Impaired placental vasculogenesis compromises the growth of sheep embryos developed in vitro

?1 BIOLOGY OF REPRODUCTION (2014) 91(1):0, 1–7
Published online before print 22 May 2014.
DOI 10.1095/biolreprod.113.113902

Impaired Placental Vasculogenesis Compromises the Growth of Sheep Embryos Developed In Vitro[1]

Antonella Fidanza,[3] Paola Toschi,[3] Federica Zacchini,[3] Marta Czernik,[3] Chiara Palmieri,[3] PierAugusto Scapolo,[3] Jacek A. Modlinski,[4] Pasqualino Loi,[3] and Grazyna E. Ptak[2,3,4]

[3]*Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy*
[4]*Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, Poland*

ABSTRACT

?2 **To evaluate how assisted reproductive technologies (ART) affect vasculogenesis of the developing conceptus, we analyzed placental and fetal development of in vitro-produced (IVP) sheep embryos. Pregnancies produced by ART carry increased risk of low birth weight, though what causes this risk remains largely unknown. We recently reported that developmental arrest of sheep conceptuses obtained by ART is most pronounced when the cardiovascular system develops (Days 20–30 of develop-**
?3 **ment). A total of 86 IVP blastocysts (2–4 per ewe) were surgically transferred to 30 recipient sheep 6 days after estrus; 20 sheep were naturally mated (control). Conceptuses were recovered from sheep at Days 20, 22, 26, and 30 of gestation and morphologically evaluated. Then, the conceptuses and part of their placentae (chorion-allantois) were fixed for histological and immunohistochemical analysis and snap-frozen in liquid nitrogen for subsequent mRNA expression analysis. Results demonstrate that the cardiovascular systems of sheep IVP conceptuses were severely underdeveloped. Pericardial and placental hemorrhages were noted in a majority (5/7) of the dead embryos. In the surviving IVP embryos, the expression of angiogenetic factors was reduced at Day 20. The placental vessels were underdeveloped on Days 20 and 22 ($P < 0.05$), though placental vasculogenesis was successfully completed on subsequent days. However, low vessel number persisted at Days 26 and 30 (4.6 vs. 5.9 and 6.64 vs. 8.70 per field, respectively; $P < 0.05$) together with reduced vessel diameter at Day 26 (46.89 vs. 89.92 μm; $P < 0.05$). In vitro production of sheep embryos induced severely impaired vasculogenesis early in gestation. This may lead to developmental programing problems, such as intrauterine growth restriction of the fetus, resulting in long-term health consequences for the offspring, such as cardiovascular diseases.**

assisted reproductive technology, developmental origins of health and disease, fetal growth, in vitro embryo culture, placenta, placental vasculogenesis, pregnancy

INTRODUCTION

The use of assisted reproductive technologies (ART) is associated with an increased incidence of pregnancy complications and decreased birth weight [1, 2]. The molecular clues underlying these events are still not well understood. Recent studies in mice and humans demonstrated that in vitro embryo development can alter transcriptional control in the placentae of embryos obtained by ART [3, 4]. Fetal growth is reliant on correct placentation, because the placenta creates the vascular network necessary for transferring maternal resources. Inadequate nutrient and gas transfer between mother and fetus can cause pregnancy complications, including fetal under- or overgrowth or even death. The risk of early abortions after human ART was previously suggested to be similar to that of naturally obtained pregnancies [5, 6]. More recent reports recognize the amplified risk of early abortions associated with ART; however, it should not be forgotten that the early miscarriage associated with ART may not result from the techniques but from the underlying health risks of subfertile patient [7]. The high frequency of embryo losses may be also related to the reduced ability of ART embryos to complete vasculogenesis. A less severe consequence of an underdeveloped placental network may be developmental adaptation (fetal programming), which permanently changes the physiology of offspring, predisposing them to cardiovascular disease later in life [8, 9].

Our recent study showed compromised placental development of sheep embryos produced in vitro [10]. Sheep are an ethically acceptable and powerful animal model [11] for the characterization of early ART pregnancies. Furthermore, the organization of villous tree structures in the sheep placentome is comparable to that of humans, confirming its usefulness as a human-like model for placental vascularization [12, 13]. The smaller size of in vitro-produced (IVP) sheep conceptuses observed in our previous study [10] suggested vascular network defects in their placentae. The placental vascular network starts to play a critical role in supporting sheep embryo growth starting from Day 20 of gestation [11]. Therefore, to clarify the cause of the intrauterine growth restriction (IUGR) frequently observed in human ART pregnancies, we studied placental vasculogenesis in sheep IVP conceptuses during this crucial stage of developmental bottleneck. In the first studied period (Days 20 and 22 of gestation), we evaluated the course of maturation of placental vessels in IVP conceptuses, whereas in the second studied period (Days 26 and 30 of gestation), we studied the completion of placental vasculogenesis. In particular, we analyzed the expression of genes regulating vasculogenesis and angiogenesis in placentae collected from IVP embryos. We also performed the immunohistochemical and histological analysis of IVP placentae to evaluate vasculogenesis and the degree of vascular network development.

[1]Supported by the European Research Council (FP7/2007–2013), Programme IDEAS GA 210103 to G.E.P; European Research Council, Programme FP7-KBBE-2012.1.3–04 GA 312097 (FECUND) to G.E.P.; MIUR/CNR, Programme FIRB GA B81J12002520001 (GenHome) to P.L. The authors are participating in the COST action FA 1201 "Epiconcept" Epigenetic and Periconception Environment. No competing interests are declared.
§2 Correspondence: E-mail: gptak@unite.it

Received: 11 September 2013.
First decision: 16 October 2013.
Accepted: 25 April 2014.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

MATERIALS AND METHODS

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co.

Oocyte Collection, In Vitro Maturation, In Vitro Fertilization, and Embryo Culture

All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609. Methods of in vitro embryo production were adapted from those previously described [14]. Briefly, sheep ovaries were collected from local slaughterhouses and transferred at 37°C to the laboratory within 1–2 h. Oocytes were aspirated with 21-gauge needles in the presence of Hepes-buffered TCM-199 medium (Gibco, Life Technologies) containing 0.005% (w:v) heparin. Then, oocytes with at least two or three layers of compact cumulus cells and uniform cytoplasm were selected for in vitro maturation (IVM). All selected oocytes were washed twice and then in vitro maturated in bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μM cysteamine, 10% fetal bovine serum (FBS; Gibco), 5 μg/ml of follicle-stimulating hormone (Ovagen; ICP), 5 μg/ml of luteinizing hormone, and 1 μg/ml of estradiol. Maturation was conducted in four-well culture plates (Nunclon) containing 0.4 ml/well of IVM medium and incubated in a humidified atmosphere of 5% CO_2 in air at 39°C for 24 h. Matured oocytes (n = 340) were partially denuded and transferred into 50-μl drops of bicarbonate-buffered synthetic oviductal fluid (SOF) enriched with 20% (v:v) heat-inactivated estrous sheep serum, 2.9 mM calcium lactate, and 16 μM isoproterenol. Ram semen was fast-thawed and washed in SOF containing 4 mg/ml of bovine serum albumin (BSA) at 200 × *g* for 5′. Fertilization was carried out at a final concentration of 5×10^6 cells/ml and left overnight in 5% CO_2 at 38.5°C. Presumptive zygotes were transferred into 20-μl drops of SOF enriched with 1% (v:v) Minimum Essential Medium nonessential amino acids (Gibco), 2% (v:v) Basal Medium Eagle essential amino acids, 1 mM glutamine, and 8 mg/ml of BSA covered with mineral oil prewashed in SOF. Cultures were carried out in a humidified atmosphere of 5% CO_2, 7% O_2, and 88% N_2 at 39°C. On Days 3 and 5 of culture (Day 0 = day of fertilization), the medium was changed. On Day 5, 10% FBS, charcoal stripped, was added to the medium. Cleavage rate was evaluated 30 h following fertilization (238/340, 70%) and blastocyst rate on Day 6 (86/238, 36%).

Embryo Transfer and Sample Recovery

Sardinian sheep were synchronized with 25-mg Chronogest sponges (Intervet). Sheep were divided into two groups: The first were recipients of IVP embryos, and the second were mated naturally (control [CTR]). At Day 6 of culture, expanded blastocysts were transferred surgically by paramedian laparotomy to recipient sheep in the IVP group 6 days after estrus as previously described [10, 14]. A total of 86 IVP blastocysts (n = 2–4 per ewe) were surgically transferred 6 days after estrus; fetuses and placentae were surgically recovered from sheep under general anesthesia on Days 20, 22, 26, and 30 of gestation for both the IVP and CTR groups as previously described [10]. Once collected in Petri dishes (90 mm) with warm Ca^{2+}/Mg^{2+} PBS containing 0.005% (w:v) heparin, fetuses were observed under the stereomicroscope to assess their vitality: Heartbeat was measured, and pictures of each fetus were taken with a reference meter. Chorion-allantoids were snap-frozen in liquid nitrogen for subsequent molecular biology analysis or fixed in 4% paraformaldehyde in PBS for histological analysis as previously reported [10].

Expression Analysis

Gene expression analysis was performed on sheep placental tissues obtained from CTR and IVP embryos (n = 6 for each group). Analyzed samples were collected on Day 20 of gestation. Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to first-strand cDNA using the QuantiTect reverse transcription kit (Qiagen). The cDNAs obtained from placental tissues were used for gene expression analysis using specific 5′-3′ primer sets: *FGF2* (NM_001009769): forward, atcaaactacaacttcaagc; reverse, aactggagtatttccttgacc; *FGF2R* (DQ908953): forward, ttgtctgcaaagtgtacagc; reverse, ttggagaccttacaaatatactcc; *VEGF* (NM_001025110): forward, tttctgctctcttgggtgcattgg; reverse, atctgcatggtgatgttgaactcc; *VEGFR1* (XM-001249768): forward, aagagagccagtataaggc; reverse, ttgaggcctcatagtgatgaatcc; *VEGFR2* (AF513909): forward, aactgtacggcaagaactgagc; reverse, aacgtgctgttcttcttgg; *ANG1* (AY881028): forward, aagatggaagtctagatttcc; reverse, ttttcatttcctatgtgg; *ANG2* (AY881029): forward, atagaaatagggaccaacc; reverse, ttcttatcttgcagtttgc; *TIE-2* (AY288926): forward, ttaccaggtggacatctttgc; reverse, ttgggccattctcctttgg; and *HOXA13* (XM_004023180.1): forward, gtggtctcgcatccttca; reverse, cctccgtttgtccttggta. The reactions were performed using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen, Life Technologies) with an ABI PRISM 7900 Real-Time PCR System (Applied Biosystems) according to the manufacturers' instructions. Twenty-five nanograms of total RNA was amplified in triplicate with 10 pmol of each primer. To avoid false-positive signals, dissociation-curve analyses and negative controls were performed into each run. Relative gene expression data were calculated using the comparative threshold cycle method (−ΔΔCt) with β-ACTIN as the reference gene amplified with the subsequent primer set (NM_001009784: forward, aatcgtccgtgacatcaagg; reverse, ttcatgatggaattgaagg).

Histological Analysis

Placental tissues were overnight fixed in 4% (w:v) paraformaldehyde and subsequently dehydrated into increasing ethanol solution, then finally cleared in xylene mixture, for 5 min in each step. Fetuses were differentially treated depending on crown-rump evaluation according to Kaufman [15]. Finally, placentae and fetuses were embedded in Paraplast. Sections (thickness, 5 μm) were used for hematoxylin-and-eosin staining. Pictures were taken using the Nikon Eclipse E600 microscope. Placental vessels were divided into three different developmental stages (stages 1, 2, and 3) according to Charnock-Jones et al. [16]. Diameter and area of matured vessels were further characterized. Vessel diameters were measured in relation to the scale bar, and vessel areas were subsequently calculated depending on vessel shape. For the heart development investigation, according to fetal longitudinal orientation, left ventricles were analyzed in all samples. For immunohistochemistry, Paraplast-embedded sections (thickness, 5 μm) of chorioallantoic membranes (IVF, n = 10; CTR, n = 8) were dewaxed, rehydrated, and stained with the following antibodies: rabbit polyclonal anti-FGF-2 (1:3200; sc-79; Santa Cruz Biotechnology), rabbit polyclonal anti-HOXA13 (1:400; ab26084; Abcam), goat polyclonal anti-PECAM-1 (1: 6400; sc-1506; Santa Cruz Biotechnology), rabbit polyclonal anti-Tie-2 (1:400; H-176/sc-9026; Santa Cruz Biotechnology), and mouse monoclonal anti-VEGF (1:200; C-1/sc-7269; Santa Cruz Biotechnology). Sections were then incubated with a biotinylated rabbit polyclonal or mouse monoclonal secondary antibody (1:100; Vector Laboratories). Staining was visualized with Universal LSAB + Kit/HRP (Dako Italia) and Dako Liquid DAB + Substrate Chromogen System.

Fetal and Vessel Measures

During surgical sample recovery, fetal pictures were taken under a stereomicroscope with a measure reference. Vessel diameters were measured from each picture using IMAL software (Image Measurement and Analysis Lab; Unix version 3.5.11). For each vessel, major and minor axes were analyzed, and the areas were deduced using the ellipses area formula. Each picture was analyzed in triplicate, and the average of the measurements was finally used. The cell layer measurements of the myocardium were taken between the outer ventricular wall and the trabecular wall [17].

Statistical Analysis

For quantitative analysis, the data are reported as the mean ± SEM. The statistical analysis was performed using Instat 6 (GraphPad Software for Science). For gene expression and vessel area, number, and myocardial cell layer measurements, scalar numbers were analyzed using the nonparametric Mann-Whitney Test (see Figs. 2A and 4, A, B, and D). For evaluation of vessel developmental stage, data expressed as percentages were analyzed using the Fisher exact test (see Fig. 3, B, C, and D). Only $P < 0.05$ was considered to be significant. Qualitative data, such as observation of hemorrhages as well as histological and immunohistochemical evaluations, were not subjected to statistical analysis.

RESULTS

Aberrant Ventricular Development of IVP Embryos

In a substantial subset of nonliving conceptuses from the IVP group (8/11), hemorrhages were observed in both pericardial (5/8) and placental tissues (3/8) (Table 1 and Fig. 1). Because placental vascularization is connected with fetal cardiovascular system, we analyzed the morphology of the hearts of collected viable conceptuses (Fig. 1B). The ventricular walls were thinner in IVP conceptuses than in CTR conceptuses only on Day 22 (7.90 ± 0.40 vs. 11.92 ± 0.87 cell layers; $P = 0.0001$); on Day

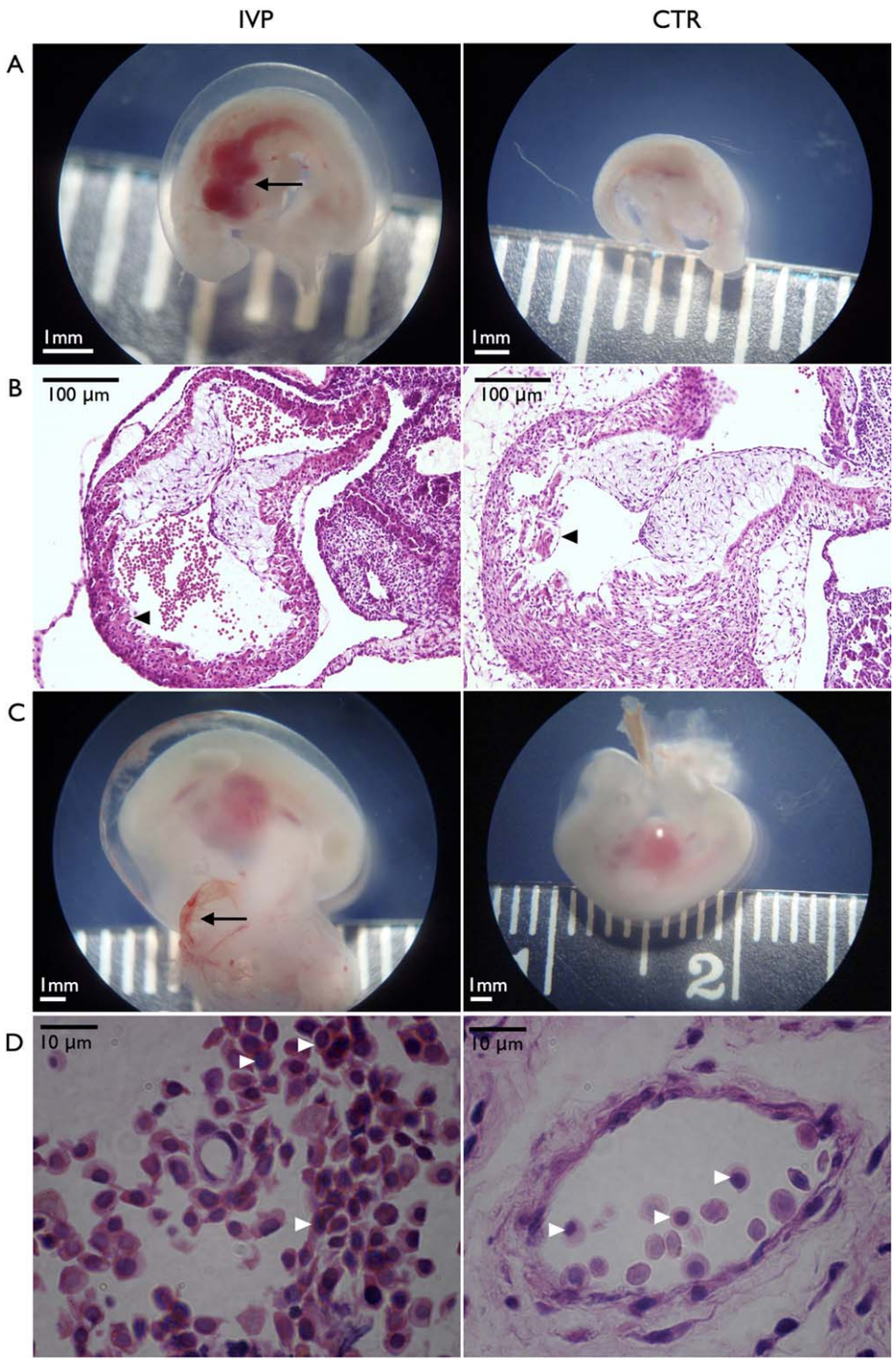

FIG. 1. General and vascular morphology is altered in IVP embryos. **A**) Pericardial hemorrhage in IVP embryo isolated at Day 22 of development (black arrow indicates the area of hemorrhage). **B**) Altered development of myocardium: reduced primitive left ventricular walls and trabeculae carnea formation (black arrowheads). **C**) Placental hemorrhage (black arrow) in IVP conceptus at Day 26 of development. **D**) Circulating red blood cells (white arrowheads) in placental tissue section (**left**) indicates a lack of vessel integrity in IVP conceptuses.

26, no differences were observed (11.00 ± 0.84 vs. 12.60 ± 1.12 cell layers; $P > 0.05$).

Vascular Development Is Reduced in Placenta from IVP Embryos

At Day 20 of development, the expression of a subset of factors regulating vasculogenesis and angiogenesis was downregulated in placentae from the IVP group (Fig. 2 and Supplemental Data S1; Supplemental Data are available online at www.biolreprod.org): FGF2, 0.20 ± 0.05; ANG2, 0.12 ± 0.05; TIE-2, 0.27 ± 0.08; HOXA13, 0.04 ± 0.02 (fold-change over CTR; $P < 0.05$). Consequently, the maturation of placental vessels (Fig. 3) and total count of vessels (Fig. 4) were reduced (Supplemental Data S1 and Table S1). High

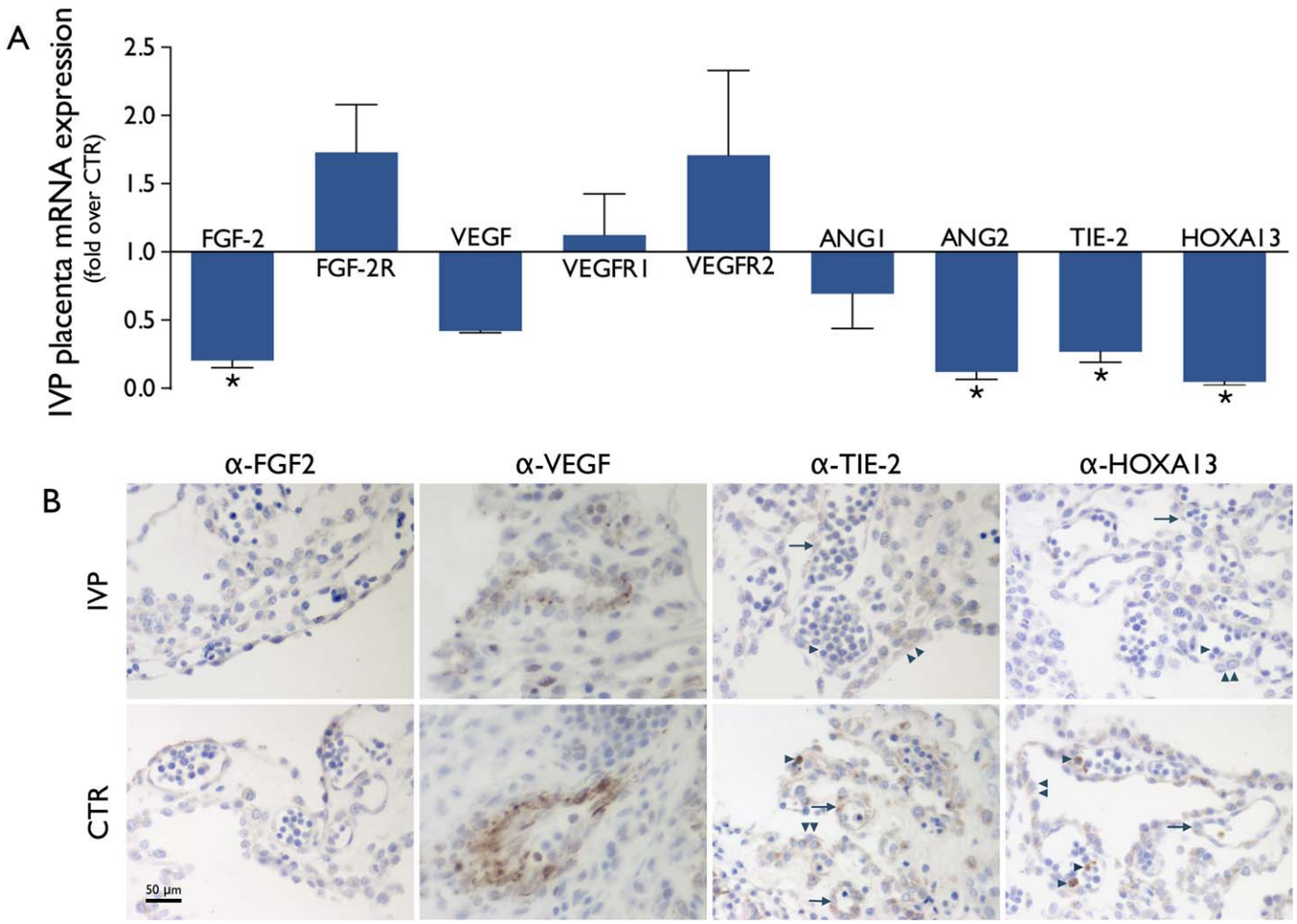

FIG. 2. Reduced expression of factors regulating vasculogenesis and angiogenesis in placentae from IVP embryos at Day 20 of development. **A**) Genes controlling the formation and maturation of placental vessels were downregulated, as evidenced by real-time PCR (*P < 0.05, IVP vs. CTR). **B**) TIE-2, VEGF, FGF2, and HOXA13 protein expression has been observed in endothelial cells (arrows), proerythroblasts (arrowheads) and allantoic cells (double arrowheads).

contents of immature placental vessels in stages 1 and 2 ($P < 0.05$) were observed at Days 20 and 22 of development in IVP conceptuses (Fig. 3). Nevertheless, an adequate maturation of placental vessels in IVP conceptuses was finally achieved on subsequent days (Fig. 3). To further investigate if any vascular defects persisted in IVP conceptuses, the placental vessel diameters were analyzed at Day 26 of development (i.e., when stage 1 of vasculogenesis was already completed; see Fig. 4A). Placental vessels from IVP conceptuses were smaller in diameter than those from CTR conceptuses (46.89 ± 14.12 vs. 89.92 ± 11.03 μm, respectively; $P < 0.05$) (Supplemental Table S1), and by extension, the area inside each vessel was also reduced in IVP conceptuses (3.27×10^3 vs. 11.66×10^3 μm^2, respectively; $P < 0.05$) (Fig. 4B and Supplemental Table S1). A significant reduction in vessel number was observed in IVP conceptuses throughout the entire studied period of vasculogenesis (Fig. 4C).

DISCUSSION

We found that placental vasculogenesis of IVP sheep embryos was severely compromised. Essentially, two time-dependent problems related to vascular development were revealed. In the first studied period (Days 20 and 22 of gestation), the maturation of placental vessels was delayed, whereas in the second studied period (Days 26 and 30 of gestation), fewer placental vessels persisted, notwithstanding the correct completion of vasculogenesis.

Vasculogenesis starts from hemangioblast differentiation in both endothelial and circulating cells [18]. The endothelial compartment must be properly developed to manage the continuous gas and molecule exchange by circulating cells between fetal and maternal tissues [19]. Here, defects in vessel integrity led to circulating cell extravasation (i.e., hemorrhage) and death of sheep IVP conceptuses.

The underdeveloped placental vascular network in IVP conceptuses, which persisted at subsequent days, may cause developmental adaptations and contribute to the fetal origin of adult disease. For example, because the heart and placental vessels develop simultaneously, hypoplasia of the myocardia of IVP embryos can have repercussions on fetal circulation, causing catabolic removal and nutrient provision to be less efficient in fetal tissues.

Vascular development depends on the expression of angiogenetic factors [16, 18]. The functional VEGF system (VEGF ligand and receptors 1 and 2) ensured correct initiation of vasculogenesis in IVP placentae. The presence of primordial vessels, as hemangioblast aggregates, demonstrates a correct primordial differentiation initiated by a proper expression of

//xinet/production/b/bire/live_jobs/bire-91-01/bire-91-01-23/layouts/bire-91-01-23.3d ■ Thursday, 12 June 2014 ■ 8:19 pm Page 4
Allen Press, Inc. ■ TOC category: 11 (Reproductive Technology)

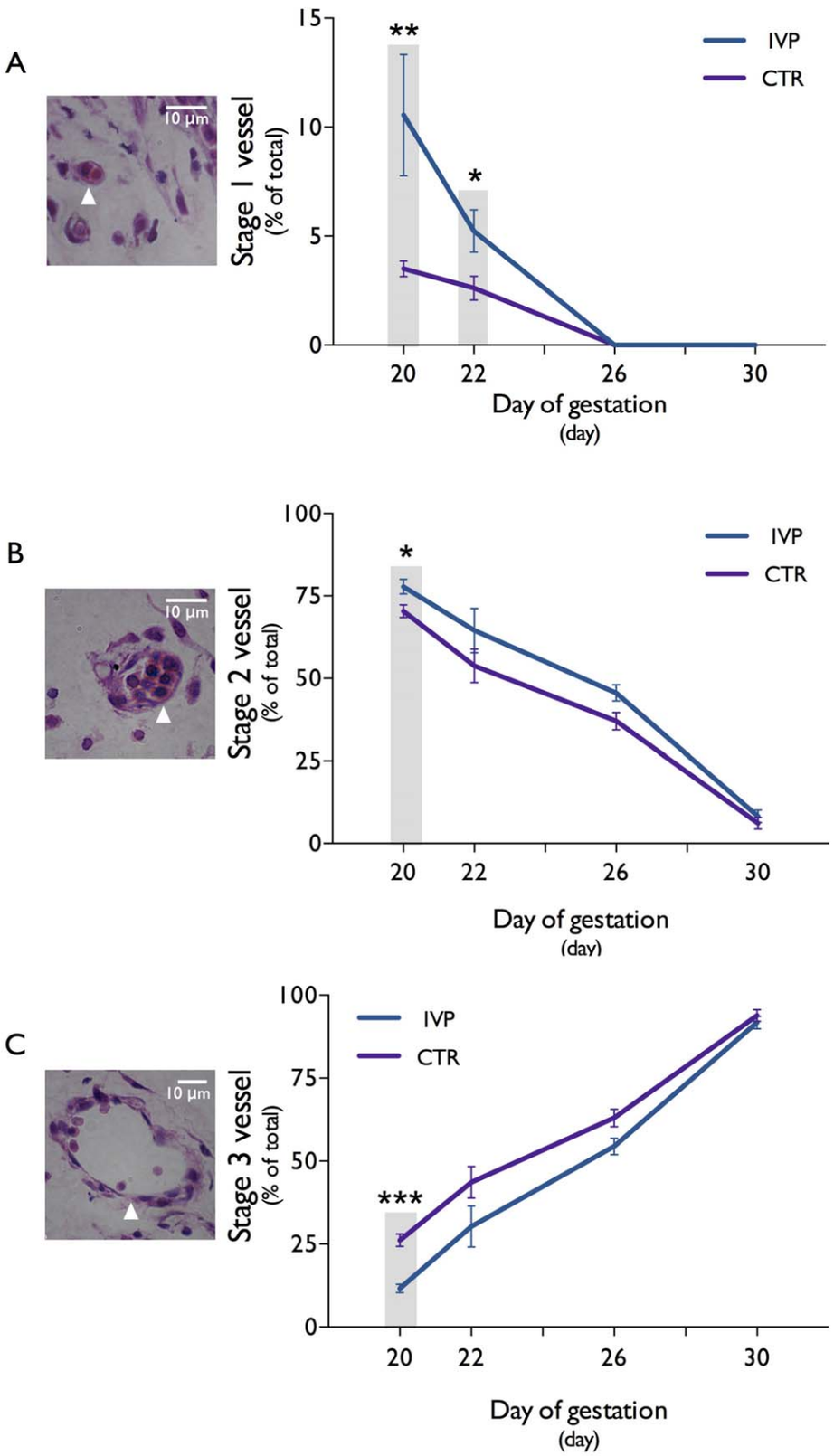

FIG. 3. Delayed maturation of vessels in early placentae from IVP embryos. **A**) Stage 1: early vasculogenesis (formation of hemangioblastic cell cords; arrowhead). **B**) Stage 2: tube formation: endothelial cells becoming flattened; additional mesenchymal cells closely apposed to the endothelial tubes; hematopoietic stem cells becoming visible in the capillary lumen (arrowhead). **C**) Stage 3: late vasculogenesis: well-formed capillaries surrounded by basal lamina and perivascular cells (arrowhead). Note a delayed vasculogenesis and a reduced number of mature vessels at the beginning of placentogenesis (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

//xinet/production/b/bire/live_jobs/bire-91-01/bire-91-01-23/layouts/bire-91-01-23.3d ■ Thursday, 12 June 2014 ■ 8:21 pm Page 5
Allen Press, Inc. ■ TOC category: 11 (Reproductive Technology)

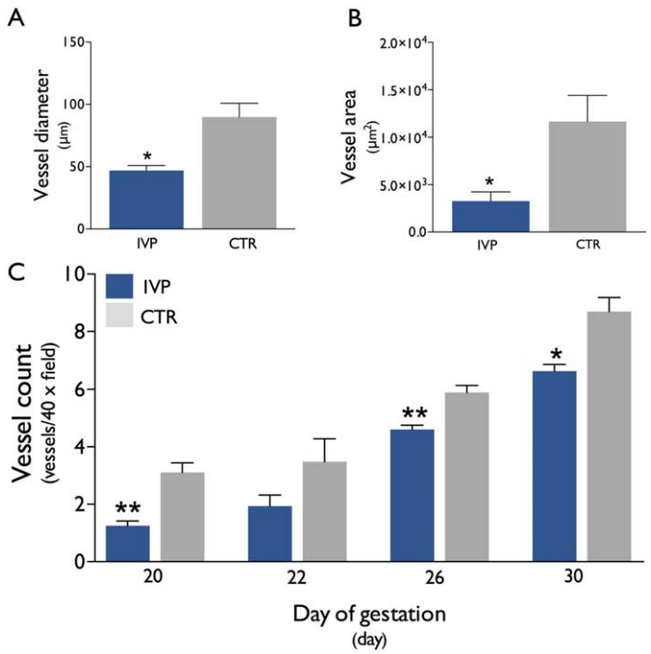

FIG. 4. Reduced vascular network in early placentae of IVP embryos. Reduced vessel diameter (**A**) and area (**B**) at the beginning of placental vasculogenesis (Day 26 of pregnancy) (*$P < 0.05$). **C**) The underdeveloped vascular network persists throughout placental vasculogenesis of IVP embryos (*$P < 0.05$, **$P < 0.005$).

VEGF, *VEGFR1*, and *VEGFR2* in IVP placentae. Both FGF2 and VEGF are needed for proliferating endothelial cells [20], but FGF2 appears to be essential for subsequent vessel remodeling [21]. So we can speculate that the reduced expression of *FGF2*, *ANG2*, and its receptor *TIE-2* are responsible for the defective remodeling/reduced vascular bed in ART-derived conceptuses, and the limited fetal growth further confirms our interpretation. Although *FGF2R* was normally expressed, the downregulation of its ligand, FGF2, is sufficient to induce impaired vessel maturation in IVP placenta. The angiogenetic factors we found downregulated in IVP fetuses are also required later in pregnancy for vessel remodeling [16]; hence, we must consider that the defective phenotype we observed in the early postimplantation phase will remain in later stages of pregnancy.

All receptors of angiogenetic factors except TIE-2 were normally expressed. This downregulation may be due to the reduced expression of its transcriptional factor *HOXA13*. HOXA13 controls vessel formation [17], with homozygous knockouts showing defective vascular pattering and expression of angiogenetic factors and heart structure, resulting in lethality of mouse embryos at the stage of embryonic development that corresponds to Day 24 in sheep [17, 22].

The aim of the present study was to compare placental vascular development in IVP and naturally conceived conceptuses. To exclude an eventual developmental delay of IVP embryos caused by the transfer procedure and ensure synchronization between development of IVP and CTR embryos, the best control should be produced by natural conception and flushing of blastocysts with their subsequent transfer to recipient ewes. However, creating such a control group in sheep (and in other mono- and biovulatory large animal models) involves serious technical difficulties. Because sheep has one or two ovulations per cycle, such experimental settings require very high numbers of animals per cycle. On the other hand, developmental delay of IVP embryos previously observed in mice in comparison to each of two controls (naturally conceived and flushed/transferred embryos) [23] confirms the validity of our control.

The placenta has a strong impact not only on fetal heart development but also on the offspring's future adulthood, because it is a programming agent for cardiovascular disease [24]. Alterations in the cardiovascular system have been observed in ART-derived individuals [25]. Their increased blood pressure could be a result of the underdeveloped vascular system found here during early pregnancy. Indeed, human IUGR impacts heart development by altering coronary, aortic, and left ventricular outflow diameters [26]. Because sheep and humans share key steps in the process of heart development [27], it is essential to investigate how ART can alter that process. Because the placental vascular system manages fetal development, further studies are needed to better understand the long-term effects of ART-induced placental vascular system alterations on offspring.

In conclusion, the application of ART in sheep embryos induced severely impaired vasculogenesis early in gestation. This may lead, in more severe circumstances, to embryo growth arrest and, in less severe ones, to fetal growth restriction throughout the pregnancy, resulting in programming susceptibility of offspring to cardiovascular diseases.

REFERENCES

1. Wang JX, Norman RJ, Wilcox AJ. Incidence of spontaneous abortion among pregnancies produced by assisted reproductive technology. Hum Reprod 2004; 19:272–277.
2. Farr SL, Schieve LA, Jamieson DJ. Pregnancy loss among pregnancies conceived through assisted reproductive technology, Unites States, 1999–2002. Am J Epidemiol 2007; 165:1380–1388.
3. Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P, Jammes H, Vaiman D. In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. PLoS ONE 2010; 5:e9218.
4. Zhang Y, Cui Y, Zhou Z, Sha J, Li Y, Liu J. Altered global expressions of human placentae subjected to assisted reproductive technology treatments. Placenta 2010; 31:251–258.
5. Steer C, Campbell S, Davies M, Mason B, Collins W. Spontaneous abortion rates after natural and assisted conception. BMJ 1989; 299: 1317–1318.
6. Pezeshki K, Feldman J, Stein DE, Lobel SM, Grazi RV. Bleeding and

TABLE 1. Collection of conceptuses collected during early placentation of IVP and CTR groups.

Day of gestation	Collected living conceptus (%)		Collected nonliving conceptus (pericardial/placental hemorrhage)		Recipient ewes	
	IVP	CTR	IVP	CTR	IVP	CTR
20	6 (27.3)	7	2 (–/–)	2 (–/–)	7	6
22	7 (26.9)	6	4 (3/1)	1 (–/–)	8	5
26	6 (28.6)	6	4 (2/2)	0	8	4
30	6 (35.3)	7	1 (–/–)	0	7	5
Total	25 (29.5)	28	11 (5/3)	3 (–/–)	30	20

//xinet/production/b/bire/live_jobs/bire-91-01/bire-91-01-23/layouts/bire-91-01-23.3d ■ Thursday, 12 June 2014 ■ 8:22 pm Page 6
Allen Press, Inc. ■ TOC category: 11 (Reproductive Technology)

spontaneous abortion after therapy for infertility. Fertil Steril 2000; 74: 504–508.
7. Sutcliffe AG, Ludwig M. Outcome of assisted reproduction. Lancet 2007; 370:351–359.
8. Barker DJ, Carl PM. Fetal undernutrition and disease in later life. Rev Reprod 1997; 2:105–112.
9. Breier BH, Vickers MH, Ikenasio BA, Chan KY, Wong WP. Fetal programming of appetite and obesity. Mol Cell Endocrinol 2001; 185: 73–79.
10. Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M, Monaco F, Loi P. Postimplantational mortality of in vitro produced embryos is associated with DNA methyltransferase 1 (DNMT1) dysfunction in placenta. Hum Reprod 2013; 28:298–305.
11. Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. Theriogenology 2008; 69:55–67.
12. Leiser R, Krebs C, Ebert B, Dantzer V. Placental corrosion cast studies: a comparison between ruminants and humans. Microsc Res Tech 1997; 38: 76–87.
13. Regnaulta TRH, Galanb HL, Parkera TA, Anthonya RV. Placental development in normal and compromised pregnancies—a review. Placenta 2002; 16:119–129.
14. Ptak G, Clinton M, Tischner M, Barboni B, Mattioli M, Loi P. Improving delivery and offspring viability of in vitro-produced and cloned sheep embryos. Biol Reprod 2002; 67:1719–1725.
15. Kaufmman MH. The Atlas of Mouse Development, rev. ed. London: Elsevier Academic Press; 1995.
16. Charnock-Jones DS, Kaufmann P, Mayhewc TM. Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation.- Placenta 2004; 25:103–113.
17. Shaut CAE, Keene DR, Sorensen LK, Li DY, Stadler HS. HOXA13 is essential for placental vascular patterning and labyrinth endothelial specification. PLoS Genetics 2008; 4:1–18.
18. Demir R, Seval Y, Huppertz B. Vasculogenesis and angiogenesis in the early human placenta. Acta Histochemica 2007; 109:257–265.
19. Desforges M, Sibley CP. Placental nutrient supply and fetal growth. Int J Dev Biol 2010; 54:377–390.
20. Goldie LC, Nix MK, Hirschi KK. Embryonic vasculogenesis and hematopoietic specification. Organogenesis 2008; 4:257–263.
21. Magnusson P, Rolny C, Jakobsson L, Wikner C, Wu Y, Hicklin DJ, Claesson-Welsh L. Deregulation of Flk-1/vascular endothelial growth factor receptor-2 in fibroblast growth factor receptor-1-deficient vascular stem cell development. J Cell Sci 2004; 117:1513–1521.
22. Stadler HS, Higgins KM, Capecchi MR. Loss of *Eph-receptor* expression correlates with loss of cell adhesion and chondrogenic capacity in *Hoxa13* mutant limbs. Development 2001; 21:4177–4188.
23. Delle Piane L, Lin W, Liu X, Donjacour A, Minasi P, Revelli A, Maltepe E, Rinaudo PF. Effect of the method of conception and embryo transfer procedure on mid-gestation placenta and fetal development in an IVF mouse model. Hum Reprod 2010; 25:2039–2046.
24. Thornburg KL, O'Tierney PF, Louey S. Review: the placenta is a programming agent for cardiovascular disease. Placenta 2010; 24:54–59.
25. Ceelen M, van Weissenbruch MM, Vermeiden JPW, van Leeuwen FE, Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. J Clin Endocrinol Metab 2008; 93:1682–1688.
26. Jiang B, Godfrey KM, Martyn CN, Gale CR. Birth weight and cardiac structure in children. Pediatrics 2006; 117:257–261.
27. Morrison JL, Botting KJ, Dyer JL, Williams SJ, Thournburg KL, McMillen IC. Restriction of placental function alters heart development in the sheep fetuses. Am J Physiol Regul Integr Comp Physiol 2007; 293: 306–313.

//xinet/production/b/bire/live_jobs/bire-91-01/bire-91-01-23/layouts/bire-91-01-23.3d ■ Thursday, 12 June 2014 ■ 8:22 pm Page 7
Allen Press, Inc. ■ TOC category: 11 (Reproductive Technology)

Queries for bire-91-01-23

1. **Author: This article has been lightly edited for grammar, style, and usage. Please compare against your original document and make changes on these pages. Please limit your corrections to substantive changes that affect meaning. If no change is required in response to a question, please write "OK as set" in the margin. Copy editor**
2. **Author: Your manuscript has been typeset to reflect its final print format. Typesetting programs may inadvertently reformat text in a way that changes the meaning, e.g., genetic nomenclature italics. Therefore, please check the nomenclature in your proof carefully to make sure there have been no inadvertent formatting errors.**
3. **Editor: Please confirm wording of summary statement: In vitro production of sheep embryos induces impaired placental vasculogenesis, which leads to fetal intrauterine growth restriction.**
4. **Author: Please clarify/confirm. In the sentence beginning "All selected oocytes...," is Nunclon the product name or the company name. If the product name, please provide the company name. Copy editor**
5. **Author: Please clarify. In the sentence beginning "Ram semen," does 5′ stand for 5 min? Copy editor**
6. **Author: Please confirm. Is the sentence beginning "Heartbeat was measured..." OK as edited for clarity and flow? Copy editor**
7. **Author: In the sentence beginning "Finally, placentae...," supplier name to cite for Paraplast? Copy editor**
8. **Author: In the sentence beginning "Sections were then...," specific antibody product designations to cite? Copy editor**
9. **Author: In the sentence beginning "Vessel diameters...," specific supplier name or URL to cite for IMAL software? Copy editor**
10. **Author: In the sentence beginning "For gene expression...," please confirm the part designations for Figure 4. The legend of Figure 4 does include a part D. Copy editor**
11. **Author: In the sentence beginning "For gene expression...," please confirm the part designations for Figure 3. The legend of Figure 3 does include a part D. Copy editor**