Development of the pig placenta[*]

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Placental insufficiency results in fetal loss, low birth weight, stillbirth, preweaning mortality and poor growth. Placental development begins at conceptus elongation, which is a primary factor controlling the size of the placenta. After elongation, the allantois develops outward from the embryo to establish the allantochorion, which defines the size of the functional placenta. During implantation, chorionic trophoblasts adhere to endometrial epithelial cells. Placental structures known as areolae develop at the openings of the endometrial glands and take up endometrial gland secreted products (histotrophe). Between day 30 and 35 of gestation, the adhered trophoblast-endometrial epithelial bilayer undergoes microscopic folding. Fetal and maternal capillaries develop adjacent to the bilayer and blood flows are arranged in a cross-countercurrent manner. Except for nutrients secreted by the glands, nutrient exchange takes place between these capillaries within these folds. By day 85, the folds deepen and become more complex, increasing surface area. The epithelial bilayer thins and capillaries indent the plane of each layer (but do not penetrate), reducing distance between capillaries. The folded bilayer is surrounded by endometrial stroma on the maternal side and placental stroma on the fetal side. The fetal-placental stroma is partially composed of glycosaminoglycans, the most abundant being hyaluronan and heparan sulfate. Changes in both hyaluronoglucosaminidase and heparanase during placental development suggest that these enzymes play a role in placental development. In addition to structural modifications, various nutrient specific transport mechanisms exist. These mechanisms are likely to be as important to transport of specific nutrients as placental size or structure.

Introduction

The number of piglets weaned per sow on a yearly basis is a useful measure of sow productivity in breeding herds. The number of fully formed piglets and the stillbirth and preweaning mortality rates influence the number of piglets weaned. Placental function influences each of these components. At its worst, placental insufficiency results in fetal loss during pregnancy. However, even when placental insufficiency is not severe enough to cause death of the fetus, it still reduces piglet birth weights, which is strongly associated with both stillbirth

rate (Tuchscherer et al. 2000) and preweaning mortality (Lay et al. 2002). In addition, most preweaning mortality occurs within 2 days of birth (England 1974), supporting the concept that poor fetal development is the root cause of most preweaning mortality. If piglets survive, their growth rates are permanently impaired (Tilley et al. 2007).

The weights of the fetus and the fetal components of the placenta (allanto- and amniochorion, hereby called "fetal-placenta") can vary widely even within the same litter, and are correlated when fetal-placental weight decreases below a threshold weight (Vallet 2000). This clearly indicates that fetal-placental weight is a primary factor contributing to the generation of runt piglets. The residual variance of fetal weights after fitting the effect of fetal-placental weight partially represents the effect of other characteristics of the placenta that determine its function (i.e., surface area, structure, vascularity). Because of the poor performance of low birth weight piglets, the goal of the swine producer is for every sow to give birth to a large litter of large, uniformly developed piglets. Because the weight of the piglet is correlated with the weight and function of the fetal-placenta, meeting this goal requires that we understand how the fetal placenta develops in terms of both its final weight and its functionality.

Fetal-placental weight and fetal weight

The relationship between fetal-placental weight and fetal weight is curvilinear (Vallet 2000), indicating that beyond a certain size fetal placenta, the fetal-placental weight ceases to influence fetal weight. Thus, fetal-placental weight is relevant primarily to underweight fetuses. Within the fetal-placental weight range that affects fetal weight, differences in fetal-placental weight do not result in fully proportional changes in fetal weight. This can be demonstrated using the relative growth rate equation of Huxley (1932) to examine relationships between fetal-placental and fetal weights. Using the data reported in Freking et al. (2007), the slopes of the relationships between log fetal-placental weights and log fetal weights were 0.20 on day 25, 0.26 on day 45, 0.44 on day 65, 0.57 on day 85, and 0.69 on day 105. A slope of 1 indicates fully proportional growth relationships. Because these slopes are uniformly less than 1, they indicate that compensatory mechanisms exist during gestation to minimize decreases in fetal weight when fetal-placental weight is decreased (i.e., a fetal sparing effect). They also indicate that these mechanisms become progressively less effective with advancing gestation. These compensatory mechanisms need not be confined to the fetal placenta, mechanisms within the uterus or the fetus could also compensate for reduced fetal-placental weight.

Relationship between conceptus elongation and fetal-placental size

It is well established that swine conceptuses undergo elongation at about the time of maternal recognition of pregnancy (Perry & Rowlands 1962, Geisert et al. 1982), reaching lengths ~ 1 m, but occupying ~ 30 cm of uterine space due to extensive folding of the endometrial lining. Elongation of the conceptus likely is a primary determinant of the amount of uterine space and maternal resources available to each developing conceptus. It also appears that most pig blastocysts cease elongation when they come into proximity with another conceptus, because individual conceptuses typically do not overlap with each other within the uterus until later in gestation (Perry & Rowlands, 1962, Ashworth et al. 1998), although exceptions to this have been noted (Crombie 1972, Bjerre et al. 2008) and this mechanism can clearly be exceeded. The initiation of elongation of blastocysts within a litter is relatively synchronous. However, a small subset of blastocysts often do not elongate with the majority of littermates. Experiments in which conceptuses were recovered just after initiation of elongation report obtaining elongated

blastocysts along with a minority population of blastocysts that remain spherical (Geisert et al. 1982, Pope et al. 1988). The mechanism that initiates elongation is not known. It has been suggested that the diversity in initiation of elongation may be explained by differences in the timing of ovulation of individual ova (Pope et al. 1988). However, later work using ultrasound measurement of ovulation suggests that the difference in time of ovulation from the first to last ovum does not explain the differences in elongation initiation (Soede et al.1992). On the other hand, it is not clear whether ova that are late to ovulate are as developmentally competent as those that ovulate earlier. Thus, although the timing of ovulation may not explain the diversity in conceptus elongation, differences in developmental competence between earlier and later ovulating ova may play a role in conceptus diversity during elongation (Xie et al. 1990).

Once initiated, elongation of the pig blastocyst occurs very rapidly, and the rate has been estimated to be ~ 45 mm/h (Geisert et al. 1982). Because this estimate was made by comparing the elongation of different blastocysts at two different times, this rate is approximate. It seems likely that elongation rate could vary between individual blastocysts, and that this variation contributes to variation in elongation success. However, methods are lacking to assess individual conceptus variation in elongation rate because once removed from the uterus for observation, the process of elongation is interrupted.

Another aspect of elongation that affects conceptus length is its spontaneous termination. The effect of this is clearly evident in Meishan pigs. Meishan conceptuses are smaller at all stages of development and terminate elongation at smaller placentas, resulting in smaller placenta (Ford 1997). This potentially limits individual conceptus interaction within the uterus, improving the outcome of elongation for that portion of the litter experiencing delays. Curiously, this would result in better uniformity of conceptus size after elongation has occurred, which has been reported in Meishans (Bazer et al. 1988). The reduction in conceptus size in Meishans is associated with reductions in uterine protein secretion during early pregnancy (Vallet et al. 1998a). Interestingly, treatment of Meishan pigs during early pregnancy with estrogen, which stimulates uterine protein secretion, increased placental size (Wilson & Ford 1999), but estrogen had no effect on placental size in European breed pigs (Vallet & Christenson 2004). This is also consistent with a lack of effect of treatment of gilts with estrogen on day 9 and 10 of gestation on elongation (Blair et al. 1991), although this treatment does result in complete embryo loss within a few days after elongation, possibly due to changes in the luminal endometrial epithelial cell glycocalyx.

Thus, the size of the uterus, the number of conceptuses, the relative timing of initiation of elongation, the relative speed of elongation, and the length of termination of elongation all interact to determine the ultimate sizes of each resulting placenta in the litter. Ideally, uniform initiation and speed of elongation would result in the uniform division of the available maternal resources among the members of any size litter. However, in the extreme, this could lead to uniform failure of all conceptuses in the litter if the division of the uterine space was such that no conceptus received an adequate amount. The diversity that occurs in elongation may provide a mechanism whereby some successful pregnancies occur even in this case.

Fetal-placental growth after elongation

A logical question to ask is whether the eventual size of the fetal placenta is fixed at elongation. The fetal placenta continues to grow in weight until day 60 to 70 of gestation, after which its growth rate decreases (Freking et al. 2007). Vonnahme et al. (2000) reported that crushing every other fetus on day 40 of gestation increased fetal-placental weights of the remaining conceptuses on day 112 of gestation, but did not affect fetal weights. In our own recent experiment (Vallet

et al. 2009), fetuses at the ovarian or cervical ends of the uterine horns were crushed on day 35 of pregnancy and adjacent fetal placenta and fetuses were compared to nonadjacent fetal placenta and fetuses on day 105. Results indicated that both the fetal placenta and fetuses were heavier adjacent to a crushed fetus, but the increase was only about 10%. In addition, fetal placentas were asymmetrical, the side of the fetal placenta adjacent to the crushed fetus was longer from umbilical junction to tip compared to the nonadjacent side. These results clearly demonstrate that (1) most of the eventual weight of the fetal-placenta is established before day 35 of pregnancy, and (2) there is a limited ability of conceptuses to benefit from the demise of an adjacent conceptus if it occurs between day 35 to 40 of gestation.

Fetal-placental structure related to function

There is clearly more to placental development than changes in size during gestation. The conceptus begins to attach to the endometrium beginning around day 13 (Keys *et al.* 1986, Stroband & Van der Lende 1990) with formal implantation occurring around day 18 (Bielanska-Osuchowska 1979). The first placental structure that develops is the yolk sac placenta, which then regresses. This is followed by the development of the allantois, which migrates into the elongated chorion. That portion of the chorion where the allantois does not reach becomes the "necrotic tip". The portion of the chorion that fuses with the allantois becomes vascularized and is the functional fetal placenta (Friess *et al.* 1980). Very little is known about factors controlling the migration of the allantois outward into the chorion, including why it stops. The chorioallantois then fills with allantoic fluid, which presses the chorioallantois against the walls of the uterus. This most likely aids in the process of implantation and also provides a mechanical stimulus for growth (Vogel & Sheetz 2006) of both the uterus and the fetal placenta until the fluid peaks around day 30 of gestation, after which the volume of allantoic fluid recedes to day 45 (Knight *et al.* 1977). Fluid volumes again increase to peak at day 60 and recede again until the volume is relatively minimal by the end of gestation. At the mouths of the uterine glands, the placental areolae develop beginning about day 15 of gestation (Bielanska-Osuchowska 1979, Leiser & Dantzer 1994), and are specialized regions of the trophoblast epithelium that take up secreted products from the uterine glands. Elsewhere on the surface of the chorioallantois, the attached trophoblast-endometrial epithelial bilayer develops microscopic folds beginning ~ day 35 of gestation (MacDonald 1976, Dantzer 1984). Using microcorrosion casts, Leiser & Dantzer (1988) reported that the maternal and fetal capillaries are arranged in net-like structures on the maternal and fetal sides, respectively, of the folded bilayer and that they are arranged in a cross-countercurrent fashion. Maternal blood enters the folded structure near the top of the folds and leaves from the bottom. Fetal blood enters the folded structure near the bottom and leaves from the top. Thus, maternal-fetal exchange takes place within the folded bilayer, because this is the area of closest contact between the maternal and fetal capillaries.

Maternal-fetal exchange given this arrangement is maximized by increased folded bilayer surface area, increased capillary density on either side of the folded epithelial bilayer, and reduction in the distance between the capillaries. During later gestation, secondary folds in the epithelial bilayer develop, increasing the maternal-fetal interaction surface area (Friess *et al.* 1980). In addition, a recent study in our laboratory (Vallet & Freking 2007) indicated that the depths of the folds increased during later gestation, accounting for much of the increase in surface area, and is greater in the fetal placentas of small fetuses, suggesting a possible compensatory mechanism for small placental size/lack of uterine space.

In regards to capillary density, microcasting of the fetal capillaries indicates that their density increases with advancing gestation (see plates 4 and 5 of MacDonald 1976). A visual appraisal of

these images suggests that little room for improvement in capillary density during late gestation may be possible. Nevertheless, other reports indicate that placental vascularity is greater in Meishan fetal placentas compared to Yorkshire placentas (Wilson *et al.* 1998) although it is not clear from this report whether the vascularity measured was only those capillaries adjacent to the folded bilayer.

Regarding the distance between capillaries, it has been reported that the capillaries adjacent to the folded bilayer become indented within the planes of both the maternal and trophoblast epithelial layer during late gestation, although both epithelial layers remain intact even during late gestation (Friess *et al.* 1980). This report indicated that the intercapillary distance is as little as 2 μm. Thus, it appears that all of the functionally relevant physical characteristics of the pig fetal placenta improve with advancing gestation.

At the same time that the folded bilayer widens, the thickness of the fetal-placental stroma outside the folds decreases (Vallet & Freking 2007). In the fetal placenta of the smallest fetuses within a litter, the average thickness of the fetal-placental stroma outside the folds is reduced at all stages of gestation compared to larger littermates and, in some cases, there is no remaining fetal-placental stroma outside the folded bilayer. We have suggested that the fetal-placental stroma external to the folded bilayer represents room for further deepening of the placental folds to further increase bilayer surface area. A consequence of this hypothesis is that in fetal placenta of small fetuses where no additional fetal-placental stromal area exists external to the folds, no further widening of the folds may be possible. This provides a possible explanation for intrauterine crowding induced fetal losses that occur during late gestation (Freking *et al.* 2007).

These ideas suggest that fold development may be a primary contributor to placental efficiency, making an understanding of fold development key to improvements in pig placental function. Interestingly, the gross morphology of the trophoblast epithelial cells within the folded bilayer of the placenta is not uniform. In this review, all references to direction and position of cell types will be after orienting sections with the fetal placenta toward the top of the image, and the maternal placenta (i.e., endometrium) toward the bottom. In this orientation, trophoblast cells at the top of the folds of the bilayer are tall columnar. Along the sides and at the bottom of the folds, trophoblast epithelial cells are cuboidal (Friess *et al.* 1980). This difference in morphology suggests differences in function, but these differences have not been explored. Friess *et al.* (1980) suggested that the tall columnar cells at the tops of the folds are involved in macromolecular transport due to their secretory appearance. Electron microscope studies by Dantzer *et al.* (1981) support this possibility, but there is no other evidence to support this suggestion. Our investigation of expression of membrane-bound folate binding protein (Kim & Vallet 2007) suggests relatively uniform expression of this gene in both cell types. A detailed exploration of gene expression and protein production by these cell types is needed to resolve differences in function between these cells. However, their position at the tops of the folds, nearest to the thinning fetal-placental stroma, suggests that these cells may play a central role in fold development. Also interesting is the proximity of these cells to incoming maternal blood given the structure of the vasculature reported by Leiser & Dantzer (1988). Do the columnar cells at the tops of the folds develop in response to the nutrient rich environment provided by preexisting or developing maternal blood vessels, or do the columnar cells induce the underlying maternal vasculature? This is a key question, because it will determine whether strategies should focus on placental vascular development or generation of tall columnar trophoblast epithelial cells.

We hypothesized that generation of the folded structure at a minimum requires modification of the fetal-placental stroma. How this is accomplished depends on its composition. Stromal extracellular matrix is composed of various proteins and glycosaminoglycans (GAG), and a previous study of fetal-placental GAG content indicated that the most abundant GAG were

hyaluronan and heparan sulfate (Steele & Froseth 1980). We confirmed and extended these observations by measuring hyaluronan in fetal-placental tissues from day 25 to 105 of gestation and localizing hyaluronan in fetal-placental tissues. Hyaluronan content of fetal-placental tissue is low on day 25 of gestation, increases dramatically by day 45, and remains high to day 105. Hyaluronan content increases further from day 85 to 105 of gestation in placenta of small fetuses. Localization indicated that hyaluronan was a component of the entire fetal-placental stromal tissue (Vallet & Freking 2006).

Given that hyaluronan is a component of the fetal-placental stroma, we hypothesized a role for hyaluronoglucosaminidase (HYAL) in the changes in the fetal-placental stroma that would be necessary for bilayer folding (Vallet *et al.* 2008). Specific primers were developed to amplify several known HYAL (Csoka *et al.* 2001) and we were successful in cloning two cDNA corresponding to pig HYAL 1 and one cDNA corresponding to HYAL 2. One of the HYAL 1 cDNAs and the HYAL 2 cDNA obtained were similar to previously described forms of HYAL 1 (Frost *et al.* 1997) and HYAL 2 (Lepperdinger *et al.* 1998). The other HYAL 1 cDNA appeared to be a novel truncated form. The truncated region is reported to contain an EGF-like domain (Chao *et al.* 2007). The consequences of this truncation in terms of function of the resulting proteins require further investigation.

Expression of each form of HYAL mRNA was examined using RT-PCR (Vallet *et al.* 2008). The full length form of HYAL 1 was similar across gestation. The truncated form of HYAL 1 increased with advancing gestation. The HYAL 2 mRNA decreased with advancing gestation. For all forms examined, expression was greater in the fetal-placental tissue of small fetuses compared to larger fetuses. We also used hyaluronan zymography (Miura *et al.* 1995) to measure hyaluronoglucosaminidase activity in placental homogenates. Two molecular weight forms were observed, one similar to those previously reported and a second, more abundant, lower molecular weight form. Both molecular weight forms of hyaluronoglucuronidase activity increased with advancing gestation and were greater in the fetal-placental tissue of small fetuses. These results are consistent with a role for hyaluronan metabolism by Hyal 1 and 2 in the development of the pig fetal placenta during gestation.

Heparan sulfate was another abundant GAG present in fetal-placental tissue (Steele & Froseth 1980). In humans, only a single functional heparanase gene exists, located on human chromosome 4 (McKenzie 2007). Human chromosome 4 is homologous to swine chromosome 8 (Rohrer 1999) in a region where a QTL for litter size is located (King *et al.* 2003). Heparan sulfates are components of proteins that make up epithelial cell basement membranes (Lopes *et al.* 2006), and we hypothesized that modifications to the structure of the epithelial bilayer would require heparanase activity because the basement membranes would be altered as part of this process (Miles *et al.* 2008). Amplification of fetal-placental total RNA using primers specific for heparanase resulted in three cDNAs related to heparanase. One of these forms corresponded to the previously described active form of heparanase (Toyoshima & Nakajima 1999), the other two coded for truncated forms. Both truncated forms are likely to be enzymatically inactive, and their role in the fetal placenta will require further experimentation. Analysis of fetal-placental total mRNA for the putative active form of heparanase indicated that expression was relatively high on day 25, was less on days 45 and 65, and increased again on days 85 through 105 of gestation (Miles *et al.* 2008). In situ hybridization localized heparanase mRNA to the cuboidal trophoblast epithelium lining the sides and bottom of the placental folds (JR Miles, unpublished observations). A closer examination of the folds indicates that the interiors of the folds are also undergoing morphological changes with the area of fetal-placental stroma within the folds becoming more extensive. Thus, it seems likely that heparanase plays a role in these changes, as well as possibly being involved in the indentation of capillaries into the plane of the epithelial bilayer that results in reductions in the maternal-fetal intercapillary distance.

Placental angiogenesis

It is obvious that extensive angiogenesis must take place on the fetal and maternal sides of the pig placenta. One of the reasons we chose to study placental hyaluronan and heparan sulfate metabolism is that fragments of both are known to be angiogenic (West *et al.* 1985, Jakobsson *et al.* 2006). Thus, expression of these genes could couple changes in fold morphology with capillary development surrounding the bilayer. There is a constellation of other genes reported to participate in one or more aspects of angiogenesis in a variety of tissues (Liekens *et al.* 2001, Bouis *et al.* 2006, Lamalice *et al.* 2007, Otrock *et al.* 2007, Ribatti *et al.* 2007). These include fibroblast growth factors (FGF; Presta *et al.* 2005), vascular endothelial growth factors (VEGF; Dai & Rabie 2007), transforming growth factors (TGF; Lebrin *et al.* 2005), and the angiopoietins (Fiedler & Augustin 2006). In addition, numerous other genes and metabolites interact with these known angiogenic factors and modulate their activity. Focusing on hyaluronan and hyaluronoglucuronidases, several proteins are known to bind hyaluronan including CD44 (Knudson *et al.* 2002), receptor for hyaluronan mediated motility (RHAMM; Savani *et al.* 2001), and other hyaladherens (Turley *et al.* 2002). Numerous proteins inhibit hyaluronoglucuronidase activity (Mio & Stern 2002). Turning to heparanase, activity requires proteolytic cleavage (Shafat *et al.* 2006). Heparan sulfate is involved in receptor binding for both basic FGF (Schlessinger *et al.* 2000) and VEGF (Jakobsson *et al.* 2006), partially explaining its role in angiogenesis. The presence of VEGF (Winther *et al.* 1999) and TGFβ (Gupta *et al.* 1998) in the pig placenta have been reported. Placental VEGF expression is correlated with vascularity of the placenta during gestation (Vonnahme & Ford 2004). Given the complexity of these interacting systems, we have just scratched the surface of regulation of placental angiogenesis in the pig.

Nutrient specific transport mechanisms

Because the pig placenta is epitheliochorial, nutrients must pass through either two sets of epithelial cell tight junctions or four sets of cell membranes and two epithelial cell cytoplasms to get from the maternal blood supply to the fetal blood supply. The cytoplasmic distance is greatly reduced during late gestation (Friess *et al.* 1980). Tight junctions are selective barriers to the passage of solutes, thus passage depends on their permeability for different solutes (Schneeberger & Lynch 2004). The phospholipid bilayer composition of cell membranes means that each membrane is a hydrophobic barrier, which prevents passage of polar molecules like sugar. One way this is dealt with is by incorporating transport proteins in the membrane to allow passage of molecules that are incompatible with the lipid environment of the plasma membranes. Generally, membrane passage can take place with (active transport) or without (facilitated diffusion) expending cellular energy. Another way to accomplish passage is through incorporation of the solute into endometrial epithelial protein, which is then secreted. This requires uptake by the fetal-placental epithelium and transport across the cell, to be released to the fetal blood stream. The following describes some of the known mechanisms facilitating transport of oxygen, glucose, amino acids, lipids and vitamins and minerals.

Oxygen

Molecular oxygen is small and nonpolar and readily diffuses. Nevertheless, the low oxygen affinity of maternal hemoglobin and the higher oxygen affinity of fetal hemoglobin facilitates the transfer of oxygen from mother to fetus (Comline & Silver 1974). In the pig fetus, embryonic hemoglobin production gives way to adult hemoglobin around day 30 of gestation, the pig does

not produce a fetal hemoglobin similar to that of humans (Weber *et al.* 1987). Nevertheless, the hemoglobin present in fetal red blood cells has a higher affinity for oxygen compared to maternal hemoglobin (Zhang *et al.* 2006). The difference in oxygen affinity is the result of differences in 2,3-diphosphoglycerate concentrations in maternal and fetal red blood cells (Sasaki & Chiba 1983). Increased concentration of 2,3-diphosphoglycerate reduces hemoglobin oxygen affinity (Kim & Duhm 1974). Concentrations of 2,3-diphosphoglycerate remain low throughout fetal life in the pig and rapidly increase after birth (Gluszak 1979). A single enzyme, 2,3 bisphosphoglycerate synthase/phosphatase, catalyzes the synthesis and degradation of 2,3-diphosphoglycerate in red blood cells (Mulquiney *et al.* 1999). The factors controlling the activity of this enzyme during fetal life and the mechanism of the dramatic increase in 2,3-diphosphoglycerate levels after birth are not known.

Glucose

Glucose and other monosaccharides are polar compounds that do not diffuse readily through lipid bilayer membranes. Two groups of glucose transporter genes exist to overcome this problem, sodium-dependent glucose transporters (gene SLC5A, protein SGLT) and facilitative glucose transporters (gene SLC2A, protein GLUT; Zhao & Keating 2007). The SGLT proteins transport glucose from low to high concentrations through the coupling of glucose with sodium transport from high to low sodium concentrations. The sodium is subsequently pumped from the cell by Na/K/ATPase, thus glucose transport by this mechanism expends cell energy. The GLUT proteins are bidirectional transport proteins that transfer solute from high to low concentration and do not expend cell energy. There appears to be no report of the investigation of either of these gene classes in the swine placenta. Nevertheless, fetal blood glucose concentrations are considerably lower than maternal blood glucose concentrations (Pere 1995, Fowden *et al.* 1997), indicating that glucose transport occurs down a concentration gradient and pointing to GLUT protein mediation of glucose transfer. Examination of the swine gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig) indicates that expressed sequence tags (EST) corresponding to sodium-glucose cotransporter 10 (SGLT5) and facilitative glucose transporters 1 (GLUT1), 2 (GLUT2), and 3 (GLUT3) have been isolated from placenta, and EST corresponding to sodium-glucose cotransporter 3 (SGLT3) and facilitative glucose transporters 1 (GLUT1), 3 (GLUT3) and 5 (GLUT5) have been isolated from uterus. The GLUT1 and 3 proteins transport glucose (Olson & Pessin 1996); GLUT2 transports glucose and fructose (Colville *et al.* 1993) but has the highest affinity for glucosamine (Uldry *et al.* 2002). The GLUT5 transporter is specific for fructose (Zhao & Keating 2007). Together, glucose transporters present in endometrial and trophoblast epithelial cells allow glucose to cross all four cell membrane barriers.

The pig placenta is fructogenic (Rama *et al.* 1973), converting a portion of the incoming glucose into fructose, and fetal plasma levels of fructose are actually higher than glucose (4.5 versus 2 mM; Pere 1995). It has been suggested that this placental ability sequesters glucose in the fetus because fructose does not escape back into the maternal circulation (Pere 1995). Conversion of glucose to fructose would also serve to maintain lower fetal glucose concentrations, widening the maternal to fetal glucose concentration gradient, and improving glucose transport to the fetus. However, the fetus does not appear to use fructose because umbilical artery and vein fructose concentrations do not differ appreciably (Pere 1995). This calls into question the utility of sequestration of glucose in the form of fructose. However, it appears that the fetal liver is also fructogenic (White *et al.* 1979). Fetal circulation in the pig is such that all the blood returning from the placenta passes through a structure similar to a ductus venosus, and has relatively intimate contact with fetal liver tissue (Dickson 1956). In addition

blood returning to the placenta is actually a mixture of blood processed by the fetus and blood returning from the placenta (Kiserud 2005). Thus fructose concentrations in the umbilical artery may not fully reflect fructose metabolism by the fetus. The fructogenic activity of the liver may mask any substantial use of fructose by the fetus because fructose may be replaced as it is metabolized. On the other hand, ^{14}C-fructose tracer studies in sheep conceptuses, which are also fructogenic, indicate that the rate of usage of fructose by the sheep conceptus is one-fifth that of glucose (Meznarich et al. 1987).

Two alternative pathways exist to generate fructose from glucose (Rama et al. 1973). The first is the Embden-Meyerhoff-Cori pathway in which glucose is first phosphorylated to glucose-6-phosphate by hexokinase, which is then converted to fructose-6-phosphate by isomerase. Alternatively, glucose can be converted to fructose through the sorbitol pathway, involving aldose reductase and ketose (sorbitol) reductase. The latter pathway has the advantage that ATP is not consumed. It has been reported that the pig placenta lacks ketose reductase (Rama et al. 1973), but ESTs for both aldose reductase and sorbitol reductase have been obtained from pig placenta. Thus, it is likely that both pathways exist in the placenta so the primary enzyme pathway controlling conversion of glucose to fructose requires investigation.

Curiously, fructose-6-phosphate participates in a pathway that generates the N-acetylglucosamine needed for hyaluronan and other GAG synthesis (Holden et al. 2003, Moussian 2008). Fructose-6-phosphate is converted to glucosamine-6-phosphate in a reaction catalyzed by glutamine fructose-6-phosphate amidotransferase (GFAT). This reaction consumes glutamine, which has the highest concentration of any amino acid in fetal plasma (Wu et al. 1995). Wen et al. (2005) indicated that the GFAT enzyme, through generation of glucosamine from fructose-6-phosphate, activates mammalian target of rapamycin (mTOR), which increases the proliferation of a human trophoblast cell line. In addition, the affinity of GLUT-2 for glucosamine is an order of magnitude higher than for glucose and fructose (Uldry et al. 2002), suggesting that glucosamine concentrations may regulate glucose and fructose transport by this transporter through competition for binding. It seems possible that the pathways that generate glucosamine from fructose, and fructose from glucose, could link GAG synthesis, trophoblast proliferation and glucose transport, and may play a role in changes in placental structure in response to reduced placental size. Clearly, these pathways and their role in placental development require further study.

Amino acids

Wu et al. (1995) reported the concentrations of amino acids in fetal and maternal circulations. Only the concentrations of cysteine, glycine, histidine, leucine, phenylalanine, and tryptophan were lower in fetal umbilical venous plasma compared to maternal plasma, and total α-amino acid concentration was greater in fetal plasma compared to maternal plasma. This suggests that amino acid transport must involve one or more active transport mechanisms. Because of interconversions among amino acids and the fact that some amino acids can be synthesized from other metabolic substrates, concentrations of individual amino acids do not allow firm conclusions on transport mechanisms.

Of the amino acids, glutamine concentrations display the greatest fetal maternal difference, and the placenta is capable of synthesizing glutamine from other amino acids (Self et al. 2004). Arginine concentrations are also unusually high in fetal fluids (Wu et al. 1996). Interestingly, arginine supplementation has been reported to improve litter size (Mateo et al. 2006, Hazeleger et al. 2007) and has been proposed to play a role in numerous functions during pregnancy (Wu et al. 2004). These include nitrous oxide (NO) generation, polyamine synthesis and creatine generation (Morris 2007, Brosnan & Brosnan 2007). While, NO (Wu et al. 1998) and polyamine

synthesis (Wu *et al.* 2005) have received some attention, creatine is thought to be responsible for shuttling ATP from the mitochondria to the cytoplasm (Paddon-Jones *et al.* 2004) and is thus essential for energy utilization in the fetus. Because low birthweight piglets have decreased survival within the first two days of life, the ability to utilize energy efficiently at birth could have a profound effect on piglet survival.

Finch *et al.* (2004) have investigated leucine uptake by placental trophoblast vesicles. They report that both sodium-dependent and independent mechanisms are present in the pig placenta, and that the sodium-dependent transport capacity of placentas of small fetuses was less than that of larger littermates. Because sodium-dependent transport requires energy to maintain the sodium gradient, this could be an adaptation to decreased availability of energy in small fetuses. Clearly, the mechanisms responsible for amino acid transport and the generation, maintenance, and reasons for the high concentrations of certain amino acids in fetal plasma warrant further investigation.

Lipid transport

Lipids are higher energy substrates than sugars, thus transport of lipids would be advantageous to the developing pig fetus. Unfortunately, when lipid transport has been measured, it has been found to be low or nonexistent (Thulin *et al.* 1989, Ramsay *et al.* 1991). However, Thulin *et al.* (1989) infused radiolabelled free fatty acids to determine transport by the placenta, which is unlikely to present lipids to the placenta in the same form that lipids typically circulate in the blood. Lipids are typically bound to lipoproteins and are delivered to tissues through receptors for the lipoprotein-lipid complexes (Herrera 2002). Thus, it may be that the capacity of the pig placenta to deliver lipids to the fetus may be underappreciated. In further support of this, certain essential fatty acids do not appear to be synthesized by animal tissues, so some mechanism for delivery of these essential fatty acids must exist (Youdim *et al.* 2000). Although piglet fat reserves are typically low at birth, one aspect of development that requires lipid is brain myelination (Youdim *et al.* 2000). Myelin membranes are specialized plasma membrane structures of nerve peripheral cells composed primarily of phospholipid (Deber & Reynolds 1991). Brain myelination begins before birth in the piglet and likely affects piglet survival after birth (Dickerson *et al.* 1971). Thus, while the currently available results suggest that lipid transport by the placenta is poor, the opportunity represented by improvements in brain function and piglet survival would seem to be good justification for further exploration of lipid transport by the pig placenta.

Vitamins and minerals

It is likely that the transport of various vitamins and minerals by the pig placenta differs with each individual nutrient. However, some general concepts can be inferred from the vitamins and minerals for which the transport mechanisms have been explored. Iron is transported to the pig fetus as a component of uteroferrin (Roberts & Bazer 1988). Pig endometrial glands secrete copious quantities (2 g per day) of uteroferrin (Buhi *et al.* 1982), which is picked up by fluid phase pinocytosis by the areolae (Raub *et al.* 1985). Substantial amounts of the iron in uteroferrin are metabolized by the placenta because circulating concentrations of acid phosphatase activity are quite low in fetal plasma (Vallet *et al.* 1996). However, the fetal liver is also capable of binding uteroferrin by mannose-6-phosphate receptors (Saunders *et al.* 1985). Finally, circulating plasma uteroferrin can also be cleared by the fetal kidney into the allantois where substantial amounts of uteroferrin are present (Baumbach *et al.* 1986). A mechanism

exists in the allantoic fluid to transfer iron from uteroferrin to transferrin, which can then return to the fetus (Buhi *et al.* 1982). The handling of uteroferrin by the pig conceptus in this way may prevent the oxidizing activity of reduced uteroferrin (Vallet 1995). Thus, iron transport via uteroferrin as a model suggests that the endometrial glands and areolae participate in the delivery of nutrients to the developing pig conceptus, and that the allantoic sac may serve as a storage and protective chamber for metabolites.

Retinol is delivered to the developing pig conceptus bound to retinol binding protein (Clawitter *et al.* 1990). Retinol plays a role in cell signaling (Duester 2008) and may also participate in providing antioxidant activity for the developing conceptus (Vallet 1995). It is likely that retinol is delivered to the fetus via the areolae in a manner similar to that of uteroferrin (Dantzer & Winther 2001).

In contrast to uteroferrin and retinol, folate transport to the developing pig conceptus likely takes place through the concerted activities of secreted and placental membrane-bound forms of folate binding protein (Kim & Vallet 2004). Uterine flushings on days 13 and 15 of the cycle or pregnancy contain large amounts of secreted folate binding protein (Vallet *et al.* 1998b). Immunohistochemical localization of the secreted form of folate binding protein indicated that it was present in endometrial glands beginning about day 13 of pregnancy until day 20 and disappears by day 35 of pregnancy (Kim & Vallet 2004). Endometrial mRNA levels for this protein do not follow this pattern (Vallet *et al.* 1999), and it has not been possible to demonstrate synthesis of secreted folate binding protein by endometrial tissue (J.L. Vallet & J.G. Kim, unpublished observations). Thus, it seems highly likely that the secreted folate binding protein found in the endometrial glands and in uterine flushings during early pregnancy may originate from serum. The placenta expresses an mRNA for a membrane-bound form of folate binding protein (Vallet *et al.* 1999), and in situ hybridization indicates that this mRNA is present in trophoblast epithelium of the placental folds (e.g., present in both tall columnar cells and in cuboidal cells) throughout gestation (Kim & Vallet 2007). Kim & Vallet (2004) have hypothesized that folate transport is thus accomplished by the sequential effects of secreted folate binding protein during early pregnancy, followed by placental membrane-bound folate binding protein during later gestation. Conceptually, transport with these characteristics is likely to be sensitive during the switchover period (i.e., during early placental development). This period of pregnancy is unusually sensitive to intrauterine crowding (Vonnahme *et al.* 2002, Freking *et al.* 2007), and it seems possible that nutrients like folate that transition from histotrophic to placental transport could contribute to the sensitivity of this period.

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