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Placental development during early pregnancy in sheep: effects of embryo origin on vascularization

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Abstract

Utero-placental growth and vascular development are critical for pregnancy establishment that may be altered by various factors including assisted reproductive technologies (ART), nutrition, or others, leading to compromised pregnancy. We hypothesized that placental vascularization and expression of angiogenic factors are altered early in pregnancies after transfer of embryos created using selected ART methods. Pregnancies were achieved through natural mating (NAT), or transfer of embryos from NAT (NAT-ET), or IVF or *in vitro* activation (IVA). Placental tissues were collected on day 22 of pregnancy. In maternal caruncles (CAR), vascular cell proliferation was less ($P<0.05$) for IVA than other groups. Compared with NAT, density of blood vessels was less ($P<0.05$) for IVF and IVA in fetal membranes (FM) and for NAT-ET, IVF, and IVA in CAR. In FM, mRNA expression was decreased ($P<0.01$–0.08) in NAT-ET, IVF, and IVA compared with NAT for vascular endothelial growth factor (*VEGF*) and its receptor *FLT1*, placental growth factor (*PGF*), neuropilin 1 (*NP1*) and *NP2*, angiopoietin 1 (*ANGPT1*) and *ANGPT2*, endothelial nitric oxide synthase 3 (*NOS3*), hypoxia-inducible factor 1A (*HIF1A*), fibroblast growth factor 2 (*FGF2*), and its receptor *FGFR2*. In CAR, mRNA expression was decreased ($P<0.01$–0.05) in NAT-ET, IVF, and IVA compared with NAT for *VEGF, FLT1, PGF, ANGPT1*, and *TEK*. Decreased mRNA expression for 12 of 14 angiogenic factors across FM and CAR in NAT-ET, IVF, and IVA pregnancies was associated with reduced placental vascular development, which would lead to poor placental function and compromised fetal and placental growth and development.

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Introduction

Placental vascularization is initiated and established early in pregnancy and supports early embryonic survival and subsequent fetal growth and development (Grazul-Bilska *et al*. 2010, 2011, 2013). Thus, the importance of placental vascular development during early pregnancy has long been recognized (Reynolds *et al*. 2010, 2013). During early pregnancy, extensive angiogenesis in maternal and fetal placental tissues progresses, accompanied by a marked increase in uterine and umbilical blood flows (Greiss & Anderson 1970, Reynolds 1986, Reynolds & Redmer 1995). Placental angiogenesis is tightly regulated by numerous growth and angiogenic factors (Mayhew *et al*. 2003, 2004*a*,*b*, Redmer *et al*. 2004, Grazul-Bilska *et al*. 2010, 2011, Reynolds *et al*. 2010, 2013).

The pattern of placental vascularization and expression of factors that influence angiogenesis during early pregnancy after natural breeding have been established for sheep (Grazul-Bilska *et al*. 2010, 2011). However, very limited data concerning placental vascularization in pregnancies established after transfer of embryos obtained through assisted reproductive technologies (ART) or pregnancies affected by environmental factors during early stages of development are available. For later stages of pregnancy, it has been demonstrated that factors such as maternal age, inadequate nutrition, environmental pollutants, and others are associated with decreased vascularization and/or blood flow in the placenta of several species (Redmer *et al*. 2004, Reynolds *et al*. 2006, 2013, Rennie *et al*. 2011). Comparison of placental development at several stages of natural pregnancies with those achieved by various ART has shown differences in fetal size, placental and fetal growth, placental steroid metabolism, global DNA methylation, and expression of selected genes in several species (Barnes 2000, Cai *et al*. 2006, Grazul-Bilska *et al*. 2006, 2013, Allen *et al*. 2008, Collier *et al*. 2009, Delle Piane *et al*. 2010, Sellers López *et al*. 2010, Esh-Broder *et al*. 2011, Tomic & Tomic 2011, Ptak *et al*. 2013). In addition, placental vascular development is dramatically altered at term in pregnancies resulting from ART in sheep (Palmieri *et al*. 2007) and cattle (Hill *et al*. 2000, Miles *et al*. 2004, Miglino *et al*. 2007), and such alterations have been

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shown to occur as early as day 70 of pregnancy in cattle (Miles *et al*. 2005). Furthermore, reduced placental vascular development and increased vascular resistance during early pregnancy have been associated with early embryonic mortality (Meegdes *et al*. 1988, Bassil *et al*. 1995).

Beyond their immediate effects on pregnancy establishment, factors influencing placental vascular development have a dramatic impact on fetal growth and development and therefore on birth weight as well as postnatal survival and growth (Reynolds & Redmer 2001, Reynolds *et al*. 2010, 2013). Moreover, it has been shown that such postnatal effects impact not only the postnatal period but also lifelong health and productivity in mammals, including humans and livestock (Nathanielsz 2006, Barker 2007, Vonnahme & Lemley 2011, Reynolds & Caton 2012).

We hypothesized that placental vascularization and/or expression of factors involved in the regulation of angiogenesis would be altered very early (2–4 weeks after fertilization) in pregnancies achieved through transfer of embryos from various sources, including those from ART, compared with natural pregnancies. To test this hypothesis, we established pregnancies using a control group that was naturally mated (NAT), as well as three groups involving i) follicle-stimulating hormone (FSH) treatment to induce multiple follicle development (Stenbak *et al*. 2001, Grazul-Bilska *et al*. 2007) combined with NAT, embryo flushing, and embryo transfer (ET) to synchronized recipients (NAT-ET); ii) transfer of embryos obtained through IVF of oocytes collected from FSH-treated ewes; and iii) transfer of embryos obtained through *in vitro* activation (IVA; i.e. parthenotes – clones containing only maternal genes) of oocytes collected from FSH-treated donors, as described before (Grazul-Bilska *et al*. 2013). Therefore, the aim of this study was to determine vascularization (e.g. vascular labeling index (LI) and the density of blood vessels) and the expression of mRNA for 14 factors involved in the regulation of angiogenesis in fetal and maternal placenta on day 22 of gestation in four pregnancy types: NAT, NAT-ET, IVF, and IVA in sheep.

Materials and methods

Animals and tissue collection

The North Dakota State University Institutional Animal Care and Use Committee approved all animal procedures used in this study. Animal use, experimental design, and methodology are described in detail in a recently published paper (Grazul-Bilska *et al*. 2013). Tissue samples for this and previous (Grazul-Bilska *et al*. 2013) studies were collected from the same animals, but different measurements were performed. Briefly, estrus was synchronized using a CIDR device for 14 days (MWI, Boise, ID, USA) for adult ewes ($n=67$; Western Range ewes, primarily Rambouillet, Targhee, and Columbia crossbred) randomly assigned to be NAT or to serve as donors or recipients. Twenty-four hours after CIDR removal, ewes ($n=10$) assigned to the NAT group were exposed to a fertile ram. For donor ewes in the NAT-ET, IVF, and IVA groups, estrus was checked twice daily using a vasectomized ram. Donor ewes ($n=3$) for the NAT-ET group were treated twice daily with FSH-P (Sioux Biochemical, Sioux Center, IA, USA): on day 13 following estrus (day 0), 5 mg/injection; day 14, 4 mg/injection; and day 15, 3 mg/injection as described before (Stenbak *et al*. 2001, Grazul-Bilska *et al*. 2006, 2012). Donor ewes ($n=22$) for the IVF and IVA groups were treated with FSH-P on days 13 and 14 after estrus, as described above. On day 15 of the estrous cycle, ewes from the NAT-ET group were exposed to a fertile ram for 48 h, but for the ewes in the IVF and IVA groups, ovaries were collected. Then, the oocytes were isolated, matured for 24 h, and fertilized or activated *in vitro*. To accomplish this, cumulus–oocyte complexes (COCs) were isolated from visible surface antral follicles >3 mm in diameter and incubated overnight in maturation medium (TCM199; Sigma; up to 30 COC/0.5 ml in a four-well Nunc culture dish) supplemented with 10% fetal bovine serum (FBS, Sigma), ovine FSH (5 μg/ml; oFSH-RP-1; NIAMDD–NIH, Bethesda, MD, USA), ovine LH (5 μg/ml; oLH-26; NIADDK–NIH), estradiol-17β (1 μg/ml; Sigma), glutamine (2 mM; Sigma), sodium pyruvate (0.25 mM; Sigma), epidermal growth factor (10 ng/ml; Sigma), and penicillin/streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin; Gibco). After denuding oocytes of cumulus cells, half of the oocytes from each ewe were used for IVF and the other half for IVA.

For IVF, capacitated semen pooled from five Western range crossbreed rams frozen and stored in liquid nitrogen (−197 °C) was thawed and viable sperm were separated in the modified sperm washing media (Irvine Scientific, Santa Ana, CA, USA) using the swim-up technique (Grazul-Bilska *et al*. 2006, 2012). Oocytes were cultured in fertilization medium prepared in our laboratory consisting of synthetic oviductal fluid (SOF), 2% heat-inactivated ovine serum collected on day 0–1 of the estrous cycle, and 1% penicillin/streptomycin in the presence of capacitated sperm ($0.5–1\times10^6$ sperm/ml) for 24 h followed by incubation (at 39 °C, 5% O_2, 5% CO_2, and 90% N_2) in culture medium until ET (Grazul-Bilska *et al*. 2003, 2006, 2012, 2013). Culture medium consisted of SOF supplemented with BSA (8 mg/ml; Sigma), glutamine (1 mM), MEM non-essential amino acids (0.01 ml/ml, vol/vol; Sigma), BME amino acids (0.02 ml/ml, vol/vol; Sigma), and penicillin/streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin). For IVA, oocytes were incubated for 5 min in TCM199 medium containing 2% FBS and ionomycin (2.5 μM; Sigma) followed by a 3-h incubation with 6-dimethylaminopurine (2 mM; Sigma). *In vitro*-activated oocytes were then transferred to culture medium and incubated until ET (Grazul-Bilska *et al*. 2003, 2006, 2013).

For the NAT-ET group, on day 5 post-mating (day 1, day of mating), embryos were flushed, evaluated using a stereomicroscope, and then transferred to synchronized recipients (three embryos at morula stage from the same donor/recipient; $n=9$). For the IVF and IVA groups, *in vitro*-generated embryos at morula stage were transferred on day 5 after fertilization or activation (day 1, day of fertilization or activation) to synchronized recipient ewes (three embryos from the same

donor/recipient; $n=10$ and 13 respectively). On day 22 after mating, fertilization, or activation, fetuses and utero-placental tissues were collected from NAT, NAT-ET, IVF, and IVA groups ($n=8$, 7, 8, and 7 ewes respectively). Pregnancy rates for NAT, NAT-ET, IVF, and IVA groups were 80, 78, 80, and 54% respectively.

Cross sections (~0.5-cm thick) of the entire gravid uterus including fetal membranes (FM, chorioallantois) were fixed by immersion in formalin for Ki67 detection or Carnoy's solution for smooth muscle cell actin (SMCA) detection and then embedded in paraffin. For total cellular RNA extraction, FM (fetal placenta) and caruncular (CAR, maternal placenta) tissues were dissected from an area close to the embryo, snap-frozen in isopentane super-cooled in liquid nitrogen, and stored at −70 °C. We chose day 22 for tissue collection because in our previous experiments, we demonstrated that between days 20 and 22, major changes in cell proliferation, vascularization, and expression of angiogenic factors occurred in fetal and maternal placenta in pregnancies achieved through NAT (Grazul-Bilska *et al.* 2010, 2011). In addition, by days 20–22 after mating, placentation is already initiated in sheep (Igwebuike 2009).

Immunohistochemistry

Immunohistochemical procedures were performed as described by Grazul-Bilska *et al.* (2010, 2011, 2013). Briefly, paraffin-embedded uterine tissues containing FM were sectioned at 4 μm and mounted onto glass slides. Sections were rinsed several times in PBS containing Triton X-100 (0.3%, v/v) and then were treated for 20 min with blocking buffer (PBS containing normal horse serum (2%, vol/vol)) followed by overnight incubation at 4 °C with specific primary antibody for Ki67 (a marker of proliferating cells; 1:500; mouse monoclonal; Vector Laboratories, Burlingame, CA, USA) or SMCA (a marker of pericytes and vascular smooth muscle cells and thus blood vessels; 1:150; mouse monoclonal; Oncogene Research Products, San Diego, CA, USA). Primary antibodies were detected using secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories). For Ki67 staining, the sections were then counterstained with hematoxylin and periodic acid-Schiff's reagent (H and PAS) to visualize cell nuclei as well as basement membranes and thus blood vessels (Reynolds & Redmer 1992). For SMCA staining, the sections were counterstained with nuclear fast red (Sigma) to visualize cell nuclei. Control sections were incubated with normal mouse IgG (4 μg/ml) in place of primary antibody.

Image analysis

For each tissue section, images were taken at 600× (Ki67 staining) or 200× (SMCA staining) magnification using an Eclipse E600 Nikon microscope and digital camera (Nikon Instruments, Inc., Melville, NY, USA) or Zeiss Imager M2 epifluorescence microscope equipped with Zeiss piezo automated stage and AxioCam HRm camera (Carl Zeiss International, Jena, Germany). Image analysis (Image-Pro Plus, Media Cybernetics, Inc., Bethesda, MD, USA) was performed for images of five to ten randomly chosen fields of CAR to determine vascular cell proliferation based on Ki67 staining, while images of five to 40 randomly chosen fields from areas containing FM or CAR were used to determine the density of blood vessels based on SMCA staining, as described previously (Borowicz *et al.* 2007, Grazul-Bilska *et al.* 2010, 2011, 2013). The LI was calculated as the percentage (%) of proliferating Ki67-positive cells out of the total number of cells within blood vessels that were marked with H and PAS/CAR tissue area.

Quantitative real-time RT-PCR

All procedures for determining the expression of mRNA of genes in ovine tissues by RT-PCR along with sequences for 14 factors involved in the regulation of angiogenesis have been reported previously (Redmer *et al.* 2005, Grazul-Bilska *et al.* 2010, 2011). Briefly, snap-frozen CAR and FM tissues were homogenized in TRI-reagent (Molecular Research Center, Cincinnati, OH, USA) and RNA was extracted according to the manufacturer's specifications. The quality and quantity of total RNA were determined via capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA). Real-time RT-PCR reagents, probes, and primers were purchased from and used as recommended by Applied Biosystems. For each sample, 30 ng total RNA was reverse transcribed in triplicate 20 μl reactions using random hexamers. Sequence-specific TaqMan probes and primers were designed using the Primer Express Software from Applied Biosystems. The ABI PRISM 7000 was used for detection of sequences amplified at 60 °C typically for 40 or 45 cycles (Applied Biosystems). Quantification was determined from a relative standard curve of dilutions of the cDNA generated from tcRNA pooled from placentomes collected on day 130 of pregnancy. Expression of each gene was normalized to expression of *18S* rRNA in a multiplex reaction using the human 18S pre-developed assay reagent (PDAR) from Applied Biosystems. The PDAR solution, which is primer limited and contains a VIC-labeled probe, was further adjusted using one-fourth the normal amount, so that it would not interfere with amplification of the FAM-labeled gene of interest. Standard curves were also generated with the multiplex solution, and the quantity of *18S* rRNA and the gene of interest were determined using each specific standard curve. The concentrations of mRNA were then normalized to *18S* rRNA by dividing each of the mRNA values by their corresponding *18S* rRNA value.

Statistical analysis

Data were analyzed using the general linear model procedure of SAS (SAS Institute 2010) with the main effect of embryo origin (i.e. NAT, NAT-ET, IVF, or IVA) and presented as means ± S.E.M. When the *F*-test was significant ($P<0.05$), differences between specific group means were evaluated using the least significant differences test (Kirk 1982). In addition, PROC CORR of SAS was used to calculate simple linear correlations between specific variables.

Results

In all pregnancy groups, Ki67 was detected in the nuclei of proliferating cells in blood vessels and other

compartments of CAR (Fig. 1), and SMCA was detected in the blood vessels in FM and CAR (Fig. 2). LI of vascular cells and the density of blood vessels in CAR and/or FM were affected by embryo origin. For CAR, the vascular LI was less ($P<0.04$) in the IVA group compared with any of the other groups, which were similar to each other (3.2 ± 1.2 vs $9.5\pm1.3\%$). In FM, the density of blood vessels was less ($P<0.01$) in IVA than in NAT or NAT-ET and was intermediate in IVF (Fig. 3A). In CAR, the density of blood vessels was less ($P<0.01$) in NAT-ET, IVF, and IVA than in NAT (Fig. 3B). In NAT, the density of blood vessels was 39.1 ± 3.5 and $39.4\pm4.3/10^6$ μm^2 in FM and CAR respectively.

In all pregnancy types, in FM and CAR, mRNA were detected for the following: vascular endothelial growth factor (*VEGF*) and its receptors, *FLT1* and *KDR*; placental growth factor (*PGF*); neuropilin 1 (*NP1*) and *NP2*; the angiopoietins (*ANGPT1* and *ANGPT2*) and their receptor *TEK*; endothelial nitric oxide synthase 3 (*NOS3*) and its receptor soluble guanylate kinase (*GUCY1B3*); hypoxia-inducible factor 1A (*HIF1A*); and fibroblast growth factor 2 (*FGF2*) and its receptor *FGFR2* (Fig. 4). Compared with NAT, mRNA expression in FM was less ($P<0.01$) for *VEGF* and *FLT1* in NAT-ET, IVF, and IVA, but *KDR* mRNA expression was similar for all groups (Fig. 4A).

Compared with NAT, *VEGF* mRNA expression in CAR was less ($P<0.01$) in NAT-ET and IVA, whereas IVF was intermediate (Fig. 4B). For *FLT1* mRNA in CAR, expression was less ($P<0.01$) in NAT-ET, IVF, and IVA compared with NAT, but *KDR* mRNA expression was similar for all groups (Fig. 4B).

Compared with NAT, mRNA expression in FM was less for *PGF* ($P<0.05$) in NAT-ET and IVF but was intermediate for IVA (Fig. 4C). Expression of mRNA in FM was less for *NP1* ($P<0.08$) in NAT-ET, IVF, and IVA compared with NAT (Fig. 4C). In addition, in FM, mRNA expression for *NP2* was greater in NAT-ET ($P<0.02$) than in IVF and IVA, and NAT was intermediate (Fig. 4C). Compared with NAT, *PGF* mRNA expression in CAR was less ($P<0.01$) in NAT-ET, IVF, and IVA, but mRNA expression for *NP1* and *NP2* was similar for all groups (Fig. 4D).

Compared with NAT, mRNA expression in FM was less ($P<0.01$) for *ANGPT1* in NAT-ET, IVF, and IVA (Fig. 4E). Expression of *ANGPT2* mRNA in FM was less ($P<0.05$) in NAT-ET and IVA but not in IVF (Fig. 4E). Expression of *TEK* mRNA was similar in FM of all groups (Fig. 4E).

Compared with NAT, mRNA expression in CAR was less ($P<0.01$) for *ANGPT1* in NAT-ET, IVF, and IVA, but *ANGPT2* mRNA expression was similar for all groups (Fig. 4F). Compared with NAT, *TEK* mRNA expression in CAR was less ($P<0.01$) for IVF and IVA but was intermediate in NAT-ET (Fig. 4F).

Compared with NAT, mRNA expression in FM was less ($P<0.03$) for *NOS3* and *HIF1A* in NAT-ET and IVA, but in the IVF group, it was either intermediate (*NOS3*) or similar (*HIF1A*) to that of the NAT group (Fig. 4G). In FM, mRNA expression for *GUCY1B3* was similar for all groups (Fig. 4G). In CAR, mRNA expression of *NOS3*, *GUCY1B3*, and *HIF1A* (Fig. 4H) and for *FGF2* and *FGFR2* (Fig. 4J) was similar for all groups.

Compared with NAT, mRNA expression in FM was less ($P<0.06$) for *FGF2* in NAT-ET, IVF, and IVA and for *FGFR2* in FM was less ($P<0.07$) in NAT-ET and IVF but intermediate for IVA (Fig. 4I).

Supplementary Tables 1 and 2 (see section on supplementary data given at the end of this article) present correlation coefficients for mRNA expression among factors involved in the regulation of angiogenesis and the density of blood vessels in FM and CAR respectively. For FM, the VEGF system members were correlated with the ANGPT and FGF systems and with eNOS; there were 39 statistically significant correlations

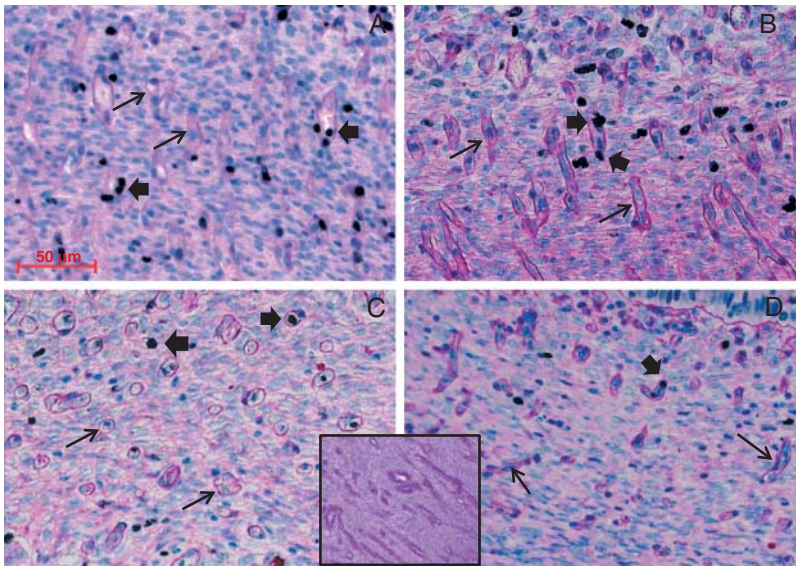

Figure 1 Representative photomicrographs of immunohistochemical detection of Ki67 (blackish nuclei, large arrows) followed by hematoxylin (bluish nuclei) and PAS (dark pink staining of basement membranes) staining in CAR from NAT (A), NAT-ET (B), IVF (C), and IVA (D) groups on day 22 of pregnancy. Small arrows indicate examples of blood vessels and large arrows point to examples of proliferating cells (Ki67-positive nuclei) within blood vessels. Bar on A (valid for B–D) = 50 μm. In inset in C/D, note the lack of positive Ki67 staining in the control sections in which non-specific mouse IgG was used in place of the primary antibody.

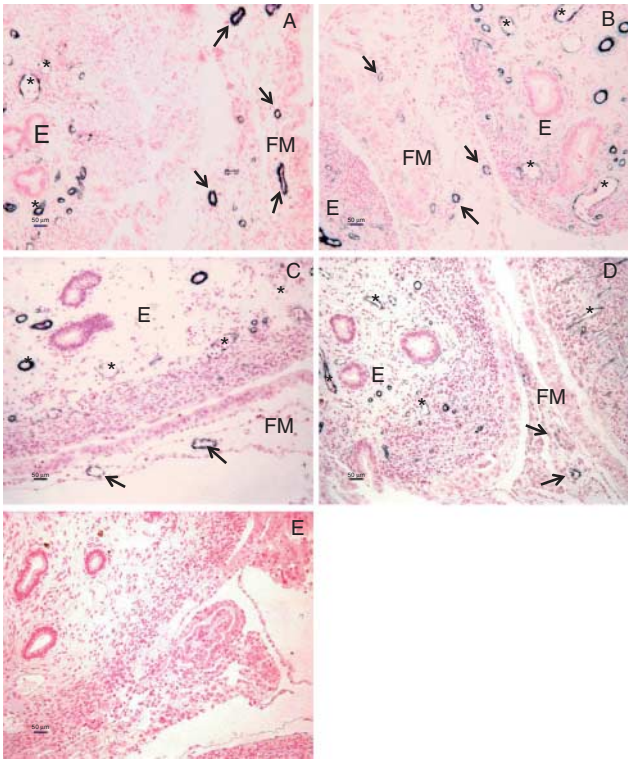

Figure 2 Representative photomicrographs of immunohistochemical detection of SMCA (blackish staining) followed by fast red (pinkish cell nuclei) staining in FM and endometrial (E) CAR from NAT (A), NAT-ET (B), IVF (C), and IVA (D) groups on day 22 of pregnancy. Arrows indicate blood vessels in FM and stars in E of CAR in A, B, C and D. Bar = 50 μm for A, B, C, D and E. In E, note the lack of positive SMCA staining in the control sections in which non-specific mouse IgG was used in place of the primary antibody.

among these factors. In addition, for FM, ANGPT2 and eNOS were correlated with blood vessel density. For CAR, the VEGF system members were correlated with the ANGPT and FGF systems, and several factors ($n=8$) were correlated with the density of blood vessels. For CAR, there were 48 statistically significant correlations among these factors.

Discussion

This study demonstrated that embryo origin and application of selected ART methods affect placental vascular development and mRNA expression of factors involved in the regulation of angiogenesis in fetal and placental tissues at a specific time point (day 22) in early pregnancy. In fact, several measurements of vascularization/angiogenesis were decreased after transfer of embryos of different origin. Interestingly, altered blood flow, vascular development, and/or expression of selected angiogenic and/or other growth factors have been demonstrated in the placenta of mid to late pregnancies compromised by factors causing fetal growth restrictions in several species (Reynolds *et al*. 2006, 2013, Vonnahme & Lemley 2011, Gourvas *et al*. 2012).

In this study, decreased vascular cell proliferation in maternal placenta was observed only in the IVA group. This demonstrates that when embryos possess only maternal genes, cell proliferation in blood vessels of CAR appears to be altered. Thus, input of the paternal genome seems to be critical for blood vessel growth and development in the maternal placenta. Previously, we have demonstrated that the total cell proliferation in fetal and maternal placental tissues for the same pregnancy types was decreased in NAT-ET, IVF, and IVA groups compared with NAT (Grazul-Bilska *et al*. 2013). Thus, it seems that embryo origin has limited effects on placental blood vessel growth but has more extensive effects on overall placental tissue growth in the early stages of pregnancy. Therefore, we hypothesize that the mechanisms of regulation of blood vessel cell proliferation differ from those involved in the regulation of proliferation of other cell types in the placenta.

A reduced density of blood vessels in FM or CAR was observed in NAT-ET, IVF, or IVA groups in this study. This may be associated with reduced overall growth measured by cell proliferation in FM and CAR reported before for the same animal models (Grazul-Bilska *et al*. 2013). In addition, the IVA group had the lowest density

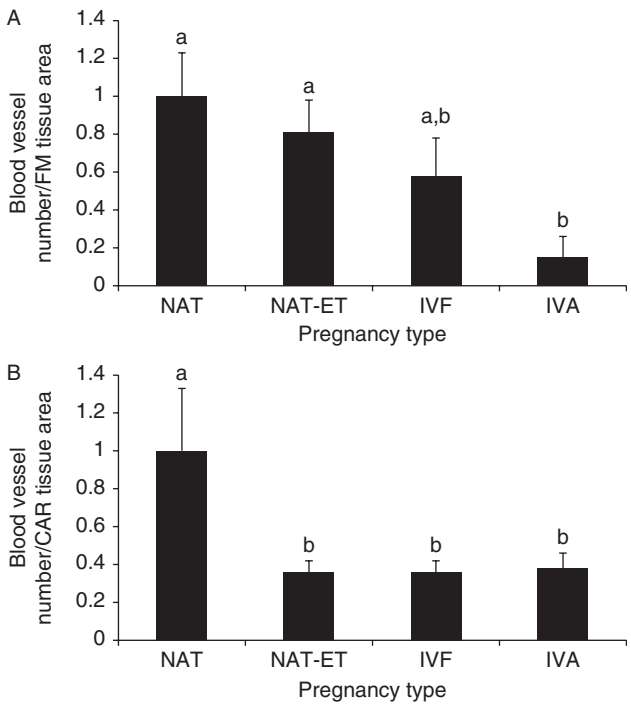

Figure 3 The density of blood vessels (based on SMCA staining) in FM (A) and CAR (B) from NAT, NAT-ET, IVF, and IVA groups on day 22 of pregnancy. Data are expressed as a fold-change compared with the NAT group, which was arbitrarily set as 1. In NAT, the density of blood vessels was 39.1 ± 3.5 and $39.4 \pm 4.3/10^6$ μm^2 in FM and CAR respectively. Means ± S.E.M. with different superscript letters differ within tissue ($^{a,b}P<0.01$).

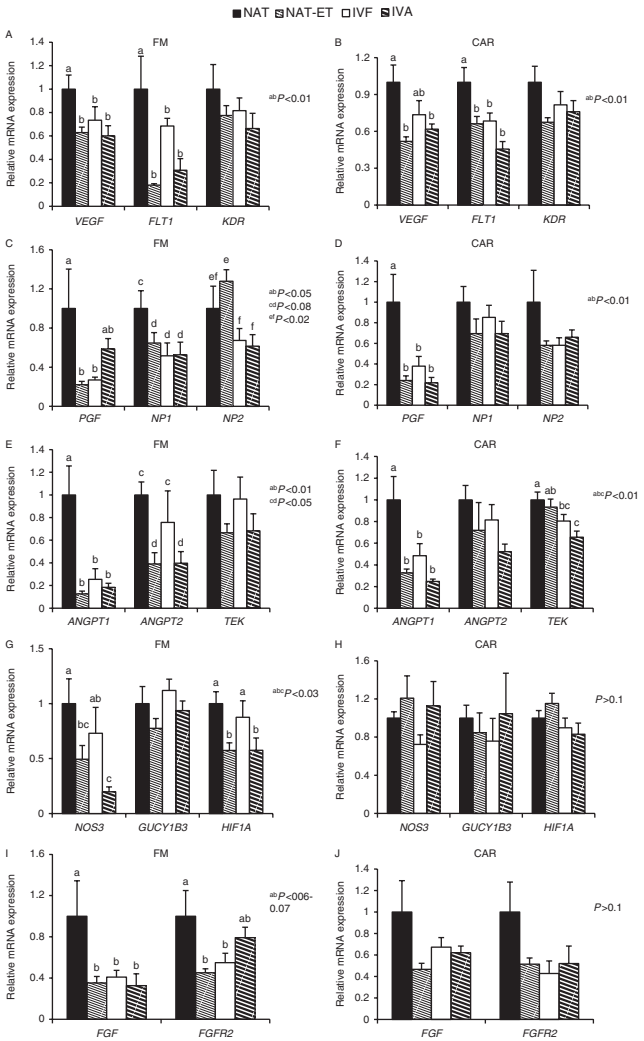

Figure 4 Expression of mRNA for *VEGF*, *FLT1*, *KDR* (A and B), *PGF*, *NP1*, *NP2* (C and D), *ANGPT1*, *ANGPT2*, *TEK* (E and F), *NOS3*, *GUCY1B3*, *HIF1A* (G and H), *FGF2*, and *FGFR2* (I and J) in FM (left column: A, C, E, G and I) or CAR (right column: B, D, F, H and J) from NAT, NAT-ET, IVF, and IVA groups on day 22 of pregnancy. Data are expressed as fold-change compared with NAT control arbitrarily set as 1. Means±S.E.M. with different superscript letters differ within a specific gene ($^{a,b,c,d,e,f}P<0.01$–0.08).

of blood vessels in FM, and a very low vascular cell proliferation in CAR. These data indicate that in the absence of the paternal genome, fetal–maternal interactions are altered causing profoundly reduced vascularization in the maternal placenta.

Reduced vascular density in maternal and fetal placenta in pregnancies achieved after transfer of embryos created through IVF or oocyte activation reported in this study indicates that angiogenesis was impaired during early pregnancy. Therefore, application of ART may lead to inadequate placental vascularization and thus blood flow at least during early gestation and may eventually contribute to compromised pregnancy.

Numerous factors are involved in the regulation of vascular function and growth in the placenta, including members of the VEGF, ANGPT, FGF, HIF families, and the NO system (Reynolds & Redmer 1995, 2001, Patan 2000, Zygmunt *et al.* 2003, Demir *et al.* 2007, Burton *et al.* 2009, Reynolds *et al.* 2010). Expression and function of these factors depend on stage of pregnancy, maternal age, and environmental and other factors (Reynolds *et al.* 2010, 2013). In this study, the mRNA expression of 12 of 14 evaluated factors involved in the regulation of angiogenesis was decreased in FM and/or CAR in NAT-ET, IVF, and/or IVA groups compared with the NAT control. Expression of mRNA for only two factors in placental tissues, *KDR* and *GUCY1B3*, remained unaffected by embryo origin in this study.

Factors belonging to the VEGF family, including VEGF, FLT1, and PGF along with NP1 and NP2, which are involved in the VEGF and PGF signaling, and also members belonging to the ANGPT family are recognized as the major angiogenic factors in placental and other tissues (Ahmed & Perkins 2000, Reynolds & Redmer 2001, Neufeld *et al.* 2002, Wulff *et al.* 2003, Reynolds *et al.* 2005, 2010, Chaballe *et al.* 2011, Koch *et al.* 2011). Expression of these factors has been demonstrated in maternal and/or fetal placenta from early to late pregnancy in sheep and other species (Reynolds & Redmer 2001, Borowicz *et al.* 2007, Grazul-Bilska *et al.* 2010, 2011, Reynolds *et al.* 2010, 2013). In our study, mRNA expression for several members of the VEGF family and NP1 or NP2 was reduced in pregnancies achieved using ART. Furthermore, in compromised pregnancies such as those involving intrauterine growth restriction (IUGR), preeclampsia, or delivery of small-for-gestational age neonates, altered placental expression of members of the VEGF or ANGPT families, vascularization, plasma concentration of PGF and VEGF, or a balance between various angiogenic and/or antiangiogenic factors from mid to late stages have all been reported for humans and other species (Ahmed & Perkins 2000, Regnault *et al.* 2002, Wulff *et al.* 2003, Redmer *et al.* 2005, 2009, Arroyo & Winn 2008, Erez *et al.* 2008). Thus, expression of members of the VEGF and ANGPT families are altered in specific pregnancy disorders at mid to late stages and early pregnancies achieved by transfer of embryos created through ART or oocyte activation. This indicates that changes in expression and possibly function of these factors may contribute to pregnancy disorders.

In FM, but not in CAR, expression of *NOS3* and *FGF2* mRNA, but not *GUCY1B3* or *FGFR2*, was affected by embryo origin in this study. Furthermore, expression of *HIF1A* mRNA in CAR but not in FM was affected by embryo origin. Members of the NO and FGF systems and HIF1A are expressed throughout pregnancy in the placenta of several species (Reynolds & Redmer 2001, Kwon *et al.* 2004, Grazul-Bilska *et al.* 2010, 2011, Patel *et al.* 2010, Reynolds *et al.* 2010, Krause *et al.* 2011).

It has been demonstrated that placental eNOS and/or HIF1A expression was reduced or altered in human preeclampsia at term (Rajakumar *et al*. 2007) and also in an ovine model of IUGR at mid to late stages (Galan *et al*. 2001, Ziebell *et al*. 2007). This indicates that expression and likely function of these factors are affected in compromised pregnancies and therefore may contribute to abnormal placental and/or fetal development.

The NO system members interact with members of VEGF, ANGPT, and FGF families, and they also play a regulatory role in placental angiogenesis, remodeling, and immunosuppression (Purcell *et al*. 1999, Ahmed & Perkins 2000, Reynolds & Redmer 2001, Mata-Greenwood *et al*. 2008, Grazul-Bilska *et al*. 2010, 2011, Reynolds *et al*. 2010, Krause *et al*. 2011). The HIF1A and other members of the HIF family are involved in regulation of placental growth, remodeling, differentiation, transport, and vascularization acting as key mediators of placental development and function and are therefore likely to be important contributors to both normal and adverse pregnancy outcomes (Pringle *et al*. 2010). Reduced expression of selected genes mentioned above may contribute to inadequate vascularization and blood flow to the fetus.

Reduced expression of several key factors regulating tissue growth and also vascular function in the placenta, including PGF, HIF1A, FGF2, and their receptors (Shimizu *et al*. 2012), probably resulted in decreased placental cell proliferation and fetal size observed in our previous study using the same animal models (Grazul-Bilska *et al*. 2013). In addition, it has been recently demonstrated that expression of several imprinted genes (e.g. IGF2, H19, and PEG1) was less in FM in pregnancies achieved after ET of embryos created *in vitro* compared with natural early pregnancies (Ptak *et al*. 2013). This indicates that impaired tissue growth is associated with changes in expression of selected growth factors, as we observed in this study, and likely with imprinted genes.

Numerous factors involved in the regulation of angiogenesis interact with each other in order to control blood vessel function and development (Reynolds & Redmer 2001, Borowicz *et al*. 2007, Grazul-Bilska *et al*. 2010, Reynolds *et al*. 2010, 2013), which is reflected by significant correlations among these factors reported for FM and CAR in this study (Supplementary Tables 1 and 2). Furthermore, correlations between eight (of 14) evaluated factors with the density of blood vessels in CAR observed in our study indicate functional associations between mRNA expression and blood vessel growth. The density of blood vessels in FM was correlated with expression of two factors, which indicates a different pattern of blood vessel growth and its regulation in fetal vs maternal placenta.

In this study, tissues were collected only at one stage of early pregnancy (day 22) after transfer of embryos of different origin and application of selected ART including estrus synchronization using CIDR devices, FSH treatment, IVF or IVA, and ET. These procedures were associated with a decrease in expression of several genes involved in the regulation of angiogenesis in FM and/or CAR. Thus, selected ART methods, including estrus synchronization, superovulatory treatments, and ET with or without IVF or IVA, which are used in animal production and human reproductive medicine, may have some negative effects on fetal and placental growth and function during pregnancy (Sinclair 2008, Laprise 2009). In addition, it has recently been demonstrated that a specific estrus synchronization protocol may affect CAR vascular development during early pregnancy in sheep (Ruiz-Gonzalez *et al*. 2013), and FSH-superovulatory treatment affected gene expression in bovine granulosa cells, which may lead to altered function (Dias *et al*. 2013). Conversely, in several experiments in which we used a similar estrus synchronization and/or FSH treatment protocol as in this study, the rates of fertilization or blastocyst formation, and the development of fetuses or offspring in sheep, seemed to be normal (Grazul-Bilska *et al*. 2003, 2006, 2013). Although others and we observed changes in placental tissue growth, vascularization, expression of selected genes, and global methylation after application of ART during early pregnancy (Grazul-Bilska *et al*. 2013, Ptak *et al*. 2013, Ruiz-Gonzalez *et al*. 2013), some compensatory mechanisms probably exist to minimize adverse effects of ART during early pregnancy and allow for normal fetal and placental growth and function throughout pregnancy and postnatal period. Alternatively, as pregnancies resulting from various ART, including IVF and somatic cell nuclear transfer (SCNT), may exhibit poor placental development and vascularization as well as abnormal/altered fetal growth and development at different stages of gestation (Hoffert *et al*. 2005, Farin *et al*. 2006, Bauersachs *et al*. 2009, Collier *et al*. 2009, Mansouri-Attia *et al*. 2009, Ptak *et al*. 2013), the effects observed during early pregnancy in this study could have long-term consequences for pregnancy outcome. To determine long-term consequences of ART application, additional studies should be undertaken using offspring created through use of selected ART.

As we have discussed in our previous studies using different embryo origin and also ART (Grazul-Bilska *et al*. 2013), the embryo affects uterine function and has an active role in initiation of pregnancy and, in turn, the uterus affects fetal growth and development (Reynolds & Redmer 1995, 2001, Barnea 2004, Spencer *et al*. 2008, Ostrup *et al*. 2011, Grazul-Bilska *et al*. 2013). In fact, abnormal embryo/fetal–maternal communication, endometrial remodeling, reduced vascularization, altered expression of angiogenic factors, and other problems during the peri-implantation and later pregnancy periods have been observed for pregnancies achieved after transfer of embryos created through SCNT in animal models (Hill *et al*. 2000, Miles *et al*. 2004, 2005, Loi *et al*. 2005, Fletcher *et al*. 2007, Miglino *et al*. 2007,

Palmieri *et al*. 2007, 2008, Campos *et al*. 2010). However, in pregnancies resulting from the transfer of embryos created through IVF, changes in endometrial vascularization, remodeling, and function were less pronounced than after transfer of embryos from SCNT (Hill *et al*. 2000, Miles *et al*. 2004, 2005, Hoffert *et al*. 2005, Miglino *et al*. 2007, Palmieri *et al*. 2007, 2008, Mansouri-Attia *et al*. 2009). As we discussed before (Grazul-Bilska *et al*. 2013), endometrial tissues possess mechanisms to adapt to embryos of different origin, which may serve as a biological sensor to meet embryonic demands or adaptation to environmental conditions (Fleming *et al*. 2004, Borowicz & Reynolds 2010, Coan *et al*. 2010). This idea is in line with our hypothesis that during pregnancy, some compensatory mechanisms may exist to reverse negative effects of embryo manipulations on placental and fetal growth.

In summary, in this study, transfer of embryos of different origins and application of ART decreased vascular cell proliferation in CAR, the density of blood vessels in FM and CAR, and expression of several factors involved in the regulation of placental angiogenesis on day 22 of pregnancy. Thus, embryo origin may have specific effects on vascular growth and likely function in the ovine placenta and fetus through regulation of tissue growth and angiogenesis, as well as other mechanisms such as epigenetic processes. As relatively few studies have focused on evaluation of selected processes in the placenta during early gestation without or with application of ART in any species, these data provide novel information concerning fetal and placental vascular growth/cell proliferation and angiogenesis in relation to embryo origin. Furthermore, these data provide a foundation for determining the expression of specific factors regulating vascular development and function of placental and embryonic tissues in pregnancies after application of ART. In addition, these data will help us to better understand placental regulatory mechanisms in compromised pregnancies and to identify strategies for rescuing such pregnancies.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0663.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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