

## **ABSTRACT**

MILES, JEREMY RICHARD. Effects of Embryo Production Systems on Angiogenesis and Development of Bovine Placentas. (Under the direction of Dr. Peter W. Farin)

Placental abnormalities have been reported following the transfer of in vitro-produced (IVP) and cloned (somatic cell nuclear transfer; SCNT) embryos in cattle and sheep. The overall objective of this research was to determine the effects of embryo production systems (IVP and NT) on angiogenesis and development of placentas during early and late gestation in cattle. Angiogenesis was assessed by the expression of vascular endothelial growth factor (VEGF), peroxisome proliferator-activator receptor-gamma (PPAR $\gamma$ ), and vascular morphometry. During late gestation (Day 222), angiogenesis and morphometry of placentas were assessed in placentas from embryos produced in vivo and in vitro using undefined, serum-supplemented medium (IVPS). During early gestation (Day 70), the effects of embryo culture media (undefined, IVPS or semi-defined modified synthetic oviductal fluid, mSOF) on angiogenesis and morphometry of placentas were evaluated. Finally, at Day 40 of gestation angiogenesis and development were compared in placentas from embryos produced in vivo, in vitro using G1.2/G2.2 media, or by SCNT.

The results described in this dissertation demonstrated that angiogenesis as well as development of placentas differed depending on embryo production system and day of gestation. During late gestation, placentas from the IVPS group had decreased percentage of placentome surface area which was associated with increased expression of PPAR $\gamma$  protein and increased blood vessels-to-placentome surface area ratios. These findings suggest that during late gestation compensatory mechanisms exist in the vascular beds of placentas from bovine embryos produced in vitro. In contrast, during early gestation (Day 70) placentas

from the IVPS group developed similar to in vivo controls; whereas placentas from the mSOF group had decreased densities of fetal and maternal blood vessels associated with a decreased expression of VEGF mRNA in cotyledons. At Day 40 of gestation, placentas from embryos produced using G1.2/G2.2 media appeared to have more compromised vascular development compared with placentas from embryos produced in vivo or by SCNT.

**EFFECTS OF EMBRYO PRODUCTION SYSTEMS ON ANGIOGENESIS AND  
DEVELOPMENT OF BOVINE PLACENTAS**

by

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## INTRODUCTION

In vitro production (IVP) and somatic cell nuclear transfer (SCNT; cloning) of embryos are important biotechnologies used for the improvement of herd genetics, development of transgenic animals, and conservation of endangered species. Normal offspring have resulted from IVP and SCNT embryos; however, developmental anomalies have also been observed following transfer of these embryos. These anomalies have ranged from an increased incidence of pregnancy loss [1-5] to abnormalities associated with the fetus [3, 6, 7], placenta [2, 4-6, 8], and offspring [4, 9], commonly referred to as Large Offspring Syndrome (LOS) [10, 11].

A variety of placental abnormalities have been reported following the transfer of IVP and SCNT embryos in cattle and sheep. Abnormalities associated with IVP embryos have included an increased incidence of hydrallantois [4, 6, 12], decreased number of placentomes [3], variations in feto-maternal contact [8, 13, 14], and allantoic malformations [15]. The production of embryos by SCNT has resulted in severe placental abnormalities including avascular development [2, 5], limited development of cotyledonary tissue [5, 16-18], placentomegaly [7], and hydrallantois [9, 19]. Inadequate development of the placental vasculature has been suggested as a primary cause for the increased pregnancy failure observed with cloned embryos [2, 5]. However, the influence of these embryo production systems on angiogenesis and development of placentas at different stages of gestation is poorly understood.

Proper development of placental vasculature is essential for adequate transfer of nutrients and exchange of gases and wastes between the mother and fetus [20]. Following the initial formation of blood vessels (vasculogenesis), extensive growth of the vasculature is

controlled by branching and nonbranching angiogenesis [21, 22]. Molecular regulation of angiogenesis in the placenta is driven by angiogenic factors such as vascular endothelial growth factor (VEGF) [20, 22] and potentially peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) [23, 24]. Alterations in the expression of VEGF and/or PPAR $\gamma$  may play an important role in placental abnormalities associated with IVP and SCNT embryos.

The overall objectives of this research were to determine the effects of embryo production systems (IVP and NT) on angiogenesis, as assessed by the expression of VEGF and PPAR $\gamma$ , and the development of placentas during early and late gestation in cattle. During late gestation (Day 222), placental angiogenesis and morphometry were assessed in placentas from embryos produced in vivo or in vitro using undefined, serum-supplemented medium (IVPS). At Day 70 of gestation, angiogenesis and morphometry were evaluated in placentas produced in vivo or in vitro using either undefined IVPS or semi-defined modified synthetic oviductal fluid. Finally, angiogenesis and development of placentas were compared at Day 40 of gestation from embryos produced in vivo, in vitro using G1.2/G2.2 media or by NT. We hypothesized that, within day of gestation, differences would be observed in angiogenesis and development of placentas from embryos produced in vitro or by NT compared with in vivo controls.



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## LITERATURE REVIEW

This review will address the procedures used for the production of bovine embryos, normal placental development, and abnormalities associated with in vitro-produced (IVP) and cloned embryos.

### IN VITRO PRODUCTION OF EMBRYOS

In vitro systems have been used to produce embryos for animal breeding, biotechnology, assisted reproduction, and research [1]. Embryo production in vitro includes the following three steps: in vitro oocyte maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC).

#### *In Vitro Oocyte Maturation*

Synchronization of nuclear and cytoplasmic maturation in oocytes is essential for successful fertilization and subsequent embryonic development [2]. Nuclear maturation of the oocyte includes germinal vesicle breakdown, chromatin condensation, spindle formation, and chromosome segregation [3]. The critical process of cytoplasmic maturation involves organelle redistribution, synthesis of new proteins, maturation of calcium-release mechanisms, and the activation of a series of kinases [2, 4].

The efficiency of development of in vitro-matured oocytes to the blastocyst stage is low compared with their in vivo-matured counterparts [5-7]. For example, Rizos et al. [7] demonstrated that bovine blastocyst development rates following IVF and IVC were lower for oocytes matured in vitro (38.9%) compared with those matured in vivo (58.2%). The low efficiency of in vitro maturation may be due to several factors. For instance, oocytes matured in vivo undergo nuclear and cytoplasmic changes that are driven by follicular

factors, many of which are still unknown [8]. Differences in cytoplasmic maturation rather than nuclear maturation may be responsible for the lower efficiency of in vitro-matured oocytes to develop to the blastocyst stage [6]. These observations have been supported by the fact that mammalian oocytes spontaneously undergo meiotic maturation in vitro when removed from antral follicles and exposed to simple media [9]. Because cytoplasmic maturation appears to be a key factor in determining the efficiency of in vitro maturation of oocytes, many different maturation media have been developed and supplemented with hormones [10], growth factors [11-13], serum [14], follicular fluid [15], and co-cultured cells [16] in an attempt to enhance cytoplasmic maturation. The low rates of blastocyst development for IVM oocytes may also be attributed to the fact that 99.9% of ovarian follicles are destined to become atretic [17]. Therefore, selection of follicles that are destined to become atretic may decrease the efficiency of in vitro maturation of oocytes.

Currently, many laboratories employ the maturation medium first introduced by Moor et al. [18], namely TCM-199 supplemented with fetal calf serum, estradiol ( $E_2$ ), follicle stimulating hormone (FSH), and luteinizing hormone (LH) [6]. However, concerns exist regarding the use of serum in maturation media because of the inherent undefined properties of serum [19]. Several laboratories have reported successful development of bovine embryos from oocytes matured in serum-free conditions [4, 11, 20-22]. Supplementation of serum-free maturation media with  $E_2$ , FSH, and LH [22] as well as epidermal growth factor (EGF) [12] has increased blastocyst development rates.

Several investigators have evaluated the effects of source of oocytes (abattoir *versus* transvaginal oocyte retrieval) [23], size of follicles [24-26], and grade of the cumulus-oocyte complexes [24, 27, 28] on development rates of blastocysts. Based on these investigations it

has been suggested that embryogenic competence of oocytes is acquired when follicles reach a diameter of greater than 2 mm [23]. Yang et al. [26] found that oocytes aspirated from medium (2-5 mm) and large (5-8) follicles had a significantly greater percentage of oocytes matured, cleaved, and developed to blastocysts compared with oocytes obtained from small follicles (1-2 mm). Most laboratories performing routine embryo production utilize oocytes from follicles 1 to 8 mm in diameter [26].

The integrity of the cumulus cells surrounding the oocyte has been related to the health of the follicle [29]. Cumulus-oocyte complexes with several layers of cumulus cells have greater development rates to the blastocyst stage [27, 28]. Blondin and Sirard [24] have also investigated the developmental potential of oocytes classified into one of six classes based on the appearance of their cumulus and ooplasm. These authors reported that oocytes of the best quality (classes 1 and 2) demonstrated high developmental competence as assessed by percent morula after 5 days of in vitro culture [24]. Interestingly, the most competent oocytes were from intermediate (class 3) oocytes that were likely from atretic follicles. These authors concluded that class 3 oocytes possessed the greatest developmental competence because they were in later phases of follicular growth, and thus had been subjected to a longer period of follicular microenvironment, and consequently were more differentiated than other classes of oocytes [24].

### ***In Vitro Fertilization***

Mammalian sperm cells must undergo capacitation and the acrosome reaction to permit fertilization of oocytes [30]. These two linked processes include an alteration of the sperm membranes (capacitation) [31, 32] and fusion of the plasma membrane with the outer acrosomal membrane (acrosome reaction). Parrish et al. [30] had demonstrated that the glycosaminoglycan, heparin, is a potent activator of capacitation in vitro. The incubation of bull sperm with heparin increased the acrosome reaction from 10% to 70% [30]. As a result, IVF medium commonly contains heparin to induce sperm capacitation and the acrosome reaction.

Preparation of thawed frozen spermatozoa prior to fertilization is necessary for removal of seminal plasma components and cyroprotectant [33]. Preparation can be performed using a Percoll density gradient [34], centrifugation [30] or the swim-up procedure into overlayed medium [35]. Sperm cells are incubated with in vitro-matured oocytes at various concentrations and lengths of time. The duration of gamete co-incubation and sperm cell concentration are important factors affecting the efficiencies of IVF [17]. Most laboratories use sperm concentrations of  $1 \times 10^6$  spermatozoa/ml of fertilization medium and a fertilization period of 20 hours postinsemination (hpi). Ward et al. [17] had demonstrated that gamete co-incubation for 10 to 20 hpi was sufficient to ensure maximum blastocyst yield. Sperm concentrations of  $0.25$  to  $1.0 \times 10^6$  spermatozoa/ml of medium have produced maximum blastocyst yield [17, 36].

The use of different sires [37], different lots of semen as well as different straws from a single bull [38] may also affect the efficiency of IVP. The presence of cumulus cells at the time of insemination facilitated fertilization of bovine oocytes and resulted in a higher yield

of blastocysts [39, 40]. It has been suggested that cumulus cells are associated with the interactions of male and female gametes inducing capacitation [41] and the acrosome reaction [42] as well as preventing premature hardening of the zona pellucida [43].

### ***In Vitro Culture***

Following fertilization of oocytes, presumptive zygotes are cultured for an extended period of time. The duration of postinsemination culture varies between species. In ruminants, embryo culture extends to the blastocyst stage ranging from 144-168 hours postinsemination (hpi) for sheep [20, 33, 44] to 168-192 hpi for cattle [33, 45]. In humans, embryos typically remain in culture for 48 to 72 hpi or until the eight-cell stage of development before transfer into a surrogate [46]. In mice, embryos are commonly cultured to the blastocyst stage which occurs about 96 hpi [47].

Culture media are complex mixtures of nutrients, macromolecules, and growth factors ranging from undefined (co-culture and/or serum supplementation), to semi-defined (bovine serum albumin [BSA] supplementation) and to chemically defined culture media [48, 49]. In the 1970's, Tervit et al. [50] demonstrated that bovine blastocysts could be produced using a medium based on the biochemical composition of sheep oviductal fluid which was referred to as synthetic oviductal fluid (SOF). This simple medium resulted in arrested embryo development, decreased embryo metabolism, and decreased viability of embryos [51]. To improve embryo development, more complex media such as TCM-199 and Menezo B2 were used in combination with co-culture of feeder cells [51, 52]. Many of these media were also supplemented with a protein source (e.g. serum and/or BSA) and energy sources (e.g. glucose, pyruvate, and lactate) [51]. Somatic cells used as feeder cells have included epithelial cells from oviducts, cumulus and granulosa cells, Buffalo Rat Liver cells, and Vero



cells [53]. These cells appeared to help the embryo overcome the 8- to 16- cell block observed when embryos were cultured in simple medium [54]. Feeder cells may also provide many embryotropic factors and remove toxic components from the medium [51, 55]. However, the use of co-culture systems can result in the introduction of pathogens such as mycoplasma, viruses, and prions [51, 56].

Protein supplementation of culture medium with serum may provide beneficial effects on the development of embryos [54]. However, serum has an undefined composition and contains a variety of macromolecules and growth factors [57]. Variation in the composition of serum may contribute to differences in results from IVP [51, 54]. Some batches of serum also result in biphasic embryo development [58]. Initially, serum may inhibit cleavage but subsequently serum can stimulate development to the morula and blastocyst stage [58]. Furthermore, developmental abnormalities of fetuses resulting from IVP embryos has been attributed to the presence of serum in culture medium [59, 60]. Bovine embryos have successfully been produced using either serum-free or serum-restricted media during the early period of culture [61-66]. Defined systems have been developed in an attempt to eliminate the problems associated with undefined systems using co-culture and/or serum supplemented media [60, 67].

An alternative to serum as a source of protein in embryo culture media is BSA. Culture media supplemented with BSA are referred as semi-defined media because they have a known chemical composition, except BSA [51, 68]. Examples of semi-defined media include SOF, CR1aa, CR2, and KSOM [51, 68]. Bovine serum albumin has been associated with beneficial effects on embryo development because of its ligand binding properties which protect embryos against the buildup of toxic components in media [54]. In addition,

BSA serves as a low-affinity, high capacity reservoir for beneficial components such as steroids, vitamins, fatty acids, and cholesterol [61]. The composition of BSA can also vary from lot to lot resulting in inconsistencies in embryo development [51, 54]. Bovine serum albumin may also have stimulatory or inhibitory effects on embryonic development [66, 69]. Because semi-defined media are cell- and serum-free, the risk of pathogen contamination is usually lower for undefined systems [51]. Furthermore, several investigators have demonstrated that developmental abnormalities of the fetus were lower following transfer of embryos produced using semi-defined media compared with undefined media using co-culture with or without serum [70-72].

Completely defined culture systems have been developed to study the nutrient requirements needed for optimal embryo production in vitro [66, 69]. Defined media are often supplemented with specific substances including essential and nonessential amino acids, growth factor(s), glutathione, glucose, pyruvate, polyvinylalcohol, hyaluronate, and EDTA [51, 65, 66, 73, 74]. Compared with undefined and semi-defined media, chemically defined media have several advantages including standardization of culture conditions and components between laboratories and limited risk of pathogen contamination [51]. Furthermore, the culture of embryos in chemically defined media has apparently resulted in a decreased occurrence of neonatal abnormalities associated with embryos produced using undefined media [75, 76]. However, the culture of embryos in chemically defined media often results in lower blastocyst development rates compared with embryos cultured in undefined or semi-defined media [58, 62, 66]. In addition, lower calving rates have been observed for embryos produced using defined media (38%) compared with embryos produced using undefined media (53%) [75, 76].

Carbohydrate and amino acid requirements change throughout development of the embryo. Prior to compaction, the ruminant embryo exhibits low levels of oxidative metabolism and oxygen consumption [77]. High concentrations of glucose have a detrimental effect on early embryo development when the capacity of the embryo to utilize glucose is limited prior to compaction [78, 79]. However, compact morula and blastocysts exhibit high levels of glycolysis and oxygen consumption [77] and increased levels of glucose are needed after compaction to support optimal development. Consequently, the predominant energy source utilized by the precompacted embryo is pyruvate and lactate alone or in combination with amino acids [80]; whereas glucose serves as the primary energy source post-compaction. The amino acid requirements of embryos differ throughout embryo development. Gardner and Lane [81] found that embryo development prior to the compact morula stage was accelerated in the presence of nonessential amino acids and glutamine; however, following compaction embryo development was enhanced by the inclusion of all 20 amino acids [73]. As a result, sequential embryo media systems have been developed to provide the appropriate amounts of protein and energy substrates for different stages of embryonic development [51]. Sequential media systems have been shown to enhance bovine embryo development compared with single semi- or chemically-defined media system [82]. For example, the sequential media system G1.2/G2.2 has recently been shown to result in blastocyst and pregnancy rates equivalent to those of undefined media with BRL cell co-culture [67].

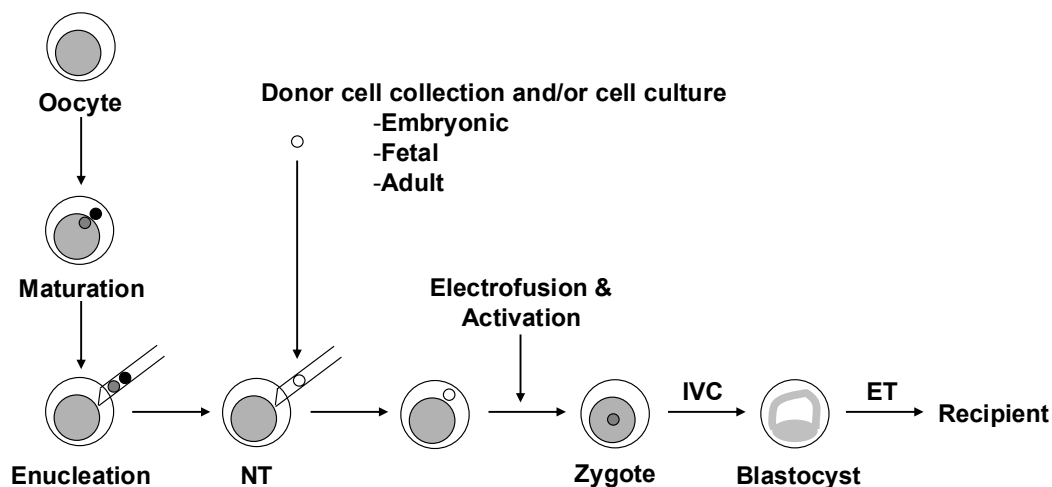
## NUCLEAR TRANSFER OF EMBRYOS

Nuclear transfer (NT) technology has been used for the improvement of animal genetics [83], the production of transgenic domestic animals [84, 85], and the conservation of rare and endangered animals [86]. Cloned offspring are genetically identical and they have been successfully produced using NT of donor cells types ranging from embryonic blastomeres to adult somatic cells [87]. The application of NT in cattle has included the generation of transgenic animals [88, 89], production of cloned bulls for use in the AI industry [90], genetic salvage of rare breeds [91], and production of interspecies clones [92].

The transfer of somatic cell nuclear transfer (SCNT) embryos has resulted in live births of mice [93], cattle [94], sheep [95], goats [96], pigs [97], rabbits [98], and cats [99]. In addition, transgenic sheep [88], cattle [100], goats [101], and pigs [89] have been produced using SCNT of genetically modified somatic donor cells. Although cloned offspring have successfully been produced by SCNT in a variety of mammalian species, the efficiency of SCNT for production of live, cloned offspring remains low. For example, blastocyst development rates for SCNT protocols in cattle vary from 18 to 43% for fetal donor cells and from 9 to 49% for adult donor cells [102]. The variation in blastocyst rates seen with SCNT is thought to be due to several factors including karyotype of donor cell, cell cycle stage of donor cells, and in vitro culture conditions [102-104]. There is also a high incidence of pregnancy loss and developmental abnormalities of the fetus, placenta, and offspring [104, 105].

Procedures involved in nuclear transfer of mammalian embryos are illustrated in Figure 1 and discussed in detail in several reviews [104-107]. Briefly, donor cells from which the cloned offspring will be generated are isolated from embryonic, fetal, or adult

sources. Oocytes are matured in vitro to the metaphase II stage using maturation techniques similar to those used for in vitro production of embryos. The metaphase II plate which contains the polar body and maternal nuclear DNA is removed (enucleated) from the oocyte. A donor cell is transferred into the enucleated oocyte. The donor cell/oocyte couplet is fused via electrical pulses and activated chemically with  $\text{Ca}^{2+}$  (ionomycin) to initiate embryonic development. Zygotes are then cultured in vitro to the blastocyst stage in similar culture systems previously described for the production of embryos in vitro. Finally, blastocysts are transferred to surrogate recipient animals.



**Figure 1.** Diagram depicting basic nuclear transfer procedures used to produce cloned offspring. Abbreviations: NT, nuclear transfer; IVC, in vitro culture; ET, embryo transfer. Adapted from Edwards et al. [104].

## IN VIVO PRODUCTION OF EMBRYOS

Multiple ovulation embryo transfer (MOET) is a powerful technique for maximizing the reproductive potential of breeding females [108]. In addition, MOET is useful for acquiring a large number of embryos for research purposes. The development of adequate in

vivo control embryos is essential for evaluation of fetuses, placentas, and offspring resulting from IVP and cloned embryos. Reproductive breeding programs routinely used for production of in vivo controls include artificial insemination and MOET.

Superovulation with follicle stimulating hormone (FSH) maximizes the number of transferable embryos per cow [109]. Several protocols are available for superovulation in cattle [110]. For instance, cows can be given FSH treatment for four days between the 9<sup>th</sup> and 13<sup>th</sup> day of estrus with prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ) given on the third day of treatment [110]. Another protocol that allows for cows to be superovulated on any day of their estrous cycle includes the use of progesterone implants and FSH [110]. In this protocol, a controlled internal drug releasing device (CIDR) containing progesterone is inserted on the first day of treatment in conjunction with an injection of Estradiol-17 $\beta$ . Starting on the fourth day of treatment, cows receive FSH daily for four days. On the third day of treatment, the CIDR is removed and PGF<sub>2</sub> $\alpha$  is administered. For both of these protocols, approximately 48 h after the first PGF<sub>2</sub> $\alpha$  injection, estrus is detected and the donor cow is artificially inseminated two times. Seven days after estrus, the embryos are recovered from the donor cow, evaluated for stage of development and grade, and then frozen or transferred into synchronized recipients [110].

The limiting obstacle for obtaining the maximum number of embryos from donor cows is the variability in response to superovulation treatment [108, 110-112]. The number of embryos recovered per cow is highly variable between animals but averages about 5 transferable embryos per cow [108, 111, 112]. Furthermore, about 20% of cows do not respond to superovulation treatment and response may decrease with subsequent FSH

treatments [108, 112]. The variability observed in response to superovulation treatment may be influenced by age, breed, lactational status, and nutritional status of the donor as well as season of treatment [110].

## **NORMAL EMBRYONIC AND FETAL DEVELOPMENT**

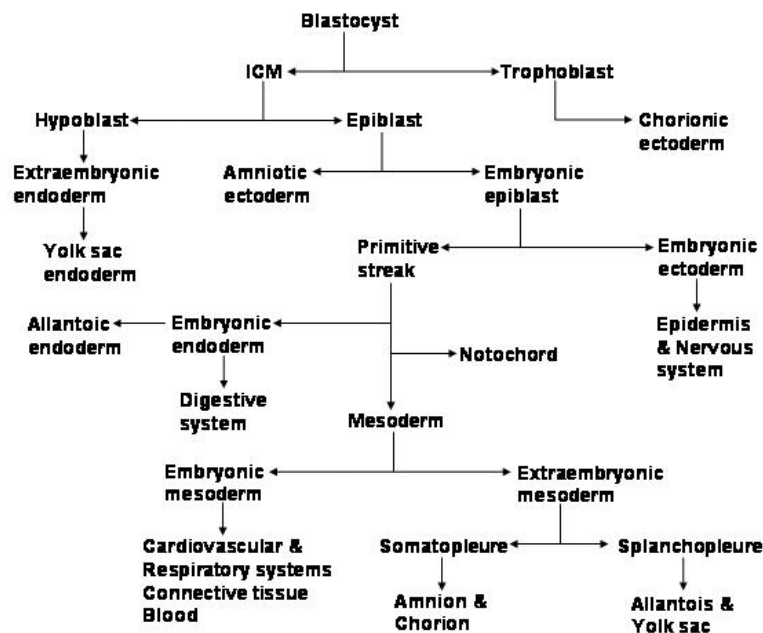
### ***Embryonic Period***

The establishment of pregnancy is influenced by proper development of the embryo during early stages of gestation. In the cow, the embryonic stage of development extends from conception to completion of organogenesis (~ Day 42 of gestation) [113], during which time the embryo undergoes a series of morphological and biochemical changes.

Following fertilization, the zygote undergoes a series of mitotic divisions called cleavage [114]. The initial cleavage results in two undifferentiated cells or blastomeres and takes approximately 30 hours postinsemination (pi) in the cow [115]. At approximately 4 days pi, the embryo has divided into 16 blastomeres and is referred to as a morula [115]. At approximately 5 days pi (~ 32-cell embryo), the cells on the outer portion of the embryo undergo compaction [114]. At the compact morula stage, two distinct populations of cells arise in the embryo, the inner and outer cells. The inner cells form gap junctions which allow for intercellular communication and clustering. The outer cells develop tight junctions that allow for fluid accumulation within the embryo [114]. The accumulation of fluid results in the formation of the fluid-filled cavity, or blastocoele. The embryo is now referred to as a blastocyst (~ 7 days pi in the cow) [115]. The blastocyst is divided into two populations of cells, the inner cell mass (ICM) and the trophectoderm or trophoblast [116]. The ICM

eventually gives rise to the embryo proper and portions of the extraembryonic membranes, whereas the trophoblast becomes the chorionic ectoderm [114].

In the cow, hatching of the blastocyst from the zona pellucida occurs between Day 8 to 11 postestrus [114]. Ruminant embryos undergo a logarithmic elongation phase during which time the embryo is transformed from a spherical shape (Day 13 pi in cattle) to a filamentous thread-like shape (Day 17 pi in cattle) [115]. During this period of elongation, the embryo undergoes extensive cellular rearrangements, referred to as gastrulation, that results in the formation of the following three germ layers: 1) the ectoderm which becomes the epidermis and nervous system, 2) the mesoderm which forms the cardiovascular and respiratory organs, connective tissues, and blood cells, and 3) the endoderm which give rise to the digestive tube and its associated organs [117]. These germ layers also contribute to the extraembryonic membranes of the placenta. Figure 2 shows the derivations of early mammalian tissues and membranes.



**Figure 2.** Schematic diagram showing the origin of tissues in the mammalian embryo. Adapted from Noden and De Lahunta [134].



During the elongation period, mononucleate cells of the trophoblast synthesize and secrete interferon-tau (IFN $\tau$ ) between Day 13 to 21 of gestation in cattle [118]. Interferon- $\tau$  signals maternal recognition of pregnancy (MRP) by blocking the secretion of PGF $_2\alpha$ , thereby preventing luteolysis [119]. Maternal recognition of pregnancy is critical for pregnancy to continue and occurs between Day 15 and 16 of gestation in the cow [114]. Following successful MRP, the bovine embryo begins the process of implantation into the maternal endometrium. Implantation begins with apposition and attachment on about Day 20 of gestation and is complete by Day 40 of gestation [120].

### ***Fetal Period***

Following successful implantation and completion of organogenesis (~ Day 42 of gestation in cattle), the fetal stage of development begins and continues to parturition [113]. This period is characterized by extensive growth of the fetus resulting in maturation of organs and the musculo-skeletal system [121]. The bovine fetus grows at an exponential rate throughout gestation with the maximal gain in fetal weight occurring between Day 230 and Day 240 of gestation [122, 123]. Fetal weight continues to increase between Day 240 of gestation to parturition (~ Day 278 to 290 for the cow [115]), but at a much slower rate [122, 123].

Growth of the bovine fetus is primarily associated with fetal genotype [124]. Other factors shown to influence fetal growth include breed of sire [125] and dam [126], sex of fetus and number of fetuses in utero [126], maternal nutrition, and heat stress [125, 127]. In addition, fetal hormones including growth hormone, insulin and insulin-like growth factor (IGF)-I and -II have been shown to influence fetal growth [127]. Growth of the bovine fetus

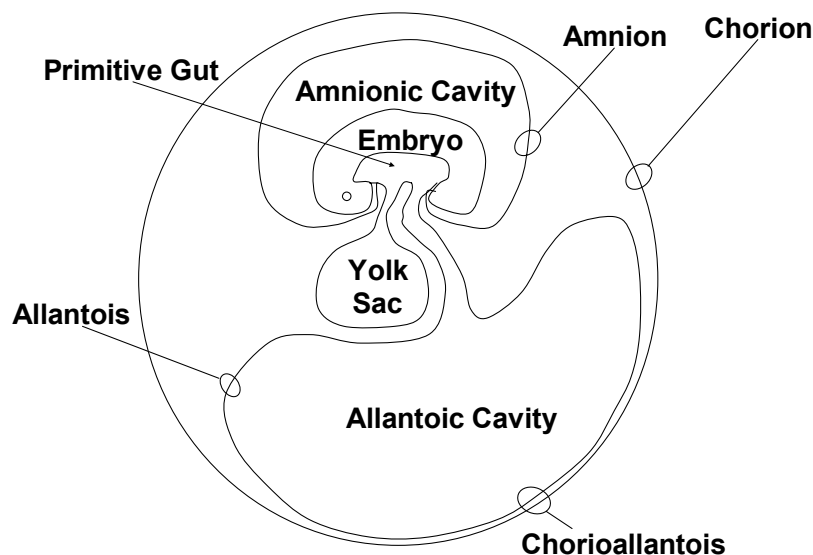
is limited by the placenta and proper utero-placental perfusion [123, 128]. Therefore, proper development of the placenta is critical for growth of the fetus and birth of a viable calf.

## **NORMAL PLACENTAL DEVELOPMENT**

The mammalian placenta develops as a transient organ that is essential for growth and survival of the fetus throughout pregnancy. The placenta serves primarily as an organ of physiological exchange providing for the transport of nutrients, gases, and waste between the fetus and mother [129]. The placenta is also a source of pregnancy-associated hormones and growth factors that play important roles in MRP as well as growth and development of the fetus [130]. In addition, the placenta is involved in immune protection of the fetus [130]. Therefore, failures in development of the placenta have been associated with complications ranging from pregnancy loss to abnormalities of the fetus and offspring [64, 104, 131].

### ***Extraembryonic Membranes***

The mammalian placenta has three developmentally distinct structures which function as organs of exchange between the developing embryo and the uterine endometrium. These placental types are named according to the extraembryonic tissues from which they are derived and include the chorionic, choriovitelline (yolk sac), and chorioallantoic placentas (Fig. 3; [132]).



**Figure 3.** Illustration of the extraembryonic membranes in eutherian mammals. Adapted from Faber [132].

The chorionic placenta is the functional exchange component of the early embryo. The chorion is derived from the trophoblast and nonvascular, somatopleure or somatic mesoderm (Fig. 2; [133, 134]). At approximately Day 13 of gestation in the cow, the amnion, composed of ectoderm and somatic mesoderm (Fig. 2), begins to fuse dorsally and eventually completely encloses the embryo in the amnionic cavity by Day 18 of gestation [134].

The chorionic placenta is then replaced by either the choriovitelline (yolk sac) or chorioallantois as the primary organ for exchange between the fetus and mother during later stages of pregnancy [132]. The yolk sac forms from primitive endoderm of the hypoblast and vascular, splancholeure or splancholeure mesoderm (Fig. 2) [134]. In ruminants, the yolk sac remains rudimentary and it is not used for exchange purposes. In most eutherian mammals, the yolk sac serves as the original source of primordial and haematopoietic stem cells [114, 135]. The yolk sac becomes fully vascularized by the end of embryonic

elongation in the cow [136]. However, the existence of the yolk sac is only transient and it is shortly displaced by the elongation of the allantoic sac [134, 136].

The allantois develops between Day 14 and 21 of gestation in cattle as an outgrowth of the embryonic hindgut (i.e. endoderm) and the vascular, splanchnopleure mesoderm (Fig. 2) [134]. The allantoic cavity serves as a site for waste deposition (i.e. urine) especially during late gestation [136]. As the allantois develops, it fuses with the avascular chorion resulting in the formation of the vascularized chorioallantois [134]. The splanchnopleure mesoderm of the allantois provides the vascular properties of the chorioallantois and also gives rise to the umbilical cord vessels [132]. Fusion of the chorioallantois usually begins around Day 23 of gestation in cattle [137]. The chorioallantois provides the major source of exchange for most eutherian mammals including ruminants. A distinguishing feature of the chorioallantois is the increased surface area at the fetomaternal junction [132]. Surface area of the chorioallantois is increased by the formation of chorionic villi that penetrate into the uterine endometrial surface, thereby, allowing fetal blood vessels to be in close proximity to the maternal blood vessels [127].

### ***Classification of the Chorioallantoic Placenta***

Because of species variation in the organization of the chorioallantois, chorioallantoic placentas are commonly classified according to gross morphology, histological morphology, and degree of tissue loss at birth [127, 136]. In ruminants, the gross morphology of the chorioallantoic placenta is classified as cotyledonary [138]. In cotyledonary placentas, chorionic villi of the cotyledon interdigitate with vascular foldings of the endometrial surface referred to as maternal crypts or caruncles [127]. In the cow, penetration of a fetal cotyledon within the convex, shaped maternal caruncle results in the formation of the placentome that

serves as the functional unit of physiological exchange between the fetus and mother [138].

The number of placentomes in the cow typically ranges from 70 to 120 [127].

Chorioallantoic placentas can also be classified histologically according to the number of tissue layers that separate maternal and fetal blood vessels [127, 136]. There is controversy regarding the exact histological classification of the ruminant placenta. Initially, the ruminant placenta was defined as syndesmochorial because of observations of intermittent loss of uterine epithelium which resulted in contact between the chorionic epithelium and uterine connective tissue [136]. Because these observations could not be confirmed by electron microscopy, the ruminant placenta was commonly classified as epitheliochorial placenta which forms the least intimate contact between the six tissue layers separating maternal and fetal blood vessels [127, 136, 138]. The ruminant placenta is more accurately classified histologically as a synepitheliochorial placenta because the binucleate cells of the chorionic epithelium migrate into and fuse with uterine epithelial cells resulting in a hybrid feto-maternal syncytium [136, 139].

Finally, chorioallantoic placentas may also be classified according to the degree of tissue lost at parturition [127, 136]. The ruminant placenta is predominantly classified as a nondeciduate because it loses no maternal tissue during parturition [127, 136]. Interestingly, placentas of ruminant animals may have some degree of decidual reaction because the maternal crypts of the caruncle undergo necrosis and maternal epithelial cells slough approximately 7 to 10 Days postpartum [138].

### ***Molecular Regulation of Placental Development***

The majority of the information regarding the molecular control of placental development has resulted from experiments with knockout mice [140]. Many of these same genes identified in the mouse have also been identified in uterine, placental, and embryonic tissues of ruminants (Table 1).

### ***Trophoblast Differentiation***

In ruminants, cells of the trophoblast include mononucleate and binucleate cells [140, 141]. As previously discussed, mononucleate cells of the trophoblast produce and secrete IFN $\tau$  to signal MRP during embryonic elongation in ruminants (Table 1) [118]. These mononucleate cells also provide the majority of the chorionic interface between the fetus and mother for nutrient exchange throughout gestation [141]. A proportion of mononucleate cells undergo nuclear divisions without cytokinesis resulting in the formation of binucleate cells that constitute approximately one-fifth of the chorionic epithelium [139]. Binucleate cells have been compared with multinucleated cells in other species such as trophoblast giant cells in mice and extravillous cytotrophoblast in humans [140]. These binucleate cells migrate into the maternal crypts and fuse with uterine epithelial cells forming a feto-maternal syncytium [136]. Because of their migratory behavior and secretion of hormones such as placental lactogen and pregnancy-associated glycoproteins, binucleate cells appear to play a key role in implantation and placentome development [136].

**Table 1.** Molecular regulators of placental development and function in ruminants.

<b>Function</b>	<b>Gene products*</b>	<b>Species</b>	<b>Tissue</b>	<b>References</b>
Trophoblast differentiation				
	Interferon-tau	Bovine, Ovine	Mononucleate cell trophoblast	[142]
	MASH2	Bovine	Blastocyst	[143]
	PPAR $\gamma$	Bovine	Blastocyst	[144]
Chorioallantoic fusion				
	DNA methyltransferase-1	Bovine	Blastocyst	[143]
	FGF receptor-2	Bovine Ovine	Blastocyst Uterine epithelium & trophoblast	[145] [146]
Implantation				
	Integrin adhesion molecules	Bovine, Ovine	Uterine epithelium & trophoblast	[147] [148]
	ECM glycoproteins	Bovine, Ovine	Uterine epithelium & trophoblast	[147] [148]
	Placental lactogen	Bovine, Ovine	Binucleate cell trophoblast	[136]
	Pregnancy-associated glycoproteins	Bovine, Ovine	Binucleate cell trophoblast	[136]
	Prolactin-related proteins	Bovine, Ovine	Binucleate cell trophoblast	[136]
Vasculogenesis & Angiogenesis				
	VEGF	Bovine Ovine	Caruncular endometrium Uterine endometrium & placenta	[149] [150]
	VEGF Receptor -1 & -2	Bovine Ovine	Caruncular endometrium Uterine endometrium & placenta	[149] [148]
	FGF-1 & -2	Ovine	Uterine endometrium & placenta	[150]
	FGF receptors	Bovine	Blastocyst	[145]
	Angiopoietin-1 & -2	Bovine Ovine	Caruncular endometrium Uterine endometrium & placenta	[149] [151]
	Angiopoietin receptors	Bovine Ovine	Caruncular endometrium Uterine endometrium & placenta	[149] [151]
	PPAR $\gamma$	Bovine	Blastocyst	[144]

\*Abbreviations: MASH2, mammalian achaeta-scute homologues-2; PPAR $\gamma$ , peroxisome proliferator-activator receptor-gamma; FGF, fibroblast growth factor; ECM, extracellular matrix; VEGF, vascular endothelial growth factor.

The molecular mechanisms regulating trophoblastic differentiation in ruminants are unknown. Studies using knockout mice have shown that mammalian achaeta-scute homologues-2 (MASH2) [152] and peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) [153] are essential for the differentiation of the trophoblast. Placentas from MASH2-deficient mice contained over abundant giant cells [152], suggesting that MASH2 functions to maintain spongiotrophoblast (mononucleate cells) and/or to block differentiation of giant cells [140]. Recently, Wrenzycki et al. [143] reported that MASH2 mRNA was expressed at higher levels in cloned bovine blastocysts compared with IVP blastocysts. Placentas from PPAR $\gamma$ -deficient mice failed to undergo terminal differentiation of the spongiotrophoblast and labyrinthine trophoblast [153]. These PPAR $\gamma$ -deficient mice also had abnormal vascular development of the placenta [153]. In the human placenta, PPAR $\gamma$  dimerizes with retinoic acid receptor- $\alpha$  and regulates differentiation of extravillous cytotrophoblast [154]. Thus, it is possible that MASH2 and PPAR $\gamma$  may also be necessary for trophoblast differentiation in ruminant species.

### ***Chorioallantoic Fusion***

Proper fusion of the chorion with the allantois is essential for adequate development of the chorioallantois. Failures of chorioallantoic fusion have been associated with abnormalities in development of the chorion or allantois [155]. In cattle, fusion of the allantois with the chorion begins during embryonic elongation [134] and is complete by Day 23 of gestation [137]. The molecular regulation of chorioallantoic fusion in ruminants is poorly understood. In mice, knockout studies have demonstrated that fibroblast growth factor receptor-2 (FGFR2) [156] and DNA methyltransferase (DNMT) [157] are essential for



chorioallantoic fusion. The expression of FGFR2 has recently been demonstrated in bovine blastocysts [145] as well as in the trophoblast and uterus of pregnant sheep [146]. The expression of mRNA for DNMT has also been found in bovine blastocysts [143]. Therefore, both FGFR2 and DNMT may play a role in chorioallantoic fusion in ruminants.

### ***Implantation***

Implantation is a complex and progressive process involving extensive tissue remodeling. Various cytokines and adhesion molecules prepare the endometrium and trophoblast for implantation [148]. In cattle, implantation begins with apposition and attachment around Day 20 of gestation and is complete by Day 40 of gestation [120]. The process involves both caruncular and intercaruncular regions of the endometrium [118]. Although the exact mechanisms of implantation are unknown, Johnson et al. [148] have proposed a working model to describe the superficial implantation observed in ruminants. In this model, the conceptus stimulates the expression, secretion, and cleavage of Osteopontin (OPN), an extracellular matrix (ECM) glycoprotein within the uterine epithelium. Osteopontin then binds to  $\alpha_V$  and  $\beta_3$  integrin heterodimer which is expressed on both the trophoblast and uterine luminal epithelium (Table 1) [148]. The binding of OPN to  $\alpha_V\beta_3$  integrins results in morphologic changes in the trophoblast and adhesion between the luminal epithelium and trophoblast [148]. In cattle, Kimmins et al. [147] found that OPN was localized to the uterine luminal epithelium and in placental tissues; however, expression of  $\alpha_V\beta_3$  integrins was limited to the uterus, suggesting that the pattern of binding of OPN to  $\alpha_V\beta_3$  integrins was different than that proposed by Johnson et al. [148]. Other ECM

glycoproteins such as collagen and laminin as well as the integrin subunits  $\alpha_2\beta_1$  and  $\alpha_6\beta_1$  have also been implicated in implantation in cattle [158].

The two primary sources of feto-maternal exchange include histotrophe, extracellular secretions between the maternal and fetal surfaces, and haemotrophe, substances carried in the maternal blood [136, 159]. Histotrophic exchange in the placenta occurs primarily via phagocytosis of secretions by trophoblast cells. In contrast, haemotrophic exchange occurs via diffusion, endocytosis, and carrier-based systems such as facilitated diffusion and active transport [136]. The embryo implants within the maternal endometrium to secure an adequate supply of oxygen and nutrients primarily by haemotrophic exchange [160]. Binucleate cells of the trophoblast produce and secrete placental lactogen, pregnancy-associated glycoproteins, and prolactin-related proteins that contribute to implantation and development of the placentome (Table 1) [161]. Following completion of implantation, histotrophic exchange shifts to predominantly haemotrophic exchange which occurs within the placentome [162].

Placentome formation begins simultaneously with implantation at about Day 20 of gestation in cattle [120, 163]. However, intimate contact between the chorionic villi and maternal caruncles occurs after Day 30 of gestation [164]. Complex placentomes with extensive secondary branching of villi are not observed until approximately Day 42 of gestation [164]. As gestation progresses, placentomes increase in size and become domed and ovoid in shape. The size of placentomes varies depending on location in the uterus [165]. Placentomes in the pregnant horn within close proximity to the fetus are much larger in those in the nonpregnant horn or at the terminal region of the placenta. By Day 170 of gestation, the placentomes are fully developed with extensive tertiary branching of the villi

[166], however, growth of the placentome continues at a slow rate until term (~ Day 280) [167].

### ***Growth Patterns of the Placenta***

Placental growth, as measured by placental weight, increases exponentially throughout gestation, similar to that of the fetus [122, 168]. Prior and Lester [122] have shown that maximal daily growth rate of the bovine placenta and cotyledons occurred by Day 207 and Day 203 of gestation, respectively. Placental weight continued to increase at a lower rate until term [122]. Interestingly, Reynolds et al. [169] found that caruncle weight increased 2.4-fold more than cotyledon weight during the last two-thirds of gestation in cattle. The DNA content of the caruncular tissue remained relatively constant from Day 100 to 250 of gestation. In contrast, the DNA content in cotyledonary tissue increased steadily throughout this period, indicating increased cellular density in the cotyledons throughout gestation [169].

### ***Circulation of the Placenta***

In ruminants, the formation of placentomes result in close contact of the fetal and maternal circulations [170]. The placenta and uterus are highly vascularized organs [167]. An important factor in determining placental exchange is the capillary architecture between maternal and fetal vasculature which determines both the direction and separation of blood flow [136]. Detailed descriptions of the fetal and maternal vasculature have been reported in a variety of species using microvascular casts [167]. Fetal and maternal vasculature have four types of theoretical blood flow relationships including concurrent, multivillous, crosscurrent, and countercurrent flow [133]. The most efficient of these relationships is the countercurrent system because blood leaving the exchange area on one side will equilibrate

with blood entering from the other side resulting in a maximum solute transfer of 100% [136]. The least efficient is the concurrent system because blood from each side flows in the same direction with a maximum solute transfer of 50% [136].

In the cow, blood flow relationships of fetal and maternal vasculature within the placentome have differential patterns depending on stage of gestation. During early- to mid-gestation (3<sup>rd</sup> and 4<sup>th</sup> month of gestation in cattle), the blood flow relationship is mainly countercurrent because the lack of capillaries in the tertiary villi allows the stem and intermediate vessels of the placentome to function in a countercurrent fashion [171]. During late gestation, blood flow in the placentome is characterized as the less efficient crosscurrent relationship because of extensive capillary beds in the tertiary villi [167]. However, the overall transfer of substances during late gestation in the cow is still high because blood flow is both countercurrent (i.e. stem and intermediate vessels) and crosscurrent (i.e. capillary beds), and because there is extensive area of exchange provided by the large number of capillaries [171].

Although the placental growth rate decreases during the third trimester in cattle, overall placental transport capacity keeps pace with fetal growth [172]. Transport capacity of the placenta (transplacental exchange) may be explained by using the Fick Principle [132] and the following two equations 1) gravid uterine uptake = uterine blood flow x ([uterine artery] – [uterine vein]) and 2) fetal uptake = umbilical blood flow x ([umbilical vein] – [umbilical artery]), where [ ] indicates the concentration of substances in the respective veins or arteries [172]. From these equations, transplacental exchange can be explained by the rate of extraction based on arterial-venous concentration differences and the rate of uterine or umbilical blood flow [172]. Uterine blood flow was found to increase three- to four-fold

during mid- to late- gestation in the cow [173]. The distribution of uterine blood flow through the caruncular endometrium varies from only ~27% in early gestation to ~82% in late gestation [174]. During late gestation in sheep, approximately 94% of the total umbilical blood flow was distributed to the cotyledons and only ~6% to the intercotyledonary chorion [175]. Umbilical blood flow also increases throughout gestation in cattle [173, 174]. Therefore, it has been suggested that the primary factor affecting transplacental exchange appears to be increased blood flow rather than arterial-venous concentration differences [172].

### ***Vasculogenesis and Angiogenesis***

Blood flow to the uterus and placenta is influenced by increased growth and development of the vascular beds and secondarily by increased vasodilation of the vessels [150, 172, 176]. Growth and development of the placental vascular beds occurs through two distinct processes, vasculogenesis and angiogenesis [177]. Vasculogenesis involves *de novo* formation of blood vessels derived from angioblasts of the mesoderm which form a primitive vascular network [177, 178]. Extensive growth of the vasculature is controlled by branching and nonbranching angiogenesis [177, 179]. Angiogenesis refers to the formation of new blood vessels from preexisting blood vessels [177-179]. Increased blood flow to the uterus and placenta by means of increased development of vascular beds is primarily associated with angiogenesis [150, 172].

The primarily angiogenic factors identified in the placenta include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and the angiopoietin proteins (ANG1 and ANG2) as well as their receptors (Table 1) [150, 177-180]. Of these angiogenic factors, VEGF appears to provide the most potent effects on vasculogenesis and

angiogenesis resulting in proliferation, migration, and sprouting of endothelial cell as well as the formation of tube-like structures [179, 181]. Angiogenesis is also regulated by antiangiogenic factors such as angiostatin and thrombospondin [177, 181]. In addition, Charnock-Jones et al. [182] identified a novel pregnancy-associated variant of the VEGF receptor, soluble Flt-1, which appears to function as a VEGF antagonist [181, 182].

Reynolds and Redmer [150] developed a model to explain angiogenesis in the maternal caruncles of the placentomes in sheep. In this model, estrogen causes the production and secretion of basic FGF (bFGF) and VEGF from glandular epithelial cells and vascular smooth muscle cells of the endometrial arterioles, respectively [150]. Both VEGF and bFGF then act on endothelial cells to induce angiogenesis. These endothelial cells also secrete nitric oxide in response to VEGF and bFGF. Nitric oxide acts on vascular smooth muscle resulting in vasodilation and stimulation of more VEGF production, thereby creating a positive feedback loop to enhance placental angiogenesis [150].

Vascular endothelial growth factor has been shown to be regulated by hypoxia [183] and estrogen [150]. Low oxygen tension causes dimerization of the hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) and HIF1 $\beta$  [184]. The HIF1 $\alpha$ /HIF1 $\beta$  complex binds to the hypoxia-inducible element in the promoter region of the VEGF gene which then upregulates VEGF mRNA [185]. Reynolds and Redmer [150] have shown that vascular smooth muscle of endometrial arterioles expressed VEGF and also contain estrogen receptors during pregnancy in sheep. Estradiol-17 $\beta$  binds to its receptor in the vascular smooth muscle resulting in upregulation of VEGF mRNA [150, 186]. Other regulators of VEGF have been shown in a variety of other tissues. For instance, PPAR $\gamma$  has been shown to upregulate VEGF

expression in human vascular smooth muscle [187] and in macrophages [188]. Furthermore, PPAR $\gamma$ -deficient mice have been shown to lack vascular development of both the placenta and the fetus suggesting that PPAR $\gamma$  may play a key role in vasculogenesis and angiogenesis (Table 1) [153].

Angiopoietins may also play a role in angiogenesis of placentas in ruminants. Recently, Regnault et al. [151] had demonstrated that both ANG1 and ANG2 were expressed in cotyledons and caruncles of sheep from Day 55 of gestation to term. These authors suggested that the function of ANG1 and ANG2 in sheep may be similar to that observed in human placentas [151]. In the human placenta, ANG1 acts synergistically with VEGF to cause maturation of the vascular network, whereas ANG2 appears to act as a VEGF antagonist, resulting in destabilization of the vascular network [179].

## **ABNORMALITIES ASSOCIATED WITH IN VITRO EMBRYO TECHNOLOGIES**

Live offspring have been produced following transfer of in vitro-produced (IVP) and cloned embryos. However, a significant number of pregnancies result in abortion and abnormalities of the fetus, placenta, and offspring, particularly pregnancies from cloned embryos [60, 64, 104, 189, 190]. Abnormalities seen in the fetus, placenta, and offspring are commonly referred to a “Large Offspring Syndrome” (LOS) [60, 190, 191]. In many cases, embryonic losses have been linked to abnormalities in placentation and embryo development [64, 131, 192, 193].

### ***Pregnancy Loss***

Pregnancy rates resulting from the transfer of bovine IVP embryos are often lower than those from embryos produced in vivo [64]. Farin and Farin [194] demonstrated that

most pregnancy loss following transfer of bovine IVP embryos occurred by Day 21 of gestation. The risk of embryonic death was 4 times greater for IVP embryos of Grade 2 (fair) compared with in vivo-produced embryos of Grade 1 (good/excellent) [194]. A further study indicated that more degenerated conceptuses were recovered at Day 17 of gestation from embryos produced in vitro (19%) compared with those produced in vivo (0%), suggesting inadequate signaling of MRP by IVP embryos [195]. McMillan et al. [196] also reported that 73% of pregnancy loss from IVP embryos occurred by Day 14 of gestation. Pregnancy rates resulting from cryopreserved IVP embryos were also decreased compared with their in vivo counterparts. For example, Hasler et al. [197] reported that pregnancy success for frozen IVP embryos was 42% compared with 67% for cryopreserved in vivo-produced embryos.

The transfer of bovine IVP embryos has also resulted in a higher percentage of abortion during the fetal period, Day 42 to parturition [191, 197-199]. For example, Agca et al. [198] reported that fetal loss following transfer of IVP embryos produced in vitro was 13% after Day 40 of gestation. In addition, cryopreserved IVP embryos had significantly higher abortion rates than fresh IVP embryos (28 *versus* 9%) [198]. Hasler et al. [199] found that the abortion rate for IVP embryos was increased compared with that for in vivo-produced embryos. Abortion rates were similar for embryos produced using B2 medium supplemented with or without serum, or TCM-199 medium with serum (13.1%, 11.9%, and 10.7%, respectively) [199]. It has been suggested that increased rates of abortion seen with IVP embryos during the fetal period may be the result of aberrant placental development [64, 198].

The efficiency of nuclear transfer (NT) for production of live offspring is extremely low ranging from 0 to 10% of reconstructed embryos [105, 200]. A major factor influencing



the low efficiency of NT is increased pregnancy loss [104]. Pregnancy rates at Day 40 of gestation from somatic cell nuclear transfer embryos ranged from 9% to 47% for fetal donor cells and 15% to 80% for adult donor cells [102]. A high percentage of early embryonic loss occurs with pregnancies from cloned embryos [104, 201], which appears to be due to a failure to signal MRP [201, 202]. Immunologic rejection may also play a role in the loss of cloned pregnancies. Hill et al. [203] found that expression of major histocompatibility complex class-I was present in cloned bovine placentas ranging from 4 to 7 weeks of gestation, but not in placentas from controls.

The transfer of cloned embryos results in a higher incidence of pregnancy loss during the fetal period than pregnancies from in vivo- or in vitro- produced embryos [104, 131, 192, 193]. Hill et al. [131] observed that pregnancy rates at Day 30 of gestation for cloned bovine embryos (45%) were comparable to in vivo controls (58%). However, survival rates at Day 90 were only 18% in the cloned group compared with 100% for controls. Fetal loss in the cloned group was 35%, 32%, and 15% at Day 30 to 40, Day 40 to 60, and Day 60 to 90 of gestation, respectively [131]. The high rate of fetal loss from Day 30 and 90 of gestation has been associated with a reduced number of placentomes and inadequate vascular development of the placenta [131, 192, 193].

A significant number of pregnancies from cloned embryos are also lost during the third trimester (Day 200 to term) [104, 204-207]. For example, Hill et al. observed that 4 of 13 pregnancies (31%) from cloned bovine embryos aborted during the 7<sup>th</sup> and 9<sup>th</sup> months of gestation. In addition, Heyman et al. [204] reported that late gestation pregnancy loss in recipients carrying embryos cloned from adult and fetal cells were 43.7% and 33.3%, respectively; whereas, no pregnancies from IVP embryos were lost during the same period.

Pregnancy loss during late gestation from cloned embryos may be associated with placental abnormalities such as hydrallantois, decreased number of placentomes, and placental edema [104, 204-206].

### ***Fetal and Neonatal Abnormalities***

The production of embryos in vitro results in a variety of fetal and neonatal abnormalities including increased weight of the fetus and offspring as well as altered development of organs [60, 64]. For example, bovine fetuses from IVP embryos during late gestation were heavier than their in vivo counterparts [194]. In sheep, fetuses at Day 125 of gestation from IVP embryos were heavier than in vivo controls [70]. Birth weights of calves [191, 197, 208] and lambs [71] resulting from IVP embryos are frequently heavier than controls. In addition, rightward shifts in the distribution of weight has been reported for fetuses [64] and calves [209] from IVP embryos compared with in vivo controls. Altered muscular development has also been observed in bovine [210] and ovine [211] fetuses from IVP embryos. An increased incidence of congenital abnormalities including cerebellar hypoplasia [208, 212] and enlargement of the heart [213] have been reported in calves resulting from the transfer of IVP embryos. Furthermore, increased mortality of newborn calves from IVP embryos has been observed and may be a direct consequence of dystocia associated with large fetuses [49, 191].

A greater frequency and broader range of fetal and neonatal abnormalities has occurred in pregnancies resulting from cloned embryos compared with those from IVP embryos [103, 206]. Fetuses [214, 215] and offspring [103, 104, 209, 216] from cloned embryos are usually larger than controls. In addition, a variety of congenital abnormalities associated with the cardiopulmonary axis, immune system, renal system, and brain function

have been observed in cloned offspring [104, 205, 217, 218]. Alterations in energy metabolism, as measured by increased plasma IGF-I and -II levels, has also been observed in cloned fetuses [219] and calves [216]. Other metabolic abnormalities reported in cloned calves were pulmonary hypertension, respiratory distress, hypoxia, hypothermia, hypoglycemia, and metabolic acidosis [104, 220].

A high incidence of perinatal and postnatal loss has been associated with pregnancies resulting from cloned embryos [201, 205]. Hill et al. [205] reported that 5 of 13 (38%) cloned fetuses were stillborn with placental edema and hydrallantois, 8 calves were born alive of which 2 calves died at Day 4 and Week 6 of age as a result of cardiopulmonary abnormalities [205]. Perinatal and postnatal mortality observed in calves from cloned embryos may also be a consequence of dystocia associated with larger fetuses at term [49].

### ***Placental Abnormalities***

Abnormal placentation has been observed following the transfer of IVP embryos in cattle [72, 194, 197, 221-223] and sheep [70]. Pregnancies from IVP embryos have resulted in higher incidence of hydrallantois in cattle [72, 197] and polyhydramnios in sheep [70]. In addition, placentas from IVP embryos were heavier than those of in vivo controls during the 3<sup>rd</sup> trimester in cattle [221, 223] and sheep [70]. However, there was no difference in the weight of placentas from IVP embryos *versus* controls during early gestation [70, 222, 223].

Farin and Farin [194] reported that bovine placentas from IVP embryos had a decreased number of placentomes per kilogram of fetal body weight compared with controls. In addition, bovine placentas from IVP embryos displayed variations in binucleate cell population compared with in vivo controls [221, 222]. During early gestation (Day 70; [222]), the volume density of binucleate cells was greater in placentomes from IVP embryos

compared to controls, whereas during late gestation (Day 222; [221]) volume density of binucleate cells was less in placentomes from IVP embryos. Increased malformations of the allantois, such as stunted or avascular allantoic growth, have also been reported in placentas from IVP embryos [202].

Variations in feto-maternal contact have been observed in bovine placentas from IVP embryos compared with in vivo controls [221-223]. During late gestation (Day 222), feto-maternal contact and fetal villous volume density were decreased in placentas from IVP embryos compared with controls [221]. In contrast, during early gestation (Day 70), no differences were observed in the volume density of fetal villi in placentas from IVP embryos compared with controls [222]. At term, bovine placentas from IVP embryos had increased feto-maternal contact as measured by cotyledonary surface area [223]. These differences in feto-maternal contact observed in placentas from IVP embryos may be attributed to differences in the time of gestation examined.

Abnormalities have also been observed in placentas from cloned embryos in mice [215, 224], sheep [193], and cattle [131, 192, 225]. Abnormal development of cotyledons was reported during the first trimester in cloned cattle [131, 192] and sheep [193]. Stice et al. [192] had observed that placentas resulting from transfer of cloned bovine embryos derived from embryonic cells had no cotyledonary development by Day 60 of gestation. Hill et al. [131] found a range of gross and histological abnormalities in bovine placentas at Days 40 and 60 of gestation from cloned embryos derived from either fetal or adult donor cells. These authors reported that 2 of 6 placentas had rudimentary cotyledons with flat, cuboidal trophoblastic epithelium, and reduced vascularization of the chorioallantois [131]. Two other cloned placentas had no observable cotyledonary development and two placentas appeared to

develop normally, but one of these placentas had hemorrhagic cotyledons [131]. In sheep, inadequate development of cotyledons, including a reduced number or lack of cotyledons, has been observed in placentas from cloned embryos derived from fetal cells [193].

During early (Day 50) to mid gestation (Day 150) in cattle, alterations in histological morphology have been observed in pregnancies from cloned embryos [219]. Increased numbers of cells in the fetal villi and caruncular endometrium as well as binucleate cells have been reported for placentomes at Day 50, 100, and 150 of gestation [219]. The increased cell numbers in placentomes from cloned embryos likely contribute to placental overgrowth [219]. Furthermore, increased spatial and temporal expression of IGF binding protein-2 and -3 (IGFBP-2 and -3), as measured by immunohistochemistry, has been demonstrated in placentomes from cloned embryos compared with AI and IVP controls [219]. In a similar study, placentas resulting from cloned embryos had a decreased number of caruncles at Day 100 of gestation compared with AI and IVP controls [214]. In contrast, increased placental weight and fluid volume were only observed in placentas from cloned embryos at Day 150 of gestation [214]. Placentas from cloned embryos were heavier than placentas from either AI or IVP controls; whereas placental fluid volume for clones was only greater than placental fluid volume from IVP embryos [214].

In late gestation and at term in cattle, placentas from cloned embryos commonly have a decreased number of placentomes, enlarged placentomes, increased placental weights as well as an increased incidence of hydrallantois and placental edema [104, 205, 216, 225]. Hill et al. [205] reported that 6 of 13 (46%) placentas from cloned embryos had hydrallantois with and without placental edema. Hill et al. [225] also reported the observation that a grossly abnormal placenta was capable of supporting a viable cloned calf to term. This

placenta had only 26 cotyledons, of which only 12 appeared functional, even though they were grossly enlarged [225]. Of the remaining cotyledons, 6 were poorly developed and 8 were degenerating [225]. Similarly, Edwards et al. [104] had reported that, during the third trimester, bovine placentas from cloned embryos often had hydrallantois, a marked reduction in placentomes, hypertrophy of cotyledons, adventitial placentation, and severe edema. In mice, placentas from cloned embryos were twice the size of placentas from controls embryos [215, 224].

### ***Potential Mechanisms Associated with the LOS Phenotype***

Although the exact mechanisms causing the LOS phenotype are unknown, several hypotheses have been proposed to explain the observations of fetal, neonatal, and placental abnormalities following the transfer of IVP and cloned embryos. A widely recognized hypothesis to explain LOS, especially in cloned embryos, is abnormal epigenetic reprogramming of the maternal and paternal genome (i.e. imprinting) during embryo development [60, 64, 103, 220, 226]. Imprinting results in monoallelic expression of specific autosomal genes [227]. Genomic imprinting plays a critical role in fetal growth and is predominantly regulated by DNA methylation and chromatin structure [227]. Normal embryonic reprogramming occurs shortly after fertilization as seen by rapid demethylation of the maternal and paternal genome [228]. The parental genome remains largely demethylated until *de novo* remethylation occurs around the time of implantation [228]. In contrast, most cloned embryos apparently fail to properly reprogram the parental genome due to the highly differentiated state of somatic cells compared with gametes [226].

Evidence supporting the hypothesis of abnormal epigenetic reprogramming has been reported for bovine embryos, fetuses, and placentas resulting from cloning and IVP. For

instance, aberrant methylation patterns have been observed in cloned bovine blastocysts including a variety of genomic repeat sequences such as satellite I and satellite II, 18S rRNA, and art-2 SINE [229]. In addition, Bourc'h et al. [230] reported that the gradual demethylation pattern normally observed during preimplantation embryo development was not observed in cloned bovine embryos. Recently, hypermethylation patterns of the satellite I repeat element and epidermal cytochrome promoter have been demonstrated in chorionic tissue from interspecies (*Bos gaurus* x *Bos taurus*) cloned embryos compared with AI (*Bos gaurus* x *Bos taurus*) controls [92]. Wrenzycki et al. [143, 231] found that cloned bovine blastocysts had increased expression of imprinted genes such as MASH2 and Xist compared to controls.

It has been suggested that fetal and placental overgrowth associated with cloned and IVP embryos may result from disruptions of the IGF system [60, 64, 103, 189, 232]. Calves from cloned embryos have increased levels of serum IGF-II, a maternally imprinted gene, immediately following birth compared with controls [216]. Increased levels of IGF-II mRNA were found in bovine fetal livers from IVP embryos at Day 70 of gestation [233]. Furthermore, decreased expression of mRNA for IGF type 2 receptor (IGF2R) was demonstrated in Day 17 bovine conceptuses from IVP embryos [234]. Fetal overgrowth has also been observed in human infants with Beckwith-Wiedeman syndrome (BWS) [235]. Interestingly, human fetal overgrowth seen with BWS share common characteristics with fetal overgrowth observed in cloned and IVP embryos such increased expression of IGF-II [216, 235]. Recent evidence has indicated that assisted reproductive technologies in humans, such as IVF and intracytoplasmic sperm injection, have been associated with an increased risk of major birth defects, specifically associated with BWS [236, 237]. In fact, the

frequency of BWS observed for children born using assisted reproductive technologies has ranged from 4% to 5%, suggesting at least a six-fold increase in BWS compared with the normal population [238, 239].

An alternative hypothesis for the LOS phenotype resulting from IVP and cloned embryos includes the aberrant expression of nonimprinted genes important for the development of embryos, fetuses, and placentas. These genes play critical roles in embryo compaction and cavitation, MRP, metabolism and growth of the fetus, muscle development, trophoblast differentiation, and implantation. For example, bovine blastocysts produced in vitro have been shown to have increased expression of mRNAs for glucose transporter (Glut-3), Glut-4, heat shock protein 70.1 (Hsp 70.1), bFGF, and IGF1R compared with in vivo controls [240]. In another study, the levels of mRNA expression for Glut-1, Glut-3, and IFN $\tau$  were decreased in IVP embryos [241]. In cloned bovine embryos, variations in gene expression have also been identified in a number of nonimprinted genes including interleukin-6, FGF4, FGF $\tau$ 2, IFN $\tau$ , and Hsp 70.1 [143, 242]. In bovine fetuses from IVP embryos, the expression of myostatin mRNA was decreased during late gestation compared with controls [210]. Furthermore, Hashizume et al. [243] reported that gene expression for heparanase, an extracellular matrix-degrading molecule, was altered in bovine placentas from cloned embryos at Day 60 of gestation. Although alternation in imprinted genes is a widely recognized hypothesis to explain LOS, aberrant expression of nonimprinted genes may also play a potential role in generating the LOS phenotype.



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## **ANGIOGENESIS AND MORPHOMETRY OF BOVINE PLACENTAS IN LATE GESTATION FROM EMBRYOS PRODUCED IN VIVO OR IN VITRO<sup>1</sup>**

### **ABSTRACT**

The objective of this study was to determine the effects of in vitro embryo production on angiogenesis and morphometry of the bovine placenta during late gestation. Blastocysts produced in vivo were recovered from superovulated Holstein cows. Blastocysts produced in vitro were obtained after culture of in vitro-matured and -fertilized Holstein oocytes. Single blastocysts from each production system were transferred into heifers. Fetuses and placentas were recovered on Day 222 of gestation (in vivo, n = 12; in vitro, n = 12). Cotyledonary and caruncular tissues were obtained for quantification of vascular endothelial growth factor (VEGF) and peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) mRNA and protein. Tissue sections of placentomes were prepared for morphometric analysis. Fetuses and placentas were heavier from embryos produced in vitro than from embryos produced in vivo. More placentas from embryos produced in vitro had an excessive volume of placental fluid. There was no effect of treatment on the expression of mRNA for VEGF and PPAR $\gamma$  in either cotyledonary or caruncular tissues. The expression of VEGF protein in cotyledons and caruncles as well as the expression of PPAR $\gamma$  protein in cotyledons were not different between the in vitro and in vivo groups. However, caruncles from the in vitro group had

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increased expression of PPAR $\gamma$  protein. The total surface area of endometrium was greater for the in vitro group compared with controls. In contrast, the percentage placentome surface area was decreased in the in vitro group. Fetal villi and binucleate cell volume densities were decreased in placentomes from embryos produced in vitro. The proportional tissue volume of blood vessels in the maternal caruncles was increased in the in vitro group. Furthermore, the ratios of blood vessel volume density-to-placentome surface area were increased in the in vitro group. In conclusion, these findings are consistent with the concept that compensatory mechanisms exist in the vascular beds of placentas from bovine embryos produced in vitro.

Key Words: developmental biology, embryo, gene regulation, in vitro fertilization, placenta

## INTRODUCTION

Proper development of the mammalian placenta is critical for determining nutrient availability to the developing fetus, regulation of gas exchange, and removal of waste products. Developmental problems of the fetus, the placenta or both have been reported following transfer of in vitro-produced (IVP) embryos in cattle [1-5] and sheep [6, 7]. In cattle, these problems have included increased pregnancy loss, oversized or malformed fetuses and calves, increased hydrallantois and other abnormalities of placental development [2, 4, 5]. Inadequate placental vascularization has also been observed in bovine [8] and ovine [9] placentas from embryos produced by somatic cell nuclear transfer. The influence of these in vitro embryo production systems on placental morphometry and angiogenesis is poorly understood.

Vascular development of the placenta is initiated by vasculogenesis and subsequently controlled by branching and nonbranching angiogenesis [10, 11]. Angiogenesis is regulated by growth factors, including vascular endothelial growth factor (VEGF; [12, 13]); and potentially, peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) which has been shown to upregulate VEGF expression [14, 15]. In sheep, expression of VEGF mRNA was greater in both cotyledonary and intercotyledonary tissues compared with caruncular and intercaruncular tissues in early and late gestation [16]. Based on immunocytochemical localization, VEGF protein was greater in fetal placental tissues during early ovine pregnancy. However, during late pregnancy, VEGF protein was found primarily in the microvessels of maternal caruncular villi [16]. PPAR $\gamma$  has been associated with angiogenesis and tissue remodeling in the mammalian placenta [17, 18] and other organs [14, 15]. For

example, in PPAR $\gamma$  knockout mice, epithelial differentiation of trophoblast tissue and placental vascular development were impaired indicating that PPAR $\gamma$  is essential for these processes [17]. Expression of PPAR $\gamma$  mRNA has been demonstrated in the villi of choriodecidual placentas of humans [18]. In the human trophoblast, PPAR $\gamma$  has been shown to dimerize with retinoic acid receptor- $\alpha$  and regulate differentiation of extravillous cytotrophoblast [19]. Alterations in expression of angiogenic factors, such as VEGF and potentially PPAR $\gamma$ , may play an important role in placental and fetal abnormalities associated with in vitro embryo production.

The overall objective of this study was to determine the effects of in vitro embryo production on the morphometry and angiogenesis of placentas during late gestation in cattle. Specifically, we compared placentas from embryos produced in vivo or in vitro for 1) gross and histological morphometry, 2) mRNA and protein expression for VEGF and PPAR $\gamma$  in cotyledonary and caruncular tissues, and 3) morphometry of blood vessels within the cotyledonary (fetal) and caruncular (maternal) components of placentomes.

## MATERIALS AND METHODS

### *Reagents and Hormones*

Tissue culture medium (M-199 with Earle's Salts) was purchased from Gibco BRL (Grand Island, NY). Equine pituitary LH (11.5 NIH LH-S1 units/mg) and porcine pituitary FSH (50 mg/vial Armour FSH Standard) preparations were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty-acid free BSA was purchased from Boehringer Mannheim (Indianapolis, IN). All other culture reagents and media were tissue culture grade and were purchased from Sigma Chemical Co.

TRI-Reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). DNase and random hexamers were purchased from Promega (Madison, WI). SuperScript II reverse transcriptase and dNTPs were purchased from Gibco BRL. PCR purification kits were purchased from Qiagen (Valencia, CA). QIAprep Miniprep system was purchased from Qiagen, Inc. (Valencia, CA). Taq polymerase was purchased from Roche Molecular Biochemical (Indianapolis, IN). SYBR green dye was purchased from Molecular Probes, Inc. (Eugene, OR). All primers for PCR and real-time PCR were custom synthesized by either Sigma-Genosys (Woodlands, TX) or Qiagen Operon (Alameda, CA). For detection of VEGF protein by Western blot and immunocytochemistry, an anti-VEGF polyclonal rabbit antibody (sc-152) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For detection of PPAR $\gamma$  protein by Western blot, an anti-PPAR $\gamma$  polyclonal rabbit antibody (107100) was purchased from Cayman Chemical (Ann Arbor, MI).

### ***Production of Embryos***

All procedures and protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at North Carolina State University. For in vivo embryo production, Holstein donor cows were synchronized using two i.m. injections of 25 mg prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ , Lutalyse<sup>®</sup>, Pharmacia & Upjohn Co., Kalamazoo, MI) 14 days apart. Donor cows were superovulated with 400 mg FSH (Folltropin<sup>®</sup>-V, Vetrapharm Canada, London, ON) administered in decreasing doses over a 4-day period beginning on Day 10, 11, 12 or 13 of the estrous cycle (Day 0 = estrus). On the morning and evening of the third day of FSH treatment, estrus was induced using two i.m. injections of 25 mg of  $PGF_{2\alpha}$ . Donors were artificially inseminated at 12 and 24 h after detection of first standing heat with thawed frozen semen from a proven Holstein bull. Embryos were collected by nonsurgical uterine flushing on Day 7 (Day 0 = first detected estrus) [2].

For in vitro embryo production, ovaries from Holstein cows were obtained at a local abattoir and held in saline with 0.75  $\mu\text{g/ml}$  penicillin for 4-6 h during transport to the laboratory. Cumulus-oocyte complexes (COC) were aspirated, matured, and fertilized in vitro as previously described [2]. Briefly, COCs were aspirated from 2-7 mm follicles and washed five times in modified Tyrode's medium (TL-Hepes). Groups of 20 to 30 COCs were matured for approximately 22 h in M-199 supplemented with 10% heat inactivated estrus cow serum (ECS), 10  $\mu\text{g/ml}$  LH, 5  $\mu\text{g/ml}$  FSH, 1  $\mu\text{g/ml}$  estradiol, 200  $\mu\text{M}$  sodium pyruvate, and 50  $\mu\text{g/ml}$  gentamicin. All cultures were incubated at 5%  $\text{CO}_2$  in air with 100% humidity. Following the maturation period, COCs were washed once and placed in fertilization medium that consisted of heparin-supplemented Tyrode's albumin lactate

pyruvate medium with 6 mg/ml fatty acid-free BSA [20]. Thawed frozen semen from the same Holstein bull used for AI of donor cows was used for in vitro fertilization. Motile spermatozoa were collected using the swim-up procedure [20] and a final concentration of  $1 \times 10^6$  spermatozoa per ml was used for fertilization in 0.75 ml of fertilization medium. Spermatozoa and COCs were coincubated for 18-20 h. Following incubation, presumptive zygotes were washed six times with TL-Hepes and placed in 1 ml M-199 supplemented with 10% ECS and 50  $\mu$ g/ml gentamicin. Embryos were incubated for a 168-h culture period and culture medium was changed at 48-h intervals.

### ***Transfer of Embryos***

Angus heifers were given two injections of 25 mg of PGF<sub>2</sub> $\alpha$  by i.m. administration 10 to 12 days apart to synchronize estrus. Grade 1 blastocysts [21] from in vivo or in vitro production systems were transferred in TL-Hepes medium singly into the uterine horn ipsilateral to the ovary bearing the corpus luteum of recipient heifers on Day 7 of the estrous cycle.

### ***Recovery of Fetuses and Placental Tissue***

At Day 222 of gestation (215 days after transfer), a total of 24 pregnant recipients (n = 12 and 12 for in vivo and in vitro, respectively) were slaughtered. Fetuses and their placentas were removed from the reproductive tracts, and physical measurements were taken including fetal weight, wet placental weight, number of placentomes, and placental fluid (amniotic plus chorioallantoic fluid) volume. Samples of cotyledonary and caruncular tissues were obtained by careful manual separation of these tissues. Tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for whole cell RNA (wcRNA) and protein

extraction. Center segments cut from whole placentomes of individual placentas were stored in 10% neutral buffered formalin for histology and immunocytochemistry. After removal of the placenta, the entire uterus was opened completely and laid flat with the endometrial surface exposed. A top-view, digital photograph of the uterine endometrial surface was taken for morphometric analysis.

### ***Processing of Tissue for RNA and Protein***

For wcRNA extraction, frozen cotyledonary and caruncular tissues were removed from storage, weighed, placed in a frozen mortar, covered with liquid nitrogen, and crushed to a fine powder. The fine powder was resuspended in TRI-Reagent (1 ml/100 mg tissue) and samples were homogenized (Brinkmann Homogenizer PT 10/35; Westbury, NY). Whole cell RNA was extracted according to the manufacturer's protocol and dissolved in diethyl pyrocarbonate- treated water. The concentration of the wcRNA was determined by absorbance at 260 nm. The quality and integrity of the wcRNA was assessed based on the ratio of absorbance at 260 and 280 nm and visualization of 28S and 18S rRNA bands in ethidium bromide-stained agarose gels (data not shown). Aliquots of approximately 30 µg of wcRNA were stored at -80°C until used for cDNA synthesis.

For protein extraction, frozen cotyledonary and caruncular tissue samples were removed from storage, weighed, placed in a frozen mortar, covered with liquid nitrogen, and crushed to a fine powder. The powder was resuspended as previously described [22] in a cold buffer (13.3 µl buffer per mg of tissue) consisting of 1% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, aprotinin (20 µg/ml), leupeptin (20 µg/ml), 1 mM PMSF, and 20 mM Hepes. Samples were stored on ice, homogenized (Brinkmann Homogenizer) and transferred



to 1.5 ml tubes. Samples were then centrifuged at  $\sim 10,000 \times g$  for 10 min at 4°C. The supernatant was collected and stored at -20°C. Total protein was quantified using bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the manufacturer's suggested protocol.

### ***Reverse Transcription and Verification of PCR Products***

Individual aliquots of wcRNA were thawed on ice and 2  $\mu$ g of each sample was treated with DNase (1.5 units) for 20 min at 37°C. Reactions were stopped by the addition of 2  $\mu$ l of 20 mM EDTA. Following DNase-inactivation, wcRNA was reverse transcribed using random hexamers and SuperScript II reverse transcriptase under conditions recommended by the manufacturer. Following cDNA synthesis, samples were purified using the Qiagen PCR purification kit as recommended by the manufacturer and stored at 4°C.

Primer sequences for VEGF-1 were designed using Oligo 4.0.2 Primer Analysis software (Plymouth, MA) and Gene Amplify 1.2 software (Madison, WI) and based on sequences reported by Leung et al. [23] (Table 1). Forward and reverse primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PPAR $\gamma$  were obtained from Leutenegger et al. [24] and Sundvold et al. [25], respectively (Table 1). VEGF-2 forward and reverse primer pair, specifically designed to detect all five VEGF isoforms [26], was used (Table 1). For verification of PCR reaction, each reaction consisted of 100 ng equivalents of cotyledonary cDNA, 1.6  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer (Roche), and 2.5 units of Taq polymerase in a 20  $\mu$ l reaction. A negative control lacking cDNA was included for each PCR assay. All PCR reactions were run in 96-well PCR plates and briefly spun before placing into an iCycler

**Table 1.** Primer sequences used for PCR analysis of bovine caruncular and cotyledonary tissues.<sup>a</sup>

Primer	Sequence	Isoform	Fragment Size (bp)
GAPDH <sup>b,c</sup>	Forward 5'-GGC GTG AAC CAC GAG AAG TAT AA		120
	Reverse 5' -CCC TCC ACG ATG CCA AAG T		
PPAR $\gamma$ <sup>b,d</sup>	Forward 5'-ATT ACC ATG GTT GAC ACA GAG	PPAR $\gamma$ 1	354
	Reverse 5' -ATG AGG GAG TTG GAA GGC TC		
VEGF-1 <sup>b</sup>	Forward 5'-ACG AAA GTC TGG AGT GTG	VEGF <sub>164</sub>	115
	Reverse 5' -TTG TTA TGC TGT AGG AAG		
VEGF-2 <sup>c</sup>	Forward 5' -TGT AAT GAC GAA AGT CTG CAG	VEGF <sub>120</sub>	186
	Reverse 5' -TCA CCG CCT CGG CTT GTC ACA	VEGF <sub>144</sub>	258
		VEGF <sub>164</sub>	318
		VEGF <sub>188</sub>	390
		VEGF <sub>205</sub>	441

<sup>a</sup> Annealing temperature for all primer sequences was 60°C.<sup>b</sup> Primers used for real-time quantitative PCR analysis.<sup>c</sup> Primer from [24].<sup>d</sup> Primer from [25].<sup>e</sup> Primer from [26].

thermocycler (Bio-Rad, Richmond, CA). Each PCR program consisted of a 90 sec hot start at 95°C, followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 60°C and primer extension for 30 sec at 72°C. Following the cycles, an additional primer extension for 5 min at 72°C was used. The primer sequences used for PCR validation with their expected product lengths and specific isoforms are shown in Table 1. The PCR products from VEGF-1, VEGF-2, GAPDH, and PPAR $\gamma$  primer pairs were verified by sequence analysis.

### ***Real-Time Quantitative PCR Analysis***

Verified GAPDH, VEGF-1, and PPAR $\gamma$  primers were used for quantification of GAPDH, VEGF, and PPAR $\gamma$  mRNA levels by real-time reverse transcription-PCR using a SYBR Green I detection system. Quantification was performed using a two-tube PCR system. Whole cell RNA was subjected to reverse transcription in a separate tube and cDNA was transferred to 96-well PCR plates for real-time PCR using the iCycler (Bio-Rad). SYBR Green I, a high affinity double-stranded DNA binding dye, was used to monitor DNA amplification [27]. For analysis of cotyledonary tissue, each PCR reaction consisted of 100 ng equivalents of cDNA, 1.6  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer, 2  $\mu$ l of 2X SYBR Green I dye, 1  $\mu$ l of 200 nM Fluorescein dye (Bio-Rad), and 2.5 units of Taq polymerase in a 20  $\mu$ l-reaction. For analysis of caruncular tissue, PCR methods were the same as those described for cotyledonary tissue, except that only 50 ng equivalents of cDNA was used. Melt-curve analysis and gel electrophoresis were used to confirm product length after amplifications were complete (data not included). In real-time PCR, the threshold ( $C_T$ ) is evaluated during the log-linear phase of the PCR amplification and is an exponential term, not a linear term [28]. Therefore,  $C_T$

values were converted to linear terms using the formula  $2^{-C_T}$  for calculation of mRNA levels for VEGF and PPAR $\gamma$  [29]. Expression of mRNA for VEGF and PPAR $\gamma$  in individual samples was analyzed as a ratio of linearized  $C_T$  values of mRNA for VEGF or PPAR $\gamma$  to the linearized  $C_T$  values of GAPDH.

Expression of mRNA in cotyledons and caruncles were analyzed separately. For each tissue type, two assays were used (in vivo, n = 6 and in vitro, n = 6 within each assay for each tissue). Each assay also contained reference samples of cotyledonary or caruncular tissue from a random control bovine placenta at approximately Day 220 of gestation (fetal crown-rump length = 75.0 cm). The random reference samples were used to determine inter- and intra-assay coefficients of variation (CV) for the linearized  $C_T$  values. The inter- and intra-assay CV for VEGF assays of cotyledons were 10.4% and 21.2%, respectively. The inter- and intra-assay CV for PPAR $\gamma$  assays of cotyledons were 7.4% and 17.1%, respectively. The inter- and intra-assay CV for VEGF assays in caruncles were 24.3% and 4.9%, respectively. The intra-assay CV for PPAR $\gamma$  assays in caruncles was 12.1%. Because the interassay CV for the two PPAR $\gamma$  assays for caruncles was greater than 25%, values from the second PPAR $\gamma$  assay were normalized to the first PPAR $\gamma$  assay.

### ***Western Blot***

Expression of VEGF and PPAR $\gamma$  was evaluated using a modified Western blot protocol [22]. Briefly, 20 mg of total protein from cotyledonary or caruncular tissues was separated on 12% (w/v) SDS-PAGE gels under nonreducing conditions. Following electrophoresis, the polyacrylamide gels were transferred to nitrocellulose membranes (Bio-Rad) using a semi-dry transfer system (Bio-Rad). VEGF and PPAR $\gamma$  antibody binding was

detected using the BM Chemiluminescence Western blotting kit (Mouse/Rabbit; Roche Applied Science, Indianapolis, IN). Nonspecific binding sites were blocked with 1% blocking solution. Blots were incubated overnight at 4°C with a 1:500 dilution of VEGF polyclonal antibody or 1:750 dilution of PPAR $\gamma$  polyclonal antibody. Specificity of the VEGF and PPAR $\gamma$  antibodies was verified using blocking peptides obtained from Santa Cruz Biotechnology, Inc. and Cayman Chemical, respectively. Blocking peptides were incubated with their respective primary antibody (5:1 and 3:1 for VEGF and PPAR $\gamma$ , respectively) for 1 h at room temperature (RT) immediately before overnight primary antibody incubations with protein bound membranes. Following primary antibody incubation, blots were washed with 0.5% blocking solution twice for 10 min each and then twice with Tris buffered saline-Tween<sup>®</sup>20 (TBST) buffer for 10 min. Following washing, the blots were incubated for 30 min at RT with a 1:500 dilution of horseradish peroxidase-label anti-mouse IgG/anti-rabbit IgG (40mU/ml). Following incubation with the secondary antibody, the blots were washed four times for 10 min in TBST buffer. Blots were incubated with a prewarmed (RT) detection solution for 1 min, exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) and binding was quantified using computer-assisted video image analysis (Optimas Visual Imaging System 6.1; Optimas Corporation, Bothell, WA).

### ***Morphometric Analysis***

Computer-assisted image analysis (Optimas Visual Imaging System 6.1) of digital photographs of the entire endometrial surface of the uterus were used to quantify total uterine and caruncular surface areas. Caruncular surface area was used to assess the proportion of

the total uterine endometrial surface area occupied by placentomes; hereafter referred to as placentome surface area.

Samples of intact placentomes were embedded in paraffin and 5  $\mu\text{m}$  sections of placentome tissues were prepared. Sections were deparaffinized, dehydrated, and stained with hematoxylin and eosin. Stereologic end points including the volume densities of fetal villi, caruncular endometrium, binucleate cells, and fetal and maternal pyknotic cells were evaluated by point count methods [30, 31] using computer-assisted image analysis. For analysis of fetal villi, maternal endometrium, and binucleate cells, 10 fields of view representing a total of  $8.12 \times 10^6 \mu\text{m}^2$  of each placentome section was examined using a 100-point grid system [31]. For analysis of fetal and maternal pyknotic cells, 10 fields of view representing a total of  $6.01 \times 10^4 \mu\text{m}^2$  from each placentome section was examined using a 256-point grid system [31].

Immunocytochemical localization of VEGF protein was used to identify vascular beds for morphometric analysis. Following deparaffinization, tissue sections were incubated for 5 min in Target Unmasking Fluid (BD Pharmingen, San Jose, CA) at  $90^\circ\text{C}$  to increase antigen availability within the tissue. Endogenous peroxidase activity was blocked using 0.5% hydrogen peroxide and nonspecific binding was blocked using normal goat serum (1:67 in PBS). Immunoreactivity for VEGF was detected using a 1:50 dilution of the VEGF polyclonal antibody. Placentome sections were incubated with primary antibody for 1 h at RT. Specificity of the VEGF antibody was verified using blocking peptide obtained from Santa Cruz Biotechnology, Inc. Blocking peptide was incubated with primary antibody (5:1) for 1 h at RT prior to primary antibody incubation with placentome sections. Biotinylated

goat anti-rabbit IgG (1:200 in PBS) was used as a secondary antibody. Following incubation with the secondary antibody, placentome sections were incubated with avidin and biotinylated horseradish peroxidase and then visualized with diaminobenzidine tetrahydrochloride containing nickel (Vector Laboratories, Burlingame, CA).

For analysis of maternal and fetal blood vessels, 20 fields of view representing a total of  $4.8 \times 10^5 \mu\text{m}^2$  of tissue was examined from each placentome section. Point count methodology with a 256-point grid system [31] was used to determine the volume densities of maternal and fetal blood vessels. For determination of total blood vessels, volume densities of maternal and fetal blood vessels were added. In order to determine the relative amount of maternal, fetal, and total blood vessels within the proportion of uterine endometrial surface area occupied by placentomes for each animal, ratios of blood vessel volume density-to-placentome surface area was determined.

### ***Statistical Analysis***

Proportional data for placental fluid volumes were analyzed using the Chi-square test [32, 33]. All other data were analyzed using general linear model procedures [32, 33] and results are reported as least-squares means  $\pm$  SEM. Means were considered statistically different at  $P \leq 0.05$  and tendencies between  $P = 0.06$  and  $P = 0.10$ .

The model for the analysis of fetal body weight, placental weight, and placental efficiency (fetal body weight/placental weight, [34]) included only the main effect of treatment because the effects of sex and the interaction of treatment by sex were nonsignificant and these effects did not increase the  $R^2$  value. For the analysis of placental fluid volume, number of placentomes, uterine and placentome surface areas, and histological

morphometry of placentas, the model included the main effects of treatment, sex of fetus, interaction of treatment by sex of fetus, and the covariate placental weight. The model for the analysis of mRNA for VEGF and PPAR $\gamma$  as well as protein for VEGF and PPAR $\gamma$  included the main effects of treatment, sex of fetus, real-time PCR assay or protein gel, all two-way interactions between main effects, and the covariate placental weight.



## RESULTS

### *Morphometry of Fetuses and Placentas*

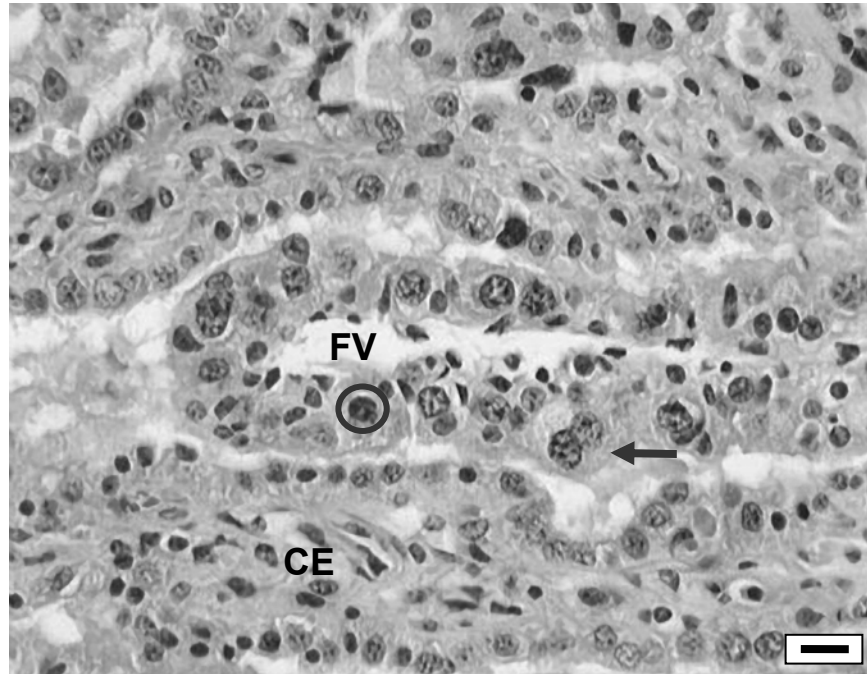
Fetuses from embryos produced in vitro were heavier ( $P = 0.03$ ) than fetuses from embryos produced in vivo (Table 2). In addition, placentas from the in vitro group tended to be heavier ( $P = 0.06$ ) than those in the in vivo group. Interestingly, placental efficiency (fetal weight/placental weight) was similar for the two treatment groups. Also, the number of placentomes was similar for the two treatment groups. No statistical difference between treatment groups was observed in placental fluid volume. However, the range of placental fluid volumes was more extreme in placentas in the in vitro group (29.5 L) compared with those in the in vivo group (7.5 L). In addition, the proportion of placentas with greater than 8.0 L (1 standard deviation above the unadjusted mean value of 6.0 L for the in vivo control group) of placental fluid volume was greater ( $P = 0.04$ ) in placentas from the in vitro group (7 of 12; 58%) compared with placentas from the in vivo group (2 of 12; 17%).

Morphometry of placentas from embryos produced either in vivo or in vitro is summarized in Table 2. Total uterine surface area was greater ( $P = 0.008$ ) for pregnancies resulting from IVP embryos compared with those from in vivo-produced embryos. However, the percent placentome surface area was less ( $P = 0.003$ ) for the in vitro group compared with the in vivo group. Figure 1 shows a section of placentome demonstrating both maternal and fetal components. Based on morphometric analysis of placentomes, volume density of fetal villi was less ( $P = 0.01$ ) in placentas from embryos produced in vitro compared with those produced in vivo. Conversely, volume density of caruncular endometrium was greater

**Table 2.** Morphometry of bovine fetuses and placentas at Day 222 of gestation from embryos produced in vivo or in vitro.<sup>a</sup>

	In Vivo	In Vitro
Number of fetuses and placentas	12	12
Body weight (kg)	17.3 ± 1.0 <sup>b</sup>	20.7 ± 1.0 <sup>c</sup>
Placental weight (kg)	2.0 ± 0.2 <sup>d</sup>	2.5 ± 0.2 <sup>e</sup>
Placental efficiency <sup>f</sup>	9.0 ± 0.5	8.9 ± 0.5
Placental fluid volume (L) <sup>g</sup>	8.0 ± 1.4	7.8 ± 1.4
Number of placentomes	81.3 ± 5.2	92.3 ± 5.2
Total uterine surface area (cm <sup>2</sup> )	2,161 ± 98 <sup>b</sup>	2,582 ± 98 <sup>c</sup>
Placentome surface area (%)	70.6 ± 2.9 <sup>b</sup>	56.1 ± 2.9 <sup>c</sup>
Caruncular endometrial volume density (%)	40.6 ± 1.4 <sup>b</sup>	46.3 ± 1.4 <sup>c</sup>
Fetal villous volume density (%)	58.8 ± 1.4 <sup>b</sup>	53.4 ± 1.3 <sup>c</sup>
Binucleate cell volume density (%)	9.0 ± 0.8 <sup>d</sup>	6.8 ± 0.8 <sup>e</sup>
Maternal pyknotic cell volume density (%)	0.7 ± 0.2	0.5 ± 0.2
Fetal pyknotic cell volume density (%)	0.4 ± 0.2	0.5 ± 0.2

<sup>a</sup> Least-squares means ± SEM.<sup>b,c</sup>  $P \leq 0.05$ .<sup>d,e</sup>  $P \leq 0.10$ .<sup>f</sup> Placental efficiency = fetal body weight/placental weight.<sup>g</sup> Amniotic plus chorioallantoic fluid.



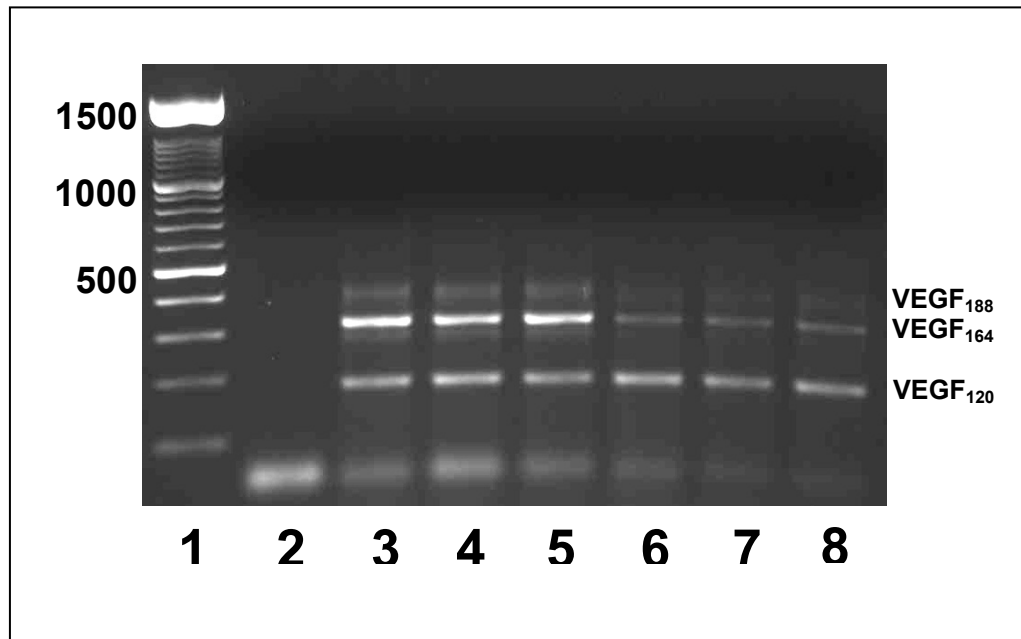
**Figure 1.** Example of a section of placentome at Day 222 of gestation from the in vivo group stained with hematoxylin and eosin that was used for assessment of volume densities for caruncular endometrium (CE), fetal villi (FV), binucleate cells (arrow), and maternal and fetal pyknotic cells (circle). Scale bar in micrograph is 10  $\mu\text{m}$ .

( $P = 0.01$ ) in placentas of the in vitro group compared with those of the in vivo group. The volume density of fetal binucleate cells tended ( $P = 0.09$ ) to be reduced in placentas of the in vitro group. There was no effect of treatment on the volume density of pyknotic cells within the fetal villi or the maternal endometrium.

### ***Expression of VEGF and PPAR $\gamma$ mRNA***

Figure 2 shows the amplification products from the VEGF-2 primer pair from cotyledonary and caruncular tissue of placentas from embryos produced in vivo or in vitro. Three bands were visualized at expected lengths corresponding to the VEGF<sub>120</sub>, VEGF<sub>164</sub> and VEGF<sub>188</sub> isoforms [26] for cotyledonary and caruncular tissue from placentas of embryos produced in vivo and in vitro. In cotyledonary tissue, VEGF<sub>164</sub> appeared to be the predominant isoform expressed. In contrast, in caruncular tissue VEGF<sub>120</sub> appeared to be expressed to a greater extent than VEGF<sub>164</sub>.

The expression of mRNA for VEGF and PPAR $\gamma$  in cotyledonary and caruncular tissues is summarized in Table 3. Based on real-time PCR, the expression of VEGF mRNA was not different in cotyledonary tissue from embryos produced in vitro compared with embryos produced in vivo. Similarly, the expression of VEGF mRNA was not different in caruncular tissue in the in vitro group compared with the in vivo group. The expression of PPAR $\gamma$  mRNA in cotyledonary tissue was not different between the in vitro group and the in vivo group. The expression of PPAR $\gamma$  mRNA in caruncular tissues was also not different in caruncular tissue from embryos produced in vitro compared with embryos produced in vivo.



**Figure 2.** Ethidium bromide-stained agarose gel of VEGF amplification products from cotyledonary and caruncular tissues at Day 222 of gestation. The gel depicts PCR products from a reaction using the VEGF-2 primer pair. Lane 1, base pair marker (100 bp ladder); Lane 2, negative control sample; Lane 3, random cotyledon sample; Lane 4, cotyledon sample in vivo group; Lane 5, cotyledon sample in vitro group; Lane 6, random caruncle sample; Lane 7, caruncle sample in vivo group; and Lane 8, caruncle sample in vitro group. The amplification products represent expected product lengths for VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> isoforms, respectively [26].

**Table 3.** Expression of VEGF and PPAR $\gamma$  mRNA and protein in bovine cotyledonary and caruncular tissues at Day 222 of gestation from embryos produced in vivo or in vitro.<sup>a</sup>

	In Vivo	In Vitro
Number of placentomes	12	12
VEGF mRNA <sup>b</sup>		
Cotyledons	0.22 $\pm$ 0.03	0.23 $\pm$ 0.03
Caruncles	0.27 $\pm$ 0.06	0.24 $\pm$ 0.06
PPAR $\gamma$ mRNA <sup>b</sup>		
Cotyledons	0.4 $\pm$ 0.2	0.5 $\pm$ 0.2
Caruncles	0.07 $\pm$ 0.02	0.05 $\pm$ 0.02
VEGF Protein <sup>c</sup>		
Cotyledons	5.5 $\pm$ 0.6	5.6 $\pm$ 0.6
Caruncles	6.1 $\pm$ 0.4	5.9 $\pm$ 0.5
PPAR $\gamma$ Protein <sup>c</sup>		
Cotyledons	6.9 $\pm$ 0.3	7.7 $\pm$ 0.3
Caruncles	7.4 $\pm$ 0.7 <sup>d</sup>	10.9 $\pm$ 0.7 <sup>e</sup>

<sup>a</sup> Least-squares means  $\pm$  SEM.

<sup>b</sup> Expressed as a ratio of mRNA for VEGF or PPAR $\gamma$  to mRNA for GAPDH.

<sup>c</sup> Expressed as signal intensity (arbitrary units).

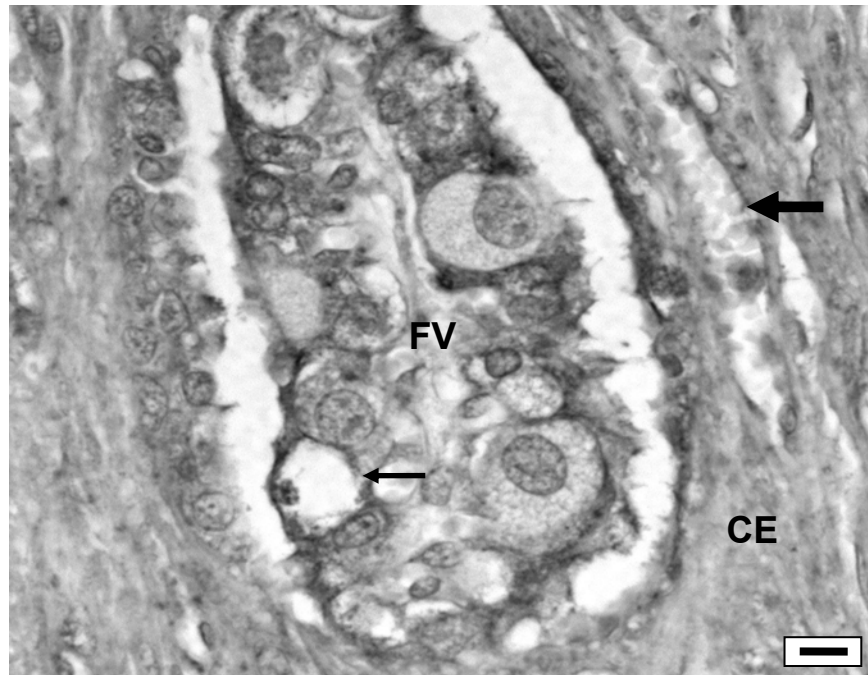
<sup>d,e</sup>  $P = 0.01$ .

### ***VEGF and PPAR $\gamma$ Protein***

Binding of VEGF and PPAR $\gamma$  antibodies to both cotyledonary and caruncular tissue proteins resulted in bands at approximately 20 kD and 50 kD, respectively (data not shown). Pre-incubation of each antibody with their respective blocking peptide eliminated the antibody signal (data not shown). The expression of protein for VEGF and PPAR $\gamma$  in cotyledonary and caruncular tissues is summarized in Table 3. The expression of VEGF protein was not different in cotyledonary tissue from the in vitro compared with the in vivo group. Also, the expression of VEGF protein in caruncular tissue was not different between the in vitro and in vivo group. The expression of PPAR $\gamma$  protein was not different in cotyledonary tissue from placentas of embryo produced in vitro compared with embryos produced in vivo. However, the expression of PPAR $\gamma$  protein in caruncular tissues was increased ( $P = 0.01$ ) for the in vitro group compared with the in vivo group.

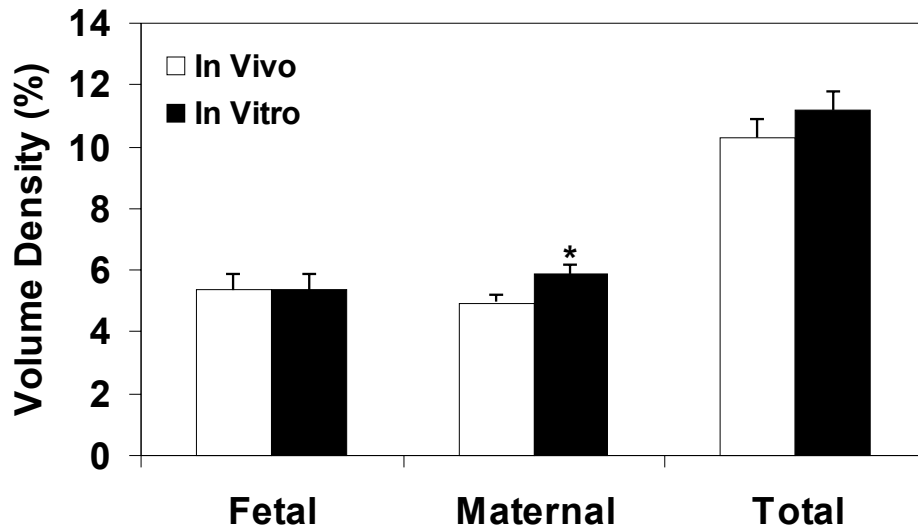
### ***Placental Vascular Morphometry***

Blood vessels within the caruncular endometrium and fetal villi were visualized using immunohistochemical staining for VEGF protein (Fig. 3). The volume density of fetal blood vessels did not differ in placentomes from embryos produced in vitro ( $5.4 \pm 0.3\%$ ) compared with embryos produced in vivo ( $5.4 \pm 0.3\%$ ; Fig. 4). The volume density of total blood vessels was not different in placentomes from embryos produced in vitro ( $11.2 \pm 0.4\%$ ) compared with embryos produced in vivo ( $10.3 \pm 0.4\%$ ; Fig. 4). In contrast, the volume density of maternal blood vessels was significantly ( $P = 0.02$ ) greater in placentomes from the in vitro group ( $5.9 \pm 0.2\%$ ) compared with the in vivo group ( $4.9 \pm 0.2\%$ ; Fig. 4). The ratio of fetal blood vessel density-to-placentome surface area was increased ( $P = 0.02$ ) in the

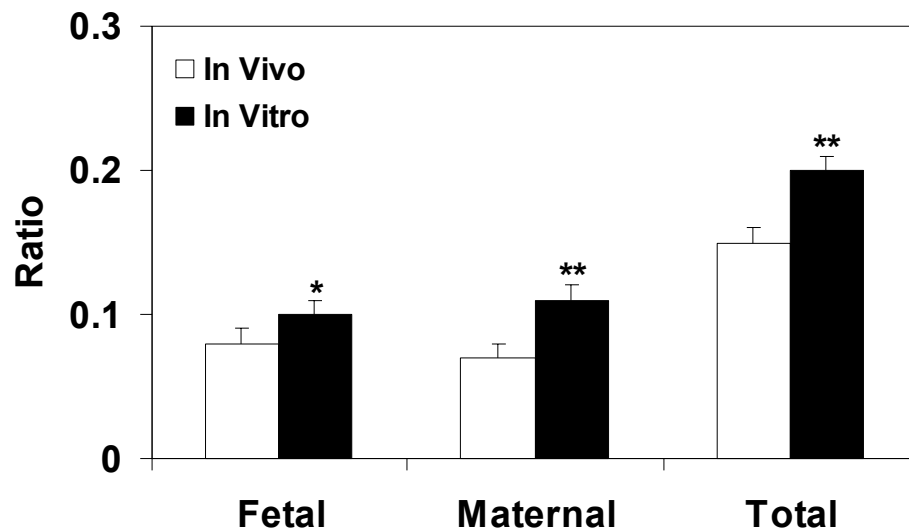


**Figure 3.** A representative transverse section of a placentome at Day 222 of gestation resulting from a bovine embryo produced in vivo. Section was incubated with a primary antibody to VEGF<sub>165</sub>. Binding of VEGF antibody was visualized by staining tissue sections with diaminobenzidine tetrahydrochloride containing nickel and was used to distinguish between vessels within caruncular endometrium (CE) (*large arrow*, note presence of red blood cells within vessel) and fetal villi (FV) (*small arrow*). Scale bar in micrograph is 10  $\mu$ m.





**Figure 4.** Volume densities of fetal, maternal, and total blood vessels (least-squares means  $\pm$  SEM) in bovine placentomes following transfer of embryos produced in vivo (n = 12) or in vitro (n = 12). \*,  $P = 0.02$ .



**Figure 5.** Ratios of fetal, maternal, and total blood vessels-to-placentome surface area (least-squares means  $\pm$  SEM) in bovine placentas following transfer of embryos produced in vivo (n = 12) or in vitro (n = 12). \*,  $P = 0.02$ ; \*\*,  $P = 0.001$ .

in vitro group ( $0.10 \pm 0.01$ ) compared with the in vivo group ( $0.08 \pm 0.01$ ; Fig 5). Similarly, the ratio of maternal blood vessel density-to-placentome surface area was increased ( $P = 0.001$ ) in the in vitro group ( $0.11 \pm 0.01$ ) compared with the in vivo group ( $0.07 \pm 0.01$ ; Fig. 5). Furthermore, the ratio of total blood vessel density-to-placentome surface area was increased ( $P = 0.001$ ) in the in vitro group ( $0.20 \pm 0.01$ ) compared with the in vivo group ( $0.15 \pm 0.01$ ; Fig. 5).

## DISCUSSION

Consistent with previous reports [1-7, 35, 36], fetuses and placentas resulting from embryos produced in vitro in the present study were heavier than in vivo controls. Pregnancies resulting from IVP embryos compared with in vivo controls had a more extreme range of placental fluid volume, with an increased proportion of placentas displaying elevation in placental fluid volume (i.e., hydrallantois). These findings are consistent with the observations of Hasler et al. [1], who observed higher incidence of hydrallantois from pregnancies resulting from IVP embryos compared with normal pregnancies. Compared with placentas from the in vivo group, placentas from the in vitro group had decreased fetomaternal contact area as measured by placentome surface area and fetal villous volume density. In contrast, placentas from the in vitro group had an increased proportional volume of blood vessels in the maternal caruncles compared with controls. In addition, the ratios of volume densities for fetal, maternal, and total blood vessels-to-placentome surface area were increased in the in vitro group. Taken together these observations suggest that placentas resulting from embryos produced in vitro may have developed compensation mechanisms in their vascular beds to ameliorate the decreased area available for fetomaternal contact.

The chorioallantoic placenta provides the major source of exchange between the developing fetus and uterine endometrium [37]. Formation of the chorioallantoic placenta results from the fusion of the nonvascular chorion with the vascularized allantoic membrane [37]. A major feature of the chorioallantoic placenta, compared with other types of mammalian placentas, is an increased surface area at the fetomaternal junction. In the cow, this increase in placental surface area occurs by the formation of chorionic villi within the

cotyledonary plaques that consist of vascular mesenchymal cones surrounded by cuboidal, mononucleate, and binucleate trophoblastic cells. These cones, or villi, interdigitate with vascular foldings of the uterine caruncular endometrium [38]. Fetal cotyledons attach to maternal caruncles to form approximately 70 to 120 placentomes that serve as the functional unit for feto-maternal exchange [39]. By Day 170 of gestation, the bovine placenta is fully developed [40]. However, placentomes continue to enlarge, resulting in the characteristic mushroom-like shape [41].

Results of the current study suggest that, during late gestation, placentas from embryos produced in vitro may be compromised relative to control placentas with respect to demands for adequate feto-maternal exchange. The observations that pregnancies from IVP embryos have larger, heavier placentas and higher incidence of hydrallantois that display a decreased placentome surface area and a decreased volume density of fetal villi support this hypothesis. These findings are in contrast to those of Bertolini et al. [5], who observed that enlarged cotyledons were associated with greater cotyledonary surface area in term placentas from bovine embryos produced in vitro compared with those produced in vivo [5]. The discrepancy in results between this study and Bertolini et al. [5] may be attributed to differences in the time of gestation examined (7 mo versus term). Our observations suggest that development is limited in placentomes in the in vitro group. Abnormal development of the placentome has also been observed in bovine placentas from nuclear transfer embryos during late gestation [42, 43].

Placentas in the in vitro group also tended to have decreased volume density of fetal binucleate cells. This observation is consistent with the suggestion that placental

development is altered in placentas from embryos produced in vitro. Fetal binucleate cells produce a variety of hormones, such as placental lactogen and pregnancy-associated glycoproteins, which play an essential role in maintenance of normal pregnancy [44].

In the normal placentome of the cow, extensive development of the vasculature occurs during late gestation [34]. In the placenta, increased blood flow results in favorable conditions that enhance exchange of materials within capillary beds [41]. Blood flow within the placenta increases by angiogenesis [16], a process that is predominantly regulated by VEGF, fibroblast growth factor (FGF) and angiopoietins [10, 45]. In the present study, we have shown that mRNA for VEGF was expressed in both cotyledonary and caruncular tissues. However, there was no effect of treatment on the levels of mRNA expression for VEGF in either cotyledonary or caruncular tissues. In addition, no differences between treatment groups were found for VEGF protein in either cotyledonary or caruncular tissues. These findings imply that, at Day 222 of gestation, bovine placentas resulting from embryos produced in vitro do not have alterations in placental angiogenesis, at least based on assessment of VEGF mRNA and protein. However, it remains possible that VEGF mRNA and protein may be altered at other stages of gestation in bovine placentas from embryos produced in vitro. Alternatively, other angiogenic factors, such as FGF or angiopoietins, may be altered in bovine placentas resulting from in vitro production of embryos.

The predominant VEGF isoform detected in late gestation bovine cotyledonary tissue in the present study was VEGF<sub>164</sub>. This finding is consistent with the report by Cheung and Brace [26], who found that VEGF<sub>164</sub> was the predominant isoform in ovine cotyledons. Interestingly, the most abundant VEGF isoform found in bovine caruncles in the present

study was VEGF<sub>120</sub>. Expression of the VEGF<sub>144</sub> or VEGF<sub>205</sub> isoforms was not detected in either cotyledons or caruncles. Together, these findings suggest that differences may exist among ruminant species in the patterns of expression of VEGF isoforms in cotyledonary and caruncular tissues.

PPAR $\gamma$ , a transcription factor, has been shown to upregulate VEGF expression in macrophages [14] and vascular smooth muscle [15]. PPAR $\gamma$  has also been shown to play a critical role in vascular development of placentas in mice [17]. Furthermore, PPAR $\gamma$  plays an important role in regulating the differentiation of extravillous cytotrophoblast in human placentas [46]. Because PPAR $\gamma$  plays a role in development and organization of the placenta, we wanted to determine if PPAR $\gamma$  was altered in bovine placentas from embryos produced in vitro or in vivo. In the present study, PPAR $\gamma$  mRNA and protein was expressed in both cotyledonary and caruncular tissues from late gestation bovine placentas. Expression of PPAR $\gamma$  mRNA was not different in either cotyledonary or caruncular tissues between the in vitro and in vivo groups. In addition, no difference between the in vivo and in vitro groups was found in cotyledonary levels of protein for PPAR $\gamma$ . In contrast, the caruncular levels of PPAR $\gamma$  protein were increased for the in vitro group compared with the in vivo group. The discrepancy between mRNA and protein for PPAR $\gamma$  in the in vitro group may be explained by an increased turnover from mRNA to protein within the caruncles. The increase of PPAR $\gamma$  protein observed in caruncular tissues of the vitro group suggests that these placentas may have enhanced vascular development compared with placentas from the in vivo group.

Fetal vascular volume densities in placentomes were similar for the in vivo and in vitro groups. Conversely, maternal vascular volume density was increased in placentomes in

the in vitro group compared with the in vivo group. Interestingly, the ratios of volume densities of fetal, maternal, and total blood vessels-to-placentome surface area were all increased in placentas from embryos produced in vitro compared with in vivo controls. These findings suggest that vascular development was enhanced in the placentomes resulting from embryos produced in vitro. Enhanced vascular development observed in these placentomes was not associated with changes in levels of mRNA or protein expression for VEGF. However, the levels of PPAR $\gamma$  protein in the caruncular tissue of placentas from the in vitro group were increased, suggesting that vascular development of these placentas may be modulated by PPAR $\gamma$  or other angiogenic factors such as FGF or angiopoietins [45]. Alternatively, mRNA and protein for VEGF may be altered at an earlier stage of gestation, thus, driving enhanced development of the placental vasculature observed in placentas from embryos produced in vitro.

In conclusion, compared with placentas from embryos produced in vivo, placentas from embryos produced in vitro appear to compensate for decreased feto-maternal contact with an increased proportion of blood vessels within the cotyledonary and caruncular tissues of the placentome. These findings are consistent with the concept that compensatory mechanisms are present during late gestation in the vascular beds of placentas from bovine embryos produced in vitro.

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## **EFFECTS OF EMBRYO CULTURE ON ANGIOGENESIS AND MORPHOMETRY OF BOVINE PLACENTAS DURING EARLY GESTATION<sup>1</sup>**

### **ABSTRACT**

The objective of this study was to determine the effects of undefined and semi-defined culture systems for in vitro embryo production on angiogenesis and morphometry of bovine placentas during early gestation. Blastocysts produced in vivo were recovered from superovulated Holstein cows and served as controls. Blastocysts produced in vitro were exposed to either serum supplemented co-culture medium (in vitro-produced with serum; IVPS) or modified synthetic oviductal fluid medium (mSOF). Single blastocysts from each production system were transferred into heifers. Fetuses and placentas were recovered on Day 70 of gestation (In Vivo, n = 6; IVPS, n = 6; mSOF, n = 6). Cotyledonary tissues were obtained for quantification of vascular endothelial growth factor (VEGF) and peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) mRNA and protein. Samples of placentomes were prepared for immunocytochemistry and histological analysis. Mean fetal body weights did not differ between the three treatment groups. However, an increased proportion of fetuses were heavier in both the IVPS and mSOF groups compared with controls. Placentas from the mSOF group were heavier and had the fewest placentomes, least placental fluid and lowest placental efficiency (fetal weight/placental weight) compared with the In Vivo and IVPS groups. There was no effect of embryo culture system on volume density of fetal villi or maternal endometrium within placentomes. The volume density of fetal binucleate cells was increased in the IVPS group compared with the In Vivo group.

Placentomes in the mSOF group had an increased volume density of fetal pyknotic cells compared with the In Vivo and IVPS groups. The expression of VEGF mRNA was lowest in cotyledonary tissue from the mSOF group compared with the In Vivo and IVPS groups. However, there was no effect of treatment on the levels of VEGF protein, expression of PPAR $\gamma$  mRNA or levels of PPAR $\gamma$  protein in cotyledonary tissue. The volume densities of blood vessels in the fetal, maternal, and combined components of the placentome were decreased in the mSOF group compared with controls. In conclusion, compared with placentas from embryos produced in vivo or in vitro using an undefined culture system, placentas from embryos produced in vitro using a semi-defined culture system exhibited a greater degree of aberrant development of the placenta during early gestation.

**Key Words:** developmental biology, embryo, gene regulation, in vitro fertilization, placenta

## INTRODUCTION

Abnormal placentation has been observed following transfer of in vitro-produced (IVP) embryos in cattle [1-4] and sheep [5]. Placental abnormalities associated with in vitro production of embryos have included an increased incidence of hydrallantois [1, 4-6], decreased number of placentomes [7] as well as variations in feto-maternal contact [3, 4], and placental vascular development [4]. Furthermore, the production of embryos by nuclear transfer (NT; cloning) has also been associated with severe abnormalities of the placenta and pregnancy loss in cattle [8-11], sheep [12, 13], and mice [14-16]. Recently, we have shown that during late gestation, bovine placentas from embryos produced in vitro had decreased feto-maternal contact as measured by surface area of placentomes and volume density of fetal villi compared with their in vivo counterparts [4]. However, placentas from embryos produced in vitro had an increased density of maternal blood vessels and an increased ratio of blood vessels-to-placentome surface area compared with controls [4]. These findings support the hypothesis that during late gestation in the cow, placentas for embryos produced in vitro have enhanced vascular development which may be compensating for decreased feto-maternal contact.

Placental angiogenesis is critical for increasing placental blood flow throughout gestation [17, 18], thereby, ensuring proper availability of nutrients to the developing fetus, regulation of gas exchange and elimination of waste products. Molecular regulation of angiogenesis in the placenta is driven by angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietins [17, 19, 20]. In sheep, VEGF and FGF act on endothelial cells to stimulate placental angiogenesis [18].



Interestingly, in our previous of late gestation placentas in the cow, no differences were found between placentas from in vivo- or in vitro-produced embryos in the levels of expression of mRNA for VEGF or the levels of protein for VEGF in either caruncular or cotyledonary tissues [4]. However, caruncular tissues from in vitro-produced embryos had increased levels of protein for peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) compared with controls suggesting that enhanced vascular development in bovine placentas may be regulated by PPAR $\gamma$  [4]. PPAR $\gamma$  has also been associated with vascular development of the placenta in mice [21] and tissue remodeling of the placenta in humans [22]. Alterations in expression of angiogenic factors, such as VEGF and potentially PPAR $\gamma$ , may initiate abnormalities in vascular development with placentas from embryos produced in vitro.

The establishment of adequate embryo culture systems is essential for the production of a live, healthy offspring following embryo transfer. Successful in vitro systems have included the use of undefined (co-culture and/or serum supplementation), semi-defined (BSA supplementation), and defined (amino acids and polyvinyl alcohol supplementation) media [23-25]. In cattle and sheep, extensive comparisons of culture systems have been made using endpoints including rate of blastocyst development [24, 26-30], embryo morphometry [31, 32], pregnancy rate [2, 33-35], fetal and placental weights [2, 5], and birth weight [34-36]. Developmental abnormalities of the fetus, placenta, and offspring have been predominantly attributed to the presence of serum in the culture medium [5, 28, 34, 37]. In sheep, placental weight was greater for placentas from embryos produced using serum supplemented co-culture medium compared with placentas from embryos produced in vivo or in vitro using

BSA supplemented medium [5]. In addition, a higher incidence of polyhydramnios ( $\geq 23\%$ ) was observed in sheep placentas derived from co-cultured embryos [5]. Similarly, a higher incidence of hydrallantois was observed in bovine pregnancies resulting from embryos produced using serum supplemented co-culture medium [1].

The overall objective of this study was to determine the effects of in vitro production of embryos using either undefined or semi-defined culture systems on the morphometry and angiogenesis of placentas during early gestation in cattle. Specifically, we directly compared placentas at Day 70 of gestation from bovine embryos produced in vivo (control group) or in vitro using either a co-culture medium supplemented with serum (undefined) or modified synthetic oviductal fluid medium supplemented with BSA (semi-defined) for 1) gross and histological morphometry, 2) mRNA and protein expression for VEGF and PPAR $\gamma$  in cotyledonary tissue, and 3) morphometry of blood vessels within the cotyledonary (fetal) and caruncular (maternal) components of placentomes.

## MATERIALS AND METHODS

### *Reagents and Hormones*

Tissue culture medium (M-199 with Earle's Salts) was purchased from Gibco BRL (Grand Island, NY). Equine pituitary LH (11.5 NIH LH-S1 units/mg) and porcine pituitary FSH (50 mg/vial Armour FSH Standard) preparations were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty-acid free BSA was purchased from Roche Applied Sciences (Indianapolis, IN). All other culture reagents were tissue culture grade and were purchased from Sigma Chemical Co.

TRI-Reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). DNase and random hexamers were purchased from Promega (Madison, WI). SuperScript II reverse transcriptase and dNTPs were purchased from Invitrogen Co (Carlsbad, CA). PCR purification kits were purchased from Qiagen (Valencia, CA). Taq polymerase was purchased from Roche Applied Sciences (Indianapolis, IN). SYBR green dye was purchased from Molecular Probes, Inc. (Eugene, OR). All primers for PCR and real-time PCR were custom synthesized by either Sigma-Genosys (Woodlands, TX) or Qiagen Operon (Alameda, CA). For detection of VEGF protein by Western blot and immunocytochemistry, an anti-VEGF polyclonal rabbit antibody (sc-152) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For detection of PPAR $\gamma$  protein by Western blot, an anti-PPAR $\gamma$  polyclonal rabbit antibody (107100) was purchased from Cayman Chemical (Ann Arbor, MI).

### ***Production of Embryos***

All procedures and protocols involving the use of cattle were approved by the Institutional Animal Care and Use Committee at North Carolina State University. For in vivo embryo production, Holstein donor cows were synchronized using two i.m. injections of 25 mg prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ , Lutalyse<sup>®</sup>, Pharmacia & Upjohn Co., Kalamazoo, MI) 14 days apart. Donor cows were superovulated with 400 mg FSH (Folltropin<sup>®</sup>, Vetrapharm Canada, London, ON) administered in decreasing doses over a 4-day period beginning on Day 10, 11, 12, or 13 of the estrous cycle (Day 0 = estrus). On the morning and evening of the third day of FSH treatment, estrus was induced using two i.m. injections of 25 mg of  $PGF_{2\alpha}$ . Donors were artificially inseminated at 12 and 24 h after detection of first standing heat with thawed frozen semen from the same proven Holstein bull. Embryos were collected by nonsurgical uterine flushing on Day 7 (Day 0 = first detected estrus).

For in vitro embryo production, ovaries of Holstein cows were obtained from a local abattoir and held in saline with 0.75  $\mu\text{g/ml}$  penicillin for 4-6 h during transport to the laboratory. Cumulus-oocyte complexes (COC) were aspirated, matured and fertilized in vitro as previously described [7]. Briefly, COCs were aspirated from 2-7 mm follicles and washed five times in modified Tyrode's medium (TL-Hepes). Groups of 20 to 30 COC were matured for approximately 22 h in M-199 supplemented with 10% heat inactivated estrus cow serum (ECS), 10  $\mu\text{g/ml}$  LH, 5  $\mu\text{g/ml}$  FSH, 1  $\mu\text{g/ml}$  estradiol, 200  $\mu\text{M}$  sodium pyruvate, and 50  $\mu\text{g/ml}$  gentamicin. All cultures were incubated at 5%  $\text{CO}_2$  in air with 100% humidity. Following the maturation period, COCs were washed once and placed in fertilization medium that consisted of heparin-supplemented Tyrode's albumin lactate pyruvate medium

with 6 mg/ml fatty acid-free BSA [38]. Thawed frozen semen from the same Holstein bull used to inseminate the donor cows for in vivo embryo production was used for in vitro fertilization. Motile spermatozoa were collected using the swim-up procedure [38] and a final concentration of  $1 \times 10^6$  spermatozoa per ml was used for fertilization in 0.75 ml of fertilization media. Spermatozoa and COCs were coincubated for 18-20 h. Following incubation, presumptive zygotes were washed six times with TL-Hepes and placed into one of two culture systems for the 168-h embryo culture period. The two culture systems used were in vitro-produced with serum (IVPS; M-199 supplemented with 10% ECS [7]) and modified synthetic oviductal fluid (mSOF; mSOF containing 0.6% BSA [32]). Embryos for the IVPS group were cultured with their cumulus investments in 1 ml of medium in an atmosphere of 5% CO<sub>2</sub> in air with 100% humidity. Culture medium for the IVPS group was changed at 48-h intervals throughout the 168-h culture period [7]. Embryos for the mSOF group had their cumulus investments removed after fertilization by moderate vortexing and embryos were cultured undisturbed in 1 ml of medium in an atmosphere of 90% N<sub>2</sub>:5%O<sub>2</sub>:5% CO<sub>2</sub> throughout the 168-h culture period [32].

### ***Transfer of Embryos***

Angus heifers were given two i.m. injections of 25 mg of PGF<sub>2</sub>α 10 to 12 days apart to synchronize estrus. Grade 1 blastocysts [39] from in vivo or in vitro production systems were transferred in TL-Hepes medium singly into the uterine horn ipsilateral to the ovary bearing the corpus luteum of recipient heifers at Day 7 of the estrous cycle.

***Recovery of Fetuses and Placental Tissue***

At Day 70 of gestation (63 days after transfer), a total of 18 pregnant recipients (n = 6 each for In Vivo, IVPS, and mSOF groups) were slaughtered. Fetuses and their placentas were removed from the reproductive tracts and physical measurements were taken including fetal body weight, wet placental weight, number of placentomes, and placental fluid (amniotic plus chorioallantoic fluid) volume. Samples of cotyledonary tissue were obtained by careful manual separation of cotyledonary tissue from the placentome. These tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for whole cell RNA (wcRNA) and protein extraction. Center segments cut from whole placentomes of individual placentas were stored in 10% neutral buffered formalin for histology and immunocytochemistry.

***Reverse Transcription and Verification of PCR Products***

Whole cell RNA from cotyledonary tissue was extracted using a previously reported protocol [4]. Briefly, wcRNA was extracted using TRI-Reagent and dissolved in diethyl pyrocarbonate-treated water. Concentrations of wcRNA were determined by absorbance at 260 nm. The quality and integrity of the wcRNA was assessed based on the ratio of absorbance at 260 and 280 nm and visualization of 28S and 18S rRNA bands in ethidium bromide-stained agarose gels (data not shown). Each sample of wcRNA was treated with DNase (1.5units) for 20 min at 37°C. Following DNase inactivation, wcRNA was then reverse transcribed using random hexamers and SuperScript II reverse transcriptase under conditions recommended by the manufacturer. Following cDNA synthesis, samples were purified using the Qiagen PCR purification kit as recommended by the manufacturer and stored at 4°C.

Forward and reverse primer sequences used to amplify mRNA for VEGF-1, PPAR $\gamma$ , and Histone H2a are found in Table 1. The VEGF-2 forward and reverse primer pair, specifically designed to detect all five VEGF isoforms [40], was also used (Table 1). Each PCR reaction consisted of 50 ng equivalents of cotyledonary cDNA from a control (In Vivo) placenta, 1.6  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer (Roche), and 2.5 units of Taq polymerase in a 20- $\mu$ l reaction. A negative control lacking cDNA was included in each PCR assay. All PCR reactions were run in 96-well PCR plates. Plates were placed into an iCycler thermocycler (Bio-Rad, Richmond CA). Each PCR program consisted of 90 sec at 95°C, followed by 35 cycles of 30 sec denaturation at 94°C and 45 sec annealing at the appropriate temperature. Following amplification cycles, a primer extension for 5 min at 72°C was used. The primer sequences used for PCR as well as the specific isoform(s) detected, annealing temperatures, and expected product lengths are shown in Table 1. The specificity of the PCR products using the VEGF-1, VEGF-2, PPAR $\gamma$ , and H2a primer pairs were each verified by sequence analysis.

### ***Real-Time Quantitative PCR Analysis***

VEGF-1, PPAR $\gamma$ , and H2a primers were used for quantification of VEGF and PPAR $\gamma$  mRNA levels by real-time reverse transcription-PCR as previously reported [4]. Briefly, each PCR reaction consisted of 50 ng equivalents of cDNA, 1.6  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer, 2  $\mu$ l of 2X SYBR Green I dye, 1  $\mu$ l of 200 nM Fluorescein dye (Bio-Rad), and 2.5 units of Taq polymerase in a 20- $\mu$ l reaction. Melt-curve analysis and gel electrophoresis was used to confirm product

**Table 1.** Primer sequences used for PCR analysis of cDNA from bovine cotyledonary tissues.

Primer	Sequence	Isoform	Annealing Temperature (°C)	Fragment Size (bp)
H2a <sup>a,b</sup>	Forward 5'-AGG ACG ACT AGC CAT GGA CGT GTG Reverse 5' -GTT CCG ATG TTA ACG ACC ACC		59	208
PPAR $\gamma$ <sup>a,c</sup>	Forward 5'-ATT ACC ATG GTT GAC ACA GAG Reverse 5' -ATG AGG GAG TTG GAA GGC TC	PPAR $\gamma$ 1	60	354
VEGF-1 <sup>a,c</sup>	Forward 5'-ACG AAA GTC TGG AGT GTG Reverse 5' -TTG TTA TGC TGT AGG AAG	VEGF <sub>164</sub>	60	115
VEGF-2 <sup>d</sup>	Forward 5' -TGT AAT GAC GAA AGT CTG CAG Reverse 5' -TCA CCG CCT CGG CTT GTC ACA	VEGF <sub>120</sub>	60	186
		VEGF <sub>144</sub>		258
		VEGF <sub>164</sub>		318
		VEGF <sub>188</sub>		390
		VEGF <sub>205</sub>		441

<sup>a</sup> Primers used for real-time quantitative PCR analysis.<sup>b</sup> Primers from [41].<sup>c</sup> Primers from [4].<sup>d</sup> Primers from [40].



length after amplifications were complete (data not included). Prior to quantification of VEGF and PPAR $\gamma$ , raw cycle threshold ( $C_T$ ) values were converted to the linear term using the formula  $2^{-C_T}$  [42]. Expression of VEGF and PPAR $\gamma$  mRNA in cotyledonary tissues was expressed relative to the expression of the endogenous control mRNA, Histone H2a. Levels of VEGF and PPAR $\gamma$  mRNAs are expressed as a ratio of linearized  $C_T$  values for the gene of interest to the linearized  $C_T$  values for H2a in individual samples.

Expression of mRNA for either VEGF or PPAR $\gamma$  in cotyledonary tissue was analyzed in one assay (In Vivo,  $n = 6$ ; IVPS,  $n = 6$ ; mSOF,  $n = 6$ ). Each assay contained reference samples of cotyledonary tissue from a random control bovine placenta at approximately Day 70 of gestation (fetal crown-rump length = 10.0 cm). The reference sample was used to determine the intra-assay coefficient of variation (CV) for the linearized  $C_T$  values. The intra-assay CV of the VEGF assay was 9.8% and the intra-assay CV of the PPAR $\gamma$  assay was 4.9%.

### ***Western Blot***

Protein was extracted from cotyledonary tissue according to Miles et al. [4]. Briefly, protein was resuspended in a cold buffer (13.3  $\mu$ l buffer per mg of tissue) consisting of 1% (v/v) Triton X-100, 2 mM EDTA  $1^{-1}$ , 2 mM EGTA  $1^{-1}$ , aprotinin (20  $\mu$ g  $ml^{-1}$ ), leupeptin (20  $\mu$ g  $ml^{-1}$ ), 1 mM PMSF, and 20 mol Hepes  $1^{-1}$ . Total protein was quantified using bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the manufacturer's recommended protocol.

Expression of VEGF and PPAR $\gamma$  protein was evaluated using a modified Western blot protocol [43]. Briefly, a total of 20 mg protein from cotyledonary tissues was separated

on 12% (w/v) SDS-PAGE gels under nonreducing conditions. Following electrophoresis, the polyacrylamide gels were transferred to nitrocellulose membranes (Bio-Rad) using a semi-dry transfer system (Bio-Rad). VEGF and PPAR $\gamma$  antibody binding was detected using the BM Chemiluminescence Western blotting kit (Mouse/Rabbit; Roche Applied Science, Indianapolis, IN). Blots were incubated overnight at 4°C with a 1:500 dilution of VEGF polyclonal antibody or 1:750 dilution of PPAR $\gamma$  polyclonal antibody. Specificity of the VEGF and PPAR $\gamma$  antibodies was verified using blocking peptides obtained from Santa Cruz Biotechnology, Inc. and Cayman Chemical, respectively. Blots were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) and binding was quantified using computer-assisted video image analysis (Optimas Visual Imaging System 6.1; Optimas Corporation, Bothell, WA).

### ***Morphometry of Placentomes***

Samples of intact placentomes were embedded in paraffin and 5  $\mu\text{m}$  sections of placentome tissues were prepared. Sections were deparaffinized, dehydrated and stained with hematoxylin and eosin. Stereological end points including the volume densities of fetal villi, caruncular endometrium, fetal binucleate cells, fetal pyknotic cells, and maternal pyknotic cells were evaluated by point count methods [44, 45] using computer-assisted image analysis (Optimas Visual Imaging System 6.1). For analysis of fetal villi, caruncular endometrium, and fetal binucleate cells, 10 fields of view representing a total of  $8.12 \times 10^6 \mu\text{m}^2$  of each placentome tissue was examined using a 100-point grid system [45]. For analysis of fetal and maternal pyknotic cells, 10 fields of view representing a total of  $6.01 \times 10^4 \mu\text{m}^2$  of each placentome tissue was examined using a 256-point grid system [45].

Immunocytochemical localization of VEGF protein was used to identify vascular beds for morphometric analysis [4]. Briefly, antigen availability within tissue sections was enhanced using Target Unmasking Fluid (BD Pharmingen, San Jose, CA) as recommended by the manufacturer. Immunoreactivity for VEGF was detected using a 1:50 dilution of the VEGF polyclonal antibody and an incubation period of 1 h at RT. Specificity of the VEGF antibody was verified using blocking peptide obtained from Santa Cruz Biotechnology, Inc. Binding of VEGF antibody within placentome sections was visualized with diaminobenzidine tetrahydrochloride containing nickel (Vector Laboratories, Burlingame, CA).

For analysis of maternal and fetal blood vessels, 20 fields of view representing a total of  $4.8 \times 10^5 \mu\text{m}^2$  of tissue was examined from each placentome section. Point count methodology with a 256-point grid system [45] was used to determine the volume densities of maternal and fetal blood vessels. For determination of total blood vessel volume density, the volume densities of maternal and fetal blood vessels were added.

### ***Statistical Analysis***

Proportional data for fetal and placental weights were analyzed using the Chi-square test [46, 47]. All other data were analyzed using general linear model procedure for analysis of variance [46, 47]. When a significant F-statistic was determined, means were separated using the Duncan multiple-range test [46, 47]. Means were considered statistically different at  $P \leq 0.05$  and tendencies between  $P = 0.06$  and  $P = 0.10$ . Results are reported as least-squares means  $\pm$  SEM.

The model for analysis of fetal body weight, placental weight, and placental efficiency (fetal weight/placental weight, [48]) included only the main effect of treatment (In Vivo, IVPS, and mSOF) because the main effects of sex of fetus and the interaction of treatment by sex of fetus were nonsignificant and these effects did not increase the  $R^2$  value. The model for analysis of placental fluid volume, number of placentomes, histological morphometry of placentas, and mRNA for VEGF and PPAR $\gamma$  included the main effects of treatment, sex of fetus, interaction of treatment by sex of fetus, and the covariate placental weight. For the analysis of protein for VEGF and PPAR $\gamma$ , the model included the main effects of treatment, sex of fetus, protein gel, all two-way interactions between main effects, and the covariate placental weight.

## RESULTS

### *Morphometry of Fetuses and Placentas*

Morphometry of fetuses and placentas resulting from the transfer of In Vivo, IVPS, and mSOF produced embryos is summarized in Table 2. The mean body weights of fetuses derived from these three sources of embryos were not different. However, the range of fetal body weight was more extreme for fetuses in the IVPS (12.9 g) and mSOF (28.2 g) groups compared with In Vivo controls (5.6 g). In addition, the proportion of fetuses heavier than 41 g (+2 standard deviations (SD) above the mean fetal body weight for the In Vivo group) was greater ( $P = 0.05$ ) in the mSOF group (3 of 6; 50%) compared with the In Vivo group (0 of 6; 0%). There was also an increased proportion of heavier fetuses in the IVPS group (2 of 6; 33%) compared with controls (0%); however, this difference in proportions was not significant ( $P = 0.12$ ).

Placentas were heavier ( $P = 0.02$ ) from the mSOF group compared with the In Vivo and IVPS groups (Table 2). The range of placental weight was also more extreme for placentas in the mSOF group (173.4 g) compared with the IVPS (76.0 g) and In Vivo (79.5 g) groups. In addition, the proportion of placentas heavier than 177 g (+2 SD above the mean placental weight for the In Vivo group) was greater ( $P = 0.05$ ) in the mSOF group (3 of 6; 50%) compared with the control group (0 of 6; 0%). The mean placental fluid volume tended to be less ( $P = 0.07$ ) in the mSOF group compared with the In Vivo and IVPS groups. Placentome number was also less ( $P = 0.02$ ) in the mSOF group compared with the In Vivo and IVPS groups. Interestingly, placental efficiency (fetal weight/placental weight) was less

**Table 2.** Morphometry of bovine fetuses and placentas at Day 70 of gestation from blastocysts produced in vivo or in vitro using either an undefined (IVPS) or semi-defined (mSOF) medium.<sup>a</sup>

	In Vivo	IVPS	mSOF
Number of fetuses and placentas	6	6	6
Fetal body weight (g)	35.9 ± 2.7 (5.6)	37.8 ± 2.7 (12.9)	39.3 ± 2.7 (28.2)
Placental weight (g)	107.4 ± 19.3 <sup>b</sup> (79.5)	87.8 ± 19.3 <sup>b</sup> (76.0)	172.3 ± 19.3 <sup>c</sup> (173.4)
Placental efficiency <sup>f</sup>	0.36 ± 0.05 <sup>b,c</sup> (0.23)	0.47 ± 0.05 <sup>b</sup> (0.38)	0.24 ± 0.05 <sup>c</sup> (0.12)
Placental fluid volume (ml) <sup>g</sup>	480 ± 45 <sup>d</sup> (296)	490 ± 49 <sup>d</sup> (310)	301 ± 54 <sup>e</sup> (239)
Number of placentomes	72.3 ± 3.0 <sup>b</sup> (25)	71.0 ± 3.3 <sup>b</sup> (42)	55.7 ± 3.6 <sup>c</sup> (44)
Endometrial volume density (%)	49.9 ± 2.6 (15.7)	50.5 ± 2.8 (18.9)	54.0 ± 3.4 (9.7)
Villous volume density (%)	50.1 ± 2.6 (15.7)	49.5 ± 2.8 (18.9)	46.0 ± 3.4 (9.7)
Fetal binucleate cell volume density (%)	2.9 ± 0.3 <sup>b</sup> (1.2)	4.3 ± 0.4 <sup>c</sup> (2.1)	3.4 ± 0.4 <sup>b,c</sup> (1.1)
Maternal pyknotic cell volume density (%)	0.29 ± 0.06 (0.27)	0.28 ± 0.07 (0.51)	0.51 ± 0.09 (0.43)
Fetal pyknotic cell volume density (%)	0.26 ± 0.04 <sup>b</sup> (0.31)	0.35 ± 0.05 <sup>b</sup> (0.24)	0.53 ± 0.06 <sup>c</sup> (0.23)

<sup>a</sup> Least-squares means ± SEM (Range).

<sup>b,c</sup>  $P \leq 0.05$ .

<sup>d,e</sup>  $P = 0.07$ .

<sup>f</sup> Placental efficiency = fetal body weight/placental weight.

<sup>g</sup> Amniotic plus chorioallantoic fluid.

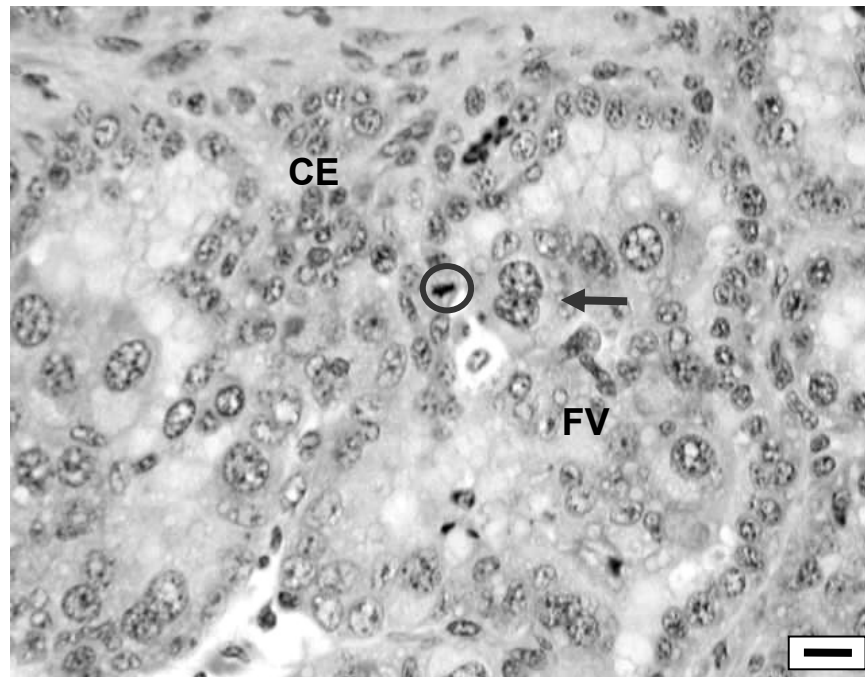
( $P = 0.01$ ) for the mSOF group compared with the IVPS group. Placental efficiency was similar for the control group compared with both the IVPS and mSOF groups.

In Figure 1 is shown the maternal and fetal components of a placentome at Day 70 of gestation. Based on morphometric analysis of placentomes, the volume densities of fetal villi as well as maternal endometrium were not different between the three groups (Table 2). However, the volume density of fetal binucleate cells was increased ( $P = 0.04$ ) in the IVPS group compared with in vivo controls. Placentomes from the mSOF group displayed an intermediate volume density of fetal binucleate cells between the In Vivo and IVPS groups. There was no effect of treatment on the pyknotic cell volume density within the maternal endometrium. However, the volume density of pyknotic cells within the fetal villi was increased ( $P = 0.01$ ) in placentas from the mSOF group compared with either the In Vivo or IVPS groups.

### ***Expression of VEGF and PPAR $\gamma$ mRNA***

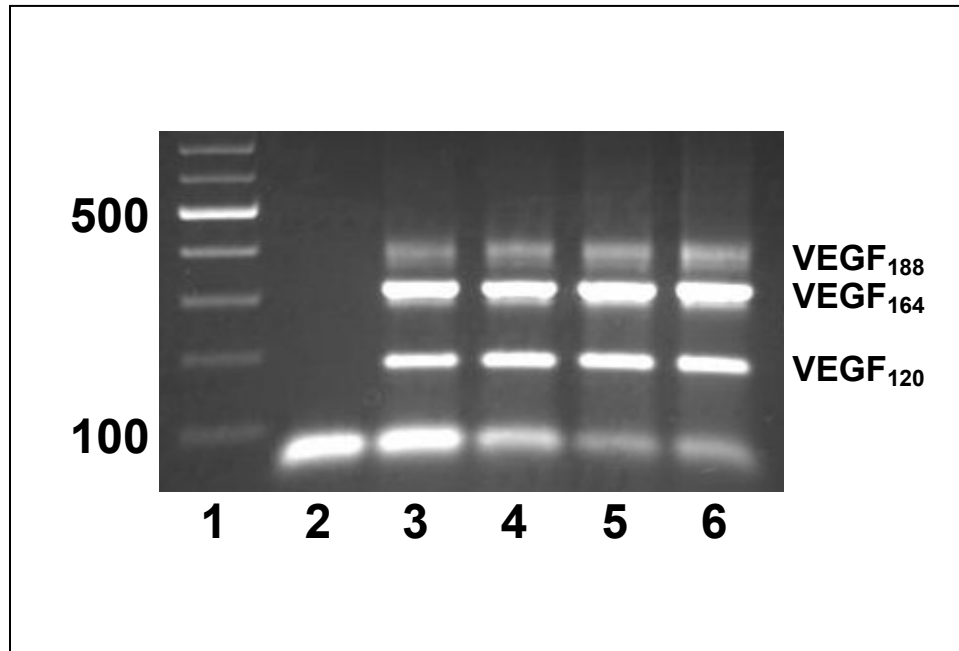
In Figure 2 is displayed the amplification products following RT-PCR using the VEGF-2 primer pair and cDNA from cotyledonary tissue of placentas from the In Vivo, IVPS, and mSOF groups. Three bands were visualized at expected lengths corresponding to the VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>189</sub> isoforms [40] for cotyledonary tissue from the In Vivo, IVPS, and mSOF groups. The predominant isoform in cotyledonary tissue from all three groups appeared to be VEGF<sub>164</sub>.

The expression of VEGF mRNA was less ( $P = 0.02$ ) in cotyledonary tissue from placentas in the mSOF group ( $0.17 \pm 0.07$ ) compared with the In Vivo ( $0.46 \pm 0.06$ ) and IVPS ( $0.50 \pm 0.06$ ) groups (Fig. 3A). In contrast, the expression of PPAR $\gamma$  mRNA was not

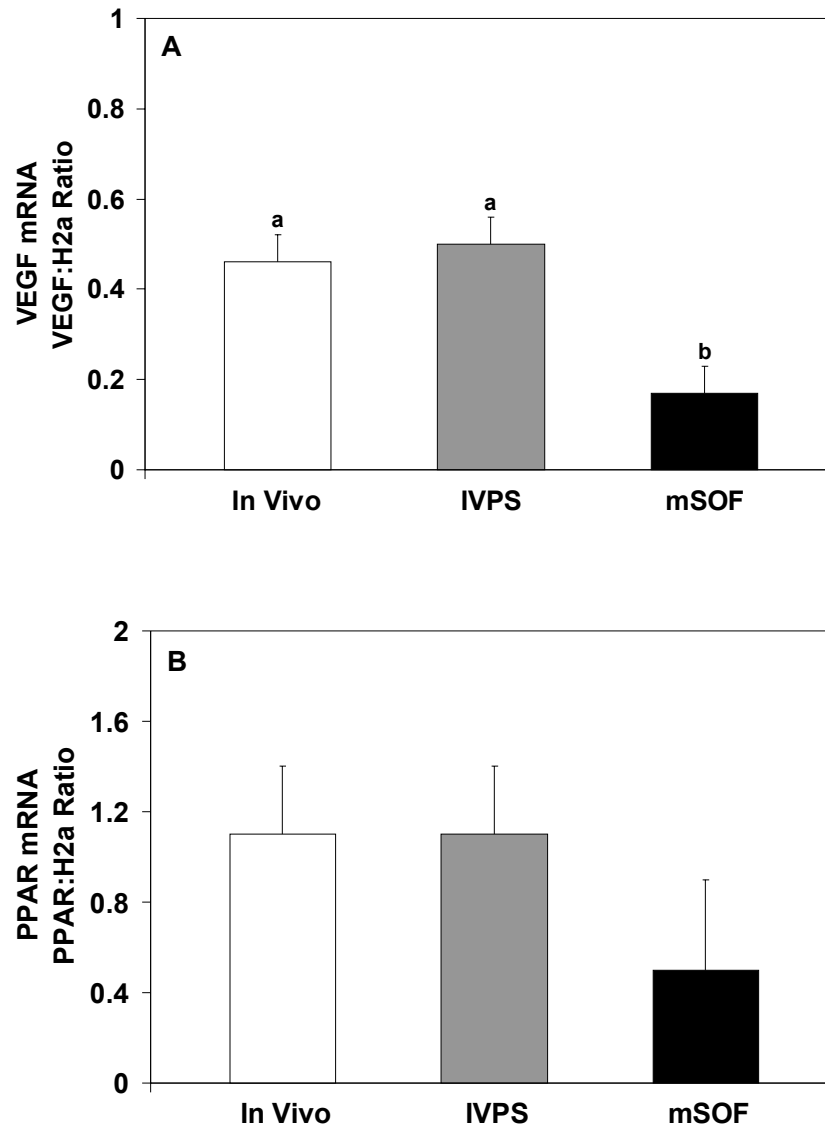


**Figure 1.** A section of bovine placentome at Day 70 of gestation from the In Vivo group stained with hematoxylin and eosin that was used for assessment of volume densities of caruncular endometrium (CE), fetal villi (FV), fetal binucleate cells (arrow), maternal pyknotic cells, and fetal pyknotic cells (circle). Scale bar in micrograph is 10  $\mu$ m.





**Figure 2.** Ethidium bromide-stained agarose gel of VEGF amplification products from bovine cotyledonary tissues at Day 70 of gestation. The gel depicts PCR products from a reaction using the VEGF-2 primer pair. Lane 1, base pair marker (100 bp ladder); Lane 2, negative control sample; Lane 3, random cotyledon sample; Lane 4, In Vivo cotyledon sample; Lane 5, IVPS cotyledon sample; Lane 6, mSOF cotyledon sample. The amplification products represent expected product lengths for VEGF<sub>120</sub> (186 bp), VEGF<sub>164</sub> (318 bp), and VEGF<sub>188</sub> (390 bp) isoforms, respectively [40].



**Figure 3.** **A)** Expression of VEGF mRNA (least-squares means  $\pm$  SEM) in bovine cotyledonary tissues of placentas from embryos produced In Vivo ( $n = 6$ ), in vitro using a serum-supplemented culture system (IVPS;  $n = 6$ ) or modified synthetic oviductal fluid (mSOF;  $n = 6$ ). **B)** Expression of PPAR $\gamma$  mRNA (least-squares means  $\pm$  SEM) in bovine cotyledonary tissues of placentas from embryos produced In Vivo ( $n = 6$ ) or in vitro using either a serum-supplemented culture system (IVPS;  $n = 6$ ) or modified synthetic oviductal fluid (mSOF;  $n = 6$ ). <sup>a,b</sup>,  $P = 0.02$ .

different in cotyledonary tissue from placentas in the In Vivo ( $1.1 \pm 0.3$ ), IVPS ( $1.1 \pm 0.4$ ), and mSOF ( $0.5 \pm 0.4$ ) groups (Fig. 3B).

### ***VEGF and PPAR $\gamma$ Protein***

Binding of VEGF and PPAR $\gamma$  antibodies to proteins in cotyledonary tissue from embryos produced either in vivo or in vitro resulted in bands at approximately 20 kD and 50 kD, respectively (data not shown). Preincubation of each antibody with their respective blocking peptide eliminated the antibody signal. The expression of VEGF protein in cotyledons was not different between the three treatment groups (Table 3). Similarly, the expression of PPAR $\gamma$  protein did not differ in cotyledons from the In Vivo, IVPS, or mSOF groups (Table 3).

### ***Placental Vascular Morphometry***

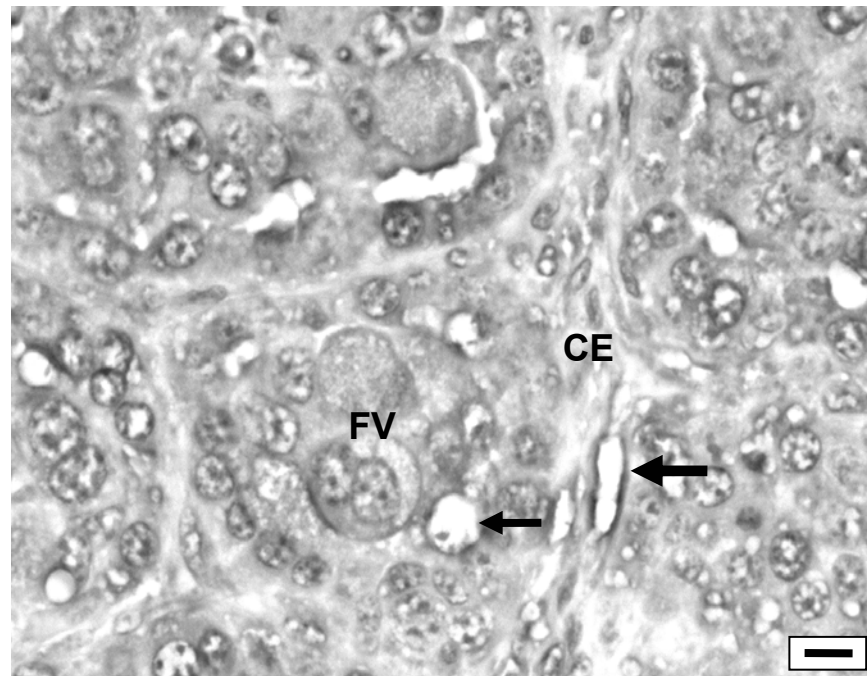
Blood vessels within the maternal endometrium and fetal villi were visualized using immunohistochemical staining for VEGF protein (Fig. 4). The volume density of fetal blood vessels was decreased ( $P = 0.03$ ) in placentomes from the mSOF group ( $4.2 \pm 0.4\%$ ) compared with the In Vivo ( $5.7 \pm 0.3\%$ ) and IVPS ( $6.1 \pm 0.4\%$ ) groups (Fig. 5). The volume density of maternal blood vessels within placentomes tended to be decreased ( $P = 0.08$ ) in placentomes from the mSOF group ( $3.5 \pm 0.4\%$ ) compared with placentomes from the In Vivo group ( $4.9 \pm 0.3\%$ ) with the density of maternal blood vessels intermediate in the IVPS group ( $4.3 \pm 0.3\%$ ; Fig. 5). Similarly, the density of blood vessels within the total placentome (maternal plus fetal components) was decreased ( $P = 0.03$ ) in the mSOF group ( $7.7 \pm 0.7\%$ ) compared with the In Vivo ( $10.5 \pm 0.6\%$ ) and IVPS ( $10.4 \pm 0.6\%$ ) groups (Fig. 5).

**Table 3.** Expression of VEGF and PPAR $\gamma$  protein in cotyledonary tissues at Day 70 of gestation from embryos produced in vivo or in vitro by either an undefined (IVPS) or semi-defined (mSOF) medium.<sup>a</sup>

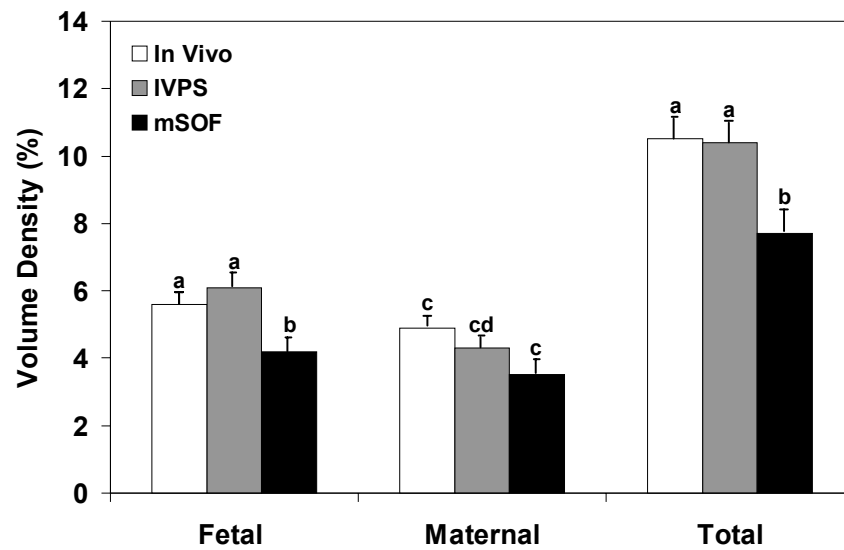
	In Vivo	IVPS	mSOF
Number of placentas	6	6	6
VEGF Protein <sup>b</sup>	9.5 $\pm$ 1.5	11.7 $\pm$ 1.5	10.6 $\pm$ 2.1
PPAR- $\gamma$ Protein <sup>b</sup>	11.3 $\pm$ 1.9	15.0 $\pm$ 2.1	10.8 $\pm$ 2.7

<sup>a</sup> Least-squares means  $\pm$  SEM.

<sup>b</sup> Values are expressed as a signal intensity (arbitrary units).



**Figure 4.** A representative transverse section of a bovine placentome at Day 70 of gestation resulting from the transfer of a blastocyst produced In Vivo. The section was incubated with a primary antibody to VEGF<sub>165</sub> with binding visualized by staining tissue sections with diaminobenzidine tetrahydrochloride containing nickel. This staining procedure was used to distinguish blood vessels within both the caruncular endometrium (CE) (*large arrow*) and the fetal villi (FV) (*small arrow*). Scale bar in micrograph is 10  $\mu$ m.



**Figure 5.** Volume densities of blood vessels (least-squares means  $\pm$  SEM) within the fetal villi, caruncular endometrium, and total placentome at Day 70 of gestation resulting from bovine embryos produced In Vivo ( $n = 6$ ) or in vitro using either a serum-supplemented (IVPS;  $n = 6$ ) or modified synthetic oviductal fluid (mSOF;  $n = 5$ ) culture media. <sup>a,b</sup>  $P = 0.03$ ; <sup>c,d</sup>  $P = 0.08$ .

## DISCUSSION

This study directly compared morphometry and angiogenesis of placentas during early gestation from bovine blastocysts produced in vivo or in vitro using either an undefined medium system (IVPS) or a semi-defined medium system (mSOF). The mean values for fetal body weight were not statistically different between the three groups. However, the range in fetal weights in both the IVPS and mSOF groups was consistent with previous reports on fetal and birth weights in cattle [2, 4, 7, 49-51] and sheep [5, 34] following transfer of embryos produced in vitro. The observation that fetal body weights from the mSOF group had the greatest range and the highest percentage of heavier fetuses compared with the In Vivo group does not support the premise that problems associated with aberrant growth of fetuses from embryos produced in vitro is primarily due to the presence of serum in culture medium [2, 3, 5, 34, 37]. Variation observed in the body weights of fetuses from embryos produced using mSOF medium could potentially be due to buildup of ammonia in undisturbed culture medium. Gardner et al. [52] suggested that excessive ammonia generated from the break down of amino acids may buildup in undisturbed culture medium. In addition, McEvoy et al. [53] has linked excessive ammonia in maternal rations to fetal oversize in sheep.

The current study demonstrated that at Day 70 of gestation bovine placentas from embryos produced using mSOF medium were heavier and had a more extreme range in weight than placentas from embryos produced in vivo and in vitro using a serum-supplemented medium (IVPS). Lee et al. [54] found no difference in the weights of placentas from embryos produced using SOF medium compared with AI at Day 50 of

gestation; however, placental weight was more variable in the SOF group. In the present study, no difference was found in placental weights between the In Vivo and IVPS groups. We have previously found that placentas resulting from IVPS embryos were heavier during late gestation than in vivo controls [4]. This discrepancy in placental weight between early (Day 70) and late (Day 222) gestation suggests that deviations in growth of placentas from IVPS derived embryos occur at different stages of gestation. The increased weight of placentas from embryos produced using serum-supplemented medium appears to occur after Day 70 of gestation.

Fetal weight is highly correlated with placental weight throughout gestation in the cow [55]. In several mammalian species including cattle the capacity of the placenta has been measured by placental efficiency (fetal weight/placental weight) [48]. In the current study, placental efficiency was lowest for the mSOF group suggesting reduced capacity of placentas from mSOF embryos to support fetal growth during gestation. Placental efficiency of the In Vivo control group was intermediate between the mSOF and IVPS groups.

Placentas from the mSOF group had less placental fluid volume and fewer placentomes compared with the In Vivo and IVPS groups. The decreased placental fluid in the mSOF group was unexpected because placental weight was greater than the In Vivo and IVPS groups and placental weight has previously been shown to be highly correlated with placental fluid volume [55]. Traditionally, chorioallantoic fluid was considered to serve as a reservoir for fetal waste because of the direct relationship between the allantois and the fetal urogenital tract [56, 57]. Accordingly, decreased placental fluid volume observed in the mSOF group may indicate abnormalities with the fetal renal system resulting in inadequate



maintenance of fetal fluid volumes [58]. Alternatively, recent evidence in sheep regarding amino acid composition of the chorioallantoic fluid suggests that this fluid is both fetal and maternal in origin and its volume is maintained via placental transport mechanism [59]. Therefore, the decrease in placental fluid observed in the mSOF group could be explained by decrease solute transfer for the maternal compartment which may be reflective of the decreased number of placentomes observed in these placentas.

No differences were observed between the In Vivo, IVPS, or mSOF groups in the volume densities of maternal endometrium or fetal villi at Day 70 of gestation. In contrast, during late gestation (Day 222) we found that the density of fetal villi was decreased in placentomes derived from IVPS embryos compared with controls suggesting decreased fetomaternal contact area [4]. Taken together, these findings indicate that fetal villous development at Day 70 of gestation is similar for placentas from embryos produced either in vivo or in vitro, and that alterations in villous formation of placentas from in vitro-produced embryos is not evident until later in gestation when more extensive branching of villi occurs [60].

The volume density of fetal binucleate cells was greater in placentomes in the IVPS group compared with the In Vivo group. These findings are consistent with a previous study in which we found increased fetal binucleate cells in placentas from embryos produced using serum-supplemented medium (IVPS), as well as serum-restricted medium, at Day 70 of gestation compared with in vivo controls [61]. In placentas from NT-derived embryos, Ravelich et al. [62] reported that binucleate cells were increased at Day 50, 100, and 150 of gestation compared with placentas from either AI or IVP embryos. Interestingly, these

authors found no difference in binucleate cells of placentas from IVP embryos compared with AI at Day 50, 100, and 150 of gestation [62]. The increased volume density of binucleate cells in placentas from the IVPS group at Day 70 of gestation imply that these placentomes may have an increased availability of placental lactogens and pregnancy-associated glycoproteins, suggesting enhanced placental development [63, 64].

Placentomes from the mSOF group had a greater volume density of fetal pyknotic cells compared with the In Vivo or IVPS groups. This finding implies that fetal villi of placentomes from mSOF embryos had increased cell death, either by apoptosis or necrosis [65, 66]. Excessive apoptosis or necrosis within the placental villi of humans at term has been associated with pre-eclampsia, a pathological condition characterized by abnormally shallow invasion of extravillous cytotrophoblast in the decidua [67]. The increased incidence of cell death observed in the fetal villi of the mSOF group may indicate compromised placental development. Alternatively, it has been suggested that apoptosis may contribute to placental remodeling in the sheep resulting in expansion of the villous architecture [68]. Therefore, the increase in cell death in fetal villi of the mSOF group may indicate enhanced remodeling of the placentome, thereby resulting in more complex villous structure.

Proper development of the placental vasculature is critical for determining the rate of placental blood flow and thereby, providing adequate exchange of respiratory gases, nutrients, and waste between the mother and fetus [18]. Vascular development of the placenta is initiated by vasculogenesis and subsequently controlled by branching and nonbranching angiogenesis [19]. Molecular regulation of angiogenesis in the placenta is driven by angiogenic factors including VEGF, FGF, and angiopoietins [17, 19, 20]. In the

present study, the levels of VEGF mRNA were lower in cotyledonary tissue in the mSOF group. This finding implies that the fetal component of the placentome had decreased angiogenesis as assessed by levels of VEGF mRNA. Interestingly, no differences due to treatment were found for VEGF protein in cotyledonary tissue. This discrepancy may be due to a lack of sensitivity of the Western blot technique to detect relatively small differences in VEGF protein levels between treatments.

The five isoforms of VEGF mRNA identified in mammalian tissues include VEGF<sub>120</sub>, VEGF<sub>144</sub>, VEGF<sub>164</sub>, VEGF<sub>188</sub>, and VEGF<sub>205</sub> [40]. In most tissues, the most abundant isoform expressed is VEGF<sub>164</sub> which has been shown to possess the greatest mitogenic and vascular permeability properties [40, 69]. In bovine cotyledonary tissue at Day 70 of gestation, VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> were detected regardless of treatment group. Furthermore, the predominant VEGF isoform detected in cotyledons at Day 70 of gestation was VEGF<sub>164</sub>. This finding is consistent with our previous report [4] in which VEGF<sub>164</sub> was the predominant isoform in bovine cotyledons at Day 222 of gestation. In sheep, Cheung and Brace [40] found that VEGF<sub>164</sub> was the predominant isoform in cotyledons.

PPAR $\gamma$  is a transcription factor that has been shown to upregulate VEGF expression in vascular smooth muscle cells [70] and macrophages [71]. Similarly, PPAR $\gamma$  null mice have been shown to be embryonic lethal due to an interference of terminal differentiation of the trophoblast and placental vascularization [21]. In the human trophoblast, PPAR $\gamma$  has been shown to regulate differentiation of extravillous cytotrophoblast [22]. Furthermore, increased levels of protein for PPAR $\gamma$  in bovine caruncular tissues from embryos produced in vitro were associated with enhanced vascular development in these placentomes [4].

Although PPAR $\gamma$  mRNA and protein were expressed in cotyledonary tissues of all three treatment groups at Day 70 of gestation, no differences due to treatment were found for either mRNA or protein levels. This finding suggests that PPAR $\gamma$  may not be associated with decreased expression of VEGF mRNA in cotyledons from the mSOF group. It is possible that PPAR $\gamma$  expression may be altered prior to Day 70 of gestation which then resulted in the observed decrease in expression of VEGF mRNA in cotyledons from the mSOF group at Day 70 of gestation. Alternatively, PPAR $\gamma$  may not be the primary regulator of VEGF in the bovine placenta, but rather VEGF in the bovine placenta may be regulated by other factors such as hypoxia or estradiol [18, 69].

The volume densities of fetal and total blood vessels in placentomes were decreased in the mSOF group compared with the In Vivo and IVPS groups. In addition, the density of maternal blood vessels in placentomes tended to be decreased in the mSOF group. These findings suggest that vascular development was deficient in both the maternal and fetal components of placentomes resulting from embryos produced using mSOF medium. This deficiency in vasculature of placentomes may have been due to decreased expression of mRNA for VEGF or perhaps other angiogenic factors such as FGF or angiopoietins [17, 18].

In summary, placentas from embryos produced using the semi-defined mSOF medium had a greater degree of aberrant development during early gestation than placentas from embryos produced in vivo or in vitro using an undefined serum-supplemented medium (IVPS). Placentas from embryos produced using mSOF were heavier and had decreased placental efficiency, fewer placentomes, and decreased placental fluid. In addition, placentomes in the mSOF group had an increased volume density of fetal pyknotic cells.

Placentomes in the mSOF group had decreased densities of blood vessels and decreased levels of VEGF mRNA in cotyledonary tissue suggesting insufficient vascular development. Taken together, these observations suggest that the use of the semi-defined mSOF medium does not appear to alleviate developmental abnormalities of the placenta associated with in vitro production of embryos, but rather may intensify these problems during early gestation.

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**ANGIOGENESIS AND DEVELOPMENT OF BOVINE PLACENTAS AT DAY 40 OF  
GESTATION FROM EMBRYOS PRODUCED IN VIVO, IN VITRO, OR  
BY NUCLEAR TRANSFER**

**ABSTRACT**

The objective of this study was to determine the effects of in vitro embryo production and somatic cell nuclear transfer on angiogenesis and morphology of bovine placentas during early gestation. Blastocysts produced in vivo were recovered from superovulated Holstein cows and served as controls (In Vivo). Blastocysts produced in vitro were cultured in G1.2/G2.2 media (IVP). Cloned embryos were produced using fetal fibroblasts from a male control fetus as donor cells and then cultured to the blastocyst stage of development in G1.2/G2.2 media (Cloned). Single blastocysts from each embryo production system were transferred into heifers. Fetuses and placentas were recovered on Day 40 of gestation (In Vivo, n = 4; IVP, n = 4; Cloned, n = 4). Chorioallantoic (CA) membrane, cotyledons, and caruncles were obtained for quantification of vascular endothelial growth factor (VEGF) and peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) mRNA. Median values for fetal body weight, eviscerated weight, heart weight, and liver weight did not differ between the three treatment groups. However, more fetuses in the IVP group were lighter (2 standard deviations less than the mean fetal body weight for the In Vivo group) than those in the In Vivo and Cloned groups. Placental weight was also reduced and amniotic vesicles tended to be smaller in the IVP group. There was no effect of embryo production system on placental efficiency (fetal body weight/placental weight), CA fluid volume, the number of cotyledons

or caruncles, and vascular score of placentas. The number of placentas with greater than 20 cotyledons tended to be less in the IVP and Cloned groups compared with the in vivo controls. Placentas from the IVP group tended to have increased avascular placental development compared with the In Vivo and Cloned group. The expression of VEGF mRNA in CA membrane or cotyledonary tissue was not different between treatment groups. However, the expression of VEGF mRNA in caruncles tended to be increased in the IVP group compared with the In Vivo group. The expression of VEGF mRNA in caruncular tissue in the Cloned group was intermediate between the In Vivo and IVP groups. The expression of PPAR $\gamma$  mRNA in CA membrane and cotyledonary tissue did not differ between the treatment groups. No expression of PPAR $\gamma$  mRNA was detected in caruncular tissue regardless of treatment group. In conclusion, at Day 40 of gestation placentas and fetuses from embryos produced in vitro were lighter than those from embryos produced in vivo or by somatic cell nuclear transfer. Placentas from embryos produced in vitro were often avascular with less than 20 cotyledons. Caruncles in the IVP group tended to have increased expression of VEGF mRNA, possibly suggesting enhanced angiogenesis for the maternal component of the placentome.

**Key Words:** culture, development biology, gene regulation, nuclear transfer, placenta



## INTRODUCTION

Normal calves have been produced following the transfer of embryos produced in vitro (IVP) or by somatic cell nuclear transfer (NT; cloning). However, pregnancies resulting from these embryo technologies have been associated with increased pregnancy loss and abnormalities of the fetus and placenta, particularly those resulting from cloned embryos [1-5]. Early embryonic loss by Day 21 of gestation is similar for bovine pregnancies resulting from IVP and cloned embryos [2]. Pregnancy loss prior to Day 21 of gestation appears to be due to inadequate signaling of maternal recognition of pregnancy [4, 6]. A high incidence of pregnancy loss occurs during the first trimester in pregnancies from cloned bovine embryos [2, 7, 8]. For example, Hill et al. [8] found that 82% of pregnancies from NT embryos aborted between Day 30 to 90 of bovine gestation, whereas none of the control pregnancies resulting from in vivo-produced embryos were lost during this period. It has been suggested that pregnancy loss observed for NT embryos late in the first trimester is associated with inadequate vascular development of the placenta and rudimentary development of the cotyledonary regions of the chorioallantoic placenta [8-10].

Proper development of the placental vasculature is critical for adequate nutrient transfer and exchange of gases and wastes between the fetus and the dam [11, 12]. Placental vascular development is initiated by vasculogenesis and subsequently controlled by branching and nonbranching angiogenesis [13]. These processes are regulated by various angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietins (ANG1 and ANG2) as well as the receptors for these angiogenic factors [12]. In sheep, VEGF and basic FGF (bFGF) stimulate endothelial cells

of caruncles to undergo angiogenesis [12]. We have previously demonstrated that expression of VEGF mRNA was decreased in bovine cotyledonary tissue at Day 70 of gestation from IVP embryos produced using a semi-defined medium compared with controls, suggesting limited angiogenesis as assessed by VEGF mRNA in placentas from IVP embryos [14].

Placentas from peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ )-deficient mice failed to undergo terminal differentiation of the spongiotrophoblast and labyrinthine trophoblast [15]. These PPAR $\gamma$ -deficient mice also have abnormal vascular development of the placenta [15]. In the human trophoblast, PPAR $\gamma$  has been shown to dimerize with retinoic acid receptor- $\alpha$  and regulate differentiation of extravillous cytotrophoblast [16]. PPAR $\gamma$  has also been shown to upregulate VEGF expression in human macrophages [17] and vascular smooth muscle [18], suggesting that PPAR $\gamma$  may play a role in angiogenesis. We had previously found that increased vascular density in caruncles during late gestation from IVP bovine embryos was associated with increased expression of PPAR $\gamma$  protein [19]. Together, these observations suggest that PPAR $\gamma$  may play a role in placental angiogenesis of cattle.

The influence of embryo production systems on angiogenesis and development of the placenta during early gestation in cattle is poorly understood. Therefore, the overall objective of this study was to assess the effects of in vitro embryo production and cloning on angiogenesis and development of bovine placentas at Day 40 of gestation. Specifically, we compared placentas from embryos produced in vivo, in vitro, or by nuclear transfer for morphology and mRNA expression for VEGF and PPAR $\gamma$  in chorioallantoic membranes as well as in the cotyledonary (fetal) and caruncular (maternal) components of the placentome.

## MATERIALS AND METHODS

### *Reagents and Hormones*

Tissue culture medium (M-199 with Earle's Salts) was purchased from Invitrogen Co. (Carlsbad, CA). Equine pituitary LH (11.5 NIH LH-S1 units/mg) and porcine pituitary FSH (50 mg/vial Armour FSH Standard) preparations were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty-acid free (FAF) BSA for in vitro fertilization was purchased from Roche Applied Science (Indianapolis, IN) and FAF BSA for cloning procedures was purchased from Sigma Chemical Co. Dulbecco's modified eagle medium (DMEM/F12) was purchased from Invitrogen Co. G1.2/G2.2 embryo culture media and G-MOPS were graciously donated by Dr. David Gardner (Vitrolife, Englewood, CO). All other culture reagents were tissue culture grade and were purchased from Sigma Chemical Co.

Puregene<sup>®</sup> DNA purification kit was purchased from Gentra Systems, Inc. (Minneapolis, MN). TRI-Reagent<sup>®</sup> was purchased from Molecular Research Center, Inc. (Cincinnati, OH). DNase and random hexamers were purchased from Promega (Madison, WI). SuperScript<sup>™</sup> II reverse transcriptase and dNTPs were purchased from Invitrogen Co. PCR purification kits were purchased from Qiagen (Valencia, CA). Taq polymerase was purchased from Roche Applied Science. SYBR green dye was purchased from Molecular Probes, Inc. (Eugene, OR). All primers for real-time PCR were custom synthesized by either Sigma-Genosys (Woodlands, TX) or Qiagen Operon (Alameda, CA).

### *In Vivo Production of Embryos*

All procedures and protocols involving the use of cattle were approved by the Institutional Animal Care and Use Committee at North Carolina State University. For in

vivo embryo production (In Vivo), Controlled Internal Drug Releasing devices (CIDR<sup>®</sup>; 1.38 g progesterone; Pfizer Animal Health, New York, NY) were inserted into the vaginas of Holstein cows followed by an i.m. injection of estradiol-17 $\beta$  (2.5 mg; Diamondback Pharmacy, Phoenix, AZ). Four days after CIDR<sup>®</sup> insertion, donor cows were superovulated with 400 mg FSH (Folltropin<sup>®</sup>, Vetrapharm Canada, London, ON) administered in decreasing doses over a 4-day period. On the morning and evening of the third day of FSH treatment, estrus was induced using two i.m. injections of 25 mg of prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ , Lutalyse<sup>®</sup>, Pharmacia & Upjohn Co., Kalamazoo, MI) with CIDR<sup>®</sup> removal at the same time as the pm injection of PGF<sub>2</sub> $\alpha$ . Cows were artificially inseminated at 12 and 24 h after detection of first standing heat with thawed frozen semen from a proven Holstein bull. Embryos were collected by non-surgical uterine flushing on Day 7 (Day 0 = first detected estrus).

### ***In Vitro Production of Embryos***

For in vitro-produced embryos (IVP), ovaries from Holstein cows were obtained at a local abattoir and held in saline solution for 4-6 h during transport to the laboratory. Cumulus-oocyte complexes (COC) were aspirated, matured, and fertilized in vitro as previously described [20]. Briefly, COCs were aspirated from 2-7 mm follicles and washed five times in modified Tyrode's medium (TL-Hepes). Groups of 20 to 30 COCs were matured for approximately 22 h in bicarbonate buffered M-199 supplemented with 10% heat inactivated estrus cow serum, 10  $\mu$ g/ml LH, 5  $\mu$ g/ml FSH, 1  $\mu$ g/ml estradiol, 200  $\mu$ M sodium pyruvate, and 50  $\mu$ g/ml gentamicin. All cultures were incubated at 5% CO<sub>2</sub> in air with 100% humidity. Following the maturation period, COCs were washed once and placed in

fertilization medium that consisted of heparin-supplemented Tyrode's albumin lactate pyruvate medium with 6 mg/ml FAF BSA [21]. Thawed frozen semen from the same Holstein bull used for AI of the donor cows was used for in vitro fertilization. Motile spermatozoa were collected using the swim-up procedure [21] and a final concentration of  $1 \times 10^6$  spermatozoa per ml was used for fertilization in 0.75 ml of fertilization medium. Spermatozoa and COCs were coincubated for 18-20 h in an atmosphere of 5% CO<sub>2</sub> in air with 100% humidity.

Following fertilization, embryos were cultured in a sequential media system based on a modified protocol from Lane et al. [22]. Briefly, presumptive zygotes were moderately vortexed to remove cumulus cells in TL-Hepes and washed three times in TL-Hepes. Embryos were then washed twice in 500 µl of G1.2 media. Groups of 20 to 30 embryos were cultured in 800 µl of G1.2 in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for a 72-h culture period. Following the 72-h culture period in G1.2 media, embryos were washed twice in 500 µl of G-MOPS and twice in 500 µl of G2.2 media. Groups of 20 to 30 embryos were cultured in 800 µl of G2.2 in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for the final 96-h culture period.

### ***Production of Nuclear Transfer Embryos***

For NT (Cloned) embryos, Holstein oocytes were matured in a similar manner as described for the IVP embryos. All cloning procedures were performed according to Piedrahita et al. [23]. Briefly, cumulus cells were removed from matured oocytes by moderately vortexing in M-199 medium with Hepes (H-199) containing 0.1% Hyaluridase and supplemented with 10% fetal bovine serum (FBS). Denuded oocytes with visible polar

bodies were washed through three micodrops of manipulation medium (H-199 + 10% FBS) and then incubated for 15 min in manipulation medium containing 5  $\mu$ l/ml Hoechst 33342. Oocytes were then placed in manipulation medium containing 7.5  $\mu$ g/ml Cytochalasin B and enucleated with a 17  $\mu$ m (external diameter) glass pipette. Following enucleation, the resulting cytoplasts were washed in manipulation medium and held in this medium until injection of donor cells.

Fetal fibroblasts were obtained from a Day 40 male Holstein fetus from the In Vivo. The donor cells were cultured in 30 mm plates and grown in DMEM/F12 medium containing 10% FBS in an atmosphere of 5% CO<sub>2</sub> in air with 100% humidity at 37°C until contact was inhibited. Fibroblasts were then trypsinized and resuspended in DMEM/F12. Donor cells were pelleted and resuspended in H-199 + 0.1% FAF BSA until used for microinjection.

Microinjection was conducted in manipulation medium supplemented with 0.1% BSA. Groups of 10 to 20 donor cells were loaded in a 17  $\mu$ m pipette (external diameter). A single donor cell was introduced through the zona pellucida and placed in the perivitelline space of recipient cytoplasts. Following injection, reconstructed embryos were washed in manipulation medium and placed in a fusion chamber with two electrodes 1 mm apart overlaid with fusion buffer comprised of 0.3 M mannitol, 0.5 mM Hepes, 0.05% FAF BSA, 0.05 mM CaCl<sub>2</sub>, and 0.1 mM MgSO<sub>4</sub>. Cell fusion was induced with two DC pulses of 210 V/mm for 10  $\mu$ sec, delivered by a BTX Electro-cell Manipulator (BTX ECM-2001, San Diego, CA). Following electrofusion, reconstructed embryos were washed in manipulation medium and fusion rates were checked 30 min later. Embryos were cultured in bicarbonate

buffered M-199 supplemented with 10% FBS in atmosphere of 5% CO<sub>2</sub> with humidity at 37°C for 4 h.

Prior to activation, embryos were washed in H-199 supplemented with 0.1% FAF BSA. Activation was induced by incubation in 30 µl drops of 5 µM ionomycin in H-199 + 0.1% FAF BSA for 4 min at 37°C. Embryos were extensively washed in H-199 + 0.3% FAF BSA and then placed in G1.2 culture medium supplemented with 2mM 6-dimethylaminopurine (6-DMAP) for 4 h. Following 4 h period in 6-DMAP supplemented G1.2 medium, embryos were washed three times in G-MOPS and then washed twice in G1.2 media. Activated embryos were cultured under conditions the same as to those described for the IVP embryos.

### ***Transfer of Embryos***

Angus heifers were given two i.m. injections of 25 mg of PGF<sub>2</sub>α 10 to 12 days apart to synchronize estrus. Grade 1 blastocysts [24] from the In Vivo, IVP, and Cloned groups were transferred in ViGro™ Holding Plus Media (AB Technology, Pullman, WA) singly into the uterine horn ipsilateral to the ovary bearing the corpus luteum of recipient heifers at Day 7 of the estrous cycle.

### ***Recovery of Fetuses and Placental Tissue***

At Day 40 of gestation (33 days after transfer), a total of 12 pregnant recipients (n = 4 each In Vivo, IVP, and NT groups) were slaughtered. Fetuses and their placentas were carefully removed from uterus and physical measurements were taken including fetal body weight, eviscerated weight, heart weight, liver weight, amniotic vesicle length, wet placental

weight, number of caruncles, number of cotyledons, and chorioallantoic fluid volume.

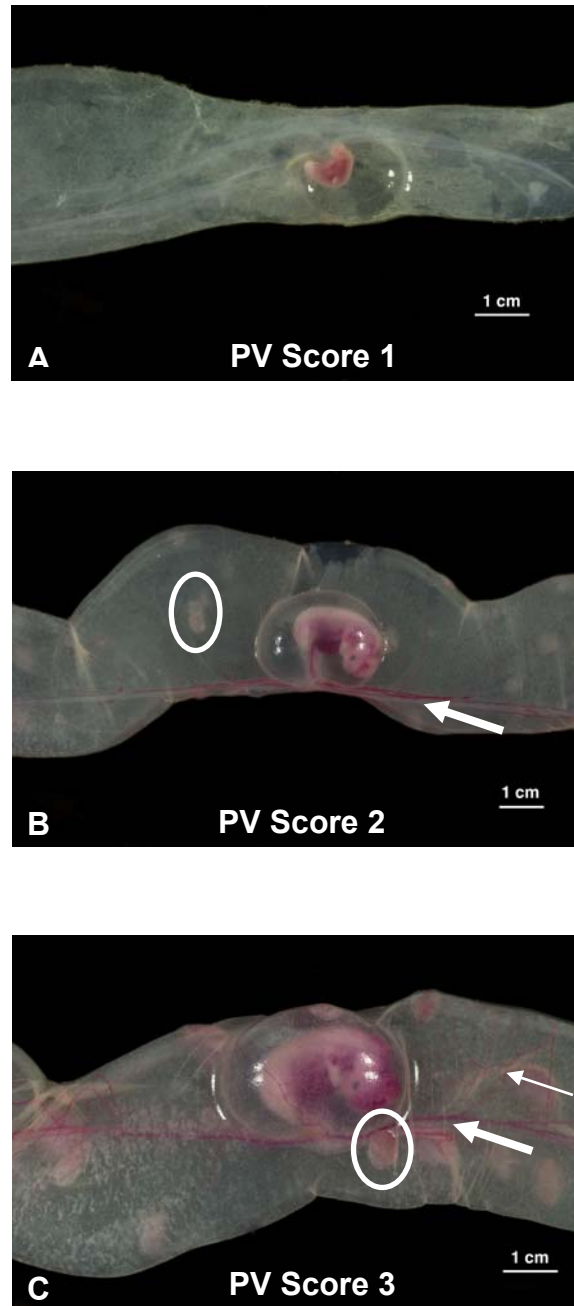
Digital photographs were taken of each placenta. Blood vessel development in placentas was assessed using the following system: Placental vascular score 1 = no vasculature, 2 = minimal vasculature (primary vessels only), and 3 = extensive vasculature (primary, secondary, and tertiary vessels; Fig. 1). Chorioallantoic (CA) membrane within close proximity of the fetus was snap frozen in liquid nitrogen and stored at -80°C for whole cell RNA (wcRNA) and genomic DNA (gDNA) extraction. Samples of caruncles and any cotyledons present were snap frozen in liquid nitrogen and stored at -80°C for wcRNA extraction.

### ***Sex Determination of Fetuses***

Sex of fetuses was determined by PCR for a Y chromosome specific region using gDNA extracted from CA Membrane. Extraction of gDNA from CA membrane was performed using the Puregene<sup>®</sup> DNA purification kit. Approximately 20 mg of tissue was homogenized in cell lysis solution and gDNA was extracted according to manufacturer's protocols. The concentration of the gDNA was determined by absorbance at 260 nm. The quality and integrity of the gDNA was assessed based on the ratio of absorbance at 260 and 280 nm and visualization of gDNA in ethidium bromide (EtBR)-stained agarose gels (data not shown). Aliquots of 20 ng/μl of gDNA were stored at 4°C until used for PCR.

Forward (5'-ACA CCA CTC TCA TCC TAC CT) and reverse (5'-AGG CTA TGC TAC ACA AAT TC) primer sequences for a Y chromosome specific region were acquired from Jarrell et al. [25]. Each 20 μl PCR reaction consisted of 100 ng of CA membrane



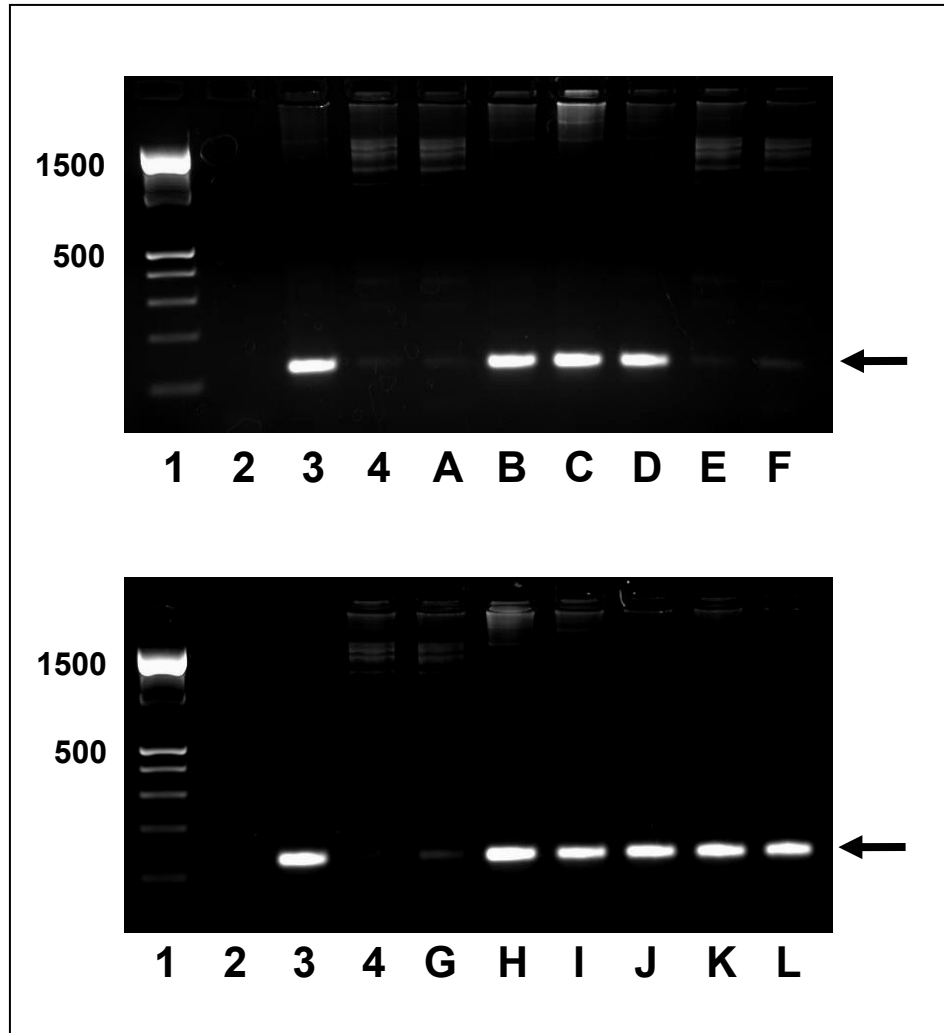


**Figure 1.** Representative bovine placentas at Day 40 of gestation of the three vascular scores from embryos produced in vivo, in vitro, or by cloning. Placental vascular (PV) score was assigned based on the extent of blood vasculature: PV score 1 = no vasculature **A**); 2 = minimum vasculature (primary vessels only) **B**); or 3 = extensive vasculature (primary, secondary, and tertiary vessels) **C**). Large arrow shows a primary vessel, small arrow shows secondary and tertiary vessels, and oval indicates cotyledons.

gDNA from each fetus, 0.8  $\mu$ M of the forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer (Roche), and 2.5 units of *Taq* polymerase. Negative controls lacking gDNA as well as positive controls of gDNA from known male and female fetuses recovered at Day 70 of gestation were included in the PCR. All PCR reactions were run in duplicate together in 96-well PCR plates. Plates were placed into an iCycler thermocycler (Bio-Rad, Richmond, CA). Reaction consisted of 25 cycles that included a 30 sec denaturation at 94°C, 60 sec annealing at 50°C, and 60 sec extension at 72°C. Fetuses were sexed according to the presence (male) or absence (female) of a 129 bp product visualized on EtBR-stained agarose gels (Fig. 2). The identity of this Y chromosome specific amplification product was verified by sequence analysis.

#### ***Reverse Transcription and Verification of PCR Products***

Whole cell RNA from CA membrane, cotyledonary, and caruncular tissues were extracted using a previously reported protocol [19]. Briefly, wcRNA was extracted using TRI-Reagent<sup>®</sup> and dissolved in diethyl pyrocarbonate-treated water. Concentrations of wcRNA were determined by absorbance at 260 nm. The quality and integrity of the wcRNA was assessed based on the ratio of absorbance at 260 and 280 nm and visualization of 28S and 18S rRNA bands in EtBR-stained agarose gels (data not shown). Each sample of wcRNA was treated with DNase (1.5units) for 20 min at 37°C. Following DNase inactivation, wcRNA was then reverse transcribed using random hexamers and SuperScript II reverse transcriptase under conditions recommended by the manufacturer. Following cDNA synthesis, samples were purified using the Qiagen PCR purification kit as recommended by the manufacturer and stored at 4°C.



**Figure 2.** Ethidium-bromide-stained agarose gel of a Y chromosome specific amplification product from bovine chorioallantoic membrane at Day 40 of gestation. Upper and Lower Gels: Lane 1, base pair marker (100bp ladder); Lane 2, negative control sample; Lane 3, positive male control; Lane 4, positive female control; Letters correspond to individual fetuses (Fetus A-D, In Vivo; Fetus E-H, IVP; Fetus I-L, Cloned). Fetal sex was determined by the presence (male) or absence (female) of the 129 bp product indicated by the arrow.

Forward and reverse primer sequences used to amplify mRNA for VEGF-1, PPAR $\gamma$ , and 18S rRNA are found in Table 1. The VEGF-2 forward and reverse primer pair, specifically designed to detect all five VEGF isoforms [26], was also used (Table 1). For VEGF-1, PPAR $\gamma$ , and VEGF-2 primers, PCR reactions consisted of 100 ng equivalents of CA membrane, cotyledonary, and caruncular cDNA from a control (In Vivo) sample, 0.8  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer (Roche), and 2.5 units of Taq polymerase in a 20  $\mu$ l reaction. For detection of 18S rRNA in CA membrane and cotyledonary tissue, PCR reactions consisted of 100 pg equivalents of cDNA from a control (In Vivo) sample, 0.3  $\mu$ M of 18S rRNA forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X buffer, and 2.5 units of Taq polymerase in a 20- $\mu$ l reaction. For detection of 18S rRNA in caruncular tissue, all components were similar to CA membrane and cotyledonary tissue except 100 ng equivalents of cDNA was used. All PCR reactions were run in 96-well PCR plates along with a negative control lacking cDNA. Plates were placed into an iCycler thermocycler. Each PCR program consisted of 90 sec at 95°C, followed by 35 cycles of 30 sec denaturation at 94°C and 45 sec annealing at the appropriate temperature. The primer sequences used for PCR as well as the specific isoform(s) detected, annealing temperatures, and expected product lengths are shown in Table 1. The specificity of the PCR products using the VEGF-1, VEGF-2, PPAR $\gamma$ , and 18S rRNA primer pairs were each verified by sequence analysis.

### ***Real-Time Quantitative PCR Analysis***

VEGF-1, PPAR $\gamma$ , and 18S rRNA primers were used for quantification of VEGF and PPAR $\gamma$  mRNA levels by real-time reverse transcription-PCR as previously reported [19].

**Table 1.** Primer sequences used for PCR analysis of cDNA from bovine chorioallantoic membrane, cotyledonary, and caruncular tissues.

Primer	Sequence	Isoform	Annealing Temperature (°C)	Fragment Size (bp)
18S rRNA <sup>a,b</sup>	Forward 5'-CTT AGA GGG ACA AGT GGC G Reverse 5' -GGA CAT CTA AGG GCA TCA CA		50	71
PPAR $\gamma$ <sup>a,b</sup>	Forward 5'-ATT ACC ATG GTT GAC ACA GAG Reverse 5' -ATG AGG GAG TTG GAA GGC TC	PPAR $\gamma$ 1	60	354
VEGF-1 <sup>a,b</sup>	Forward 5'-ACG AAA GTC TGG AGT GTG Reverse 5' -TTG TTA TGC TGT AGG AAG	VEGF <sub>164</sub>	60	115
VEGF-2 <sup>c</sup>	Forward 5' -TGT AAT GAC GAA AGT CTG CAG Reverse 5' -TCA CCG CCT CGG CTT GTC ACA	VEGF <sub>120</sub> VEGF <sub>144</sub> VEGF <sub>164</sub> VEGF <sub>188</sub> VEGF <sub>205</sub>	59	186 258 318 390 441

<sup>a</sup> Primers used for real-time quantitative PCR analysis.<sup>b</sup> Primers from [19].<sup>c</sup> Primers from [26].

For detection VEGF and PPAR $\gamma$  in CA membrane, cotyledons, and caruncles, each PCR reaction consisted of 100 ng equivalents of cDNA, 0.8  $\mu$ M of the appropriate forward and reverse primers, 16  $\mu$ M dNTPs, 2  $\mu$ l of 10X PCR buffer, 2  $\mu$ l of 2X SYBR Green I dye, 1  $\mu$ l of 200 nM Fluorescein dye (Bio-Rad), and 2.5 units of Taq polymerase in a 20  $\mu$ l reaction. For detection of 18S rRNA in CA membrane and cotyledons, all components were similar to VEGF and PPAR $\gamma$  PCR except that reactions consisted of 100 pg equivalents of cDNA and 0.3  $\mu$ M 18S rRNA forward and reverse primers. For detection of 18S rRNA in caruncles, all components were similar to VEGF and PPAR $\gamma$  PCR except that the reaction consisted of 0.3  $\mu$ M 18S rRNA forward and reverse primers. Melt-curve analysis and gel electrophoresis were used to confirm product length after amplifications were complete (data not included). Prior to quantification of VEGF and PPAR $\gamma$ , raw cycle threshold ( $C_T$ ) values were converted to the linear term using the formula  $2^{-C_T}$  [27]. Expression of VEGF and PPAR $\gamma$  mRNA in placental and caruncular tissues were expressed relative to the expression of 18S rRNA. Levels of VEGF and PPAR $\gamma$  mRNAs are expressed as a ratio of linearized  $C_T$  values for the gene of interest to the linearized  $C_T$  values for 18S rRNA in individual samples.

Expression of mRNA for either VEGF or PPAR $\gamma$  in CA membrane and caruncular tissue was analyzed individually in one assay (In Vivo, n = 4; IVP, n = 4; Cloned, n = 4). In cotyledonary tissue, expression of mRNA for either VEGF or PPAR $\gamma$  was also analyzed individually in one assay (In Vivo, n = 3; IVP, n = 1; Cloned n = 2). Each assay contained reference samples of appropriate tissue from a control (In Vivo) placenta. The reference sample was used to determine the intraassay coefficient of variation (CV) for the linearized

C<sub>T</sub> values. For CA membrane, the intraassay CV of the VEGF assay was 17.0% and the intraassay CV of the PPAR $\gamma$  assay was 4.9%. For cotyledonary tissue, the intraassay CV of the VEGF assay was 9.8% and the intraassay CV of the PPAR $\gamma$  assay was 4.9%. For caruncular tissue, the intraassay CV of the VEGF assay was 14.7%. No expression of PPAR $\gamma$  mRNA was detected in any of the caruncular tissue samples.

### ***Statistical Analysis***

Proportional data for fetal body weight, CA fluid volume, number of cotyledons, and presence of placental blood vessels were analyzed using the Chi-square test [28, 29]. The effect of treatment (In Vivo, IVP, and Cloned) was analyzed using the Kruskal-Wallis test [29, 30]. Medians were considered statistically different at  $P \leq 0.05$  and tendencies between  $P = 0.06$  and  $P = 0.10$ . When the  $P \leq 0.10$  for the Kruskal-Wallis test, medians were separated using the two-sided all-treatment multiple comparisons based on pairwise rankings (Dwass, Steel, Crithlow-Fligner) [30].

## RESULTS

### *Morphology of Fetuses and Placentas*

The percentage of male fetuses was 75% (3 of 4 fetuses) in the In Vivo group, 25% (1 of 4 fetuses) in the IVP group, and 100% (4 of 4 fetuses) in the Cloned group (Fig. 2). Median values for fetal body weight, eviscerated weight, heart weight, and liver weight were not different between the three treatment groups (Table 2). However, the proportion of fetuses less than 2 standard deviations (SD) below the mean fetal body weight for the In Vivo group (0.6 g) was greater ( $P = 0.03$ ) in the IVP group (3 of 4; 75%) compared with both the In Vivo (0 of 4; 0%) and Cloned (0 of 4; 0%) groups. Two of the three female fetuses from the IVP group were also grossly underdeveloped compared with the only female from the In Vivo group (Fig. 3). Median placental weight was also lowest ( $P = 0.05$ ) in the IVP group compared with the In Vivo and Cloned groups (Table 2). Amniotic vesicle length tended ( $P = 0.07$ ) to be shorter for placentas in the IVP group. Placental efficiency (fetal body weight/placental weight) was not different between the In Vivo, IVP, and Cloned groups. There was no effect of treatment on the median values for the CA fluid volume. However, the proportion of placentas less than 2 SD below the mean CA fluid volume for the In Vivo group (15 ml) was greater ( $P = 0.03$ ) in the IVP group (3 of 4, 75%) compared with either the In Vivo (0 of 4; 0%) or Cloned (1 of 4; 25%) groups.

There was no effect of treatment on the median number of cotyledons and caruncles (Table 2). The proportion of placentas with one or more cotyledons present was not different between to the In Vivo (3 of 4; 75%), IVP (1 of 4; 25%), and Cloned (2 of 4; 50%) groups. However, the proportion of placentas with greater than 20 cotyledons tended ( $P = 0.09$ ) to be



**Table 2.** Morphology of bovine fetuses and placentas at Day 40 of gestation from blastocysts produced in vivo, in vitro (IVP), or by nuclear transfer (Cloned).<sup>a</sup>

	In Vivo	IVP	Cloned
Number of fetuses and placentas	4	4	4
Fetal body weight (g)	1.02 (0.72, 1.23)	0.26 (0.12, 1.03)	0.96 (0.64, 1.29)
Eviscerated weight (g)	0.92 (0.62, 1.14)	0.23 (0.12, 0.93)	0.87 (0.57, 1.07)
Heart weight (g)	0.02 (0.01, 0.02)	0.02 (0.01, 0.02)	0.03 (0.02, 0.10)
Liver weight (g)	0.07 (0.06, 0.1)	0.07 (0.05, 0.08)	0.07 (0.05, 0.13)
Placental weight (g)	16.4 (11.0, 25.6) <sup>b</sup>	10.8 (3.5, 15.7) <sup>c</sup>	32.5 (11.4, 35.3) <sup>b</sup>
Amniotic vesicle (mm)	26.5 (23.0, 29.0) <sup>d</sup>	21.5 (20.0, 27.0) <sup>e</sup>	28.0 (23.0, 28.0) <sup>d</sup>
Placental efficiency <sup>f</sup>	0.06 (0.05, 0.08)	0.04 (0.01, 0.07)	0.04 (0.02, 0.07)
Chorioallantoic fluid (ml)	61.0 (35.0, 86.0)	5.50 (4.5, 50.0)	48.5 (6.0, 80.0)
Number of caruncles	59.0 (53, 65)	63.0 (60, 76)	69.0 (55, 105)
Number of cotyledons <sup>g</sup>	15.5 (0, 27)	0.0 (0, 17)	5.0 (0, 11)
Placental vascular score <sup>h</sup>	3.0 (2, 3)	2.0 (1, 3)	2.5 (2, 3)

<sup>a</sup> Median (minimum, maximum).

<sup>b,c</sup>  $P = 0.05$ .

<sup>d,e</sup>  $P = 0.07$ .

<sup>f</sup> Placental efficiency = fetal body weight/placental weight.

<sup>g</sup> In Vivo, n = 3; IVP, n = 1; Cloned, n = 2.

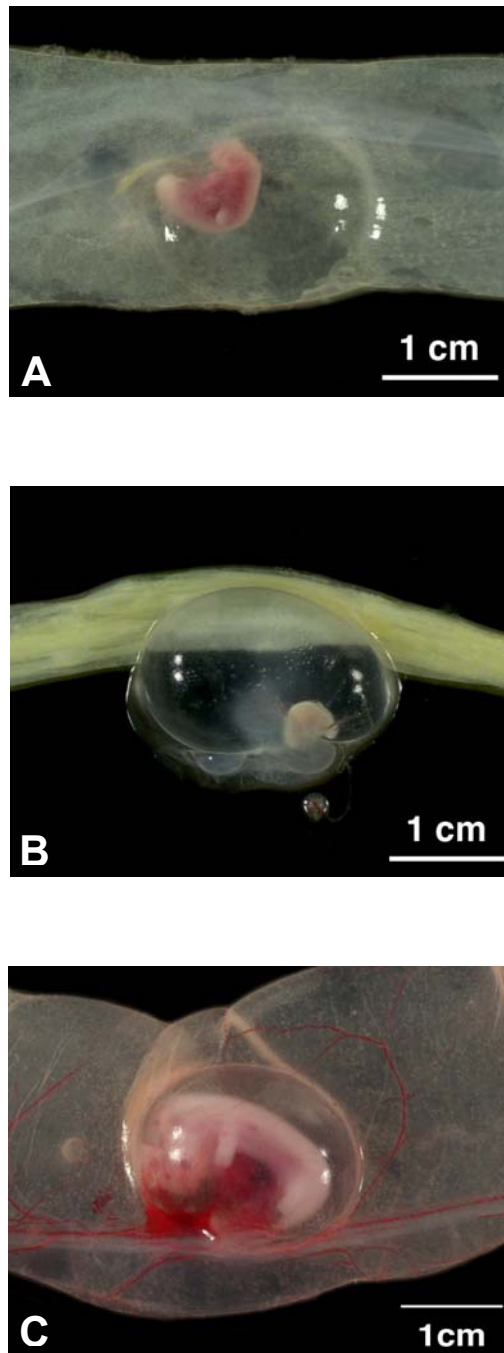
<sup>h</sup> Placental vascular score: 1 = no vasculature, 2 = minimal vasculature, and 3 = extensive vasculature.

decreased in the IVP (0 of 4; 0%) and Cloned (0 of 4; 0%) groups compared with the In Vivo group (2 of 4; 50%). One of the four placentas from the IVP group did not undergo proper fusion of the chorioallantois (Fig. 3). The extent of blood vessel development in placentas assessed by placental vascular score (Fig. 1), was not different between the In Vivo, IVP, and Cloned groups. However, the proportion of placentas with no observable vessels tended ( $P = 0.09$ ) to be greater for placentas in the IVP group (2 of 4; 50%) compared with the In Vivo (0 of 4; 0%) and Cloned (0 of 4; 0%) groups.

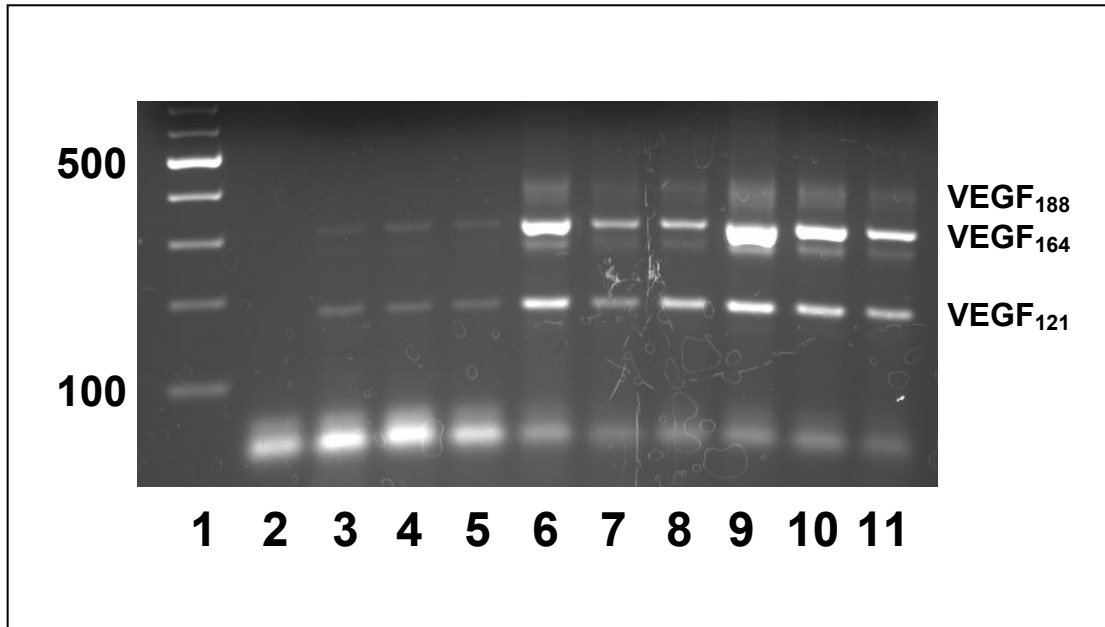
#### ***Expression of VEGF and PPAR $\gamma$ mRNA***

In Figure 4 is displayed the amplification products following RT-PCR using the VEGF-2 primer pair and cDNA from CA membrane, cotyledonary, and caruncular tissues of placentas from the In Vivo, IVP, and Cloned groups. Only two faint bands were visualized at the expected length corresponding to the VEGF<sub>120</sub> and VEGF<sub>164</sub> isoforms [26] for caruncular tissue from the In Vivo, IVP, and Cloned groups. Three bands were visualized at expected lengths corresponding to the VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> isoforms [26] for CA membrane and cotyledons from the In Vivo, IVP, and Cloned groups. The predominant isoforms in CA membrane and cotyledons from all three treatment groups appeared to be VEGF<sub>164</sub> and VEGF<sub>120</sub>.

The expression of mRNA for VEGF and PPAR $\gamma$  in CA membrane, cotyledons, and caruncles is summarized in Table 3. The expression of VEGF mRNA in CA membrane or cotyledons was not different between the three treatment groups. However, the expression of VEGF mRNA in caruncular tissue tended ( $P = 0.07$ ) to be increased in IVP group compared



**Figure 3.** Bovine fetuses recovered at Day 40 of gestation. **A)** and **B)** underdeveloped female fetuses from embryos produced in vitro (IVP) compared with **C)** a female fetus from an embryo produced in vivo group.



**Figure 4.** Ethidium bromide-stained agarose gel of VEGF amplification products from caruncular tissue, chorioallantoic (CA) membrane, and cotyledonary tissue at Day 40 of gestation. The gel depicts PCR products from a reaction using the VEGF-2 primer pair. Lane 1, base pair marker (100 bp ladder); Lane 2, negative control sample; Lane 3, caruncle sample In Vivo group; Lane 4, caruncle sample IVP group; Lane 5, caruncle sample Cloned group; Lane 6, CA membrane sample In Vivo group; Lane 7, CA membrane sample IVP group; Lane 8, CA membrane sample Cloned group; Lane 9, cotyledon sample In Vivo group; Lane 10, cotyledon sample IVP group; Lane 11, cotyledon sample Cloned group. The amplification products represent expected product lengths for VEGF<sub>120</sub> (186 bp), VEGF<sub>164</sub> (318 bp), and VEGF<sub>188</sub> (390 bp) isoforms, respectively [26].

**Table 3.** Expression of VEGF and PPAR $\gamma$  mRNA in placentas on Day 40 of gestation from embryos produced in vivo, in vitro (IVP), or by nuclear transfer (Cloned).<sup>a</sup>

	In Vivo	IVP	Cloned
Number of placentas	4	4	4
VEGF mRNA <sup>d</sup>			
Chorioallantoic membrane	1.3 (0.9, 2.1)	1.4 (1.0, 2.6)	1.6 (0.5, 2.3)
Cotyledonary tissues <sup>e</sup>	0.3 (0.2, 0.6)	1.0 (1.0, 1.0)	0.4 (0.4, 0.4)
Caruncular tissues	0.1 (0.1, 0.1) <sup>b</sup>	0.2 (0.2, 0.2) <sup>c</sup>	0.1 (0.1, 0.3) <sup>bc</sup>
PPAR $\gamma$ mRNA <sup>d</sup>			
Chorioallantoic membrane	2.3 (0.4, 3.0)	3.8 (2.6, 6.5)	0.9 (0.5, 6.0)
Cotyledonary tissues <sup>e</sup>	0.8 (0.3, 2.3)	2.6 (2.6, 2.6)	1.1 (0.9, 1.3)
Caruncular tissues	No expression	No expression	No expression

<sup>a</sup> Median (minimum, maximum values).

<sup>b,c</sup>  $P = 0.07$

<sup>d</sup> Values are expressed as a ratio to 18S rRNA mRNA expression.

<sup>e</sup> In Vivo, n = 3; IVP, n = 1; Cloned, n = 2.

with the In Vivo group. Caruncular tissue in the Cloned group displayed an intermediate level of VEGF mRNA expression compared with the In Vivo and IVP groups. The range in the expression for VEGF mRNA for caruncles was greater in the Cloned group (0.25 relative units) compared with the In Vivo (0.05 relative units) and IVP (0.08 relative units) groups. The expression of PPAR $\gamma$  mRNA in CA membrane and cotyledonary tissue was not different between the In Vivo, IVP, and Cloned groups. Interestingly, no expression of PPAR $\gamma$  was detected in any of the caruncular tissues.

## DISCUSSION

In the current study, both angiogenesis and the morphology of bovine placentas were compared in placentas from embryos produced in vivo, in vitro, or by cloning. A number of studies have established that fetuses [14, 19, 20, 31], placentas [14, 19, 32], and offspring [7, 32-36] derived from IVP embryos are consistently heavier than their in vivo counterparts. In the current study, no differences were observed in the median values for fetal body weight between the three treatment groups at Day 40 of gestation. However, a greater percentage of fetuses in the IVP group were smaller compared with fetuses in the In Vivo group and Cloned group. In addition, placentas in the IVP group had lower placental weight, shorter amniotic vesicles, and proportionally less chorioallantoic fluid volume compared with placentas in the In Vivo and Cloned groups. These findings are consistent with those of Bertolini et al. [32], who observed a decreased crown-rump length (CRL) in bovine fetuses from IVP embryos compared with in vivo controls at Day 37, 44, 51, and 58 of gestation. These authors had suggested that, first, the growth of fetuses from IVP embryos is retarded during the first trimester and, second, that there appears to be a point of inflexion from retarded to enhanced growth between Day 65 and 72 of gestation [5, 32]. The growth retardation observed in fetuses and placentas in the IVP group compared with the In Vivo controls could have also resulted from the greater percentage of the female fetuses in the IVP group compared with the In Vivo and Cloned groups. Female bovine fetuses have been shown to weigh less than their male counterparts throughout gestation [37].

In cattle [2, 38], sheep [1], and mice [39], offspring resulting from cloned embryos are frequently larger than their in vivo counterparts. In the present study, no difference in

fetal growth was observed between the In Vivo and Cloned groups at Day 40 of gestation. This finding is consistent with the findings of Lee et al. [40], who observed no differences in growth of fetuses at Day 50 of gestation in bovine pregnancies resulting from NT versus AI and IVP controls. However, increased variability in fetal weights and a greater proportion of heavier fetuses were observed at Day 100 and 150 of gestation in fetuses from embryos produced by NT compared with controls [40]. In contrast, Chavatte-Palmer et al. [41] reported shorter CRL in bovine fetuses from cloned embryos at Day 34, 50, and 64 of gestation compared with AI and IVP controls. These findings suggest that excessive growth of bovine fetus from embryos produced by cloning does not occur until sometime after Day 50 of gestation.

Rudimentary development of cotyledonary tissues has been observed in placentas from cloned cattle [8, 9] and sheep [10] during the first trimester of gestation. In addition, Dindot et al. [42] found that placentas from *Bos gaurus* x *Bos taurus* cloned embryos had no visible cotyledons at Day 40 of gestation compared with AI *Bos gaurus* x *Bos taurus* control placentas which all developed cotyledons. In the current study, the number of cotyledons and the proportion of placentas with at least one or more cotyledons present were not different between the three treatment groups. However, the proportion of placentas with greater than 20 cotyledons tended to be decreased in placentas from IVP and cloned embryos compared with placentas from in vivo-produced embryos. These findings suggest that placentas from embryos produced in vitro or by cloning have delayed or limited development of cotyledonary tissue compared with placentas from embryos produced in vivo.



Proper development of the placental vasculature is essential for providing adequate transfer of nutrients and exchange of gases and waste between the mother and the fetus [12]. Inadequate vascular development of the placentas has been suggested as a primary cause for the increased pregnancy loss observed following the transfer of cloned bovine [8, 9] and ovine [10] embryos. No differences due to treatment were found for placental vascular score at Day 40 of gestation. However, more placentas in the IVP group had no observable blood vessels compared with placentas in the In Vivo and Cloned groups. This finding suggests that placentas resulting from IVP embryos may have either delayed or limited vascular development compared with placentas from in vivo or cloned embryos.

Formation of blood vessels is initiated by vasculogenesis and subsequently controlled by branching and nonbranching angiogenesis [13, 43]. Molecular regulation of vasculogenesis and angiogenesis is driven by angiogenic factors such as VEGF, FGF, and angiopoietins [13, 43]. Although VEGF mRNA was expressed in CA membrane and cotyledonary tissue from all three treatment groups at Day 40 of gestation, no differences between treatment groups were found for expression of VEGF mRNA either in CA membrane or cotyledons. These findings imply that, at Day 40 of gestation, placentas resulting from embryos produced in vitro or by cloning do not have alterations in placental angiogenesis based on assessment of VEGF mRNA. However, it is possible that VEGF protein and/or the receptors for VEGF, Flt-1 and Flk-1 [44], may be altered in bovine placentas from embryos produced in vitro or by cloning at Day 40 of gestation. Other angiogenic factors, such as FGF or angiopoietins, may also be altered in bovine placentas

resulting from IVP or NT. Alternatively, VEGF mRNA may be altered at other stages of gestation in bovine placentas from embryos produced in vitro or by cloning.

In a previous study at Day 70 of gestation [14], we demonstrated that expression of VEGF mRNA was decreased in cotyledonary tissue from embryos produced in vitro compared with in vivo controls. The discrepancy in VEGF mRNA expression from Day 40 and Day 70 of gestation may have resulted from differences in culture media or stage of gestation. In the previous study (Day 70), we used a synthetic oviductal fluid medium [14], whereas in the present study (Day 40), we used G1.2/G2.2 media.

In the present study, the level of VEGF mRNA expression tended to be increased in caruncular tissues in the IVP group compared with the In Vivo group. This finding implies that at Day 40 of gestation the maternal component of the placentome from embryos produced in vitro had increased angiogenesis as measured by VEGF mRNA. We did not measure the level of VEGF protein in caruncular tissues; therefore, it is possible that VEGF protein may also be altered in caruncular tissue from embryos produced in vitro. No differences were found in the levels of VEGF mRNA expression between the IVP and Cloned group. This finding is consistent with the findings of Hoffert et al. [45], who found no differences in mRNA expression of VEGF or its receptors in caruncular tissue at Day 30 of gestation from bovine embryos produced in vitro or by cloning.

Five isoforms for VEGF mRNA have been identified in mammals and include VEGF<sub>120</sub>, VEGF<sub>144</sub>, VEGF<sub>164</sub>, VEGF<sub>188</sub>, and VEGF<sub>205</sub> [26]. At Day 40 of gestation in the cow, VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> were detected in CA membrane and cotyledons regardless of treatment group. It appears that the predominant isoforms expressed in these

tissues were VEGF<sub>164</sub> and VEGF<sub>120</sub>. This finding is consistent our previous findings in which VEGF<sub>164</sub> was the predominant isoform in bovine cotyledons at Days 70 [14] and 222 [19] of gestation. Only VEGF<sub>120</sub> and VEGF<sub>164</sub> mRNA isoforms were faintly detected in caruncular tissue regardless of treatment group. This finding is also consistent with our previous findings in which VEGF<sub>120</sub> and VEGF<sub>164</sub> isoforms were the only isoforms detected in caruncular tissue at Day 222 of gestation [19].

PPAR $\gamma$  has been associated with vascular development of the placenta in mice [15]. PPAR $\gamma$  has also been shown to upregulate VEGF in macrophages [17] and vascular smooth muscle [18]. PPAR $\gamma$  has also been shown to play an important role in regulating the differentiation of extravillous cytotrophoblast in the human placenta [46]. Furthermore, we previously found that PPAR $\gamma$  protein was associated with increased vascular development of the placentome from embryos produced in vitro [19]. Although PPAR $\gamma$  mRNA was expressed in CA membrane and cotyledons from placentas at Day 40 of gestation, no differences were found between treatment groups in the expression of PPAR $\gamma$  mRNA in CA membrane and cotyledons. In contrast, no expression of PPAR $\gamma$  mRNA was detected in caruncular tissue in any of the treatment groups. Therefore, it is possible that PPAR $\gamma$  may not be the primary regulator of VEGF in bovine caruncles, but rather VEGF in caruncles may be regulated by other factors such as hypoxia or estradiol [12, 44].

In conclusion, at Day 40 of gestation, placentas from embryos produced in vitro were smaller than those from embryos produced in vivo or by somatic cell nuclear transfer. Placentas in the IVP group also had fewer placentas with greater than 20 cotyledons and an increased percentage of avascular placentas. Furthermore, the expression of VEGF mRNA

was increased in caruncular tissues from IVP embryos compared with in vivo controls, suggesting that angiogenesis was enhanced in the maternal component of the placentome. Taken together, these findings suggest that the tendency for increased expression of VEGF in the caruncular endometrium may reflect compensation in the vasculature of the maternal component of the placentome to alleviate limited or delayed cotyledonary growth and vascular development of placentas from the IVP embryos at Day 40 of gestation.

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## GENERAL CONCLUSIONS

Altered development of placentas resulting from in vitro-produced (IVP) and cloned (somatic cell nuclear transfer, SCNT) embryos has been associated with increased pregnancy loss and the Large Offspring Syndrome phenotype [1-4]. The research described in this dissertation directly compared placentas from bovine embryos produced in vivo, in vitro, and by SCNT for vascular development and morphology. The goal of this research was to gain a better understanding of the influence of these embryo production systems on angiogenesis and development of placentas during early (Day 40 and Day 70) and late (Day 222) gestation in cattle.

During late gestation, fetuses and placentas from embryos produced in vitro using undefined, serum-supplemented medium were heavier than in vivo controls. In addition, an increased proportion of placentas from the in vitro group had excessive placental fluid volume compared with placentas from the in vivo group. These findings support previous reports indicating that fetuses and placentas from embryos produced in vitro are associated with increased weight [5-7] and placental fluid volume [6, 8, 9].

Placentas from IVP embryos also had decreased feto-maternal contact as measured by placentome surface area and fetal villous volume density. Placentas from the in vitro group also had an increased blood vessel density in the caruncular endometrium compared with the in vivo group. In addition, the ratios of volume densities for fetal and maternal blood vessels-to-placentome surface area were increased in placentomes from the in vitro group. These findings suggest that blood vessels in the placentome may be compensating for the decreased feto-maternal contact in these placentas.

The expression of VEGF mRNA and protein in caruncles or cotyledons did not differ between the in vivo and in vitro groups at during late gestation. These findings suggest that bovine placentas from IVP embryos do not have alterations in placental angiogenesis, at least based on assessment of VEGF mRNA and protein. It remains possible that other angiogenic factors, such as fibroblast growth factor (FGF) or angiopoietins, may be important regulators of angiogenesis in bovine placentas resulting from IVP embryos. There was no effect of treatment on the expression of PPAR $\gamma$  mRNA in either caruncles or cotyledons or the expression of PPAR $\gamma$  protein in cotyledons. However, the expression of PPAR $\gamma$  protein was increased in caruncles from the in vitro group, indicating the PPAR $\gamma$  may play a role in vascular development of these placentas.

At Day 70 of gestation, we found that fetuses from embryos produced in vitro using either an undefined (IVPS) or a semi-defined (mSOF) media had a more extreme range in fetal body weight compared with fetuses from embryos produced in vivo. This finding is consistent with previous reports on fetal body weight in cattle [2, 5] and sheep [6] following transfer of embryos produced in vitro. Interestingly, fetal body weight from the mSOF group had the highest percentage of heavier fetuses compared with the in vivo group. This observation does not support the premise that aberrant growth of fetuses from embryos produced in vitro is primarily due to the presence of serum in the culture medium [2, 10]. Placentas from the mSOF group were also heavier and had a more extreme range in placental weight compared with placentas from the in vivo and IVPS groups. In addition, placental efficiency (fetal body weight/placental weight), a measure of placental capacity, was lowest

in the mSOF group. Finally, placentas from the mSOF group had less placental fluid and fewer placentomes compared with the in vivo and IVPS groups.

There was no effect of treatment on the volume densities of caruncular endometrium or fetal villi at Day 70 of gestation. However, placentomes from the mSOF group had a greater volume density of fetal pyknotic cells compared with the in vivo and IVPS groups. Thus, fetal villi of placentomes from the mSOF group had increased cell death, either by apoptosis or necrosis. Placentomes from the mSOF group also had decreased densities of blood vessels in the fetal and maternal components of the placentome. This finding implies that vascular development was reduced in placentomes resulting from embryos produced using mSOF medium.

The expression of PPAR $\gamma$  mRNA or protein in cotyledonary tissue did not differ between treatment groups at Day 70 of gestation. Although no differences were observed between treatment groups in the cotyledonary expression of VEGF protein, the levels of VEGF mRNA were decreased in cotyledonary tissue in the mSOF group compared with the in vivo and IVPS groups. This finding implies that the fetal component of the placentome had decreased angiogenesis as assessed by VEGF mRNA.

Finally, fetuses from embryos produced in vitro using G1.2/G2.2 media (IVP) were proportionally smaller compared with fetuses produced in vivo or by SCNT (Cloned) at Day 40 of gestation. In addition, placentas from the IVP group had decreased placental weight, smaller amniotic vesicles, and proportionally less chorioallantoic fluid volume compared with placentas from the in vivo and Cloned groups. These findings support the findings of Bertolini et al. [7], who reported that fetuses from bovine embryos produced in vitro had

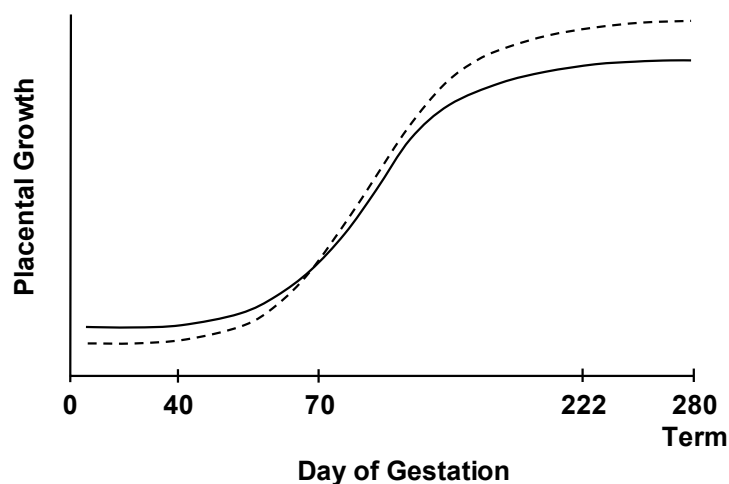
retarded growth at Day 37, 44, 51, and 58 of gestation. The proportion of placentas with greater than 20 cotyledons also tended to be decreased for placentas in the IVP and Cloned group. This finding suggests that placentas from embryo produced in vitro or by NT may have delayed or limited development of cotyledons compared with in vivo controls. Furthermore, placentas from the IVP group tended to have more avascular placentas, suggesting that these placentas may have limited or delayed vascular development.

There was no effect of embryo production system on the expression of mRNA for VEGF or PPAR $\gamma$  in chorioallantoic membrane and cotyledons at Day 40 of gestation. In contrast, the level of VEGF mRNA expression tended to be increased in caruncles from the IVP group compared with the in vivo group, indicating increased angiogenesis in the maternal component of the placentome. This finding suggests that increased angiogenesis in the caruncular endometrium may reflect compensation for the limited or delayed vascular development of placentas from IVP embryos at Day 40 of gestation.

In conclusion, measurements of placental angiogenesis and development varied by embryo production system and day of gestation. We determined that, during late gestation, compensatory mechanisms exist in the vascular beds of placentas from embryos produced in vitro using undefined media. At Day 70 of gestation, placentas from embryos produced in vitro using semi-defined medium (mSOF) had reduced vascular development. Furthermore, at Day 40 of gestation, placentas from embryos produced using a sequential media system (G1.2/G2.2) appeared to have more compromised development than placentas from embryos produced in vivo or by cloning.

## PROPOSED GROWTH PATTERNS OF PLACENTAS FROM BOVINE EMBRYO PRODUCED IN VIVO OR IN VITRO

The results described in this dissertation have allowed us to propose potential growth patterns of placentas based on weights of placentas from embryos produced in vivo or in vitro regardless of embryo culture medium. However, the exact growth patterns of placentas from embryos produced in vitro or by SCNT could not be determined for each embryo production system because we did not investigate each of these production systems at each stage of gestation. Figure 1 shows the proposed growth patterns of placentas from embryos produced in vivo or in vitro.



**Figure 1.** Proposed growth patterns of bovine placentas throughout gestation as measured by weights of placentas from embryos produced in vivo (solid line) or in vitro (dashed line).

During early gestation at Day 40, placentas from embryos produced in vitro using sequential G1.2/G2.2 media had delayed or limited growth compared with placentas from embryos produced in vivo (Table 2, page 164). By Day 70 of gestation, growth patterns of placentas were either similar for placentas from embryos produced in vitro using serum-

supplemented medium or enhanced for placentas from embryos produced in vitro using semi-defined mSOF medium compared with in vivo controls (Table 2, page 120). During late gestation at Day 222, however, placentas from embryos produced in vitro using serum-supplemented medium had increased growth compared with placentas from embryos produced in vivo (Table 2, page 84).

Several hypotheses can be proposed to explain the discrepancy in placental growth patterns between early and late gestation observed from embryos produced in vitro. For instance, a greater percentage of female fetuses were observed at Day 40 of gestation in the in vitro group compared with the in vivo controls. Placental weights could have been decreased at Day 40 of gestation as a result of greater number of female fetuses. In cattle, female fetuses have been shown to weigh less than their male counterparts [11]. During later stages gestation (Day 70 and 222), the in vivo and in vitro groups had equal distributions of males and females. Alternatively, pregnancies at Day 40 of gestation from the in vitro group with lighter and less developed placentas may have been lost sometime between Day 40 and 70 of gestation resulting in similar or enhanced placental growth beyond Day 70 of gestation. Finally, the differential growth pattern of placentas from embryos produced in vitro may be the result of developmental compensation during later stages of gestation (Day 70 and 222) to alleviate delayed or limited placental development during early gestation (Day 40). This finding supports the hypothesis of Bertolini et al. [4, 7] in which these authors have suggested a point of inflexion from retarded to enhanced growth between Day 65 and 72 of gestation from bovine embryos produced in vitro. In conclusion, the results of this



dissertation indicate that placentas from embryos produced in vitro have a differential pattern of growth compared with in vivo controls.

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## **APPENDICES**

## **APPENDIX I**

### **MEDIA COMPOSITION FOR IN VITRO PRODUCTION OF EMBRYOS**

**Table A1.** Composition of Tyrode's albumin lactate pyruvate based media (TL HEPES, Sperm TL and Fertilization Media).

Component	TL-HEPES	Sperm TL	Fertilization Media
NaCl	114.0 mM	110.0 mM	114.0 mM
KCl	3.2 mM	3.1 mM	3.2 mM
NaHCO <sub>3</sub>	25.0 mM	25.0 mM	25.0 mM
NaH <sub>2</sub> PO <sub>4</sub> • H <sub>2</sub> O	0.34 mM	0.29 mM	0.34 mM
CaCl <sub>2</sub>	2.0 mM	2.0 mM	2.0 mM
MgCl <sub>2</sub> • 6H <sub>2</sub> O	0.5 mM	0.5 mM	0.5 mM
Sodium lactate	10.0 mM	10.0 mM	10.0 mM
HEPES	10.0 mM	10.0 mM	--
Pyruvate	0.25 mM	1.0 mM	1.0 mM
BSA fatty acid free	--	--	0.6% (6 mg/ml)
BSA fraction V	0.3% (3 mg/ml)	0.3% (3 mg/ml)	--
Gentamicin	50 µg/ml	50 µg/ml	--
Heparin	--	2 µg/ml	--

**Table A2.** Composition of modified synthetic oviductal fluid (mSOF) medium.

Component	Concentration
NaCl	107.6 mM
KCl	7.1 mM
KH <sub>2</sub> PO <sub>4</sub>	1.1 mM
CaCl <sub>2</sub>	2.2 mM
MgCl <sub>2</sub> • 6H <sub>2</sub> O	1.0 mM
NaHCO <sub>3</sub>	25.0 mM
Sodium Lactate	5.0 mM
Hepes	25.0 mM
Sodium Citrate	0.5 mM
Pyruvate sodium salt	0.3 mM
L-Glucose	1.5 mM
Gentamicin	50 µg/ml
MEM non-essential amino acids	1% (v/v)
BSA fatty acid free	0.6% (6 mg/ml)

**Table A3.** Composition of G1.2/G2.2 media.

Component	G1.2 (mM)	G2.2 (mM)
NaCl	90.08	90.08
KCl	5.5	5.5
NaH <sub>2</sub> PO <sub>4</sub>	0.25	0.25
MgSO <sub>4</sub>	1.0	1.0
NaHCO <sub>3</sub>	25.0	25.0
CaCl <sub>2</sub>	1.8	1.8
Glucose	0.5	3.15
Lactate	10.5	5.87
Pyruvate	0.32	0.1
EDTA	0.01	--
Taurine	0.1	--
Alanyl-glutamine	0.5	1.0
Alanine	0.1	0.1
Arginine	--	0.6
Asparagine	0.1	0.1
Aspartate	0.1	0.1
Cystine	--	0.1
Glutamate	0.1	0.1
Glycine	0.1	0.1
Histidine	--	0.2
Isoleucine	--	0.4
Leucine	--	0.4
Lysine	--	0.4
Methionine	--	0.1
Phenylalanine	--	0.2
Proline	0.1	0.1
Serine	0.1	0.1
Threonine	--	0.4
Tryptophan	--	0.5
Tyrosine	--	0.2
Valine	--	0.4
Ca pantothenate	--	0.0042
Choline chloride	--	0.0072
Folic acid	--	0.0023
Inositol	--	0.01
Niacinamide	--	0.0082
Pyridoxal	--	0.0049
Riboflavin	--	0.0003
Thiamine	--	0.003



## **APPENDIX II**

### **LIST OF PUBLICATIONS**

**Table A4.** List of publications during the PhD program.**Refereed Papers**

Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. 2004. Angiogenesis and morphology in late gestation bovine placentas from embryos produced in vivo or in vitro. *Biol Reprod* 71:1919-1926.

Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. Effects of embryo culture on angiogenesis and morphology of bovine placentas in early gestation. (In Preparation).

Miles JR, Piedrahita JA, Farin CE, Estrada JL, Alexander JE, Farin PW. Angiogenesis and development of bovine placentas at day 40 of gestation from embryos produced in vivo, in vitro or by nuclear transfer. (In Preparation).

Rodriguez KF, Blomberg LA, Miles JR, Alexander JE, Zuelke KA, Farin CE. Use of serial analysis of gene expression (SAGE) for the identification of candidate genes associated with gonadotropin-induced oocyte maturation in the mouse. (In Preparation).

Farin PW, Miles JR, Farin CE. 2004. Pregnancy loss associated with embryo technologies in cattle. *Proc. 23rd Ann. World Buiatrics Congress, Quebec, Canada, Vol. 34, Number 1*:62-64.

**Abstracts**

Miles JR, Farin CE, Stockburger EM, Rodriguez KF, Alexander JE, Farin PW. 2002. Expression of vascular endothelial growth factor mRNA in bovine placentas from embryos produced in vivo or in vitro. *Biol Reprod* 66 (Suppl 1):235 (Abst. 339).

Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. 2003. Angiogenesis in late gestation bovine placentas from embryos produced in vivo or in vitro. *Proc of the North Carolina State University, College of Veterinary Medicine Annual Research Forum, Raleigh, NC.* (Abst. 10).

Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. 2004. Vascular morphometry of bovine placentomes in late gestation from embryos produced in vivo or in vitro. *Reprod Fertil Dev* 16 (1,2):259 (Abst. 279).

Dindot SV, Farin PW, Farin CE, Miles J, Alexander E, Walker S, Long C, Piedrahita JA. 2004. Analysis of epigenetic modifications and genomic imprinting in nuclear transfer derived *Bos gaurus* x *B. taurus* concepti. *Reprod Fertil Dev* 16 (1,2):140 (Abst. 35).

Rodriguez KF, Blomberg LA, Miles JR, Alexander JE, Zuelke KA, Farin CE. 2004. Use of serial analysis of gene expression (SAGE) for the identification of candidate genes associated with gonadotropin-induced oocyte maturation in the mouse. *Biol Reprod (Special Edition)*:128 (Abst. 157).