1 Maternal Environment and Placental Vascularization in Small Ruminants

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17 **A B S T R A C T**

18 Uteroplacental development is a crucial step facilitating conceptus growth. Normal placental
19 development comprises extensive placental angiogenesis to support fetoplacental transport;
20 meeting the metabolic demands of the fetus. Compromised pregnancies due to maternal stressors
21 such as over or undernutrition, maternal age or parity, altered body mass index or genetic
22 background result in altered vascular development of the placenta. This negatively affects
23 placental growth, placental function and ultimately results in poor pregnancy outcomes.
24 Nonetheless, the placenta acts as a sensor to the maternal stressors and undergoes modifications,
25 which some have termed placental programming, to ensure healthy development of the
26 conceptus. Sex steroid hormones such as estradiol-17 β (**E2**) and progesterone (**P4**), chemokines
27 such as chemokine ligand twelve (**CXCL12**) and angiogenic/vasoactive factors such as vascular
28 endothelial growth factors (**VEGF**), placental growth factor (**PlGF**), angiopoietins (**Ang**), and
29 nitric oxide (**NO**) regulate uteroplacental development and hence are often used as therapeutic
30 targets to rescue compromised pregnancies. Interestingly, the presence of sex steroid receptors
31 have been identified in the fetal membranes (developing fetal placenta). Environmental steroid
32 mimetics known as Endocrine Disrupting Compounds (**EDCs**) disrupt conceptus development
33 and to lead to transgenerational impairments by epigenetic modification of placental gene
34 expression, which is another area deserving intense research efforts. This review attempts to
35 summarize current knowledge concerning intrinsic and extrinsic factors affecting selected
36 reproductive functions with the emphasis on placental development.

37 ***Keywords:***

38 Placenta, conceptus, vascular development, compromised pregnancies, maternal stressors,
39 endocrine disrupting compounds

1. Introduction

The critical events that occur during early pregnancy include dramatic growth and development of the embryo/fetus as well as growth and vascularization of the placenta [1-4]. It is not surprising, therefore, that the majority of embryonic loss, amounting to 30 to 50% of fertilized oocytes, occurs during early pregnancy in all mammals studied, including humans [1-4].

Utero-placental vascularization, as reflected by dramatic vascular growth (angiogenesis) along with vascular remodeling, is an essential component of increased placental blood flow. Increased placental blood flow is critical to placental function, which supports fetal growth and development [1, 3, 5-10]. Compromised pregnancy encompasses any pregnancy in which fetal growth (and, by inference fetal development) are abnormal [2, 9] . Compromised fetal growth/development are usually accompanied by altered placental growth and development [2, 9, 11]. Practically, most compromised pregnancies involve poor fetal growth/development, reflected by low birth weight.

We and others have shown that alterations in placental vascular development and function underlie the negative effects of many factors, including maternal factors, on fetal growth. Maternal factors include malnutrition, age, body mass index, and genetic background, and external factors including environmental stress (e.g., high altitude, heat stress), psychosocial stress, multiple fetuses, medical conditions (e.g., polycystic ovary syndrome), lifestyle choices (e.g., alcohol consumption, smoking), and assisted reproductive technologies [1, 2, 12, 13].

Placental vascular development begins very early in pregnancy and determines, to a large degree, placental function – that is, the magnitude of the increase in placental blood flow and thus nutrient transport to the fetus [1, 10, 14, 15]. Many of the same factors that negatively

impact fertility and fetal growth and development also have similar consequences for placental growth and development. For example, in numerous models of compromised pregnancy in sheep reduced fetal weight near term is associated not only with reduced placental size but also reduced

Table 1. Changes in Fetal and Placental Weights, Uterine and Umbilical Blood Flows, and Placental (CAR = Caruncular or Maternal Placental; and COT = Cotyledonary or Fetal Placental) Vascularity in Various Models of Compromised Pregnancy in Sheep.¹

Model	Day of gestation ²	Fetal weight	Placental weight ³	Uterine blood flow	Umbilical blood flow	Vascularity
Overfed Adolescent	130-134	↓20-28%	↓45%	↓36%	↓37%	↓59% (total capillary vol., COT) ⁴
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 ⁵	130	↓43%	↓47%	----	----	↑28-43% (cap. area density, CAR and COT, respectively)
Heat-stressed Adult	133-135	↓42%	↓51%	↓26%	↓60%	----
Multiple Pregnancy ⁶	140	↓30%	↓37%	↓23%	----	↓30% (total cap. vol., COT)
High Dietary Se	135	NSE	↓24%	----	----	↑20% (cap number density, COT)
Hypoxic (Hypobaric) Stress ⁷	140	NSE	----	↓35%	----	↑19% (cap. area density, CAR)

¹Table adapted from Reynolds et al., 2010a; cap. = capillary; CAR = caruncular (maternal placenta); COT = cotyledonary (fetal placental/ villous); and NSE = no significant effect.

²Length of gestation = approximately 145 days; thus, all measurements taken from late (0.9 to 0.99) of pregnancy..

³Placental weight = total placentome weight.

⁴Day 50 only, which was well before decreased fetal or placental weight, which was observed on day 130 but not on days 50 or 90 of gestation. .

⁵Genotype represents highly prolific (approx. 4 offspring per pregnancy) vs. relative low prolificacy (approx. 1.2 offspring per pregnancy) breeds of sheep - the high prolificacy is associated with relatively small individual fetal and placental weights but a compensatory increase in placental vascularity (see Fig. 2) to support the much larger total fetal and placental mass.

⁶Singletons vs. triplets.

⁷The increased placental vascularity is reflected by normal fetal weight despite a 35% decrease in uterine blood flow.

gravid uterine (maternal placental) and umbilical (fetal placental) blood flows as well as altered placental vascular development and function [4, 14] (**Table 1**). In ewes that are nutrient-restricted during pregnancy, the normal decrease in uterine arterial resistance that accompanies pregnancy does not occur and umbilical vascular resistance is increased [2]. These observations indicate that placental vascular function also is compromised.

In humans, reduced placental vascular development and increased vascular resistance during early pregnancy is associated with early embryonic mortality [16, 17]. In human intrauterine growth restricted (**IUGR**) fetuses, although gravid uterine blood flow increases exponentially as it does in average size for gestational age (**AGA**) fetuses, the magnitude of increase is dramatically reduced [18]. Additionally, in humans IUGR is reflected by dramatically reduced placental (villous) development including that of the capillary beds, but this does not occur in

pre-eclamptic pregnancies [19]. Altered angiogenesis, altered expression of selected angiogenic factors or reduced blood flow were also observed in the placenta in several pregnancy disorders including early intrauterine embryonic death, pre-eclampsia, placenta accretia, IUGR or diabetes in several species [8, 20-25]. Thus, the data available from sheep models of compromised pregnancy are in agreement with those from other mammals.

a. Altered placental vascularization/vascular function as an initiating event in placental dysfunction

The majority of placental growth occurs before that of the fetus, and a strong correlation exists between the placental mass and fetal size at birth in all species studied [1, 14, 26-28]. Transplacental exchange, which meets the metabolic demands of the fetus, mainly depends on increased uterine and umbilical blood flows, which in turn depend on uteroplacental vasculature development [1, 14]. Maternal stressors such as undernutrition or overnutrition, which lead to placental dysfunction, also result in abnormal fetal outcomes [29].

In sheep, overnourishment during pregnancy promotes rapid maternal growth whereas nutrient supply to the gravid uterus is decreased. This leads to a decrease in placental mass and premature delivery of low birth weight lambs [30-32]. These pregnancies are also characterized by reduced blood flow and nutrient uptake resulting in fetal hypoxia, hypoglycemia, hypoinsulinemia and asymmetric growth restriction [32, 33]. For example, Wallace et al. [34] demonstrated that overnourishing pregnant adolescent ewes resulted in compromised fetoplacental size at term. In a follow-up study, on day 81 of gestation placental mRNA expression of angiogenic factors including VEGF, Ang1 and 2, eNOS and VEGFR was decreased in overfed ewes, well ahead of the decrease in fetal or placental sizes, suggesting ‘programming’ of placental vascular growth and subsequent function [35]. Another study

demonstrated that global nutrient restriction during early gestation followed by re-alimentation increased mRNA expression of PlGF and Flt-1 in caruncular and cotyledonary tissues [36].

In pregnant mice, the placenta may act as a sensor to maternal undernutrition by adapting to maintain healthy fetal development (i.e., placental programming) [37]. In this study, on day 19 of pregnancy placental and fetal weight were reduced in undernourished mice, with a concomitant decrease in volume and surface area of the labyrinthine zone responsible for nutrient transfer. However, unidirectional maternal-fetal clearance of glucose and amino acids were maintained due to a compensatory increase in expression of glucose and amino acid transporters [37]. A complete understanding of the factors regulating vascular functions as an initiating event in placental dysfunction requires more studies using a variety of animal models [11].

2. Placental Vascular Development and Function

a. Normal pattern

One of the earliest events marking embryonic/placental development is the establishment of functional fetal and placental circulations. In sheep, blood vessels in fetal membranes (chorioallantois) were detected as early as day 18 post-conception [15]. In marmosets, increased endometrial endothelial cell proliferation and blood vessel diameter was observed on week 2 of pregnancy [38], and in rats increased endothelial cell proliferation was observed on day 3 of pregnancy [39]. In humans, extensive vascular remodeling of the endometrium is initiated during the first week and completed on week 20 of gestation [40, 41]. These angiogenic events are initiated very early in pregnancy to support not only placental function and fetal growth but also are critical to successful pregnancy establishment [42].

Data from our lab have demonstrated significant increase in placental angiogenesis during early pregnancy in sheep [10, 15]. Compared to non-pregnant controls, proliferation of

endothelial cells (as measured by labelling index using Ki67, a marker of cell proliferation) increased 7- to 20-fold on days 14–20, and 23- to 34-fold on days 22–30 of pregnancy. Angiogenesis also occurred during this time-frame. Capillary area density increased 1.3-fold on day 16 and 1.5- to 1.9-fold on days 20–30. Capillary surface density increased 1.4- to 1.6-fold on days 20–30 of pregnancy, and capillary size (cross sectional area per capillary) increased 1.5-fold on days 16 and 22, and 2.2- to 3-fold on days 22–30 of pregnancy [10]. In sheep, a two-fold increase in endometrial vascular volume is observed in gravid uterine horn by day 24 of pregnancy, whereas the non-gravid uterine horn demonstrates increased angiogenesis by day 30 post-mating [6].

Differences in the patterns of microvascular growth are observed between maternal and fetal placenta throughout gestation in sheep [43]; (Fig.

Figure 1. Models of vascular growth and remodeling in maternal and fetal placenta from day 50 to day 140 of gestation in sheep. Taken from Borowicz et al., 2007.

1). The vascular density of maternal placental tissues increases gradually throughout gestation in contrast to that of the fetal placental cotyledons, which grows slowly until midgestation but demonstrates dramatic vascular growth during the last third of gestation, in parallel with the dramatic increase in fetal growth [43]. In addition, the caruncular (maternal placental) capillary beds grow primarily by an increase in capillary size (cross sectional area per capillary) along with modest, 3-fold, increases in capillary number and surface density. In contrast, cotyledonary (fetal villous) capillary beds grow predominantly by vessel branching, resulting in a 12-fold increase in capillary number density and 6-fold increases in capillary area and surface densities [43].

149 Additionally, compared with nonpregnant endometrium, during early gestation (days 30 and
150 50 of pregnancy) the maternal placental capillary bed forms a capillary plexus just under the
151 uterine luminal epithelium where the endometrium contacts the fetal chorioallantois [44]. By the
152 last third of pregnancy, maternal caruncular tissues consist of large capillaries, with diameters of
153 20 microns or more [43]. This type of capillary system facilitates a low velocity, ‘irrigation’
154 pattern of blood flow, which promotes oxygen and nutrient delivery to and waste removal from
155 the fetus [45]. The fetal placental microvasculature on the other hand is composed of highly
156 branched and abundant small (approximately 5 microns in diameter) capillaries, resulting in a
157 system suite to rapid transport [45].

158 The differences in microvasculature are also demonstrated as differences in flow velocity
159 between the maternal and fetal placenta of sheep. During the last two-thirds of gestation,
160 umbilical blood flow increases 2-3 times faster than the uterine blood flow [4, 8, 14, 45].
161 Additionally, due to a much greater degree of branching, fetal placental capillaries have more
162 surface area for nutrient absorption than maternal placental capillaries [4, 14, 45].

163 Although ovine placental microvascular architecture is ideally suited for both nutrient
164 delivery, on the maternal side, and nutrient uptake and transport, on the fetal side, this
165 arrangement does not appear to be unique to sheep. For example, there are several striking
166 similarities in placental function between the sheep and the cow, which is another placentomal,
167 or cotyledonary, mammal [1, 36]. The capillary plexus that forms on the maternal portion of the
168 placenta also has been observed in pigs early in pregnancy[46], although to our knowledge this
169 has not been examined later in pregnancy. In primates, including humans, the chorioallantois is
170 so invasive that a portion of the maternal endometrium is eroded, and the fetal villi are bathed in
171 maternal blood; this arrangement thus represents the ultimate in a low-velocity, irrigation-flow
172 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 [1, 8].

During the last half of gestation in sheep and cattle, increased uterine blood flow accounts for a 3- to 6-fold increase in total gravid uterine oxygen uptake. In contrast, umbilical blood flow accounts for a 16-fold increase in oxygen uptake by the ovine and bovine fetus[45]. The dramatic differences in microvascular growth and architecture can explain these differences in the magnitude of increase of gravid uterine compared with umbilical blood flows and help explain how the proportion of nutrient and oxygen uptake by the gravid uterus that is shuttled to the fetus increases 2- to 4-fold whereas the proportion utilized by the placenta declines during the last half of gestation [4, 8, 14, 45].

Placental vascular cell proliferation and angiogenesis coincide with the period of implantation and establishment of pregnancy [10, 15]. Several factors including sex steroid hormones, growth factors (e.g., vascular endothelial growth factors [VEGF] and fibroblast growth factors [FGF]), prostaglandins, chemokines and osteopontins regulate vascular cell proliferation and are involved in initial attachment/implantation of fetal membranes to uterine epithelium, as well as placental growth and development [3, 47-51]. Thus, in addition to its role in providing for exchange of nutrients and wastes between the maternal system and the conceptus, initiation of placental angiogenesis also helps to prepare uterine environment for the process of implantation and establishment of pregnancy.

b. Early onset of placental vascular defects

As shown in Table 1, placental vascular defects are observed in compromised pregnancies, which result in impaired fetal growth and development. There are various maternal factors such as

maternal age, body mass index, nutritional stress, multiple fetuses, medical conditions (such as Polycystic Ovarian Syndrome), environmental factors (high altitude, heat stress), lifestyle choices (e.g., alcohol consumption, smoking) and assisted reproductive techniques (ART) which negatively affect pregnancy [2, 4, 9, 14]. The factors affecting fetal outcomes also affect placental vascular development [2, 4, 9, 14].

As mentioned previously, increased blood flow to the placenta is important for pregnancy establishment and fetal development, and compromised pregnancies are inevitably associated with decreased placental blood flow and reduced fetal nutrient and oxygen uptake, resulting in fetal mortality and morbidity [8, 9, 34, 52]. Data from our lab have demonstrated that on day 22 of pregnancy in sheep, vascular cell proliferation and density of blood vessels in fetal membranes and maternal caruncular tissues were significantly reduced in pregnancies achieved through selected ART methods such as embryo transfer (**ET**) after in-vitro activation (**IVA**, parthogenesis) or in-vitro fertilization (**IVF**) as opposed to natural mating [53]. Several studies have demonstrated that pregnancies achieved through IVF have less pronounced defects in placental vascularization than those achieved by somatic cell nuclear transfer (**SCNT**) in several species [54, 55].

Immunohistochemical studies of placenta in pregnancies achieved through SCNT in ovine models exhibited decreased expression of markers of endothelial maturation such as von Willebrand Factor (**vWF**, Factor VIII), smooth muscle cell actin (**SMCA**), calponin and cluster of differentiation (**CD**) 34, indicating immature vasculogenesis in placenta [56]. Microvasculature of bovine fetal placenta at term demonstrated impaired capillary development after transfer of cloned embryos compared to control pregnancies, and scanning electron microscopy images of blood vessel casts revealed that the fetal microvasculature failed to form dense capillary loops and sinusoidal dilations after transfer of cloned embryos [57]. Furthermore,

decreased fetal villi formation was observed in bovine placenta after transfer of embryos created through IVF [58]. However, in these pregnancies the volume of blood vessels in maternal caruncles and the ratio of blood vessel volume density to placentome surface area were increased, indicating a compensatory mechanism in placental vascularization [59]. Thus, maternal placental tissues not only act as biological sensors and respond differently to pregnancies achieved through natural mating, SCNT or IVF but also may reverse the negative effects of embryo manipulations on placental and fetal growth due to existing compensatory mechanisms [2, 11, 14, 55, 60]. For example, in pregnancies achieved under environmental stress such as hyperthermia and/or hypoxia coiled, randomly branched with sinusoidal dilated placental microvascular architecture has been reported, which may be a compensatory mechanism to facilitate increased oxygen supply to the developing fetus [61, 62]. Exception to this compensatory mechanism is observed in case of multiple fetuses. A study on placental vascular development in late pregnancy in ewes bearing single, twin or triplet fetuses demonstrated decreased cotyledonary capillary volume with increase in fetus number [63].

In humans, placental vascular defects have been associated with adverse fetal outcomes [29]. Analysis of placental vascularization using 3D power Doppler method demonstrated decreased vascularization index of the placenta in pregnancies achieved through IVF compared to natural pregnancies [64]. Decreased placental perfusion is associated with fetal growth restriction in humans [65-67]. Fetal growth restriction in humans has also been associated with increased umbilical pulsatility index and placental vascular resistance [68-71]. Due to the mutual dependence of placental blood flow and fetal outcomes, it has been suggested that pharmacological agents which target vascular tone may ameliorate fetal growth restriction [72-74]. Phosphodiesterase 5 specific- inhibitors, which include sildenafil, tadalafil and vardenafil (marketed under the trade names Viagra, Cialis and Levitra, respectively), enhance the

vasodilatory actions of nitric oxide (NO) by inhibiting breakdown of cGMP and thereby causing sustained relaxation of vascular smooth muscle cells. These compounds might be possible therapeutic choices [74-76]. In addition, nutritional supplements in the form of citrulline (arginine precursor), arginine or tetrahydrobiopterin (BH4, a cofactor for NOS) can be considered as therapeutic remedies to impaired fetal outcomes [52, 77-79].

1. Mechanisms and Consequences

a. Genetic differences

Maternal effects on placental vascularization may be due to endogenous, genetic, as well as exogenous effects. In terms of genetic effects, breed differences in sheep can have a dramatic effect on both fertility (i.e., the ability to conceive and successfully establish a pregnancy) and placental vascularization.

In a recent study, we utilized Romanov sheep, which are well known to be highly prolific and early maturing compared to more standard European or American production breeds [80, 81].

Using reciprocal ET, singleton pregnancies

were evaluated in the following groups 1)

Romanov embryos in Romanov dams (**R-**

R), 2) Romanov embryos in Columbia

dams (**R-C**), 3) Columbia embryos in

Romanov dams (**C-R**), and 4) Columbia

embryos in Columbia dams (**C-C**). Results

from this study demonstrated that the

Romanovs placenta was 22% more vascular

Figure 2. Vascularity of placenta in Romanov and Columbia ewes at day 130 of pregnancy. *P < 0.05 for breed differences. Borowicz, Redmer, Grazul-Bilska and Reynolds, unpublished.

($P < 0.05$) at day 130 compared with the Columbia placenta (**Fig. 2**). Specifically, the area density of capillaries, which represents the proportion of tissue area or volume occupied by capillaries [43, 82], was 30.4% greater in caruncle (maternal placenta) and 42.9% greater in cotyledon (fetal placenta) in Romanov vs. Columbia pregnancies (Borowicz, Redmer, Grazul-Bilska and Reynolds, unpublished).

Because placental vascularization is initiated early in gestation, it is important to elucidate if vascularization differences noted on day 130 also occur during the first 30 days of pregnancy. Recently, we expanded our sheep fertility model to include Dorset ewes and demonstrated that first service conception rates for Dorsets were 24.4% compared to 70% for Romanov, although

Table 2. Conception Rates and Lambs Born to Dorset and Romanov Ewes.

Breed	First-service conception, %	Bred at second service, %	Total conception, %	Total lambs born per ewe	Live lambs born per ewe
Dorset	24.4	51.2	82.4	1.20	1.12
Romanov	70.0	20.0	89.7	2.11	2.00

N = 41 Dorset and 40 Romanov. Ewes were bred in the summer of 2013 at a CIDR-synchronized estrus (first service) or the following estrus (second service) by rams of their own breed. Rams were fitted with marking harnesses, and breeding marks were recorded twice daily. All conception data are based on ultrasound evaluations at days 35, 55, 85, and 120 after mating and subsequent lambing. SEM = 0.085 and 0.160 for total and live lambs born/ewe for Dorset and Romanov, respectively. Redden, Crane, Reynolds, and Caton, unpublished.

total conception rate was similar (**Table 2**). In addition, as we expected, lambing rate of Romanovs was nearly twice that of Dorsets (**Table 2**).

b. Diet effects

As mentioned above, maternal dietary intake profoundly affects placental vascular development and function (**Table 1**). Maternal diet during the peri-conceptual period also has dramatic effects on fertility and embryonic development. Inadequate diet affects oocyte quality, which is critical for fertilization, early embryonic development, pregnancy outcome and

offspring health [83-90]. In several studies the importance of diet and/or body adiposity during pre- and peri-conceptual periods in humans and animal models have been clearly recognized [2].

Both underfeeding and overfeeding sheep for 60 days before oocyte collection resulted in diminished oocyte quality, leading to decreased rates of IVF and poor embryonic development to blastocyst stage [88, 90]. In addition, overfed sheep had increased serum insulin, leptin and **P4** concentration whereas underfed sheep had increased **E2** but decreased insulin-like growth factor (**IGF**) 1, insulin and leptin concentration [90, 91]. Other studies reported decreased rates of IVF and/or early embryonic development in ewes fed a low- (nutrient-restricted) or high- (ad libitum intake) energy diet during the peri-conceptual period [92, 93]. Conversely, others have shown that an altered plane of nutrition during the peri-conceptual period had either positive or no effects on blastocyst formation and/or embryo health in sheep [93], [87]. Interestingly, pre-mating nutrition altered mRNA expression in oocytes and follicular cells, which may account for reductions in reproductive performance of ewes fed restricted diets [94].

In cows, the effects of diet fed before and during peri-conceptual period on oocyte quality measured by rates of IVF and blastocyst formation, and cell number per blastocyst depend on animal body condition (i.e., adiposity) and diet composition [95, 96]. High levels of feeding improved postfertilization embryonic development for animals in low body condition, but reduced postfertilization embryonic development for animals in good body condition [95]. Hyperinsulinemic cows fed a high-energy diet with good body condition produced fewer oocytes and had lower blastocyst yield after IVF [95]. In addition, dairy cows that are fed a high-protein and high-energy diet are frequently in negative energy balance characterized by low fertility, perhaps due to low-quality oocytes and embryos [97, 98] In dairy cows, dietary factors, altered endocrine and metabolic function, and genetic factors contribute to diminished oocyte and embryo quality [97, 99].

Maternal undernutrition during the peri-conceptual period in sheep results in altered fetal growth trajectory, altered fetal hypothalamic-pituitary-adrenal axis (**HPAA**) development, accelerated maturation of fetal adrenal glands, altered fetal pancreatic development, altered insulin signaling and amino acid metabolism, all leading to an increased rate of premature birth [100-103]. How maternal underfeeding during during the peri-conceptual period affects offspring growth and development is unknown. Recently, Sinclair et al. [12] demonstrated that specific maternal dietary components such as vitamin B and methionine fed to ewes during the peri-conceptual period affect DNA methylation, insulin resistance and blood pressure in the offspring.

Overall, the available data indicate that diet during the peri-conceptual period may have short-term (e.g., effects on oocyte quality and thus the rates of fertilization and early embryonic development) and/or long-term (e.g., effects on offspring development and timing of birth, and thus developmental programming) effects. These observations also clearly demonstrate a role of diet in epigenetic regulation [12, 104].

c. Molecular mechanisms

i. Angiogenic factors

Early pregnancy is marked by upregulation of several angiogenic factors, which parallels vascular development and tissue growth in the uteroplacenta. Expression of mRNA is upregulated for endothelial nitric oxide synthase (**eNOS**; which is responsible for conversion of arginine to NO), placental growth factor (**PlGF**), angiopoietins (**ANG**) 1 and 2, ANG receptor TEK vascular endothelial growth factor (**VEGF**) receptors Flt 1 and KDR, fibroblast growth factor receptor (**FGFR**) 2IIIc, and hypoxia-inducible factor (**HIF**) 1 α during early pregnancy in ewes compared to non-pregnant controls. Expression of mRNA for PlGF increased 5-fold on day 24 and 8- to 10-fold on days 26-30 of pregnancy. Expression of Flt1 increased approximately

2.5-fold on days 20-28 and 3.8-fold on day 30 of pregnancy. KDR increased by 2-fold or greater on days 18-30 of pregnancy and FGFR2IIIC increased 1.5-fold on day 16 of pregnancy [10].

Similarly, in the fetal placenta PlGF increased from 3.4-fold to 35-fold from days 16-22 to days 24-30 of pregnancy. VEGF mRNA expression increased by 2-fold on days 28-30 compared to days 16-20. Flt1 mRNA increased 5- to 50-fold on days 28-30 compared to days 16-24. KDR mRNA expression was 2- to 11-fold greater on days 20-24 than on days 16-18 and 26-30[15] . Moreover, during early pregnancy the pattern of expression of some of the angiogenic factors (such as eNOS, ANG1, ANG2 and HIF1A) differed, whereas others (e.g. VEGF, VEGFR1, VEGFR2, TEK and FGF2) were similar from mid to late gestation [43]. This suggests a differential role of angiogenic factors in regulation of placental microvascular development at different stages of pregnancy.

Correlation studies have indicated a positive association of eNOS with ANG system members (ANG1, ANG2 and TEK) in maternal placenta. Based on a previous study, it can be speculated that ANG might be involved in NO release by maternal placental cells [105]. In the fetal placenta, a positive correlation was observed between mRNA expression of eNOS and the VEGF and FGF2 systems, which indicates potential interactions among these factors in regulation of vascular development of the fetal placenta [15].

Protein and mRNA expression of VEGF, PlGF, FGF2 and their corresponding receptors have been detected in extraembryonic tissues in monkeys, humans and cows at different stages of early pregnancy; however, their mechanism of action in regulating placental angiogenesis still needs to be elucidated [106-109].

As mentioned previously, ART often leads to compromised pregnancies and impaired placental vascular development. A study on the expression of angiogenic factors in fetal membranes of embryos developed by ART demonstrated decreased maternal placental mRNA

expression of VEGF and its receptor Flt1, PlGF, ANG1 and 2, eNOS, HIF-1 α , FGF2 and its receptor FGFR2 compared to natural pregnancy. In addition, ANG2 and eNOS expression in fetal membranes were correlated with blood vessel density, and in caruncular tissues the expression of members of the VEGF system correlated with those of the ANG and FGF systems [15].

The expression of VEGF was also studied in hyperthermia-induced ovine models of placental insufficiency (PI) with IUGR. Placental expression of VEGF receptor on day 90 of gestation was significantly decreased in hyperthermic ewes compared to thermoneutral ones [62]. In addition, both absolute uterine and umbilical blood flows were reduced in this model[110]. These data suggests that impaired angiogenic growth factor expression during the period of maximum growth may contribute to placental insufficiency leading to IUGR.

In addition, in pre-eclampsia which is another case of compromised pregnancy, decreased expression of placental VEGF and PlGF was observed in humans [111-114]. In humans, during late gestation in compromised pregnancies such as pre-eclampsia or IUGR, increased eNOS expression in the syncytiotrophoblast and increased NO concentration in blood has been reported [115, 116]. Nitric oxide is a potent vasodilator and is known to increase uterine blood flow. Interestingly, a study of the levels of NO metabolite (**NOx**) throughout gestation demonstrated a biphasic pattern of increase on NOx in ewes; moreover, NOx levels were increased in multiple pregnancies [117] . It has been demonstrated that E2 contributes to vasodilation by NO-dependent mechanisms as reflected by uterine venous cGMP [118]. However, intra-arterial L-NAME, which is an inhibitor of NOS, decreased basal cGMP secretion by 66% but did not affect uterine blood flow in sheep [118].

ii. Chemokines

A challenge with understanding early gestational complications in livestock is the lack of knowledge of specific genes and pathways regulating fetal and maternal communication, leading to successful implantation and placental development, including angiogenesis. We have investigated a potential role of chemokines in these processes and thus in fetal survival during early gestation in ruminants.

Chemokines are well known regulators of immune cell function and migration, and act through seven-transmembrane G-protein coupled receptors on the cell surface [119-121]. To date, approximately 50 members of the chemokine (which is a combined word for chemotactic cytokines) family have been identified. Chemokines can be divided into two main categories based on placement of amino acids (designated by X) relative to the n-terminal cysteine residues (designated by C). These categories are the CXC and the CC chemokines [122]. The predominant category researched thus far in ruminants is the CXC chemokines [123]; therefore the CXC chemokines will be discussed in this review.

In ruminants, it is hypothesized that CXC chemokines promote recruitment of immune system cells needed for embryo implantation as well as angiogenesis for placental vascularization [51, 123, 124]. Interferon- γ inducible protein (IP-10), also known as chemokine ligand ten (**CXCL10**) increases in pregnant ewes compared to non-pregnant ewes during early gestation (days 14, 17, 20 and 25)[125]. Additionally, mRNA for its receptor, CXCR3, increases on days 17 and 20 and on day 20 in pregnant compared to non-pregnant ewes and goats, respectively. Localization of CXCR3 via immunofluorescence also revealed specific staining in trophoblast cells on day 17 of pregnancy [123]. In ovine endometrial explant cultures, CXCL10 increases in response to interferon tau (**IFN τ**), the primary maternal recognition signal secreted by the trophoblast in ruminants [125]. To further delineate how CXCL10 may promote implantation in ruminants during early pregnancy, chemotaxis assays with CXCL10 treatment

were conducted utilizing isolated peripheral blood mononuclear cells (PBMC) from cyclic (day 15) goats [126]. Following CXCL10 stimulation, the migration of PBMC increased, as did mRNA expression of the cytokine interleukin (IL) 10 and a natural killer (NK) cell and macrophage marker, CD11b. These studies suggest that an increase in IFN τ can stimulate an increase in CXCL10 in the endometrium, which in turn can promote the recruitment of specific immune cells to help maintain an ideal environment for trophoblast development, conceptus attachment and fetal-maternal communication. Although CXCL10 binds to CXCR3, this receptor also has high affinity for CXCL9 and CXCL11. Similar to CXCL10, CXCL9 mRNA increases on days 17, 20 and 25 of gestation in endometrium, and CXCL11 mRNA increases on day 17 of pregnancy compared to cyclic ewes. Additionally, CXCL9 and CXCL11 proteins increase in uterine flushings on day 17 in pregnant ewes compared to day 14 pregnant or non-pregnant ewes [123]. Expression of these chemokines may therefore be important during the implantation period in ruminants.

Interestingly, the expression pattern of CXCL9, CXCL10 and CXCL11 follows similar to that of another chemokine, CXCL12, which exhibits increased expression on days 18 and 20 of gestation compared to non-pregnant ewes in

endometrial tissue [51, 124]. In addition mRNA for its receptor, CXCR4 also increases on days 18, 20, 22, and 25 of gestation (**Fig. 3**). In these two studies an increase in CXCL12 and CXCR4 was also present in fetal membranes (chorion or chorioallantois) during the time frame of fetal

attachment (**Fig. 4**). Furthermore, CXCL12 immunofluorescence revealed predominant localization within trophoblast cells compared to the endometrium [51]; **Fig. 5**). CXCL12-CXCR4 may promote trophoblast migration, and activation of CXCR4 by CXCL12 is known to drive synthesis of two important angiogenic factors, VEGF and FGF2 [25, 32]. Our studies have shown that treatment of ovine trophoblast cells with CXCL12 increases mRNA expression of VEGF and FGF2 in (Quinn et al., 2014; **Fig. 6**). Thus, CXCL12-CXCR4 may promote placental development and vascularization, and therefore be equally important as other chemokines in establishment of pregnancy in ruminants.

Connections between the CXCL9-CXCL10-CXCL11 and CXCL12 pathways has yet to be

Figure 5. Immunofluorescence localization of (A) CXCL12 (yellowish) in ovine trophoblast (TR) and uterine luminal epithelium (ULE) with minimal staining in glandular epithelium (G); (B) CXCR4 (pinkish) in TR, ULE, and G. Both micrographs are from day 26 of gestation. Taken from Quinn et al., 2014 [51].

elucidated in ruminants, but could provide more insight into how these chemokine signaling mechanisms may correlate with each other to promote fetal attachment and immune cell regulation during early pregnancy. Because these chemokine pathways may promote migration of immune cells into the uterus, it is important to address in future research which immune cells express its receptors (CXCR3 or CXCR4) and how this association affects pregnancy establishment. This could then further our understanding of the communication between the maternal and fetal interface in ruminants.

iii. Steroids and steroid receptors

Sex steroids are important regulators of uterine blood flow and dramatically affect uterine vascular tone [127]. When chronically ovariectomized ewes are administered E2, a 6- to 10-fold increase in uterine blood flow occurs within 90 min [5,6]. In addition, chronic (14-day) administration of E2 to ovariectomized ewes mimics many of the cardiovascular and hemodynamic changes seen during pregnancy, including increased cardiac output, blood volume, and uterine blood flow, and decreased systemic vascular resistance and mean arterial pressure [7].

Estrogens also regulate endometrial (maternal placental) angiogenesis, angiogenic factor expression, and nitric oxide synthase expression in several species [6,11,12,15-18]. Administration of physiological levels of E2 to ovariectomized ewes increases VEGF expression

Figure 6. Effects of CXCL12 on mRNA expression of VEGF (top) and FGF2 (bottom) in ovine trophoblast cells. Taken from Quinn et al., 2014 [51].

by 30-fold within 4 h and size of the endometrial capillary bed by 2-fold within 24 h [15,16]. A time course experiment on ovariectomized ewes demonstrated that E2 increases microvascular volume by 1.8 fold within 24 hours of administration and remains constant for 72 hours [128]. In a similar experiment, physiological levels of E2 when administered to ovariectomized ewes increased expression of VEGF by 30 fold with a concomitant increase in the size of capillary bed within 24 hours. Moreover, increased expression of mRNA for eNOS, PIGF, HIF, and ANG were reported following E2 administration [129, 130].

Progesterone is critical for maintenance of pregnancy, in part due to its effects on uterine function, and thereby, along with interferon tau, on embryonic survival and growth [19-25]. Additionally, P4 moderates the vasoactivity of estrogens [8-14].

Combined treatment with E2 and P4 in ovariectomized ewes increased endometrial proliferation by both hypertrophy and hyperplasia [131]. Similarly co-administration of E2 and P4 in ovariectomized ewes increased the expression of estrogen receptor ER β in uterine artery endothelium [132].

During early pregnancy in sheep, expression of the classical nuclear estrogen and P4 receptors (ER and PR, respectively) decreases in several uterine compartments except the vasculature, where it remains elevated and undoubtedly drives both blood flow and angiogenesis in the utero-placenta [5,7,34-38]. A study on the distribution of ER and PR protein demonstrated a spatial and temporal distribution of the receptors in the uteroplacenta during early pregnancy in sheep [133]. Immunostaining of ewe uteroplacenta from early pregnancy indicated decreased expression of ER and PR in luminal epithelium and endometrial glands as pregnancy advanced. However, a sustained or transient increase in ER and PR was observed in deep glands, deep stroma and myometrium, which was inversely correlated to cell proliferation [133]. Another study demonstrated that in pregnant ewes endometrial ER mRNA decreased between days 11-15

but increased slightly between days 15-25. A differential expression of PR in ovine endometrium has been reported with a decreased expression on day 11 which increased on days 11-17 and decreased again on days 17-25 [134]. The decrease in sex steroid receptors in endometrial epithelium also occurs in humans and seems to be critical for successful pregnancy establishment [39].

Paracrine regulation of sex steroid receptor expression is suggested by the observation that E2 treated, ovariectomized cows had increased expression of ER and PR in endometrium compared to P4 treated cows [135]. Moreover continued PR expression in the luminal epithelium of the uteroplacenta is required for the implantation process, and downregulation of PR is initiated by uterine factors secreted after embryo implantation [136].

Despite their importance, only a few studies have evaluated sex steroid receptor expression in utero-placenta during early pregnancy. Recent data from our laboratories have demonstrated that the day of pregnancy affects the expression of both membrane and nuclear ER and PR. For example in caruncular tissues, mRNA expression of nuclear PR was increased on days 16-18 of pregnancy compared with days 24-30 of pregnancy [137]. Similarly, caruncular expression of nuclear ER α mRNA was greater on days 14 and 16 compared with days 18-30 of pregnancy. In contrast, expression of membrane PR- α mRNA was decreased across all days of pregnancy compared with non-pregnant controls. Interestingly we have also demonstrated the expression of steroid receptors in chorioallantois. For chorioallantois (fetal placenta), expression of ER- α , nuclear PR, and membrane PR was increased between days 18 and 22 compared with days 16 or 24-30 of pregnancy.

Our study was also the first to demonstrate the effects of ART on steroid receptor expression on uteroplacenta across the days of pregnancy [137];(**Table 3**). In caruncular tissues, mRNA expression of nuclear ER- α and nuclear PR was reduced by 60-80% in pregnancies achieved

after transfer of embryos created through IVF and IVA (i.e., parthenotes which have only maternal genome). For chorioallantois, membrane ER (*GPER1*) mRNA was dramatically

Table. 3. Pattern of changes of mRNA expression for steroid receptors in uteroplacental tissues during early pregnancy after natural breeding (days 14 to 30), and after application of ART (day 22) including transfer of embryos created in vivo, in vitro or through oocyte activation (e.g., parthenotes) based on data published by Reynolds et al., (2015).

Receptor	Uteroplacenta from pregnancies after natural breeding		Uteroplacenta from pregnancies after application of ART	
	Maternal	Fetal	Maternal	Fetal
ESR alpha	Greater on day 14 than days 18-30	Greater on day 20 than any other day	Affected by ART	Affected by ART
ESR beta	Lower during early pregnancy than non-pregnant controls	Not expressed	Not affected by ART	Not affected by ART
GPER1	Not changed during early pregnancy	Not changed during early pregnancy	Not affected by ART	Affected by ART
Nuclear PGR	Greater on days 16-18 than 24-40	Greatest on days 20-22	Affected by ART	Not affected by ART
Membrane PGR alpha	Lower during early pregnancy than non-pregnant controls	Greatest on day 20	Not affected by ART	Not affected by ART
Membrane PGR beta	Greatest on days 18-22	Greatest on days 18 and 28-30	Not affected by ART	Not affected by ART
Membrane PGR gamma	Greatest on days 18-20	Greatest on days 20	Not affected by ART	Not affected by ART

increased in chorioallantois; by 18-fold in the embryo transfer group and by 8-fold in the IVF group [138].

iv. Epigenetic Alterations

For sheep, we and others have recently demonstrated that vascularization, expression of several angiogenic factors, receptors for P4 and estrogens, and DNA methyl transferases (**DNMT**), as well as global DNA methylation and markers of growth of utero-placental tissues all were altered early in pregnancy after transfer of embryos obtained through natural mating, IVF or IVA [44, 139]. These observations suggest that changes in DNA methylation or histone modifications observed in embryos of several species (cattle, mice, sheep) after ART [140-144] that continue during the critical period of placentation, probably contribute to poor placental vascularization and function later in pregnancy, ultimately leading to altered fetal growth/development and poor

pregnancy outcomes associated with ART [4, 44]. Such epigenetic defects leading to altered placental gene expression and development have been observed in IVF and especially in SCNT clones not only in sheep but in cattle and mice as well [56]. These defects include altered expression of imprinted genes, which are thought to be critical for normal placental development [139, 145, 146].

2. Conclusions and Future Directions

a. Regulation of placental vascular development/angiogenesis.

Nitric oxide is a major regulator of placental blood flow as well as placental vascular development/angiogenesis (**Fig. 7**) [147, 148]. Expression of NO receptor namely soluble guanylate cyclase (sGC, also known as GUCY1B3) is elevated in uterine arteries during pregnancy [149, 150]. In case of pregnancies with multiple fetuses, circulating levels of NO and its metabolites are elevated compared to that of single fetuses [117]. The basal production of NO

Figure 7. Schematic of angiogenic factors and vasoactive factors thought to regulate utero-placental vascular development and function, as well as potential therapeutic strategies proposed to optimize placental growth, vascular development, and vascular function.

decreases fetoplacental vascular resistance during pregnancy [147]. Moreover NO, regulates uterine blood flow by stimulating the production of VEGF from vascular smooth muscle and capillary pericytes [151]. The sex steroids also play a vital role in placental angiogenesis. In overfed, adolescent ewes, which is a model of compromised pregnancy, decreased systemic estradiol levels during the last half of gestation have been reported [152]Vonnahme et al., unpublished]. Other factors including bradykinin, relaxin, and hydrogen sulfide have been proposed to modulate uterine vascular function [153-155]. However, how these various factors and their mediators are modulated such that vascular development and function of maternal and placental blood flows are optimized remains to be determined and should be an area of intensive study in the future.

b. Potential therapeutic/management approaches to regulating placental vascular development and function.

A hallmark of compromised pregnancies is impaired uteroplacental blood flow and vascularization along with reduced expression and action of vasoactive and angiogenic factors [9]. Hence therapeutic strategies targeting placental blood and vascular development could be used to rescue compromised pregnancies and fetal growth restrictions (**Fig. 6**)[9, 14, 71-73]. Phosphodiesterase 5 (**PDE5A**)-specific inhibitors are one of the best candidates to target, as they enhance the vasodilatory action of NO by inhibiting the breakdown of cGMP, second messenger for NO, causing prolonged relaxation of vascular smooth muscle cells. It is marketed as sildenafil, tadalafil and vardenafil (marketed under the trade names Viagra, Cialis and Levitra, respectively)[9]. Sildenafil has demonstrated increased basal and estrogen-induced uterine blood flow in ovariectomized ewes [156]. On administration of sildenafil, women having growth restricted fetuses exhibited vasorelaxation of myometrial arteries [74].

Various nutraceutical approaches are also proposed for the management of placental function. Arginine, which is the immediate precursor of NO, has also been used as a management approach to regulate placental vascular development. Arginine is metabolized to ornithine, proline, and nitric oxide which perform many physiological functions. Nitric oxide is a vasodilator and angiogenic factor, whereas both ornithine and proline are substrates for placental synthesis of polyamines, which regulates protein synthesis and angiogenesis. Additionally, arginine, leucine, glutamine, and proline activate the mammalian target of rapamycin (mTOR) cell-signaling pathway to enhance protein synthesis and cell proliferation in placentae. Intravenous and oral administrations of Arginine have been suggested as a therapeutic strategy to improve placental blood flow in compromised pregnancies. Dietary supplementation with 0.83% l-arginine to gilts on d 14 to 28 or d 30 to 114 of gestation increased the number and litter birth weight of live-born piglets. In addition, supplementing the gestation diet with 0.4% l-arginine plus 0.6% l-glutamine enhanced the efficiency of nutrient utilization, reduced variation in piglet birth weight, and increased litter birth weight [157]. In gilts, dietary supplementation with 1% Arg-HCl between days 30-114 of gestation increased fetal survival [158]. In addition, supplementing 1-3% Arg-HCl to diet for female rats either throughout entire pregnancy (21 days) or between 1-7 days of gestation increased embryonic survival and birth litter size by 30% [159]. Feeding Arg-free diets to pregnant rats resulted in increased fetal resorptions, IUGR, increased perinatal mortality and decreased number of live fetuses [160]. Dietary Arg supplementation (0.2 or 2% Arg in drinking water) prevented hypoxia-induced fetal growth retardation in rats [161]. During early gestation, increased levels of arginine have been reported in porcine allantoic fluid (a reservoir of nutrients) which is synchronized with rapid placental growth [157]. In humans, Arginine decreased the expression of Bax and enhanced the expression of bcl-2, which may be associated with reduced placental apoptosis and improved placental

function and fetal development [162]. Similarly, IV administration of Arg increased fetal growth in ovine models of both undernutrition-induced and naturally-occurring IUGR [78]. Arginine treatment of ewes between days 100 and 121 increased percentage of live birth and also fetal body weight [79]. Intravenous maternal L-arginine administration to twin-bearing ewes during late pregnancy had a greater proportion of more type B placentomes, which possess increased placental vascularity and increased placental nutrient transport capacity [163]. In dairy heifers, Arg supplementation between early to mid-pregnancy reduced uterine blood flow, but did not change other parameters that could influence placental and fetal growth such as heart rate, maternal concentration of plasma AAs, or P4 synthesis; furthermore, infusion sites (blood vs. peritoneum) of Arg render different metabolic responses [164]. In a rat model of preeclampsia induced by chronic inhibition of NO synthesis, intravenous administration of Arg (0.16 g/kg body wt/day) from gestational day 10 through term, reversed the disease-associated symptoms such as hypertension, IUGR, proteinuria, and renal glomerulus injury [165]. Oral administration of Arg (3 g daily for 4 weeks) beginning at 29 weeks of gestation to women with preeclampsia increased NO synthesis, reduced blood pressure, prolonged pregnancy, improved fetal wellbeing and enhanced fetal growth [166]. Also, administration of Arg (20 g/day intravenously for 5 days followed by 4 g/day orally for 2 weeks) to women with gestational hypertension prolonged pregnancy, reduced blood pressure, and decreased the frequency of babies with low birth weights [167]. Similarly, on oral administration of Arg (3 g/day for 7 days) to women with threatened preterm labor increased pulsatility indexes of middle cerebral arteries and altered fetal-placental blood flow distribution [168].

Citrulline is a precursor of arginine and the substrate for NO synthesis via any of the various NOS [169]. Maternal undernutrition from day 28 to day 78 of gestation in sheep was associated with 23-30% decrease in arginine and citrulline in maternal plasma, fetal plasma, and allantoic

fluid with concomitant fetal growth retardation [9, 170]. Further, underfed ewes demonstrated 32-36% decrease in the concentrations of biopterin (an indicator of de novo synthesis of tetrahydrobiopterin [BH₄], which is an essential cofactor for NOS) in fetal plasma, and amniotic and allantoic fluids (G. Wu, unpublished results, as referenced in [9]) indicating reduced bioavailability of BH₄ for NO production in the conceptus. These affects the fetoplacental NO synthesis and uterine blood flow [170, 171].

Another nutraceutical approach to be considered is that of folate as folate concentrations also affect placental development. Lower folate concentration is associated with lower placental weight and birthweight and increased risk of small for gestational age (SGA) fetuses, prematurity and pre-eclampsia [172]. In humans, folic acid increased vascular density in cultured explants at gestational age 7 week as determined by anti-CD31 immunostaining. Furthermore, folic acid increased vascular density as determined by anti-CD31 immunostaining in cultured placental explants at gestational age 7 week [173]. Dietary N-carbamylglutamate (NCG) is also known to affect reproductive performance. 0.05% NCG supplemented gilts reproduced piglets which had higher birth weight than the control pigs and increased arginine and ornithine in placenta. They also demonstrated increased expression of angiogenic factors such as eNOS, VEGF-A, PlGF and ANG-2 in chorioallantois [174]. Polyamines (putrescine, spermidine and spermine) are essential for placental growth and angiogenesis [157]. Concentration of polyamines in placentomes and intercaruncular endometrium in ewes increased on Day 40 of gestation. In ovine allantoic and amniotic fluids, increased polyamines were reported during early (Days 40-60) and late (Days 100-140) of gestation [175].

c. Sex steroid role(s) in conceptus development, including Endocrine Disrupting Compounds (EDCs).

Major sex steroids P4 and E2 besides regulating placental development and function also modulates conceptus development. The concentration of P4 affects embryonic survival during early pregnancy in cattle [176, 177]. Moreover, P4 advanced elongation of the blastocyst to a filamentous conceptus on Day 12 of pregnancy in ewes, and this effect was blocked by administering RU486, a P4 receptor antagonist [178]. P4 is critical for achieving embryo-maternal synchrony, and exogenous administration of P4 in fresh IVF cycles improves pregnancy outcomes [179, 180]. The current dogma is that the sex steroids, including P4, affect conceptus development indirectly by acting on the endometrium to modulate the endometrial transcriptome/proteome [181-183], thereby ‘customizing’ histotrophic secretions [50, 184], increasing endometrial receptivity and stimulating secretion of pregnancy recognition signals (interferon tau), cytokines(GM-CSF), and growth factors from the conceptus [49, 185-188]. This dogma is supported by the observation that in vitro exposure of bovine embryos to P4 did not affect the proportion of embryos developing to blastocyst stage, blastocyst cell numbers or gene expression. Furthermore, in vitro exposure to P4 did not affect embryo elongation after transfer to synchronized recipients and recovery on day 14 in cows. However, transfer of in vitro-derived embryos to a P4-primed uterine environment resulted in a four-fold increase in conceptus length on Day 14 [189]. In addition E2 is secreted by pig conceptus on days 10 to15 of pregnancy which is essential for the establishment of pregnancy [190]. Increased histotrophic secretions is reported after release of E2 from conceptus on day 11 of pregnancy in pigs [191]. Placental also acts in a paracrine manner on the endometrial epithelium and upregulates the expression of specific growth factors, including IGF-1 and FGF-7 which in turn act on trophoectoderm to stimulate cell proliferation and development [192].

On the other hand, increasing evidence suggests the presence of sex steroid receptors on oocytes, fertilized eggs and fetal membranes (FM) of various species including as mice, cows,

pigs, sheep and rhesus macaques [138, 189, 193-198]. A study recently published from our lab has clearly demonstrated mRNA expression of nuclear and membrane E2 and P4 steroid receptors in FM that decreased for the majority of receptors studied as early pregnancy progressed [138]. The differential expression of ER and PR in oocytes and fertilized eggs of mouse which declined at 2-cell stage and reached its lowest level at 5- to 8-cell stage has been reported [194]. ER mRNA was undetectable at morula stage but reappeared at blastocyst stage [194]. PR mRNA was undetectable until the blastocyst stage [193-195]. Presence of genomic and non-genomic PRs were detected in bovine cumulus oocyte complexes which increased significantly in cumulus cells and decreased in oocytes after in vitro maturation [189, 198]. PRs have also been detected in embryos or FM from pig and rhesus macaques [196, 197]. In human FM, P4 receptor (PR)-A and B are expressed in the cytoplasm of amniotic epithelial cells, trophoblast, and uterine decidual cells, and PR-C is expressed in human amnion [199, 200]. However the physiological relevance of the sex steroids on FM requires future studies.

One of the accepted postulates in mammalian reproduction is that pregnancy requires the unique support of P4 and that conceptus development is largely dependent on P4 actions on the uterus. Recently, [201] reported the paradigm-shifting observation that 5 α -dihydroprogesterone (**DHP**), a progesterone metabolite, is a biopotent endogenous P4 receptor agonist and can support pregnancy establishment in mares in the absence of P4. Evidence of pregnancies carried to term in the presence of extremely low levels of P4 have been reported in humans with congenital hypobetalipoproteinemia [202], pregnant elephants [203] and rock hyrax [204]. Evidence of increased levels of alternative steroids/steroid conjugates such as 5 α -reduced steroids including DHP and allopregnenolone, pregnenolone, and pregnenolone isomers in maternal and fetal circulation has been widely reported [205-207]. The non-uniform distribution

of steroid conjugates in amniotic fluid, as well as fetal skin and lungs, indicates specific regulation of fetal development by these steroid conjugates [208, 209].

d. Environmental steroids

In addition, various environmental steroid mimetics have been shown to affect conceptus development across numerous species [210-214]. They are often known as EDCs, which may cross the placental barrier to disrupt conceptus development in humans as well as in animals [215]. EDC contamination of the follicular microenvironment is associated with decreased fertilization rate and diminished competence of oocytes to mature into high quality embryos [216]. Polychlorinated biphenyls (**PCBs**), a type of EDC alter AhR signaling in rabbit blastocysts [217]. PCBs increased force and frequency of uterine contractions in bovine myometrium and affected ovum fertilization and blastocyst implantation in cows [218]. PCBs are also known to affect implantation associated genes, increased VEGF and COX-2 expression and altered placental-embryonal interface [219]. Diethylhexylphthalate (**DEHP**) and its metabolites, which are yet another form of EDC, have been reported to decrease placental expression of PPAR gamma genes and alter trophoblastic differentiation in humans [220]. Researches also have reported transgenerational epigenetic effects of EDC, and some of the genes epigenetically modulated by EDCs are imprinted [221, 222]

Hence, to conclude the maternal environment exerts a profound effect on conceptus development. Future research should be aimed at modulating various factors of the maternal environment to rescue compromised pregnancies and impaired conceptus development.

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