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Uteroplacental vascular development and placental function: an update

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ABSTRACT The importance of the placenta and its vascular development to fetal growth and development has been appreciated since ancient times. Based on numerous studies in humans and animal model organisms in the last 2-3 decades, normal placental angiogenesis is critically important to ensure adequate blood flow to the placenta and therefore to provide the substrates that support normal fetal growth. Placental angiogenesis is abnormal at term in compromised pregnancies (those in which fetal growth is altered), including those resulting from maternal nutritional or environmental stress, maternal age, increased numbers of fetuses, maternal or fetal genotype, or the use of assisted reproductive technologies (e.g., cloning by somatic cell nuclear transfer). We and others have recently shown that these defects in placental vascular development occur quite early in pregnancy and may therefore presage compromised fetal growth and development. The challenges will be to find biomarkers of abnormal placental angiogenesis and to develop therapeutic strategies to "rescue" placental vascular development and thus fetal growth in compromised pregnancies. Animal models will be essential in meeting these challenges.

KEY WORDS: *placenta, angiogenesis, pregnancy, fetal growth, regulation, therapeutic*

The [umbilical] vessels join on the uterus
like the roots of plants and through them
the embryo receives its nourishment.
Aristotle. On the Generation of Animals *(ca. 340 B.C.)*

Introduction

As pointed out in this issue (Longo and Reynolds, 2010), the importance of the placenta and its vascular development to fetal growth and development has been appreciated since ancient times, as the quotation from Aristotle, above, makes abundantly clear. More recently, Longo (1972) put a modern spin on this concept when he stated, "The fetal 'lifeline' thus includes an adequate maternal placental circulation and supply of blood nutrients, a placenta that transports and metabolizes various substances properly and a functional fetal placental circulation."

Perhaps based in part on the study of bird embryos (the Egyptians and probably also the Chinese developed a system for the artificial incubation of bird eggs as early as 3,000 B.C.E.; Needham, 1934), the ancient investigators recognized the correct function of the placenta and umbilical cord. These studies also led the ancient investigators to recognize that early development of the chick and mammals had many similarities. This revelation led to the use of the developing chick as the most important model organism for embryological research in vertebrates from that time forward, and established the importance of animal models in obstetric research. Thus, animal models have been central to the study of the placenta and the placental circulation since the earliest times, and much of our knowledge of placental vascular development continues to be derived from comparative studies in animals (Kaufmann *et al.,* 2004; Reynolds *et al.,* 2005b; Longo and Reynolds, 2010). Fortunately, the power of the comparative approach in solving complex biological problems is widely recognized and has been not only widely applied but highly successful.

Based on numerous studies in humans and animal model

Abbreviations used in this paper: ANGPT, angiopoietin; eNOS, endothelial nitric oxide synthase; FGF, fibroblast growth factor; NO, nitric oxide; VEGF, vascular endothelial growth factor.

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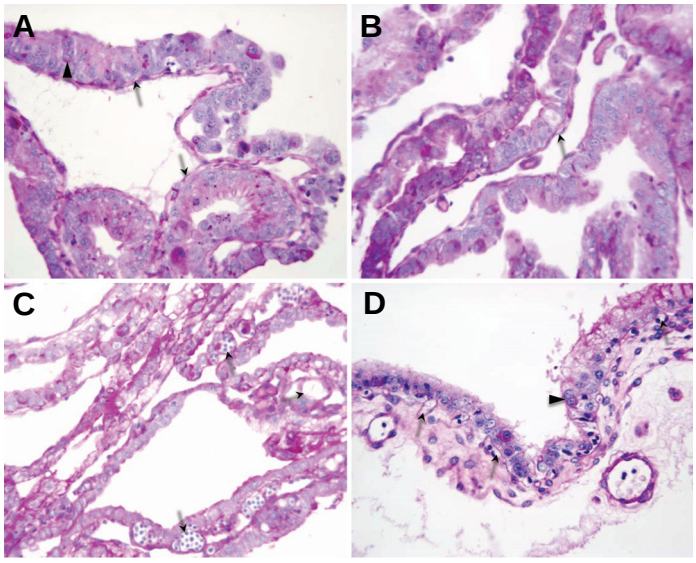

Fig. 1. Chorioallantois (fetal placenta) *on day 24 of pregnancy from* **(A)** *IVF,* **(B,C)** *parthenogenetic, and* **(D)** *normal sheep embryos. (Arrows indicate placental capillaries; arrowheads indicate binucleate cells in (A,D); Borowicz, Grazul-Bilska, Ptak, Loi, Palmieri, Della Salda, and Reynolds, unpublished)*

organisms, adequate blood flow to the placenta is critical for normal fetal growth. It is therefore not surprising that conditions that affect fetal growth, such as maternal and fetal genotype, increased numbers of fetuses, maternal nutrient excess or deprivation, or environmental stress, have similar effects on placental growth and are associated with reduced rates of fetal oxygen and nutrient uptakes, as well as reduced placental angiogenesis and blood flow (Vonnahme *et al.,* 2001, 2002; Anthony *et al.,* 2003; Wallace *et al.*, 2005; Reynolds *et al.,* 2006). Similarly, in highly compromised pregnancies established using assisted reproductive technologies, such as somatic cell nuclear transfer, placental angiogenesis is also highly compromised at term (Palmieri *et al.*, 2007), and this defect in placental vascular development seems to occur quite early in pregnancy (Borowicz *et al.*, unpublished, Fig. 1).

The critical role of the placental blood supply in humans is confirmed by the observation that intrauterine growth restriction in third-trimester pregnancies is characterized by impaired uterine (maternal placental) and umbilical (fetal placental) blood flows, leading to reduced fetal nutrient uptakes as well as fetal hypoxia, hypoglycemia and asymmetric organ growth (Pardi *et al.,* 1993; Marconi *et al.,* 1996; Ferrazi *et al.,* 2000; Konje *et al.,* 2003). In addition, increased uterine vascular resistance and reduced uterine blood flow during early pregnancy can be used as predictors of high-risk pregnancies and are associated with fetal growth retardation (Trudinger *et al.,* 1985; North *et al.,* 1994).

More than a decade ago, we presented a review in which we discussed the current evidence for the importance of placental vascular development to placental function and fetal/neonatal growth and development (Reynolds and Redmer, 1995). In the present review, we will provide an 'update' of what we have learned in the intervening decade concerning placental blood flow and vascular development.

An update

Throughout this article, we will emphasize data in ruminants, for which there are numerous well established models of compromised pregnancy, but we also will discuss the relevance to other species, including humans, where applicable. Ruminant models are relatively unique in that, macroscopically, the ruminant placenta comprises 60 to 100 discreet structures, or units, known as placentomes (Ramsey, 1982; Mossman, 1987). Each of these placentomes consists of maternal caruncular and fetal cotyledonary portions, which interdigitate and thus are in close apposition, thereby facilitating transplacental exchange of nutrients, respiratory gases, and wastes (Fig. 2; Ramsey, 1982). In mammals with epitheliochorial placentation (moles, manatees, whales, horses, pigs, cattle, sheep, and a few prosimians), the chorioallantois (the 'definitive' placenta of eutherian mammals) is minimally invasive, and thus the uterine epithelium remains intact during pregnancy (Ramsey, 1982; Mossman, 1987; Wooding and Flint, 1994).[1] Because of this, the ruminant placenta is an ideal model for studying placental development because the maternal and fetal portions of the placenta remain closely associated but intact, and thus one can evaluate each tissue separately (Ramsey, 1982; Reynolds & Redmer, 1995; Reynolds *et al.,* 2005 a,b). In addition, as described later, although the epitheliochorial placenta of ruminants and other ungulates is quite different anatomically from the hemochorial placenta of most mammals, functionally they appear to operate quite similarly.

Further evidence for the importance of placental angiogenesis and blood flow

As we indicated in our 1995 review, based on the Fick principle, placental transport capacity, also termed placental uptake, can be calculated as:

Uptake = blood flow x [A - V],

where [A - V] represents the arteriovenous concentration difference (represented as [v – a] for the umbilical vein minus umbilical arterial, or fetal, uptake; Reynolds and Redmer, 1995). Thus, transplacental exchange could increase by increasing the rate of extraction (the A-V concentration difference) or by increasing the rate of blood flow, or both.

Based on numerous studies, it seems that increased blood flow is critical to increased transplacental exchange throughout gestation (Meschia, 1983; Reynolds *et al.*, 1986, 2006; Metcalfe *et al.*, 1988; Reynolds and Redmer, 1995). For example, in cattle from mid to late gestation, oxygen extraction by the gravid uterus increases only 0.4-fold, whereas uterine blood flow increases approximately 4.5-fold (Reynolds *et al.*, 1986). Thus, increased uterine blood flow accounts for most of the 5- to 6-fold increase in

[1]Note that the sheep has now been reclassified as a subset of epitheliochorial, termed 'synedesmochorial' because the maternal epithelium is 'invaded' and modified by the trophoblast to form a syncitium. Additionally, although there are similarities in placental function between the epitheliochorial and hemochorial (as in rodents, insectivores, and anthropoids) types, there also are some functional differences such as transport of immunoglobulins across the hemochorial but not the epitheliochorial chorioallantoic placenta (Wooding and Flint, 1994).

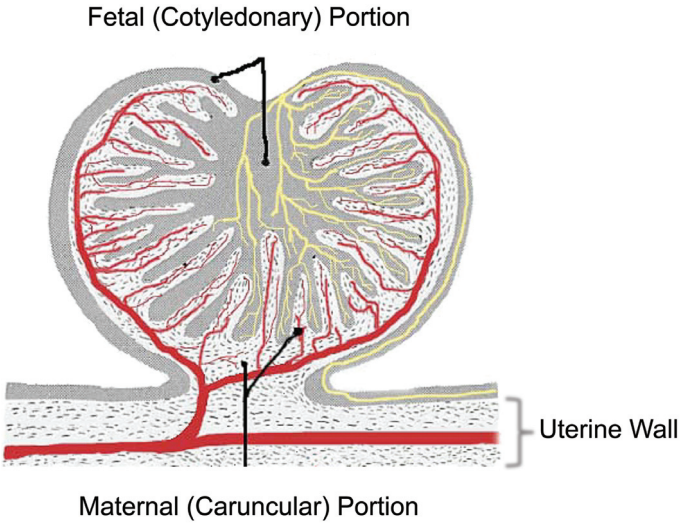

Fig. 2. Schematic representation of the sheep placentome. *Taken from Reynolds* et al.*, 2005b.*

total gravid uterine oxygen uptake. The 16-fold increase in oxygen uptake of the bovine fetus from mid to late gestation also can be accounted for primarily by the increased rate of umbilical blood flow (Reynolds *et al.*, 1986). Similarly in sheep, gravid uterine oxygen extraction increases approximately 0.4-fold from mid to late gestation, whereas uterine blood flow increases approximately 3-fold (Meschia, 1983). Moreover, the large increases in gravid uterine and fetal uptakes of glucose, lactate, and amino acid nitrogen from mid to late gestation in cattle seem to depend primarily on large increases in uterine and umbilical blood flows because the A-V concentration differences for these nutrients remain relatively constant (Reynolds *et al.*, 1986; Reynolds and Redmer, 1995).

Thus, adequate blood flow to the placenta is critical for normal fetal growth. This does not, however, minimize the importance of concomitant increases in the abundance of specific transporters and an increase in the maternal to fetal concentration gradient also seem to be important components of increased transplacental exchange, at least for those substances that are diffusion limited (or transporter dependent), such as glucose and amino acids (Bell *et al.*, 1999). Nevertheless, gravid uterine and umbilical glucose uptakes, which provide for about 60% of fetal metabolic needs (Reynolds *et al.*, 1986; Bell *et al.*, 1999), are reduced approximately in proportion to the reduction in placental mass and blood flows in pregnancies compromised nutritionally or by environmental heat stress (Reynolds *et al.*, 1985, 2006).

Based on the concept that chronic increases in blood flow to any growing tissue depend on vascular growth, or angiogenesis, Meschia (1983) reasoned "the large increase of blood flow to the uterus during pregnancy … results primarily from the formation and growth of the placental vascular bed." Numerous studies have subsequently confirmed that angiogenesis is indeed a major component of the increase in placental blood flow throughout gestation, and establishment of functional fetal and placental vascular beds is one of the earliest events during embryonic/placental development (Reynolds and Redmer, 1992, 1995; Magness, 1998; Charnock-Jones *et al.*, 2004; Kaufmann *et al.*, 2004; Mayhew *et al.*, 2004; Reynolds *et al.*, 2005a,b; Borowicz *et al.*, 2007). In fact, not only does the sustained increase in gravid uterine and umbilical blood flows depend on development of the placental vascular beds, but placental growth itself depends on placental angiogenesis because tissue growth of any magnitude normally cannot occur in the absence of vascular growth (Bassingthwaighte and Goresky, 1984; Hudlicka, 1984). This dependence on vascular development results from the high metabolic demands associated with tissue growth and the limited ability of respiratory gases, nutrients, and metabolic wastes to diffuse through the extracellular compartment (Bassingthwaighte and Goresky, 1984; Adair *et al.*, 1990). Thus, growth and development of the vascular beds are critical components of tissue growth and function, including that of the uteroplacenta, and as mentioned, the importance of vascular development to placental function has long been recognized. However, research on placental vascular growth has comprised primarily descriptive histological studies, whereas only a handful of quantitative studies of placental angiogenesis have been reported (Kaufmann *et al.*, 2004; Mayhew *et al.*, 2004; Reynolds *et al.*, 2005b).

The evaluation of placental angiogenesis has utilized primarily 2 methodologies: (1) classical histological methods involving embedding, sectioning, and staining, followed by stereological analysis (more recently, using confocal microscropy and computerized image analysis) of the microcirculation, and sometimes involving perfusion fixation (Borowicz *et al.*, 2007; Vonnahme *et al.*, 2007); and (2) vascular casting procedures utilizing perfusion with a casting medium such as Batson's No. 17, followed by digestion of the tissue and scanning electron microscopic evaluation of the remaining microvascular cast (Hafez *et al.*, 2007).

To evaluate uteroplacental angiogenesis during early preg-

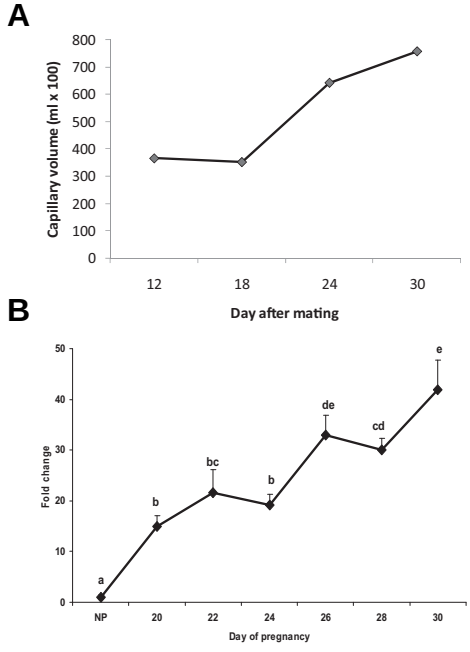

Fig. 3. Maternal placental angiogenesis from days 12-30 of pregnancy in sheep *(equivalent to days 22 to 54 in humans).* **(A)** *Total capillary vascular volume and* **(B)** *capillary labeling index (relative rate of cell proliferation) of maternal placental tissues through day 30 of early pregnancy in sheep.*

nancy, we have used simple, classical histological methods, with immersion fixation followed by paraffin embedding and sectioning of the fixed tissues. We have been able to use these methods because the tissues are still relatively 'normal' and, thus, the vessels comprising the microcirculation (arterioles, capillary beds, and venules) are more easily identified than those during mid to late pregnancy, which we will discuss in the next paragraph. These methodologies have been described in detail by Reynolds and Redmer (1992). Although we have utilized several more specific methods to identify the microvessels in other tissues, such as immunohistochemistry for Factor VIII, smooth muscle cell alpha-actin, or histochemistry using specific lectins (Jablonka-Shariff *et al.*, 1993; Redmer *et al.*, 2001), these methods have not worked as well for the fetal placenta. Thus, we have used primarily periodic acid-Schiff's reagent, which is a more general staining method for basement membranes, to identify the placental microcirculation (Hudlicka, 1984; Reynolds and Redmer, 1988; Reynolds and Redmer, 1992; Borowicz *et al.*, 2007).

In contrast with those during early pregnancy, the microcirculatory beds of the uteroplacenta later in pregnancy are highly interdigitated, and the microvessels, especially those of the fetal cotyledons, or villi, are much more difficult to identify, both because some of the capillaries seem to be so immature that they do not readily stain using classical microvascular markers (Factor VIII, lectins, etc.) and also because they seem to collapse easily, probably because of the high water content of the placental tissues. Thus, to solve these problems, we have developed perfusion fixation techniques similar to those we have previously described (Jablonka-Shariff *et al.*, 1996). These placental perfusion fixation methods that we have developed have been described in detail by Borowicz *et al.* (2007) and Vonnahme *et al.* (2007).

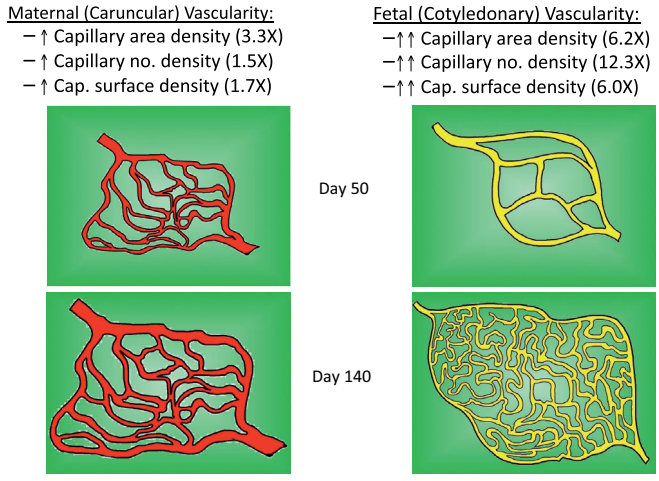

Fig. 5. Model of angiogenesis *in the sheep placenta throughout the last two-thirds of gestation (taken from Borowicz* et al.*, 2007).*

Using these methods, we have shown that total capillary volume of the maternal placenta in sheep increases dramatically after day 18 of pregnancy, in conjunction with dramatic capillary growth (Fig. 3). This growth of the placental microvascular beds, including both the maternal and fetal placental tissues, continues throughout pregnancy (Fig. 4). These quantitative histological methods also have allowed us to develop a model of placental microvascular development (Fig. 5). In this model, the caruncular (maternal placental) capillary beds grow primarily via an increase in capillary size (area per capillary), with only small increases in capillary number or surface densities, resulting in a modest, 3-fold increase in capillary area density. In contrast, the cotyledonary (fetal villous) capillary beds grow primarily by branching, resulting in a large, 12-fold increase in capillary number density, accompanied by a decrease in capillary size. The relatively large, 6-fold increase in capillary area and surface densities of the fetal villi (Fig. 5) can be explained by this branching pattern of growth.

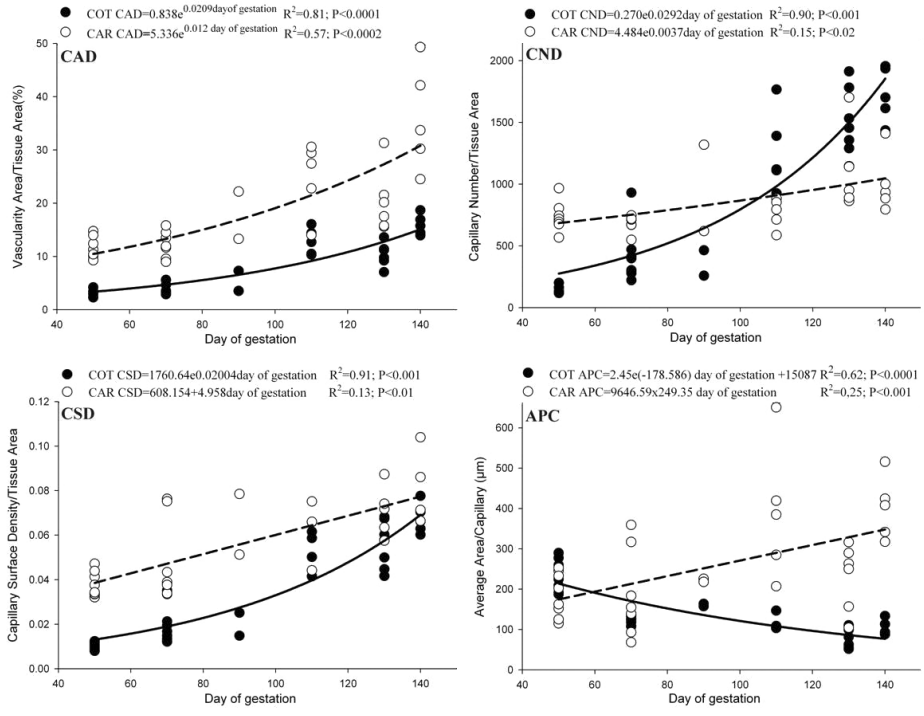

Fig. 4. Regressions of vascularity measures *for caruncular (CAR, maternal) and cotyledonary (COT, fetal villus) placental tissues throughout the last two thirds of gestation in sheep (taken from Borowicz* et al.*, 2007).*

Our empirical model of angiogenesis in the caruncles and cotyledons is corroborated by studies of the physiological and anatomical constraints on morphology of vascular beds (Reynolds *et al.*, 2005c). For example, according to "Murray's Law," the diameter (D) of a parent vessel is equal to the sum of the cube roots of the diameters of its branches; i.e., $(D_{parent})^{1/3} = (D_{branch\ 1})^{1/3} + (D_{branch\ 2})^{1/3} + \ldots + (D_{branch\ n})^{1/3}$ (Murray, 1926; Hutchins *et al.,* 1976; West *et al.,* 1997). Assuming 2-fold branching (i.e., two branches per parent vessel, or a bifurcated system) and using Murray's law, we

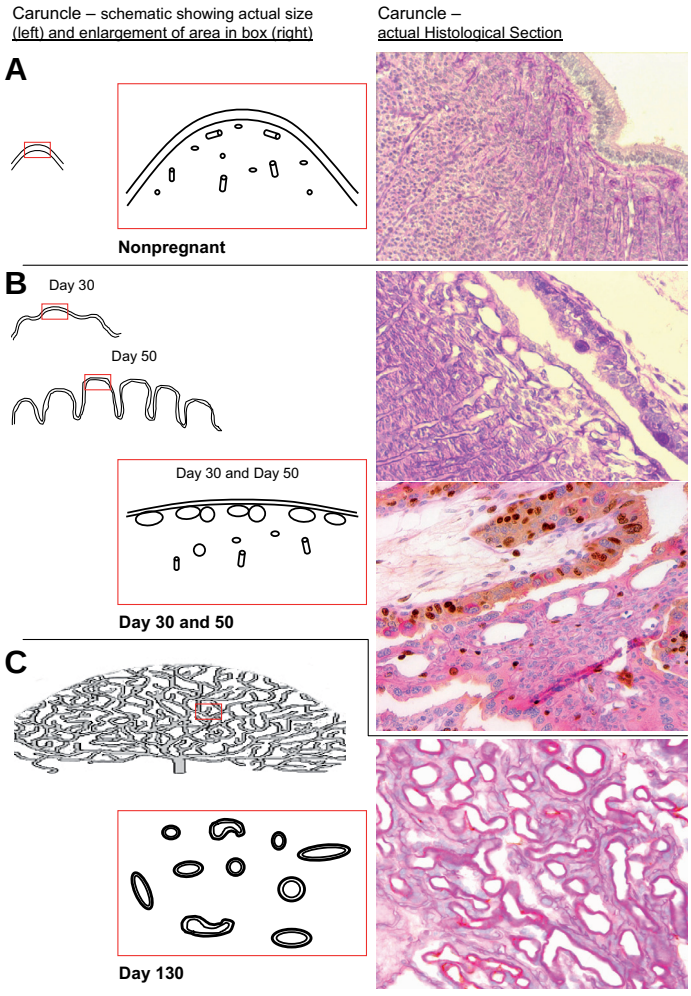

Fig. 6. Developmental changes in the microvascular architecture of the maternal placenta (caruncle) throughout gestation in sheep. (A) *Nonpregnant,* **(B)** *Day 30 and 50 of gestation, and* **(C)** *Day 130 of gestation (length of pregnancy approximately 145 days; taken from Reynolds* et al., *2005c).*

determined that the number of generations of capillary branches required to go from the smallest caruncular and cotyledonary arterioles (mean D = 80 μm) to the average diameter of the caruncular and cotyledonary capillaries at day 140 of gestation was 10 for the maternal caruncles and 15 for the fetal cotyledons, which would result in 2^5, or 32-fold, more vessels in the cotyledons compared with the caruncles (Reynolds *et al.*, 2005c; Borowicz *et al.*, 2007; Vonnahme *et al.*, 2007). Thus, our empirical model supports the conclusion that the fetal placental capillary beds are much more highly branched than those of the maternal placenta.

This difference in the pattern of growth between the fetal and maternal placental capillary beds is associated with additional, and dramatic, differences in their microscopic anatomy and, most likely, in their physiological function. As shown in Fig. 6, from very early in gestation the maternal placental capillary bed begins to form a 'capillary plexus,' initially at the surface of the uterine lumen where the caruncle contacts the fetal chorioallantois (compare nonpregnant caruncle with a caruncle on day 30 and 50 of gestation; Fig. 6). By the beginning of the last third of pregnancy (about day 90 to 100 of gestation in sheep), the maternal caruncular tissue is composed primarily of these large 'capillaries,' many exhibiting diameters of 20 μm or more (Reynolds *et al.*, 2005c; Borowicz *et al.*, 2007). In contrast, the diameter of the fetal capillaries decreases throughout gestation, resulting in an average capillary diameter of 4.5 μm by the end of gestation (Reynolds *et al.*, 2005c; Borowicz *et al.*, 2007). In addition, our recent electron microscopic evaluation of vascular casts has confirmed this dramatic difference in microvascular architecture between the fetal and maternal placental tissues (Fig. 7).

The microvascular architecture of the maternal placental compartment is characterized by a preponderance of very large capillaries, which dictates a low-velocity, 'irrigation-flow' or slowly percolating type of system, which is designed primarily as a delivery (and, conversely, a waste-removal) system (Reynolds *et al.*, 2005a,c; Borowicz *et al.*, 2007). In contrast, the microvascular architecture of the fetal placenta, which is highly branched and composed primarily of abundant small capillaries, is designed as a high velocity, rapid transport system (Reynolds *et al.*, 2005c; Borowicz *et al.*, 2007).

The suggested flow and transport differences between the maternal and fetal placental circulations agree with various functional observations. For example, umbilical blood flow increases at 2- to 3-times the relative rate (proportional or percentage increase per day) as that of uterine blood flow during the last two-thirds of gestation (Reynolds and Redmer 1995; Reynolds *et al.*, 2005a,b, 2006). Moreover, because of increased branching and thus larger numbers of capillaries per unit of tissue (Fig. 5), the surface area available for exchange is greater in fetal cotyledonary villi compared with maternal caruncles during the last third of pregnancy (Reynolds *et al.*, 2005a,b). Additionally, the thickness of the barrier between the fetal and maternal capillaries also may be reduced throughout gestation (Faber and Thornburg, 1983; Longo 1987). Taken together, these observations help to explain why in normal pregnancies the proportion of the nutrients and oxygen taken up by the gravid uterus that is transported to the fetus increases by 2- to 4-fold from mid to late gestation (and, conversely, the proportion that is utilized by the placenta decreases by 2- to 4-4-fold), essentially keeping pace with the rate of fetal growth (Reynolds and Redmer, 1995; Reynolds *et al.*, 2005c).

Thus, although the placental microvascular architecture is ideally suited for both nutrient delivery, on the maternal side, and nutrient uptake and transport, on the fetal side, this arrangement does not appear to be unique to sheep. For example, there are several striking similarities in placental function between the sheep and the cow, which is another placentomal, or cotyledonary, mammal (Reynolds and Redmer, 1995; Vonnahme *et al.*, 2007). The capillary plexus that forms on the maternal portion of the placenta also has been observed in pigs early in pregnancy (Assheton 1906; King *et al.,* 1982), although to our knowledge this has not been examined later in pregnancy. In primates, including humans, the chorioallantois is so invasive that a portion of the maternal endometrium is eroded, and the fetal villi are bathed in maternal blood (Ramsey, 1982); this arrangement thus represents the ultimate in a low-velocity, irrigation-flow system. It is interesting to note that this 'hemochorial' type of placenta (signifying that the chorion, or outer layer of the chorioallantois, is bathed in maternal blood) is widespread among mammals, being

present not only in the vast majority of primates but also in rodents, insectivores, and bats, which together comprise 95% of the more than 4,000 species of mammals (Ramsey, 1982; Nowak, 1991).

As summarized in Table 1, in sheep studied during late gestation, uterine or umbilical blood flows, or both, are reduced in every model of compromised pregnancy in which they have been evaluated. These models of compromised pregnancy include overfed adolescents, underfed adolescents and adults, as well as environmental heat-stress, hypoxic stress, and multiple fetuses. These observations agree with those in women, in which placental perfusion is reduced in pregnancies with growth restricted fetuses (Poston, 1997; Moore *et al.*, 2004; Redmer *et al.*, 2004; Huppertz and Peeters, 2005).

In these various models of compromised pregnancy, although placental vascular development also is decreased in some instances, it is increased in others (Table 1). Interestingly, in two of the models in which placental vascularity is increased (high dietary Se, or hypoxic stress), there was no effect on fetal size, suggesting an adaptive placental response that preserves the fetal nutrient supply. In the other model exhibiting increased placental vascularity (Romanov vs. Columbia genotype), the animals were subject to long-term genetic selection, resulting in increased litter size. This latter case resembles that of Meishan and Yorkshire pigs, in which the Meishans exhibit increased litter size and weight associated with increased placental vascularity and VEGF expression (Biensen *et al.*, 1998; Wilson *et al.*, 1998; Vonnahme and Ford, 2004).

Regulation of placental angiogenesis

As we mentioned, angiogenesis refers to the formation of new vascular beds, and is a critical process for normal tissue growth and development, including that of the placenta (Reynolds and Redmer, 1995, 2001; Reynolds *et al.*, 1992, 2005a,b, 2006). Although numerous molecules have been implicated in the regulation of vascular growth, recent observations have led to the identification of the major factors regulating vascularization in the placenta and elsewhere (Yancopoulos *et al.*, 2000; Koh *et al.*, 2002; Charnock-Jones *et al.*, 2004; Reynolds *et al.*, 2005a; Borowicz *et al.*, 2007). These angiogenic factors include the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin (ANGPT) protein families, as well as their respective receptors. In terms of regulating uteroplacental angiogenic factor expression, estrogen seems to be a key player; in ovariectomized rats or ewes, endometrial VEGF, FGF, ANGPT1, and ANGPT2 mRNA are strongly upregulated within a few hours after estrogen treatment, in association with a dramatic increase in uterine vascularization and blood flow (Culllinan-Bove *et al.*, 1993; Magness, 1998; Reynolds *et al.*, 1998; Johnson *et al.*, 2006), as is expression of the mRNA for endothelial nitric oxide synthase (eNOS, or NOS3), which produces nitric oxide (NO) and

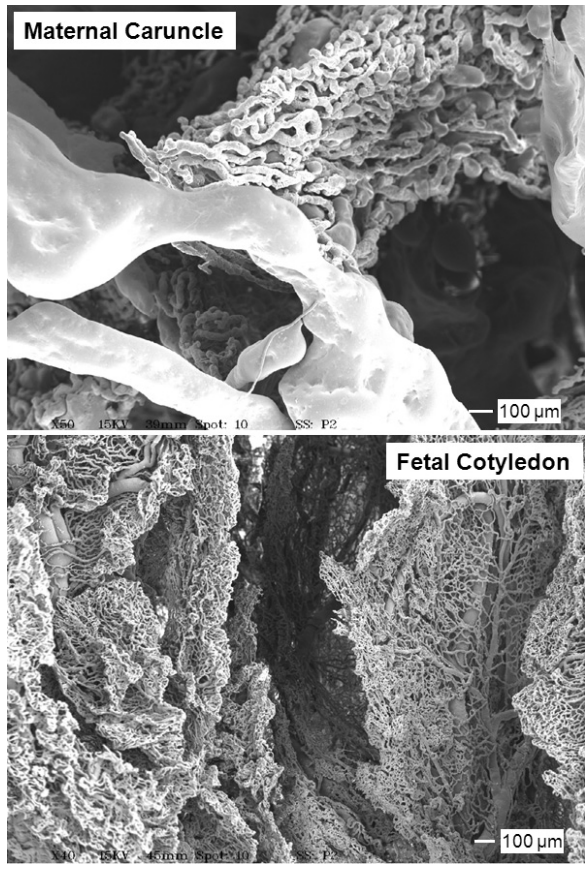

Fig. 7. Scanning electron photomicrographs of placental microvascular casts. *Day 130 [0.9] of pregnancy; Hafez, Borowicz, Reynolds and Redmer, unpublished.*

TABLE 1

CHANGES IN FETAL AND PLACENTAL WEIGHTS, UTERINE AND UMBILICAL BLOOD FLOWS, AND PLACENTAL VASCULARITY IN VARIOUS MODELS OF COMPROMISED PREGNANCY IN SHEEP[1]

Model	Day of gestation[2]	Fetal weight	Placental weight	Uterine blood flow	Umbilical blood flow	Vascularity[3]
Overfed Adolescent	130-134	↓20-28%	↓45%	↓36%	↓37%	↓31% (total capillary vol.)
Underfed Adolescent	130	↓17%	NSE	----	----	↓20% (cap. area density, CAR)
Underfed Adult	130-144	↓12%	---	↓17-32%	NSE	↓14% (cap. area density, CAR)
Adolescent vs. Adult	135	↓11%	↓29%	----	----	----
Genotype	130	↓43%	↓47%	----	----	↑36%
Heat-stressed Adult	133-135	↓42%	↓51%	↓26%	↓60%	----
Multiple Pregnancy	140	↓30%	↓37%	↓23%	----	↓30% (total cap. vol., COT)
High Dietary Se	135	NSE	↓24%	----	----	↑20% (cap number density, COT)
Hypoxic (Hypobaric) Stress	140	NSE	----	↓35%$^{\Delta}$	----	↑(cap. area density, CAR & COT)

[1]Table adapted from Reynolds *et al.* (2006). [2]Length of gestation = approximately 145 days. [3]cap. = capillary; CAR = caruncle (maternal placenta); COT = cotyledon (fetal placenta/villus).

TABLE 2

STANDARD PARTIAL REGRESSION COEFFICIENTS[1] ILLUSTRATING THE RELATIONSHIPS OF VARIOUS ANGIOGENIC FACTOR mRNA (INDEPENDENT VARIABLES) WITH MEASURES OF CARUNCULAR (MATERNAL PLACENTAL) VASCULARITY (DEPENDENT VARIABLES) OF SHEEP THROUGHOUT PREGNANCY[2]

Angiogenic factor[3]	Measure of vascularity[4]			
	CAD	CND	CSD	APC
VEGF	**0.711**[5] (0.0001)	-0.126 NS	0.309 NS	**0.694** (0.014)
VEGFR1	-0.331 NS[6]	0.112 NS	-0.034 NS	-0.292 NS
VEGFR2	0.295 NS	0.318 NS	0.348 NS	0.017 NS
FGF	**-0.197** (0.032)	-0.168 NS	-0.225 NS	-0.267 NS
NOS3	**0.722** (0.0001)	0.287 NS	**0.725** (0.005)	0.454 NS
GUCY1B3	0.010 NS	-0.186 NS	-0.117 NS	0.226 NS
ANGPT1	**-0.432** (0.025)	-0.011 NS	-0.191 NS	-0.603 NS
ANGPT2	**0.581** (0.002)	-0.176 NS	0.228 NS	**0.951** (0.003)
TIE2	**-0.356** (0.031)	-0.289 NS	-0.373 NS	-0.041 NS
HIF1A	**-0.424** (0.003)	-0.527 NS	**-0.549** (0.012)	-0.125 NS

[1]This procedure calculates standard partial regression coefficients, also termed β, which provide a measure of the relative contribution of the various independent variables to each dependent variable (Wright, 1934). The data for the regressions were taken from Borowicz *et al.* (2007); and the overall R^2 = 0.92, 0.52, 0.80, and 0.76 for CAD, CND, CSD, and APC, respectively.

[2]Days of pregnancy were days 50, 70, 90, 110, 130, and 140 (n = 5 ewes per day, except for day 90, for which n = 2; Borowicz *et al.*, 2007). Relative expression of angiogenic factor mRNA was by quantitative, real-time PCR, as described by Borowicz *et al.* (2007).

[3]VEGF = vascular endothelial growth factor; VEGFR1 = VEGF receptor-1 (FLT1); VEGFR2 = VEGF receptor-2 (KDR); FGF = fibroblast growth factor-2 (basic FGF); NOS3 = endothelial nitric oxide synthase; GUCY1B3 = soluble guanylate cyclase (NO receptor); ANGPT1 = angiopoietin-1; ANGPT2 = angiopoietin-2; TIE2 = angiopoietin receptor; and HIF1A = hypoxia-inducible factor-1alpha.

[4]CAD = capillary area density, CND = capillary number density, CSD = capillary surface density, and APC = area per capillary (Borowicz *et al.*, 2007).

[5]Standard partial regression coeffiencent (β) with *P*-value in parentheses (significant β are shown in bold).

[6]NS = not significant (P > 0.05).

thus is an important regulator of both angiogenesis and vasodilation (Rosenfeld *et al.*, 1996; Magness, 1998; Vagnoni *et al.*, 1998; Reynolds and Redmer, 2001; Redmer *et al.*, 2005; Borowicz *et al.*, 2007).

To evaluate the relationships among placental angiogenesis and expression of angiogenic factors in the sheep placenta, we recently determined the mRNA expression of a suite of placental angiogenic and vasoactive factors and also measured placental angiogenesis in the same ewes at 10- to 20-day intervals throughout the last two-thirds of gestation (Borowicz *et al.*, 2007). To determine the relative contribution of these various factors to placental vascular development (that is, capillary area density, capillary number density, capillary surface density, and area per capillary), we have utilized a multiple regression procedure similar to Wright's (1934) method of 'path coefficient analysis.' This procedure calculates standard partial regression coefficients, which provide a measure of the relative contribution of the various independent variables to each dependent variable. As shown in Table 2, application of this methodology has provided some intriguing results. For example, although VEGF, NOS3, and ANGPT2 mRNA levels show a strong positive relationship, mRNA of several of the factors (for example, FGF, ANGPT1, and TIE2) show a strong negative relationship to capillary area density of the maternal placental (caruncular) tissues. Similarly, for capillary surface density, NOS3 mRNA shows a strong positive relationship, whereas that of HIF1A exhibits a strong negative relationship (Table 2). For area per capillary (capillary size), both VEGF and ANGPT2 mRNA show a strong positive relationship (Table 2). Surprisingly, none of the angiogenic or vasoactive factors evaluated showed a positive relationship to any of the measures of vascularity in the fetal placental (cotyledonary) tissues (Table 3). It seems obvious that much more work of this type needs to be done, and especially for more stages of pregnancy as well as in compromised pregnancies, to more fully understand the relationship between expression of angiogenic factors and the placental microvascular architecture.

'Rescue' of placental angiogenesis and blood flow in compromised pregnancies

Fetal growth restriction, resulting in low birth weight, occurs in 7 to 8% of human pregnancies in the United States, and is associated with increased perinatal mortality and morbidity (NLM, 2002a,b; NVSR, 2004). Because of the importance of placental blood flow to placental function, and the recognition that placental size, uteroplacental blood flows and angiogenesis, and expression of angiogenic and vasoactive factors are reduced or altered in compromised pregnancies, it has been suggested that therapeutic agents that target placental blood flow and vascular development could be used to ameliorate fetal growth restriction (Godfrey, 2002; Ahmad and Ahmed, 2005; Wu *et al.*, 2004; Wareing *et al.*, 2005; Reynolds *et al.*, 2006).

Altered placental growth and vascular development has been associated with altered expression of the genes for the major angiogenic factors and their receptors (Redmer *et al.*, 2005; Reynolds *et al.*, 2005a,b, 2006; Luther *et al.*, 2007; Vonnahme *et al.*, 2007, 2008). In addition, placental explants from preeclamptic human pregnancies exhibit increased production and release of soluble VEGF receptor-1, which binds to and thereby inhibits the activity of VEGF ligands (Ahmad and Ahmed, 2005). Thus, placental angiogenic and vasoactive factors might serve as therapeutic targets in compromised pregnancies in humans (Godfrey, 2002; Ahmad and Ahmed, 2005; Reynolds *et al.*, 2005a,b, 2006).

Nitric oxide is an important regulator of blood flow to the uterus in the nonpregnant state and also during pregnancy (Magness, 1998). Expression of both eNOS and GUCY1B3 (also known as soluble guanylate cyclase), which serves as the receptor for NO and thus mediates its effects in vascular smooth muscle, are elevated in uterine arteries during pregnancy (Itoh *et al.*, 1998; Vagnoni *et al.*, 1998; Zheng *et al.*, 2000; Magness *et al.*, 2001; Joyce *et al.*, 2002). In addition, basal production of NO contributes to low fetoplacental vascular resistance during pregnancy (Sladek *et al.*, 1997). Circulating NO and its metabolites are elevated in pregnancies with multiple compared with single fetuses (Vonnahme *et al.*, 2005). Placental expression of eNOS is reduced in some models of compromised pregnancy, including various conditions associated with intrauterine growth restriction in humans (Bird *et*

al., 2003; Maul *et al.*, 2003; Wu *et al.*, 2004; Redmer *et al.*, 2005). Moreover, NO, produced by endothelial cells, and VEGF, produced primarily by vascular smooth muscle and capillary pericytes, may interact by stimulating each other's expression (Ahmed and Perkins, 2000; Reynolds and Redmer, 2001). Thus, impaired placental syntheses of NO may provide a unified explanation for fetal growth retardation in both underfed and overfed sheep models of fetal growth restriction (Wu *et al.*, 2004).

Thus, perhaps some of the best candidates for therapeutic agents to rescue placental blood flow and angiogenesis are the phosphodiesterase 5 (PDE5A)-specific inhibitors, which include sildenafil, tadalafil, and vardenafil (marketed under the trade names Viagra, Cialis, and Levitra, respectively). These pharmacological agents enhance the vasodilatory action of NO by inhibiting the breakdown of cGMP, the second messenger for NO, thus causing sustained relaxation of vascular smooth muscle (Michel, 2006). Sildenafil enhanced both basal and estrogen-induced increases in uterine blood flow in ovariectomized ewes (Zoma *et al.*, 2004). Sildenafil also has been shown to stimulate vasorelaxation of myometrial arteries from women whose fetuses were growth restricted (Wareing *et al.*, 2005). Thus, PDE5 inhibitors do indeed seem a likely therapeutic agent to increase uteroplacental perfusion, and perhaps also placental angiogenesis, in compromised pregnancies, and this suggestion is supported by preliminary studies (Reynolds *et al.*, 2006).

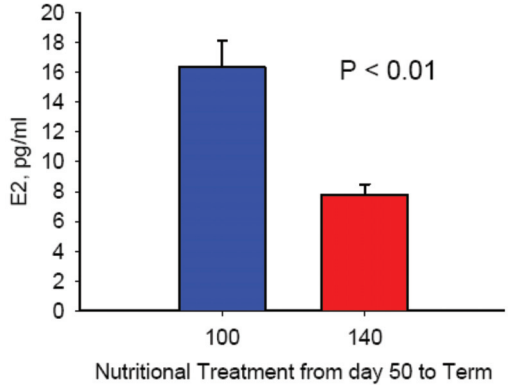

Fig. 8. Estradiol concentrations in dams fed to 100 or 140% of dietary intake requirements from day 50 after mating until term. *Vonnahme, Redmer, Reynolds and Caton, unpublished data.*

In addition to alterations in PDE5A activity, nutriceutical approaches may also be used to manipulate the NO system. Citrulline is a precursor of arginine, which is the substrate for NO synthesis via any of the various NOS (Flynn *et al.*, 2002). Fetal growth retardation induced by maternal undernutrition from day 28 to day 78 of gestation in sheep was associated with a decrease in arginine and citrulline in maternal plasma, fetal plasma, and allantoic fluid by 23 to 30% at Day 78 of gestation (Kwon *et al.*, 2004). Further, concentrations of biopterin (an indicator of *de novo* synthesis of tetrahydrobiopterin [BH_4], which is an essential cofactor for NOS) in fetal plasma, and amniotic and allantoic fluids, were reduced by 32 to 36% in underfed ewes (G. Wu, unpublished results, as referenced in Reynolds *et al.*, 2006), indicating reduced availability of BH_4 for NO production in the conceptus. These changes could impair placental and fetal NO synthesis, thereby resulting in reduced placental blood flow, in underfed ewes (Bell and Ehhardt 2002; Kwon *et al.*, 2004).

In view of the critical roles of the arginine-dependent metabolic pathways, intravenous or oral administration of arginine may provide a therapeutic strategy to enhance uterine and placental blood flow in compromised pregnancies, thereby improving fetal growth. Indeed, Xiao and Li (2005) recently reported that daily intravenous infusion of arginine for 7 days during late gestation (week 33), to women with unknown causes of fetal intrauterine growth restriction, resulted in a 6.4% increase in birth weight at term. Similarly, dietary supplementation of arginine throughout gestation resulted in a 24% increase in live litter weight in pigs (Mateo *et al.*, 2007), and supplementation of arginine to lactating sows increased piglet body weight gain by 11% through day 21 postpartum (Mateo *et al.*, 2008).

As mentioned above, estrogen appears to be the primary regulator of uteroplacental angiogenesis via its strong upregulation of angiogenic and vasoactive factors (Rosenfeld *et al.*,

1996; Magness, 1998; Vagnoni *et al.*, 1998; Culllinan-Bove *et*

TABLE 3

STANDARD PARTIAL REGRESSION COEFFICIENTS[1] ILLUSTRATING THE RELATIONSHIPS OF VARIOUS ANGIOGENIC FACTOR mRNA (INDEPENDENT VARIABLES) WITH MEASURES OF COTYLEDONARY (FETAL PLACENTAL) VASCULARITY (DEPENDENT VARIABLES) OF SHEEP THROUGHOUT PREGNANCY[2]

Angiogenic factor[3]	Measure of vascularity[4]			
	CAD	CND	CSD	APC
VEGF	-0.067[5] NS[6]	0.200 NS	0.086 NS	-0.128 NS
VEGFR1	-0.231 NS	-0.086 NS	-0.134 NS	-0.011 NS
VEGFR2	-0.175 NS	-0.033 NS	-0.126 NS	0.006 NS
FGF	-0.239 NS	**-0.229** (0.026)	-0.203 NS	0.122 NS
NOS3	0.204 NS	0.051 NS	0.083 NS	0.304 NS
GUCY1B3	0.043 NS	-0.009 NS	-0.005 NS	-0.082 NS
ANGPT1	-0.066 NS	**-0.271** (0.010)	-0.173 NS	0.208 NS
ANGPT2	0.509 NS	0.088 NS	0.348 NS	0.090 NS
TIE2	0.206 NS	0.108 NS	0.182 NS	-0.117 NS
HIF1A	-0.228 NS	-0.130 NS	-0.190 NS	-0.143 NS

[1]This procedure calculates standard partial regression coefficients, also termed β, which provide a measure of the relative contribution of the various independent variables to each dependent variable (Wright, 1934). The data for the regressions were taken from Borowicz *et al.* (2007); and the overall R^2 = 0.85, 0.96, 0.91, and 0.88 for CAD, CND, CSD, and APC, respectively.

[2]Days of pregnancy were days 50, 70, 90, 110, 130, and 140 (n = 5 ewes per day, except for day 90, for which n = 2; Borowicz *et al.*, 2007). Relative expression of angiogenic factor mRNA was by quantitative, real-time PCR, as described by Borowicz *et al.* (2007).

[3]VEGF = vascular endothelial growth factor; VEGFR1 = VEGF receptor-1 (FLT1); VEGFR2 = VEGF receptor-2 (KDR); FGF = fibroblast growth factor-2 (basic FGF); NOS3 = endothelial nitric oxide synthase; GUCY1B3 = soluble guanylate cyclase (NO receptor); ANGPT1 = angiopoietin-1; ANGPT2 = angiopoietin-2; TIE2 = angiopoietin receptor; and HIF1A = hypoxia-inducible factor-1alpha.

[4]CAD = capillary area density, CND = capillary number density, CSD = capillary surface density, and APC = area per capillary (Borowicz *et al.*, 2007).

[5]Standard partial regression coeffiencent (β) with *P*-value in parentheses (significant β are shown in bold).

[6]NS = not significant (P > 0.05).

al., 1993; Magness, 1998; Reynolds *et al.*, 1998; Johnson *et al.*, 2006). Recently, in one of our models of compromised pregnancy; namely, the overfed, adolescent ewe, we have found dramatically reduced maternal systemic estradiol levels during the last half of gestation (Wallace *et al.*, 2008; Vonnahme *et al.*, unpublished, Fig. 8). Whether the reduced maternal estradiol levels are due to increased metabolism or reduced placental production of estrogens remains to be determined. Nevertheless, reduced circulating estradiol is a likely culprit in the reduced placental blood flow and angiogenesis observed in this model (Table 1), leaving open the possibility of estrogen replacement therapy to rescue placental function and thus fetal growth and development in this type of compromised pregnancy (Wallace *et al.*, 2008).

Summary and challenges

Nearly 15 years ago, we reviewed the current state of knowledge concerning uteroplacental vascular development, its contribution to placental function, and, ultimately, its importance to fetal and neonatal growth and development. In the intervening period, we have learned much more about the critical role of placental vascular development in placental function in both normal pregnancy as well as numerous models of compromised pregnancy, including those in which fetal or placental growth, or both, are reduced due to abnormal maternal dietary intake, increased numbers of fetuses, maternal or fetal genotype, maternal age, or maternal environmental stress. We also have learned much about the factors that regulate placental vascular growth, or angiogenesis. Based on these observations, we suggest several potential therapeutic approaches to 'rescuing' placental vascular development, and thus fetal and placental growth and development.

Although we have learned much in a relatively short time, we obviously have much more to do. For example, we need a much better understanding of when and where and how changes in angiogenic factor expression and the process of angiogenesis itself occur in various compromised pregnancies, and the effects of various therapeutic agents not only on fetal and placental growth and development but also on postnatal outcomes. It seems equally obvious that animal models of compromised pregnancy will be critical to solving these problems, which have large socioeconomic consequences.

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