in culture and infected with an adenovirus vector expressing EGFP. Panel A: Hemotoxylin stained BNC. Panel B: Direct fluorescence microscopy of BNC expressing EGFP. Nuclei were stained with DAPI. 400X magnification.

As noted in the introduction, the methods used to generate two trophoblast cell lines from cattle blastocysts have been described. One cell line (CT-1 cells; Talbot *et al.* 2000) was generated by plating 10-11 dpc hatched blastocysts on a feeder layer of STO mouse fibroblast cells. The second cell line (BT-1 cells; Shimada *et al.* 2001) was generated by culturing blastocysts on collagen-coated dishes, in the absence of a feeder cell, but cell growth was accelerated when cultured in the presence of bovine endometrial fibroblast-conditioned medium. Both cell lines express interferon-τ, but the BT-1 cells have been shown to generate BNC (Nakano *et al.* 2002), and express PL and pregnancy-associated glycoproteins (Ushizawa *et al.* 2005). It was demonstrated that the expression of metalloproteinases and tissue inhibitors of metalloproteinases by BT-1 cells could be regulated by exposure to several cytokines, including tumor necrosis factor-α and interleukin-1α, demonstrating the responsiveness of BT-1 cells to exogenous stimuli (Hirata *et al.* 2003). In a similar fashion, exposure of CT-1 cells to granulocyte-macrophage colony-stimulating-factor resulted in increased interferon-τ expression (Michael *et al.* 2006). Furthermore, Ezashi *et al.* (2008) demonstrated that both Distal-less 3 (DLX3)

and ETS2 are expressed by CT-1 cells and that transfection of CT-1 cells with small interfering RNA designed to target DLX3 resulted in reduced interferon-τ production. The summation of the various studies reported with either BT-1 or CT-1 cells suggest that these cell lines may be quite useful in examining ruminant trophoblast gene regulation and function. It has yet to be clearly demonstrated how efficiently these cells can be transfected for transcriptional regulation studies and/or genetic manipulation of function.

In contrast to the BT-1 and CT-1 cell lines, which were derived from outgrowths of hatched blastocyts, Farmer *et al.* (2008) reported the generation of oTr-1 and oTr-F cell lines from 15 dpc sheep conceptuses. The elongated conceptuses were minced, and cultured either on plastic (oTr-1) or on collagen coated dishes (oTr-F). These cell lines were established and used to investigate the function of galectin 15 (LGALS15) in trophectoderm migration and attachment (Farmer *et al.* 2008). LGALS15 promoted oTr proliferation and migration, and the effect of LGALS15 on migration could be inhibited with a c-Jun N-terminal kinase inhibitor, but not with a Rho-kinase inhibitor. Seeking an *in vitro* cell culture system to investigate regulation and function of proline-rich 15 (PRR15), a nuclear protein with peak expression at 15-16 dpc in sheep conceptuses (Purcell et al. 2009), we obtained oTr-1 cells developed in Dr. R.C. Burghardt's laboratory (Texas A&M University) from Dr. T.E. Spencer (Texas A&M University). Analysis of PRR15 and interferon-τ mRNA isolated from the oTr-1 cells by qRT-PCR indicated that the expression of these two genes was considerably lower in oTr-1 cells, when compared to 15 dpc conceptus mRNA. Subsequently, we generated a series of oTR cell lines (oTR-17, -18, -19, -23 and -25) from 15 dpc sheep conceptuses using the methods of Farmer *et al.* (2008), and found that when these cells are cultured on plastic, PRR15 and interferon-τ mRNA concentrations were quite low. Consequently, we compared the expression of PRR15 by oTR cells when cultured on plastic or various concentrations of Matrigel® for 48 hours. As evidenced in Figure 2, PRR15 mRNA concentration increased significantly when oTR cells were plated on a 1 mm cushion of Matrigel®, at a concentration of 6 mg/ml. There is a notable phenotypic change in the oTR cells following plating on Matrigel®, as evidenced in Figure 3. During the first hour of culture on Matrigel® the cells begin to migrate and by 3 hours have formed clusters of cells (Figure 3), which continue to condense and invade into the matrix. Expression of PRR15 mRNA increases in a time-dependent manner when oTR cells are cultured on Matrigel®, until 36 hours, at which time its mRNA concentration plateaus. The phenotypic behavior of the oTR cells cultured on Matrigel® is similar to what we observe with human ACH-3P cells (Hiden *et al.* 2007), and what has been reported for first trimester human cytotrophoblast cells (Librach *et al.* 1991) and mouse trophoblast stem cells (Lei *et al.* 2007), suggesting greater inter-species trophoblast cell similarity than might be anticipated.

From this discussion, it is obvious that there are a number of ruminant-specific trophoblast cell isolation and culture systems now available for use in assessing placental gene function *in vitro*. There are differences in gestational age at isolation, subsequent culture conditions, and possibly in responsiveness. All of these approaches utilize continual culture and passage of primary cells, which opens the door for passage related changes in cell phenotype, responsiveness, and usefulness. While a number of "markers" have been used to assess human cytotrophoblast cells and the various cell lines available (Hiden *et al.* 2007; Hannan *et al.* 2010), as yet a similar strategy has not been fully developed for assessing ruminant trophoblast cell lines. This will require investigators reaching consensus as to which markers should be used, and the sharing of reagents. Regardless, the availability of these ruminant trophoblast cell lines should be quite useful in assessing placental gene function in ruminants, but the results from such studies should always be put in context with what we know about *in vivo* function.

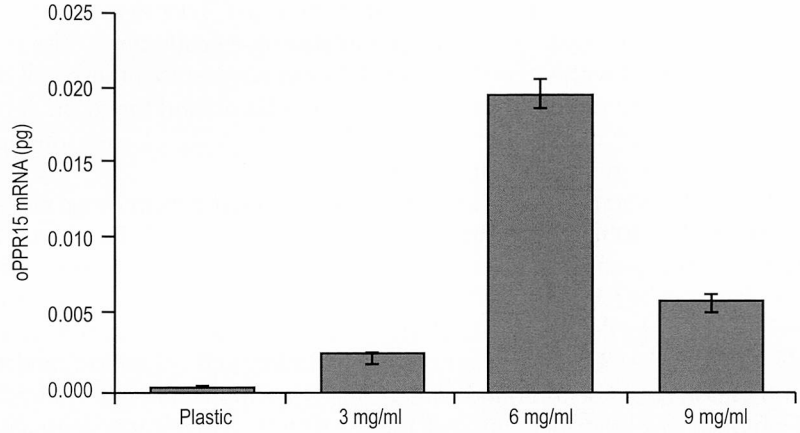

Fig. 2. PRR15 mRNA concentration in oTR cells cultured on Matrigel®. oTR-17 cells were plated on plastic culture dishes, or on dishes coated with 3, 6 or 9 mg/ml of Matigel® for 48 hours. PRR15 mRNA concentration was assessed by qRT-PCR as described in Purcell *et al.* (2009).

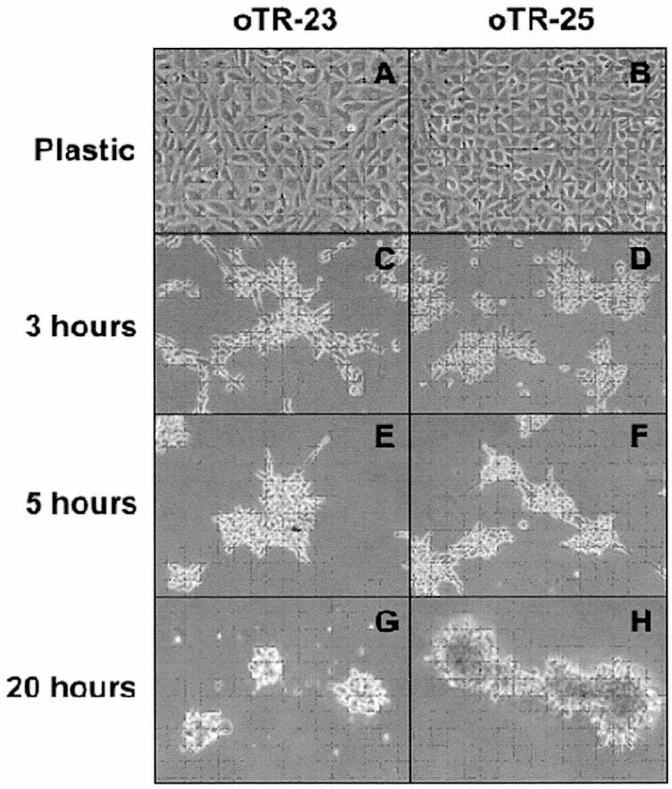

Fig. 3. Phenotypic change in oTR cells cultured on Matrigel®. oTR-23 and oTR-25 cells were cultured on plastic culture dishes, or on dishes coated with 6 mg/ml of Matrigel®. Panels A & B: oTR-23 and -25 cells grown on plastic. Picture was taken just before harvesting the cells for plating onto Matrigel®. Panels C & D: oTR-23 and -25 cells following 3 hours of culture on Matrigel®. Panels E & F: oTR-23 and -25 cells following 5 hours of culture on Matrigel®. Panels G & H: oTR-23 and -25 cells following 20 hours of culture on Matrigel®. 100X magnification.

In vivo assessment of placental gene function

In vivo assessment of placental function

While our understanding of the function of specific genes expressed within the ruminant placenta may lag behind other species, especially rodents, we do have a better understanding of actual placental function in ruminants than we do in other species. This results from the ability to surgically place and maintain catheters in both the maternal and fetal vasculature in sheep (Meschia *et al.* 1965; Battaglia *et al.* 1968), allowing repetitive sampling of both maternal and fetal blood under non-stressed conditions. Placement of catheters in both maternal and fetal vessels (Figure 4) not only allows for infusion of substrate, but also simultaneous sampling of arterial-venous concentration differences on both sides of the placenta, allowing application of the Fick principle (Meschia *et al.* 1980). Simultaneous sampling of arterial-venous differences, along with measures of blood flow in uterine and umbilical vessels, provides for the determination of uteroplacental utilization and or metabolism (Hay 1991). This approach has been applied extensively to placental uptake and utilization of O_2, glucose and amino acids, but as inferred in Figure 4, it can also be applied to other tissues (e.g., hind limb skeletal muscle, liver, etc.) within the fetus (Anderson *et al.* 2001; Teng *et al.* 2002). At mid gestation, uteroplacental O_2 consumption by the placenta accounts for approximately 80% of total O_2 uptake by the uterus (Bell *et al.* 1986), and during late gestation, uteroplacental O_2 consumption is essentially equivalent to O_2 consumption by the fetus (Meschia *et al.* 1980). Of the oxygen consumed by the placenta, 90% can be accounted for by oxidative phosphorylation of glucose (Meschia *et al.* 1980), as 80% of the glucose taken up by the uteroplacental unit is consumed (Hay 1991). These data demonstrate how metabolically active the placenta is, and can only be determined under steady state conditions. Simultaneous collection of maternal and fetal arterial and venous blood samples can be collected in ruminants, an experimental approach that is not permissible in humans or feasible in rodents. This experimental approach can also be applied to placental hormone production and secretion in ruminants, as Schoknecht *et al.* (1992) demonstrated that the PL entry rate into fetal sheep circulation increases with increasing gestational age even though fetal concentrations of PL plateau by 90 days of gestation (Kappes *et al.* 1992).

As methods develop to alter specific gene expression in the ruminant placenta (see below), the utility of being able to assess placental function *in vivo* will become advantageous. For example, it has been demonstrated *in vivo*, using two distinct sheep models of intrauterine growth restriction (Wallace *et al.* 2005), that placental delivery of O_2, glucose and amino acids to the umbilical circulation is impaired. The ability to ablate the expression of specific nutrient transporters within the placenta, incorporating steady-state *in vivo* transfer studies into the experimental paradigm, would provide considerable insight into the role of individual placental transport mechanisms. Additionally, the ability to enhance or ablate the expression of specific placental hormones, followed by *in vivo* assessment of the physiological impact using the approaches outlined above, could finally provide a direct assessment of the physiological role and necessity of these hormones.

In vivo assessment of gene function

Transgenesis in mice has been used for approximately 30 years to examine the effect of "adding" genes. Similarly, homologous recombination in mouse embryonic stem cells as a means of functionally deleting genes has been used to study specific gene function for over 20 years. Unfortunately, comparable efficient methods have not been forthcoming for ruminants, especially

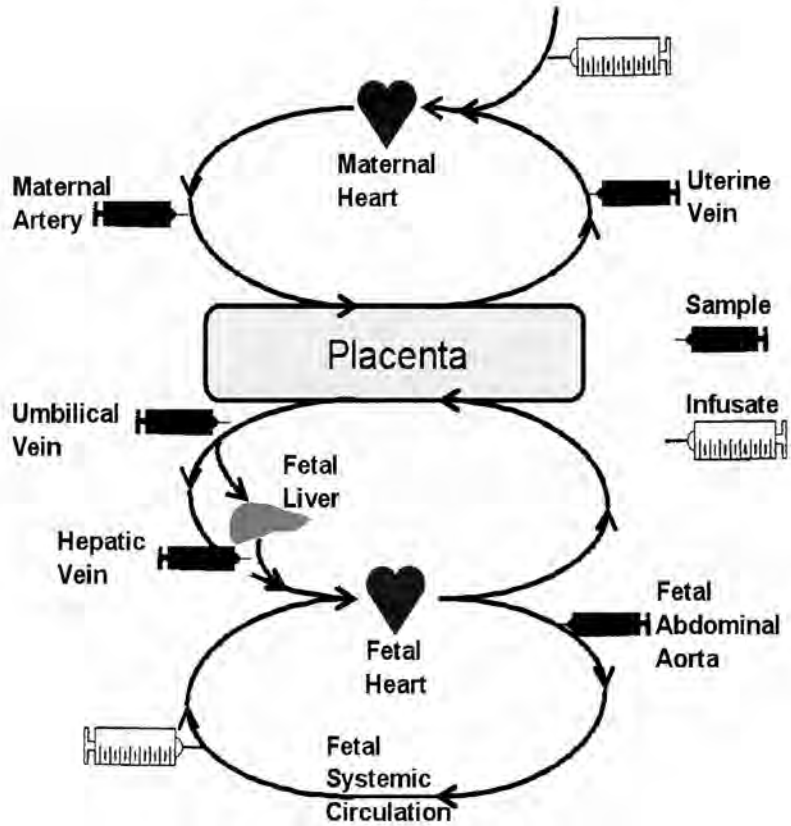

Fig. 4. Schematic representation of *in vivo* preparation of pregnant sheep used to measure utero-placental and fetal uptake and metabolism of O_2 and nutrients. Infusate can be administered to either maternal or fetal circulation, and blood samples drawn simultaneously from either circulation.

for genes expressed by the placenta. Consequently, until recently, there have not been viable approaches available to directly assess the function of genes expressed in the ruminant placenta. Dunlap *et al.* (2006) reported the successful use of morpholino oligonucleotides, infused into the uterine lumen on 8 dpc, to examine the impact of inhibiting the expression of the endogenous retrovirus enJSRV *env* within sheep conceptuses. Antisense morpholiono oligonucleotides utilize morpholine rings in their structure in place of deoxyribose, and are designed to hybridize with RNA sequences encompassing the translational start site, or an exon/intron splice-acceptor site (Summerton 1999). Hybridization at these sites provides steric blocking of translation and or pre-mRNA splicing in an RNase H independent fashion (Summerton 1999). By infusing antisense morpholino oligonucletides designed against enJSRV *env*, Dunlap *et al.* (2006) demonstrated that enJSRV *env* plays an important role in trophectoderm growth and differentiation in elongating sheep conceptuses. This is a useful approach to study early events in conceptus growth and placentation. However, since the approach relies on transient transfection, efficacy of morpholino oligonucleotide mediated gene "knockdown" is lost as the conceptus/placenta proliferates.

Steric blocking of translation or pre-mRNA splicing interferes with production of the gene product of interest, but does not target the mRNA for destruction. Naturally occurring micro RNA (miRNA) provide a mechanism of defense against virus infection and or endogenous gene regulation (He and Hannon 2004), through RNA interference (RNAi). As depicted in Figure 5, primary miRNA transcripts are processed in the nucleus by the RNase III activity of Drosha, yielding pre-miRNA with a characteristic hairpin-loop structure, that are exported out of the nucleus. The pre-miRNA are further processed in the cytoplasm by the RNase III activity of Dicer, yielding a 22-base pair duplex, or mature miRNA. The miRNA serves as the "guide" sequence incorporated into the RNA-induced silencing complex (RISC). When RISC complexes with the target transcript, Argonaute, a component of RISC that possesses RNase H activity, cleaves the mRNA thus targeting it for nuclease-mediated degradation. If there is imperfect complementarity between the "guide" sequence and the target, Argonaute may not cleave the target, but rather recruit additional proteins that ultimately inhibit translation of the target (Figure 5). Furthermore, many endogenous miRNA target the 3'-untranslated regions of mRNA, such that the miRNA-RISC complex represses translation, rather than initiating mRNA degradation.

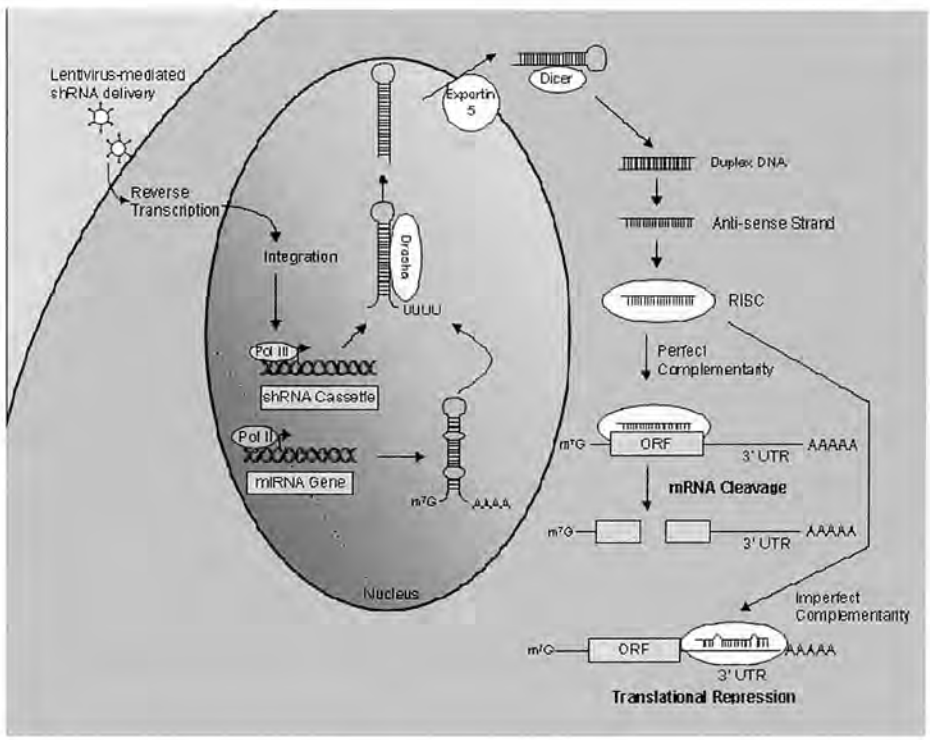

Fig. 5. Schematic representation of the generation, processing and function of miRNA or shRNA. Both miRNA and shRNA are processed in the nucleus by Drosha, and further processed in the cytoplasm by Dicer, yielding a 22 base pair duplex. The antisense strand of the duplex complexes with RISC, acting as a "guide" sequence for hybridization with the "target" transcript. If there is perfect complementarity between the "guide" and "target sequences, the "target" sequence undergoes cleavage and degradation. With incomplete complementarity, translation of the "target" sequence is inhibited by the RISC complex.

Based on our knowledge of how endogenous miRNA are generated, processed and function (He and Hannon 2004), this information has been used to develop and use exogenous RNAi to modify gene expression. Short-hairpin RNA (shRNA) introduced into cells (Paddison *et al.* 2002) are processed in the same fashion as endogenous miRNA. Cassettes encoding a shRNA often consist of inverted repeats separated by a loop sequence and followed by a short poly(T) track to terminate transcription (Paddison *et al.* 2002). Typically, the shRNA-encoding cassette is inserted downstream of a RNA polymerase III promoter (Paddison *et al.* 2004), within the targeting vector of choice. The targeting vector can be a simple plasmid used for transfection of cells *in vitro*, or it can be a viral vector, such as adenovirus or lentivirus. Adenoviral vectors have numerous advantages, primarily the ease in obtaining high titer stocks of virus and the infectivity of the virus, but suffer from the fact that they only provide for transient expression, such that the "effect" may be washed out over time in a rapidly proliferating tissue such as the ruminant placenta. By contrast, lentiviral vectors provide for stable integration into the host genome making them ideal for "long-term" transgenesis. Lentiviral constructs are often easier to generate than adenoviral constructs, but it is more difficult to generate high-titer stocks of lentivirus.

As noted above, when shRNA are introduced into cells, either *in vitro* or *in vivo*, the shRNA is processed in the cell like endogenous miRNA (Paddinson *et al.* 2002), providing for targeted-degradation of mRNA or translational repression of that mRNA (Figure 5). However, not all shRNAs work, and it is not uncommon to design and test 3 to 5 shRNA before identifying one that effectively "knocks down" specific-gene expression. Furthermore, sufficient validation of a shRNA is needed to verify that it is not inducing generalized repression of multiple mRNA or off-target effects, or that its introduction into cells is not triggering interferon induction and an innate immune response. Often, investigators will scramble the shRNA sequence, to render it non-functional, and use that as the control. The drawback of that approach is that it is difficult to know that the scrambled shRNA is capable of interacting with Dicer and RISC, which would yield the most robust control. Alternatively, one can introduce a functional shRNA as the control. For example, a functional shRNA that targets a mRNA transcript not expressed by the cell or tissue of interest. Purcell *et al.* (2009) utilized a shRNA that targeted human PRR15, but due to three nucleotide mismatches with the sheep mRNA sequence, it did not alter sheep PRR15 expression, providing a robust control for their subsequent *in vivo* experiments.

Hofmann *et al.* (2004) infected cattle oocytes with a lentiviral construct leading to the expression of EGFP in calves derived from *in vitro* fertilization. Similarly, they infected fetal fibroblasts with the recombinant lentivirus and used these as donor cells for somatic cell nuclear transfer. Both approaches underscore the potential for lentiviral-mediated transgenesis in ruminants. Lentiviral-mediated expression of shRNA targeting the prion protein in goats and cattle was accomplished either by somatic cell nuclear transfer (goats) or injection of the lentivirus into the perivitelline space of oocytes (cattle), prior to *in vitro* fertilization (Golding *et al.* 2006). These two reports confirmed the potential for lentiviral-mediated transgenesis, as well as shRNA-mediated RNAi in ruminants. Recently, we reported (Purcell *et al.* 2009) the infection of day 8 sheep blastocysts with a lentiviral construct designed to target the mRNA encoding PRR15, a nuclear protein expressed by the elongating ruminant conceptus. We utilized a replication-deficient lentiviral construct generated by Rubinson *et al.* (2003), such that when hatched blastocysts were exposed to the virus, only the outer trophectoderm would be infected. Exposing three blastocysts to 300,000 transforming units of the virus in 100μl drops for 4 to 6 hours resulted in uniform infection of the trophectoderm. Infected blastocysts were then surgically transferred to synchronized recipient ewes. The lentivirus construct used (LL3.7; Rubinson *et al.* 2003) contains an EGFP expression cassette driven by the cytomegalovirus

promoter, and an insertion site for the shRNA cassette downstream of the mouse U6 promoter, a RNA polymerase III promoter. As reported in Purcell *et al.* (2009), infection of blastocysts with a lentivirus that did not contain a shRNA cassette, or with one containing a shRNA cassette that targets human PRR15 mRNA (discussed earlier), did not impact conceptus development by 15 dpc, and there was uniform expression of EGFP in the conceptus trophectoderm. In contrast, infecting blastocysts with the lentivirus expressing the shRNA targeting sheep PRR15 mRNA resulted in conceptus demise or a failure to develop by 15 dpc. While we continue to investigate the exact function of PRR15, our results demonstrate the utility of lentiviral-mediated transgenesis in the ruminant placenta. Furthermore, while our efforts were ongoing, Georgiades *et al.* (2007) and Okada *et al.* (2007) reported trophoblast-specific lentiviral-mediated gene transfer in mice, again by infecting the hatched blastocysts. These three reports (Geogiades *et al.* 2007; Okada *et al.* 2007; Purcell *et al.* 2009) provide the "proof of concept" that lentiviral-mediated transgenesis can be directed to the placenta specifically. Since lentiviral constructs are stably integrated into the host genome, any derivative of the trophectoderm should express the "transgene" throughout the remainder of gestation, providing the opportunity to directly assess the function of genes expressed by the ruminant placenta. Furthermore, this approach could be expanded to "gain of function" experiments, where a transgene would be expressed under the control of a tissue-specific promoter, or under the control of a "regulated" promoter that could be "turned on or off" at various times during gestation.

Conclusions

Historically we have gained considerable insight into the physiological function and processes of the placenta from studies using ruminants. This is especially true for sheep which have been used extensively for *in vivo* studies assessing placental functions affecting fetal-placental development. However, it has been difficult to directly assess specific gene function in the ruminant placenta. The generation of cattle or sheep trophoblast cell lines is providing ruminant-specific assessment of gene function and regulation *in vitro*. Furthermore, reports of lentiviral-mediated transgenesis specifically in the trophoblast lineage provide the potential to expand assessment of gene function in the placenta *in vivo*. The use of RNAi *in vivo* is a powerful tool in determining the importance of specific genes expressed in the placenta, and when coupled with maternal and fetal catheterization, the impact on placental and fetal physiology can be assessed simultaneously. Results from these *in vivo* approaches coupled with *in vitro* investigations using the appropriate ruminant trophoblast cell line could yield powerful strategies for uncovering new biological functions and underlying molecular mechanisms. With these new approaches in hand, the future for assessing gene function in the ruminant placenta is bright.

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