Peri- and Postnatal Effects of Prenatal Adenoviral VEGF Gene Therapy in Growth-Restricted Sheep 1

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ABSTRACT

Uterine artery (UtA) adenovirus (Ad) vector-mediated overexpression of vascular endothelial growth factor (VEGF) enhances uterine blood flow in normal sheep pregnancy and increases fetal growth in the overnourished adolescent sheep model of fetal growth restriction (FGR). Herein, we examined its impact on gestation length, neonatal survival, early postnatal growth and metabolism. Singleton-bearing ewes were evenly allocated to receive Ad.VEGF-A$_{165}$ (5×10^{10} particles/ml, 10 ml, n = 17) or saline (10 ml, n = 16) injected into each UtA at laparotomy (0.6 gestation). Fetal growth was serially monitored (blind) by ultrasound until delivery. Lambs were weighed and blood was sampled weekly and a glucose tolerance test performed (68-day postnatal age). Hepatic DNA/RNA was extracted at necropsy (83-day postnatal age) to examine methylation status of eight somatotropic axis genes. *IGF1* mRNA and protein expression were measured by RT-PCR and radioimmunoassay, respectively. All pregnancies remained viable following Ad.VEGF-A$_{165}$ treatment. Fetal abdominal circumference and renal volume were greater in the Ad.VEGF-A$_{165}$ group compared with the saline group at 21/28 days ($P \leq 0.04$) postinjection. At delivery, gestation length ($P = 0.07$), lamb birthweight ($P = 0.08$), umbilical girth ($P = 0.06$), and plasma glucose ($P = 0.09$) tended to be greater in Ad.VEGF-A$_{165}$-treated lambs. Levels of neonatal intervention required to ensure survival was equivalent between groups. Absolute postnatal growth rate ($P = 0.02$), insulin area under the curve ($P = 0.04$) and carcass weight at necropsy ($P = 0.04$) were increased by Ad.VEGF-A$_{165}$ treatment. There was no impact on markers of insulin sensitivity or methylation/expression of key genes involved in somatic growth. Ad.VEGF-A$_{165}$ gene therapy increased fetal growth in a sheep FGR model, and lambs continued to thrive during the neonatal and early postnatal period.

gene therapy, intrauterine growth restriction (IUGR), metabolism, sheep, vascular endothelial growth factor

INTRODUCTION

Fetal growth restriction (FGR), a condition in which the fetus fails to achieve its genetically predetermined growth potential, remains a leading cause of stillbirth and neonatal death [1].

Survivors of FGR are susceptible to a wide range of neonatal complications [2] and, thereafter, are at increased risk of cardiovascular and metabolic disease in later life [3], including insulin resistance and type II diabetes mellitus.

FGR is commonly caused by uteroplacental insufficiency, which is characterized by reduced uterine blood flow (UBF). UBF represents a potential target therapeutically. Our research group has previously demonstrated that localized adenovirus (Ad)-mediated overexpression of vascular endothelial growth factor (VEGF) in the uterine arteries (UtAs) of midgestation pregnant sheep produces a sustained increase in UBF, which appears to be mediated by increased endothelial nitric oxidase synthase expression short term, and perivascular adventitial angiogenesis long term [4, 5]. Moreover, we recently showed that maternal UtA Ad.VEGF-A$_{165}$ gene therapy significantly increased ultrasonographic fetal growth and reduced the proportion of fetuses with marked FGR in an ovine paradigm of FGR induced by overnourishment of adolescent ewes [6]. Our previous study was electively terminated in late pregnancy to allow for detailed analysis of fetal outcomes. In this new study, using similar methods, we explored the potential impact of maternal UtA Ad.VEGF-A$_{165}$ gene therapy on gestation length, perinatal survival, and wellbeing.

The overnourished adolescent sheep paradigm of FGR replicates the main features of human FGR, including a major reduction in UBF as a key underlying defect [7–9]. Overnourishment adversely impacts nutrient partitioning in the adolescent mother and promotes her own growth at the expense of the conceptus. This results in uteroplacental growth restriction of varying degrees, leading to impaired fetal nutrient supply. Approximately 50% of lambs born to overnourished mothers are defined as markedly FGR, in which birth weight is more than two standard deviations below the mean birth weight of normally grown fetuses of contemporaneous control-intake ewes [10, 11]. This model also replicates the prematurity and increased perinatal mortality rates associated with human FGR. Typically, spontaneous delivery occurs 3–5 days early in

individual studies, the amount of colostrum harvested immediately after birth is hugely attenuated, and, in severe cases, lactation and the formation of an adequate ewe-lamb bond is delayed [12–14]. In the absence of human intervention, this has profound consequences for the smallest and most premature lambs [15], which can largely be prevented by a proactive regimen of intensive neonatal care. Once released from the nutritionally constrained prenatal environment, FGR lambs exhibit rapid catch-up growth (increased fractional growth rates) with evidence of increased adiposity and reduced bone mineral density (BMD) at 11 wk of age (weaning) when assessed by dual-energy X-ray absorptiometry (DEXA) [15]. At 7 wk of age, fasting plasma glucose and insulin levels are increased, which represents a reversal of the pattern observed in late pregnancy, at which stage growth-restricted fetuses are hypoglycemic and hypoinsulinemic relative to normally growing fetuses [16–19]. Glucose-stimulated insulin secretion (given by the area under the curve [AUC] of plasma insulin over time following a standardized i.v. glucose bolus), fasting insulin-to-glucose and insulin AUC-to-glucose AUC ratios are also increased, which is indicative of relative insulin resistance [11].

The impact of FGR on postnatal growth and metabolism represents an intrauterine programming effect, which may at least partially involve epigenetic alterations of genes, leading to changes in gene expression in the absence of any modification of the DNA base sequence [20]. The most extensively studied epigenetic marker to date is DNA methylation, which occurs selectively at sites where a cytosine (C) base precedes a guanine (G), termed CpG dinucleotides. CpGs cluster in regions called CpG islands, which are frequently located within or close to the promoter regions of genes. Relative hypomethylation is associated with increased gene expression, while hypermethylation leads to reduced expression or even gene silencing. Several rodent models of FGR are characterized by changes in DNA methylation [21–23] in various genes, including insulin-like growth factor 1 (*IGF1*), which is the principal determinant of early postnatal growth [24]; however, there is a paucity of data in ovine paradigms of FGR. Arguably, the sheep is a more clinically relevant model for human pregnancy, given the potential to study singleton gestation, comparable maternal size and adiposity, maternal-to-fetal weight ratio, gestation length, birth weight, similar organogenesis for all major body systems, equivalent rates of prenatal protein accretion and fat deposition, and relative maturity at birth.

We hypothesized that prenatal maternal Ad.VEGF-A$_{165}$ gene therapy, through effects on fetal growth, would influence gestation length, increase birth weight, and mitigate the negative impact of FGR on neonatal growth and metabolism. Our aims were to: 1) determine the effects of midgestation Ad.VEGF-A$_{165}$ UtA injection in FGR sheep pregnancies on gestation length and lamb birth weight; 2) assess safety by comparing the level of neonatal care required to ensure lamb survival between treated and untreated groups; 3) examine whether prenatal maternal Ad.VEGF-A$_{165}$ treatment is able to mitigate the metabolic phenotype previously observed in early postnatal life; and 4) evaluate the potential impact on the somatotropic axis by measuring DNA methylation of key genes with relevance to postnatal growth and metabolism.

MATERIALS AND METHODS

Experimental Animals

Animal procedures were approved by the UK Home Office, under the Animals (Scientific Procedures) Act 1986 and by local ethics committee review. Ewes were housed in individual pens under natural lighting conditions at the Rowett Institute of Nutrition and Health (57°N, 2°W). Embryos (high-

quality early morulae) were derived from seven superovulated adult donor ewes (Scottish Blackface × Border Leicester) 4 days after insemination by a single sire (Dorset Horn) and synchronously transferred into the uteri of 44 adolescent recipient ewes (Dorset Horn × Mule) of similar age, weight (43.4 ± 1.99 kg), and adiposity (body condition score, 2.3 ± 0.08) in order to establish exclusively singleton pregnancies of precisely known gestational age and maximum genetic homogeneity, as described previously [25]. Following embryo transfer and throughout gestation, adolescent recipients were offered a complete diet ad libitum in order to restrict placental and fetal growth [26]. The diet provided 12 MJ metabolizable energy (ME) and 140 g crude protein/kg, and ewes consumed (on average) twice the estimated ME requirements for optimal fetoplacental growth in this genotype. Viable pregnancies were confirmed using transabdominal ultrasound in 33 of 44 ewes, giving an overall pregnancy rate of 75%.

Ultrasound Assessment

At 79 ± 0.1 days (0.5) gestation (term = 145 days) ewes underwent detailed ultrasound examination (while awake and upright) incorporating baseline measurements of fetal abdominal circumference (AC) and renal volume (RV), which we have previously shown are the strongest markers of ovine fetal growth [27, 28]. Ultrasound scans were repeated at approximately weekly intervals on four occasions between 101 ± 0.1 and 133 ± 0.3-days gestation. All examinations were performed by a single operator accredited in advanced obstetric ultrasound (DJC) blind to treatment allocation using a Logiq 400 CL machine (GE, Milwaukee, WI) and a 5.0 MHz curvilinear probe.

Gene Therapy Administration

At 88 ± 0.7-days gestation ewes underwent a midline laparotomy. Anesthesia was induced with 5 mg/kg i.v. propofol and maintained by inhaled isoflurane. Ewes were randomized (by J.M.W. and on the basis of baseline ultrasound measurements of AC and RV) to receive bilateral UtA injections of Ad.VEGF-A$_{165}$ vector (n = 17) or saline (n = 16) as a control treatment. The main trunk of each UtA was occluded digitally and injected distal to the occlusion site with 10 ml normal saline with or without 5×10^{11} particles first generation replication deficient Ads (E1, E3-deleted, titre determined at optical density at 260 nm; Ark Therapeutics Oy, Kuopio, Finland) containing the VEGF-A$_{165}$ gene, as previously described [29]. The UtA occlusion was maintained for a total of 5 min postinjection to minimize systemic vector spread.

Lambing and Neonatal Care

From 135 days gestation (the earliest point commensurate with live birth in the overnourished adolescent paradigm) ewes were supervised 24 h/day and allowed to spontaneously labor. A standardized proactive regimen of neonatal care was used in anticipation of very high rates of neonatal mortality in this FGR model (up to 62%) due to both prematurity and impaired passive immunity and/or nutrient intake secondary to inadequate colostrum supply [16]. Maternal outcomes (labor complications, postpartum hemorrhage) and the types and degree of immediate neonatal care provided were prespecified and documented by caregivers (D.J.C., R.P.A., J.S.M.) blind to the treatment group. After birth and any necessary resuscitation, lambs were dried and weighed. As soon as possible (and always within 10 min), venous blood was sampled into a heparinized tube and immediately centrifuged at $2000 \times g$ for 20 min at 4°C to facilitate plasma harvest. At the same time, heparinized whole venous blood was analyzed immediately using an OSM 3 Hemoximeter (Radiometer Medical A/S, Copenhagen, Denmark) to measure hemoglobin and hemoximetry, and the hematocrit was determined in duplicate using a tube reader after centrifugation for 3 min at 12 000 rpm (Micro Hematocrit MK-IV; Hawksley and Sons Ltd., Lancing, Sussex, U.K.). Next, measurements of umbilical girth and biparietal head diameter were made using string and a ruler, and calipers, respectively. After delivery, 10 IU i.v. oxytocin (Intervet UK, Ltd., Milton Keynes, U.K.) was administered to each ewe in order to induce milk letdown. The udder was stripped and the total volume of colostrum was determined before being fed back to the lamb by bottle or feeding tube. A small sample of colostrum was retained for quantification of immunoglobulin (Ig) G, energy, and nutrient content (see below). It is recognized that lambs require at least 50 ml/kg birth weight to acquire sufficient antibody protection [30]. Accordingly, when maternal colostrum yield fell below this minimum requirement, the difference in volume was provided to the lamb in the form of pooled donor colostrum of known IgG and nutrient content. Finally, at 1 h of age, after allowing for stabilization of physiological parameters, vital signs were recorded, including respiratory rate, heart rate, and rectal temperature. Following delivery of the placenta (comprising fetal cotyledons and membranes), its weight was measured and cotyledons were dissected, counted, and weighed. All lambs

were weighed at 4-h intervals for the first 3 days and 8-h intervals between 4 and 7 days of life. Any lambs that failed to spontaneously suckle or gain weight over an 8-h period were provided with supplementary colostrum, ewe milk, or formula milk appropriate for stage and for as long as required, and the total number and frequency of these supplementary feeds were recorded. All lambs routinely received intramuscular vitamin E and selenium supplementation and prophylactic antibiotics for a total of 5 days. To reduce the risk of infection, each lamb's navel was dipped in iodine at birth and at 12 h of age. In addition, at 24 h of age, paired maternal and fetal venous blood samples were taken, processed, and stored for quantification of plasma IgG levels.

Postnatal Investigations

Following the initially intensive period of neonatal care, serial measurements of lamb body weight and umbilical girth were taken at weekly intervals until ~12 wk of age in order to determine their absolute and fractional growth rates. Fractional growth rates (% per day) were calculated by expressing the live weight gain between birth and necropsy as a proportion of lamb birth weight and dividing by the time interval between birth and necropsy. Venous blood was sampled at weekly intervals for determination of serial plasma insulin and IGF1 concentrations. At 8 days of age, an additional venous blood sample was acquired to measure serum biochemistry (sodium, potassium, chloride, urea, and creatinine) and liver function tests (alanine transaminase [ALT], aspartate transferase [AST], gamma-glutamyl transferase [GGT], glutamate dehydrogenase [GLDH], alkaline phosphatase [ALP] and albumin). At 68 ± 0.5 days of age, all lambs underwent an i.v. glucose tolerance test, as previously described [14]. Briefly, following a 3-h fast, lambs were blood sampled at -20, -10, 0, $+5$, $+10$, $+15$, $+20$, $+25$, $+30$, $+45$, $+60$, $+90$, and $+120$ min relative to a standardized i.v. glucose bolus (0.25 g/kg), and the resultant plasma was analyzed for glucose and insulin. Fasting levels and AUC for insulin and glucose were determined and additionally used to calculate a number of established markers of insulin resistance, including the Homeostatic Model Assessment (HOMA) index, which was calculated as the product of the fasting glucose level (mass concentration in mg/dl) and the fasting insulin level, divided by the appropriate constant (405). Thereafter, at 74 ± 0.4 days postnatal age, lambs underwent a noninvasive assessment of body composition using DEXA under general anesthesia, which was induced and maintained using isoflurane in a mixture of oxygen and nitrous oxide. Lambs were imaged lying prone in a Norland XR-26 Mark II analyzer (Norland Corp., Fort Atkinson, WI). Lambs were standing and eating within 15 min of general anesthesia ceasing. Semiautomated analyses were repeated three times for accuracy (coefficient of variation, <5%).

Necropsy and Tissue Sampling

At 83 ± 0.2 days postnatal age, one further blood sample was taken for final measurements of insulin and IGF1 plus a single time point assessment of plasma leptin. Thereafter, all lambs were humanely killed by an i.v. injection of pentobarbital sodium (20 ml) and underwent postmortem examination. All major internal organs were examined macroscopically and weighed. Samples of hepatic tissue were snap frozen in isopentane chilled with liquid nitrogen and stored at $-80°C$ pending DNA and RNA extraction.

Laboratory Analyses

Plasma glucose levels were determined in lamb blood samples using a dual biochemistry analyzer (Model 2700; Yellow Springs Instruments, Yellow Springs, OH), and variation between duplicates was <5%. Plasma insulin, IGF1, and leptin levels were determined using double antibody radioimmunoassays, as previously described [31–33]. The limits of sensitivity for the insulin, IGF1, and leptin assays were 0.08, 0.04, and 0.05 ng/ml, respectively, and intra- and interassay coefficients of variation ranged between 6.0% and 9.8%. Colostrum and plasma IgG content were determined in duplicate using an ovine-specific ELISA, as described previously [27]. Colostrum samples were diluted 1:100 000, while maternal and lamb plasma was diluted 1:30 000. Intra- and interassay coefficients of variation were <10%. Colostral fat, protein, and lactose concentrations were determined in duplicate by previously described methods [34, 35].

DNA Extraction and Bisulphite Sequencing

DNA methylation was quantified in the following eight genes of interest in relation to postnatal growth and metabolism, as previously described [36]: insulin (INS), IGF1, IGF2, H19, IGF receptor 1 (IGF1R) and 2 (IGF2R), growth hormone receptor (GHR), and insulin receptor (INSR). Briefly, 25 mg of hepatic tissue from each lamb was lysed and homogenized. DNA was

extracted, quality checked, and quantified, then 400 ng from each lamb was treated overnight with sodium bisulphite, which selectively degrades unmethylated C bases into uracil (that is subsequently converted to thiamine [T]) and leaves methylated C bases intact. Percentage methylation was quantified at 50 individual CpG sites across the 8 genes by pyrosequencing, which measures the C:T ratios in the modified sample to determine the original proportions of methylated versus unmethylated template DNA.

RNA Extraction and RT-PCR

An ovine-specific probe and primer set was custom designed using Primer Express version 3.0 (Applied Biosystems, Foster City, CA) using a published mRNA sequence for IGF1, hepatic mRNA expression of which has been shown to differ by gender in this FGR model [36]. For each lamb, 30 mg hepatic tissue was lysed and homogenized. RNA was extracted using the RNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and quantified and quality checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Next, 1 ng of each sample was reverse transcribed to cDNA using Superscript VILO MasterMix (Invitrogen, Carlsbad, CA). The resultant cDNA was diluted with 480 μl RNAse-free water, combined with the required volumes of Taqman PCR Mastermix, forward primer (5′-TGTACTGTGCGCCTCTCAAG-3′), reverse primer (5′-TGGGCATGTCGGTGTGG-3′), and probe (5′[6FAM]-TCGGCCCGCTCAGTC-[TAMRA] 3′), and run on a Taqman 7900HT fast real-time PCR system (Applied Biosystems). The individual sample mRNA expression was expressed relative to the geometric mean of the sample's internal beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content, which was found to represent the most stable combination of housekeeping genes for this particular species and tissue type using a geNorm kit (Primerdesign Ltd., Southampton, U.K.). Ct values for all three genes were first converted to mass (ng mRNA) using separate standard curves. Then, to derive the relative gene expression, the quantity of IGF1 was divided by the geometric mean of ACTB and GAPDH.

Statistical Analyses

An a priori power calculation was performed based on an anticipated 20% increase in fetal size following Ad.VEGF-A$_{165}$ treatment. Assuming interanimal variability of 607 g, it was estimated that 16 animals per group would be required to detect a 20% increase in birth weight (719 g) with 90% power. Results were analyzed using the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL). After confirming normality and equality of variance using Q-Q plots and Levene test, respectively, comparisons between Ad.VEGF-A$_{165}$ and saline groups were made using Student t-test. In anticipation of gender differences during postnatal life, general linear model (GLM) was used to examine for putative effects of prenatal Ad.VEGF-A$_{165}$ treatment, gender, and their potential interaction. Wherever significant male versus female differences or interactions were detected, results are presented as four groups in the tables. GLM was further utilized to examine for differences between colostrum/milk supplemented and nonsupplemented Ad.VEGF-A$_{165}$ and saline-treated lambs. Categorical data were compared between groups using the chi-square test. Correlations were assessed using Pearson product moment test. All data are presented as mean $\pm$ SEM unless otherwise stated. Formal statistical significance was taken as $P < 0.05$ and a tendency towards a statistically significant difference was taken as $P = 0.05$–0.1.

RESULTS

There were no adverse maternal or fetal complications during surgery or postoperatively, and all ewes remained in good health throughout the study. There was one intrauterine death (at 132 days gestation) of a saline-treated fetus that had tracked extremely small on serial ultrasound examination; the resultant stillborn lamb weighed 1496 g. All remaining pregnancies continued uninterrupted and culminated in live births at 141 ± 0.2 days (range, 136 to 145 days gestation, Table 1). Two lambs (<2.5 kg), both in the saline group, required delivery by Caesarean section due to failure of cervical dilatation; all other lambs were born vaginally. One lamb in the Ad.VEGF-A$_{165}$ group was euthanized on welfare grounds at 27 h of age after sustaining multiple rib fractures, having been crushed by its mother. Complete datasets were collected on 31 surviving lambs.

TABLE 1. Pregnancy outcome data and selected physical, metabolic, and hematological parameters at the time at birth.

Parameter	Saline (n = 16)	Ad.VEGF-A$_{165}$ (n = 17)	P value
Lamb birth weight, g (range)	3433 ± 303.3 (1496–6050)	4114 ± 230.4 (2620–5540)	0.08
Gestation length, days (range)	140 ± 0.8 (132–144)	142 ± 0.4 (138–145)	0.07
Total fetal placental weight, g (range)	298 ± 20.2 (186–438)	289 ± 19.2 (164–416)	0.78
Cotyledon weight (g)	78 ± 8.7	76 ± 6.34	0.85
Cotyledon number	81 ± 4.8	89 ± 3.4	0.20
Mean cotyledon weight (g)	0.86 ± 0.066	0.96 ± 0.100	0.37
Birth weight-to-placental weight ratio	13.1 ± 0.64	14.5 ± 0.42	0.07
Male-to-female sex ratio	8:8	9:8	0.87
Birth weight adjusted to 142 days (g)	3449 ± 283.6	4083 ± 226.1	0.09
Birth weight adjusted to 145 days (g)	3634 ± 298.1	4300 ± 238.1	0.09
Biparietal head diameter (mm)	63.6 ± 1.72	67.4 ± 1.17	0.07
Umbilical girth (mm)	341 ± 15.3	376 ± 9.6	0.06
Glucose (mg/dl)	38.0 ± 4.13	51.4 ± 6.02	0.09
Hemoglobin (g/dl)	13.5 ± 0.38	13.3 ± 0.43	0.73
Hematocrit (%)	45.0 ± 1.25	44.1 ± 1.61	0.68
Methemoglobin (g/dl)	1.83 ± 0.056	1.84 ± 0.043	0.86
Carboxyhemoglobin (g/dl)	3.56 ± 0.106	3.49 ± 0.113	0.63
Venous oxygen saturation (%)	48.9 ± 3.40	47.4 ± 2.71	0.73
Venous oxygen content (mmol/L)	8.81 ± 2.700	8.4 ± 0.639	0.66
Insulin (ng/ml)	0.46 ± 0.067	0.44 ± 0.047	0.79
IGF1 (ng/ml)	0.12 ± 0.015	0.15 ± 0.017	0.29

Fetal Growth

Figure 1, A and B, shows serial ultrasound measurements of the fetal AC and RV, respectively, from 76 ± 0.1 until 133 ± 0.3 days gestation. Compared to equivalent presurgery baseline values, measurements of both parameters increased to a greater degree in Ad.VEGF-A$_{165}$ relative to saline groups, and were larger at 108 ± 0.1 days gestation (AC, 221 ± 2.7 vs. 211 ± 3.5 mm, $P = 0.02$; RV, 5.14 ± 0.193 vs. 4.37 ± 0.165 cm^3, $P < 0.01$) and 116 ± 0.1 days gestation (AC, 239 ± 2.6 vs. 226 ± 3.9 mm, $P = 0.02$; RV, 6.4 ± 0.22 vs. 5.6 ± 0.28 cm^3, $P = 0.04$), corresponding to 21 and 28 days postinjection, respectively. There were no significant differences in AC between groups at 133 ± 0.3 days gestation, when the measurements were more variable; however, there remained a trend towards greater RV values in the Ad.VEGF-A$_{165}$ versus the saline groups at this final time point (8.6 ± 0.42 vs. 7.5 ± 0.38 cm^3, $P = 0.06$).

Pregnancy Outcome

Lamb birth weight, gestational age at delivery, and placental data are presented in Table 1 and Figure 2. Both lamb birth weight and gestation length tended to be greater in Ad.VEGF-A$_{165}$ versus saline groups ($P = 0.08–0.09$). To assess whether this apparent shift in birth weight was solely attributable to the mean increase in gestation of 2 days, individual lamb birth weights were adjusted to term for this genotype (145 days) and to the mean gestational age at delivery (142 days) using the following formula, as previously described [7]: adjusted birth weight = birth weight × 1.01305 per day of gestation. The statistical trend towards a difference in lamb birth weight between the groups remained following correction for gestation length using either approach. Notably, there were no very small lambs (<2.5 kg) born following Ad.VEGF-A$_{165}$ treatment (0 of 17), compared with 4 of 16 (25%) after saline injection ($P < 0.05$). The gender distribution was even within groups and for the study overall (17 males and 16 females in total). There were no significant gender effects on any parameters during fetal life or at birth. The birth weight-to-placental weight ratio tended to be increased in the Ad.VEGF-A$_{165}$ group compared with the saline group ($P = 0.08$), indicating enhanced placental efficiency, and there was also a trend toward higher plasma glucose concentrations shortly after birth ($P = 0.09$), suggestive of increased placental nutrient transfer. Measurements of abdominal girth and biparietal diameter also tended to be higher for Ad.VEGF-A$_{165}$-treated lambs ($P = 0.06–0.07$). There were no significant group differences in plasma IGF1 or insulin levels at birth; however, plasma insulin demonstrated a moderate correlation with lamb birth weight in the saline group ($r^2 = 0.69$, n = 15, $P < 0.01$), but not in the Ad.VEGF-A$_{165}$ group ($r^2 < 0.01$, n = 17, $P = 0.98$). By contrast, plasma IGF1 levels were positively correlated with birth weight and total placental weight, irrespective of treatment group ($r^2 = 0.47$ and $r^2 = 0.38$, respectively; n = 38, $P < 0.01$ each). There were no significant differences in quantity or quality of colostrum (in terms of individual nutrient or IgG concentration/content) between the two groups (Table 2). No differences in the length of labor or maternal blood loss at delivery were observed between groups (data not shown).

Neonatal Care

There were no statistically significant differences between the Ad.VEGF-A$_{165}$ group and saline group in the level of neonatal care needed to ensure survival, including the number of lambs requiring the following interventions: use of a heating lamp (1 vs. 7); supplementary feeding beyond initial feed at birth (10 vs. 7); use of donor colostrum (4 vs. 5); oxygen (0 vs. 1); steroids (0 vs. 1); respiratory stimulants (0 vs. 1); or additional antibiotics (0 vs. 1). Among lambs requiring supplementary feeding, there were no differences between the Ad.VEGF-A$_{165}$ group and saline group in the number of additional feeds required (6 ± 3.0 vs. 13 ± 5.4, $P = 0.27$) or total duration of feeding (64 ± 24.0 vs. 70 ± 24.2 h, $P = 0.86$). Lambs requiring feeding for longer than 48 h (n = 7) were of lower birth weight relative to other lambs in the study (2931 ± 467.3 vs. 4114 ± 173.9 g, $P < 0.01$), indicating that small size at birth was a strong predictor of the need for prolonged supplementary feeding. The final supplementary feed was given at 7 days of age to the smallest surviving lamb in the study, which was born to a saline-treated ewe, and weighed only 1720 g at birth. Postnatal growth trajectory was unaffected by supplementation; thus, there were no differences in overall weight gain between birth and necropsy in supplemented and nonsupplemented lambs within the Ad.VEGF-A$_{165}$ group (28.5 ± 1.40 vs. 31.2 ± 1.06 kg, $P = 0.36$) or the saline group (32.2 ± 0.90 vs. 34.4 ± 1.14 kg, $P = 0.55$). Irrespective of treatment group, by 24 h, there were no
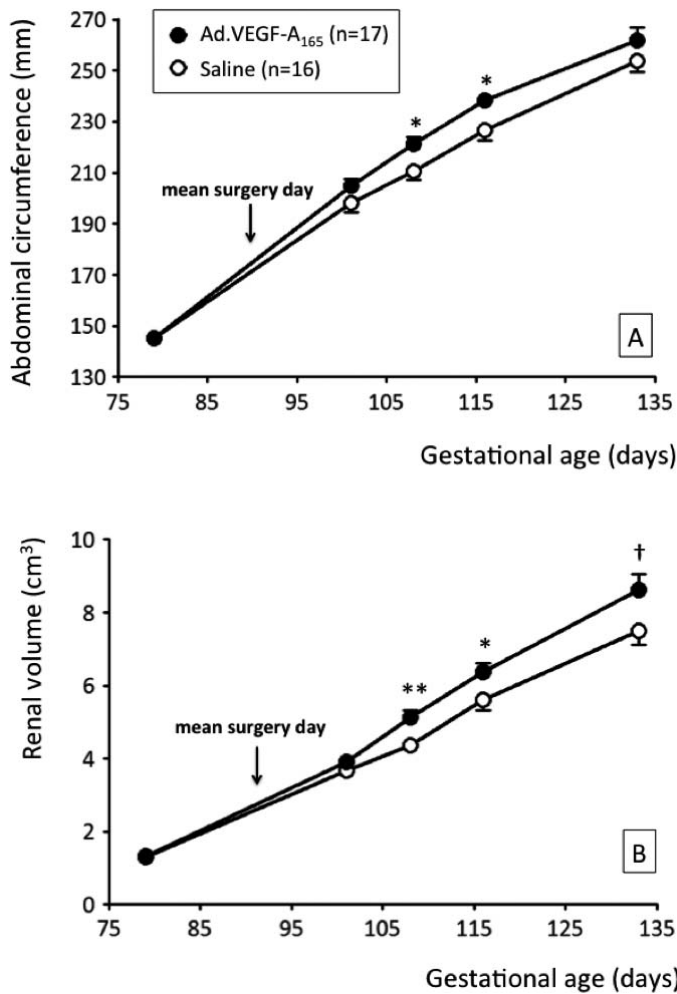
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FIG. 1. Ultrasound assessment of fetal growth. Serial ultrasound measurements of fetal AC (**A**) and renal volume (RV; **B**) at regular intervals between 76 ± 0.1 and 133 ± 0.3 days gestation in singleton-bearing adolescent ewes overnourished to generate FGR that received bilateral UtA injections of Ad.VEGF-A$_{165}$ (closed circles, n = 17) or saline (open circles, n = 16) at 88 $\pm$ 0.7 days gestation (indicated by arrow). Symbols denote gestational time points at which there were statistically significant differences or trends between groups: $^{\dagger}P = 0.05-0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$.
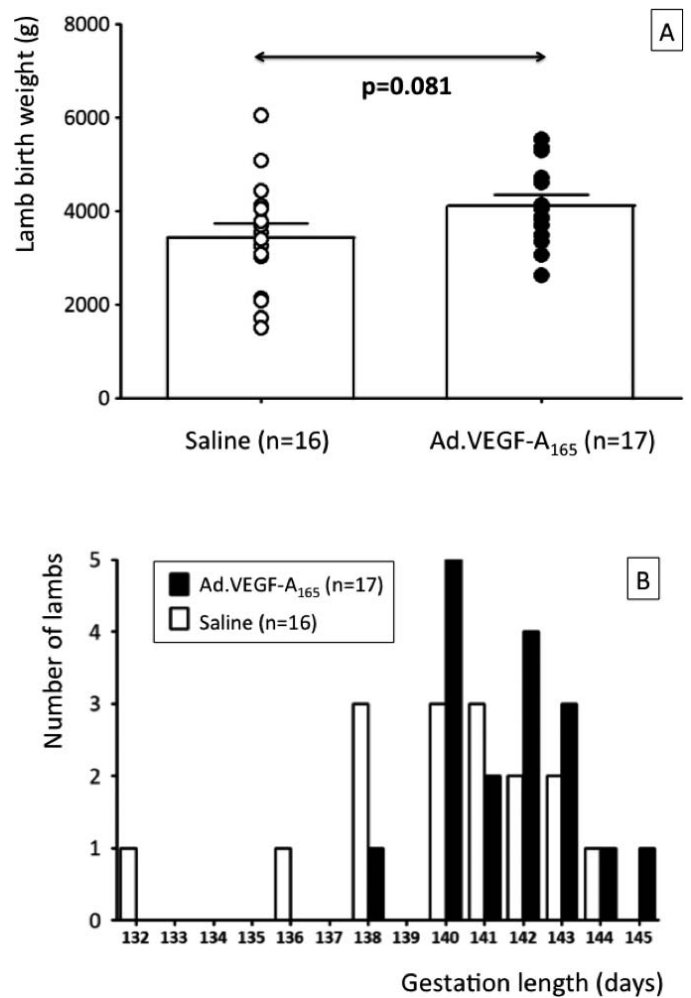

FIG. 2. Lamb birth weight and gestation length. **A**) Dot plot illustrating individual birth weights following spontaneous delivery at 141 ± 0.2 days gestation in singleton-bearing adolescent ewes overnourished during pregnancy to generate prenatal growth restriction and receiving bilateral UtA injections of Ad.VEGF-A$_{165}$ (closed circles, n = 17) or saline (open circles, n = 16) at 88 $\pm$ 0.7-days gestation. **B**) Histogram showing number of lambs delivering per day between 132 and 145 days gestation. The single (saline-treated) lamb that delivered at 132 days gestation was stillborn. The remaining 32 lambs were live born.

differences in plasma IgG levels between supplemented and nonsupplemented lambs (24.3 ± 3.13 vs. 21.3 ± 12.25 mg/ml, $P = 0.45$), indicating that the neonatal care regimen was effective in conferring passive immunity to lambs with low colostrum availability at birth. There were also no differences between the Ad.VEGF-A$_{165}$ group and saline group in any vital signs at 1 h of age, specifically, heart rate (246 ± 6.2 vs. 232 ± 13.3 beats min^{-1}, $P = 0.34$), respiratory rate (72 ± 2.7 vs. 71 ± 3.1 beats min^{-1}, $P = 0.96$), and temperature (39.9 ± 0.14 vs. 39.8 ± 0.20°C, $P = 0.76$). At 8 days of age, there were no significant abnormalities or differences between the Ad.VEGF-A$_{165}$ group and the saline group in serum concentrations of sodium (149.2 ± 0.46 vs. 149.2 ± 0.34 mmol/L, $P = 0.98$), potassium (6.0 ± 0.13 vs. 6.2 ± 0.12 mmol/L, $P = 0.34$), chloride (105.6 ± 0.50 vs. 106.1 ± 0.42 mmol/L, $P = 0.51$), urea (7.9 ± 0.56 vs. 7.4 ± 0.66 mmol/L, $P = 0.62$), creatinine (50.3 ± 1.08 vs. 50.9 ± 1.29 μmol/L, $P = 0.71$), ALT ($4.2 \pm .0.34$ vs. 3.9 ± 0.25 IU/L, $P = 0.56$), AST (53.3 ± 3.34 vs. 51.6 ± 1.10 IU/L, $P = 0.64$), GGT (306 ± 30.2 vs. 289 ± 34.9 IU/L, $P = 0.72$), GLDH (15.0 ± 3.09 vs. 27.4 ± 12.40 IU/L, $P = 0.35$), ALP

(1011 ± 86.1 vs. 1034 ± 81.4 IU/L, $P = 0.85$), or albumin (27.7 ± 0.25 vs. 27.6 ± 0.45 g/L, $P = 0.85$).

Postnatal Growth Rates

Figure 3 shows serial measurements of lamb body weight and umbilical girth, as well as plasma levels of insulin and IGF1 from birth until necropsy at 83 ± 0.2 days of age. There was a trend toward increased body weight in the Ad.VEGF-A$_{165}$ group relative to the saline group between 14 and 35 days of age ($P = 0.07$ each) and, subsequently, live weight was significantly increased in the Ad.VEGF-A$_{165}$ group versus the saline group from 42 to 82 days postnatal age ($P = 0.02-0.04$). Consequently, absolute postnatal growth rate from birth until necropsy was increased in the Ad.VEGF-A$_{165}$ group versus the saline group (397 ± 8.7 vs. 362 ± 11.7 g/day, $P = 0.02$). In contrast, fractional growth rate did not differ (10.0 ± 0.61 vs. 10.7 ± 0.76%/day, $P = 0.48$). There were no differences in absolute or fractional growth rate between male and female lambs at any stage ($P > 0.1$). Measurements of umbilical girth

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TABLE 2. Colostrum nutrient and IgG content immediately after birth and IgG concentrations in paired maternal and lamb plasma samples at 24 h of age.

Parameter	Saline (n = 16)	Ad.VEGF-A$_{165}$ (n = 17)	P value
Colostrum yield (ml)	266 ± 39.1	314 ± 48.5	0.45
Colostrum butterfat concentration (g/100 g)	7.74 ± 1.274	7.43 ± 0.958	0.85
Total colostrum butterfat content (g)	23.0 ± 4.42	23.0 ± 3.74	1.00
Colostrum lactose concentration (g/100 g)	1.54 ± 0.15	1.72 ± 0.12	0.40
Total colostrum lactose content (g)	5.03 ± 0.92	4.46 ± 0.79	0.63
Colostrum crude protein concentration (g/100 g)	18.5 ± 1.11	19.3 ± 1.06	0.57
Total colostrum crude protein content (g)	46.4 ± 6.75	56.6 ± 8.17	0.35
Total colostrum energy content (kJ)	662 ± 95.9	769 ± 117.5	0.50
Colostrum IgG concentration (mg/ml)	50.8 ± 3.48	56.8 ± 5.74	0.40
Total colostrum IgG content (g)	12.7 ± 1.84	15.9 ± 2.20	0.28
Lamb plasma IgG (mg/ml) at 24 h	22.9 ± 1.85	23.1 ± 4.06	0.98
Maternal plasma IgG (mg/ml) at 24 h	12.3 ± 0.79	12.0 ± 1.81	0.88

were greater in the Ad.VEGF-A$_{165}$ group compared with the saline group at 56, 77, and 82 days ($P = 0.02$–0.03) and tended to be greater at 7, 14, 42, 63, and 70 days postnatal age ($P = 0.06$–0.1). Plasma insulin concentrations increased approximately five-fold by the end of the first week of life, at which point levels tended to be higher in the Ad.VEGF-A$_{165}$ group compared with the saline group (2.99 ± 0.30 vs. 2.30 ± 0.26

ng/ml, $P < 0.1$). Insulin concentrations subsequently stabilized over the next 11 wk and did not significantly differ by treatment group or by lamb gender at any point. By contrast, plasma IGF1 levels tended to be higher in male versus female lambs at 7 days of age ($P = 0.06$), and thereafter were significantly greater at all time points, except one (Day 21), from 14 days onwards. However, there were no statistically
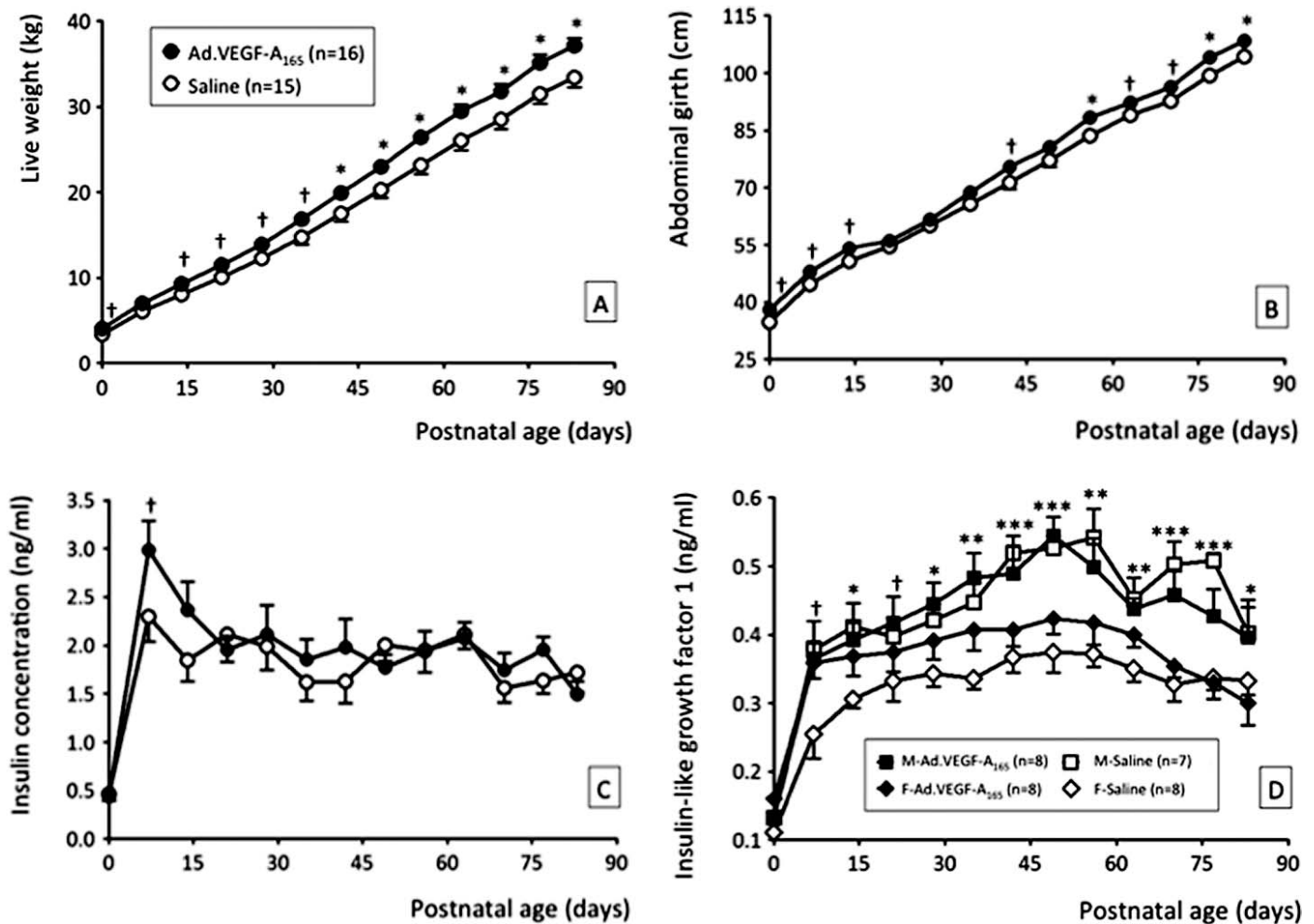
FIG. 3. Postnatal growth rates (weight and girth) and serial measurements of plasma insulin and IGF1. Serial measurements of live weight (**A**), umbilical girth (**B**), and plasma insulin (**C**) and IGF1 (**D**) levels measured by radioimmunoassay, at weekly intervals from birth until 83 ± 0.2 days of age in 31 surviving prenatally growth-restricted male (M) and female (F) lambs whose overnourished adolescent mothers received bilateral UtA injections of Ad.VEGF-A$_{165}$ (closed circles, n = 16) or saline (open circles, n = 15) at 88 ± 0.7-days gestation. Symbols denote time points at which there were statistically significant differences or trends toward differences between groups: [†]$P = 0.05$-0.1; *$P < 0.05$.

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FIG. 4. Intravenous glucose tolerance test. Serial measurements of plasma glucose (**A**) and insulin (**B**) at regular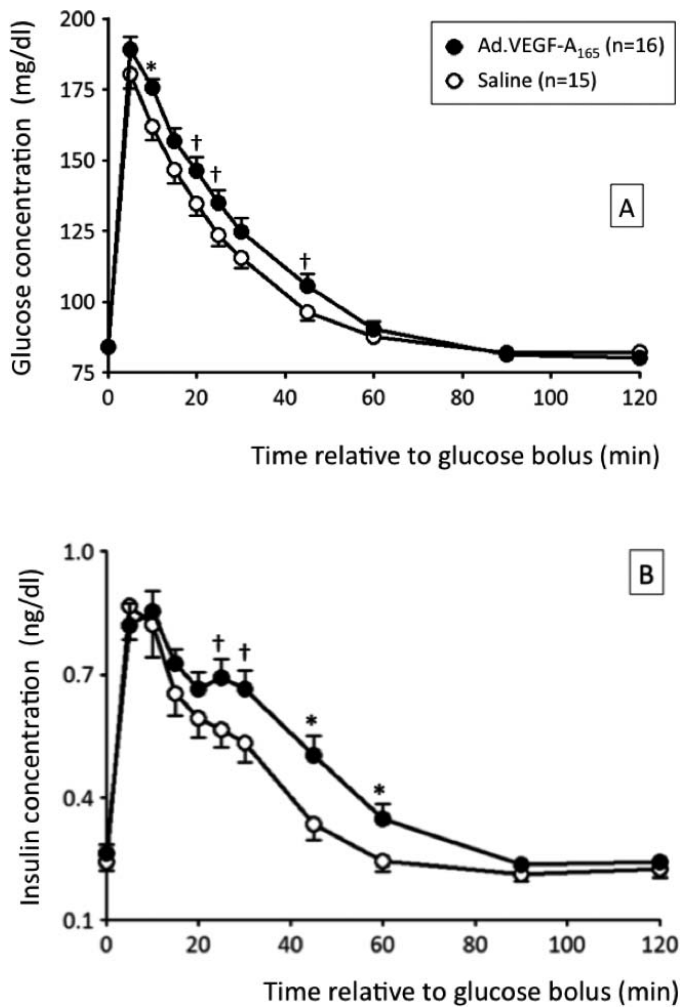 intervals up to 120 min following a standardized i.v. glucose infusion (0.25 mg/kg) administered at 68 ± 0.5 days postnatal age in 31 surviving prenatally growth-restricted lambs whose overnourished adolescent mothers received bilateral UtA injections of Ad.VEGF-A$_{165}$ (closed circles, n = 16) or saline (open circles, n = 15) at 88 ± 0.7 days gestation. Symbols denote time points with statistically significant differences or trends toward a difference between the groups: $^{\dagger}P = 0.05$–0.1; *$P < 0.05$.

significant differences between the Ad.VEGF-A$_{165}$ group and the saline group at any stage.

Glucose Tolerance Test

Figure 4 depicts the glucose and insulin responses to an i.v. 0.25 mg/kg glucose bolus administered at 68 ± 0.5 days postnatal age. Fasting (baseline) levels were taken as the arithmetic mean of three samples drawn at −20, −10, and 0 min relative to the glucose load. There were no significant gender differences in either metabolite at any stage of the test. Baseline glucose and insulin levels did not differ between the Ad.VEGF-A$_{165}$ group and the saline group (84.0 ± 2.01 vs. 84.2 ± 1.27 mg/dl, $P = 0.92$, and 1.42 ± 0.120 vs. 1.33 ± 0.108 ng/ml, $P = 0.6$, respectively), and there were no differences in any of the following markers of insulin resistance: fasting insulin-to-glucose ratio (0.40 ± 0.025 vs. 0.37 ± 0.030, $P = 0.45$); fasting glucose-to-insulin ratio (2.63 ± 0.159 vs. 2.96 ± 0.243, $P = 0.27$); insulin AUC-to-glucose AUC ratio (1.08 ± 0.137 vs. 1.04 ± 0.190, $P = 0.86$); glucose AUC-to-insulin AUC ratio (1.09 ± 0.106 vs. 1.48

± 0.288, $P = 0.21$); and HOMA index (130 ± 15.4 vs. 118 ± 10.2, $P = 0.51$). Following the glucose bolus, insulin AUC was greater in the Ad.VEGF-A$_{165}$ group relative to the saline group (112 ± 10.5 vs. 82 ± 12.1 ng/min/ml, $P = 0.04$). By contrast, despite differences at various individual time points (see Fig. 4A), glucose AUC did not differ between the Ad.VEGF-A$_{165}$ group and the saline group (2645 ± 246.8 vs. 2051 ± 241.3 mg/min/dl, $P = 0.43$).

Lamb Body Composition

At assessment by DEXA, there were no differences between the Ad.VEGF-A$_{165}$ group and the saline group in fat mass (3.02 ± 0.339 vs. 2.43 ± 0.350 kg, $P = 0.22$) or percentage body fat (9.3 ± 1.01 vs. 8.0 ± 1.14%, $P = 0.38$). By contrast, the following parameters reflecting musculoskeletal mass tended to be increased in the Ad.VEGF-A$_{165}$ group compared with the saline group: BMD (0.51 ± 0.010 vs. 0.48 ± 0.010 g/cm, $P = 0.06$); bone mineral content (BMC; 857 ± 27.2 vs. 783 ± 27.8 g, $P = 0.07$); and lean tissue mass (28.7 ± 0.77 vs. 26.3 ± 0.94 kg, $P = 0.07$).

Table 3 details brain and major visceral organ weights (from necropsy at 12 wk of age) in absolute and relative (per kg carcass weight) terms. In keeping with their significantly greater body weight, Ad.VEGF-A$_{165}$ lambs demonstrated significantly increased absolute liver weights, but these differences were no longer significant when expressed per kg carcass weight, indicating a proportionate shift in hepatic growth. Absolute perirenal fat weight was significantly greater in the Ad.VEGF-A$_{165}$ group versus the saline group and tended to remain higher when expressed per kg carcass. Moreover, there was a significant gender × treatment interaction, with the highest values being observed in the female lambs that had received prenatal Ad.VEGF-A$_{165}$ treatment. Independent of gender, absolute adrenal weight tended to be less, and relative adrenal weight was significantly lower in the Ad.VEGF-A$_{165}$ group versus the saline group. In the latter group, relative adrenal weight was inversely correlated with gestation length ($r^2 = -0.43$, n = 15, $P = 0.02$). This relationship was not seen in the Ad.VEGF-A$_{165}$ group ($r^2 = -0.06$, n = 16, $P = 0.38$). There were no other significant differences in any lamb organ weights, but the residual carcass (reflecting the muscle and skeletal mass) was heavier in the Ad.VEGF-A$_{165}$ group.

Measurements of plasma leptin and IGF1 prior to necropsy did not differ between the Ad.VEGF-A$_{165}$ group and the saline group, but, compared with females, male lambs demonstrated higher IGF1 (0.40 ± 0.03 vs. 0.31 ± 0.02 ng/ml, $P = 0.04$) and lower leptin concentrations (2.51 ± 0.36 vs. 4.48 ± 0.56 ng/ml $P = 0.02$). There were no treatment or gender differences in insulin levels at necropsy ($P > 0.05$).

DNA Methylation of Somatotropic Axis Genes

Table 4 details the mean DNA methylation across multiple individual CpG sites in eight genes of interest in relation to postnatal growth and metabolism. There were no significant differences between the Ad.VEGF-A$_{165}$ group and the saline group in these averages, or at any individual CpG dinucleotides (data not shown). DNA methylation of *INS* was greater in males versus females (88.7 ± 0.23 vs. 87.0 ± 0.50%, $P < 0.01$), while methylation of the *IGF1* gene tended to be lower (84.3 ± 0.16 vs. 84.8 ± 0.22%, $P = 0.05$). Irrespective of treatment group, *INS* methylation correlated negatively with (fasting) baseline insulin levels measured prior to glucose challenge ($r^2 = -0.186$, n = 31, $P = 0.02$).

TABLE 3.   Necropsy parameters at 83 days of age.

Parameter	F-saline (n = 8)	M-saline (n = 7)	F-Ad.VEGF A$_{165}$ (n = 8)	M-Ad.VEGF A$_{165}$ (n = 8)	P values[a]		
					Treatment	Gender	Interaction
Body weight (kg)	31.9 ± 1.43	35.3 ± 1.76	36.9 ± 1.10	37.4 ± 1.53	**0.02**	0.20	0.32
Carcass weight (kg)	19.3 ± 1.04	20.5 ± 1.05	25.7 ± 0.91	25.4 ± 1.00	**0.04**	0.87	0.33
Brain weight (g)	69.6 ± 1.11	71.2 ± 2.95	72.5 ± 0.91	72.5 ± 2.17	0.29	0.69	0.71
Relative brain weight (g/kg carcass weight)	3.66 ± 0.23	3.49 ± 0.08	3.43 ± 0.14	3.37 ± 0.15	0.11	0.81	0.39
Liver weight (g)	560 ± 24.4	630 ± 24.7	651 ± 20.2	659 ± 22.9	**0.02**	0.10	0.19
Relative liver weight (g/kg carcass weight)	29.2 ± 1.22	30.9 ± 0.77	29.2 ± 0.79	30.8 ± 1.61	0.97	0.15	0.97
Weight of adrenals (g)	1.78 ± 0.050	1.84 ± 0.051	1.61 ± 0.069	1.76 ± 0.085	0.08	0.14	0.57
Relative adrenal weight (g/kg carcass weight)	0.094 ± 0.006	0.091 ± 0.005	0.072 ± 0.002	0.083 ± 0.005	**<0.01**	0.37	0.13
Perirenal fat weight (g)	308 ± 36.4	226 ± 33.7	498 ± 53.7	233 ± 26.1	**0.02**	**<0.01**	**0.03**
Relative perirenal fat weight (g/kg carcass weight)	15.8 ± 1.30	10.9 ± 1.42	21.8 ± 1.71	10.7 ± 1.01	0.06	**<0.01**	**0.04**
Weight of kidneys (g)	106 ± 5.2	124 ± 7.3	124 ± 5.4	122 ± 6.1	0.25	0.20	0.12
Relative kidney weight (g/kg carcass weight)	5.53 ± 0.27	6.06 ± 0.17	5.54 ± 0.12	5.67 ± 0.33	0.43	0.17	0.39
Pancreas weight (g)	33.8 ± 2.34	38.9 ± 2.26	39.3 ± 1.13	39.5 ± 2.06	0.16	0.19	0.23
Relative pancreas weight (g/kg carcass weight)	1.75 ± 0.09	1.90 ± 0.06	1.78 ± 0.10	1.87 ± 0.05	0.97	0.14	0.72
Spleen weight (g)	135 ± 11.8	155 ± 20.2	149 ± 12.2	149 ± 6.7	0.81	0.42	0.46
Relative spleen weight (g/kg carcass weight)	7.06 ± 0.74	7.48 ± 0.72	6.56 ± 0.35	6.68 ± 0.26	0.24	0.63	0.79
Plasma IGF1 (ng/ml)	0.32 ± 0.02	0.39 ± 0.04	0.29 ± 0.01	0.39 ± 0.05	0.63	**0.04**	0.74
Plasma insulin (ng/ml)	1.66 ± 0.34	1.79 ± 0.20	1.31 ± 0.09	1.69 ± 0.17	0.34	0.29	0.59
Plasma leptin (ng/ml)	4.07 ± 0.92	2.43 ± 0.59	4.91 ± 0.99	2.62 ± 0.74	0.52	**0.02**	0.68

[a] P values below the threshold for formal significance (P < 0.05) are indicated in bold.

Table 5 details the mRNA expression data for *IGF1*, expressed relative to the geometric mean of *GAPDH* and *ACTB*. There was no significant effect of prenatal Ad.VEGF-A$_{165}$ gene therapy; however, irrespective of treatment group, *IGF1* was greater in males versus females (1.10 ± 0.01 vs. 1.06 ± 0.01, $P < 0.05$). This difference is in the opposite direction to the DNA methylation data (as expected) and in agreement with the higher plasma IGF1 levels in male versus female lambs in serial blood samples taken between 14 and 82 days postnatal age.

DISCUSSION

In the present study, which used a balanced design whereby groups were equivalent in terms of key ultrasound parameters of size prior to surgery, we observed a significant increase in ultrasonographic fetal growth velocity (measured by a single operator blinded to treatment group) in the growth-restricted fetuses of overnourished adolescent ewes receiving bilateral injections of Ad.VEGF-A$_{165}$ into the UtAs at midgestation compared with injections of an inactive substance (saline). No

serious adverse effects were encountered following gene therapy administration in the limited number of observations made. All Ad.VEGF-A$_{165}$-treated pregnancies continued until term and followed an uncomplicated course. After spontaneous delivery near to term, lamb birth weight and abdominal girth (which directly reflects the ultrasonographic AC measurement) tended to be higher in the Ad.VEGF-A$_{165}$ group compared with the saline group, in agreement with the late-gestation ultrasound findings. Furthermore, the mean increase in birth weight in the Ad.VEGF-A$_{165}$ group versus the saline group was 681 g (~20%) in keeping with the 18% increase in ultrasonographic measurements of fetal size and anticipated size of effect used for the a priori power calculation (719 g; 20%). Physical measurements at the time of birth, including biparietal head diameter, also tended to be greater in the Ad.VEGF-A$_{165}$ group versus the saline group, suggesting a proportionate increase in brain and somatic growth.

Gestation length tended to be increased in the Ad.VEGF-A$_{165}$ group compared with the saline group. Importantly, the tendency towards higher birth weight in the Ad.VEGF-A$_{165}$ group was not solely explained by this modest prolongation of pregnancy,

TABLE 4.   Percentage DNA methylation of eight somatotropic axis genes in lamb hepatic tissue at 83 days postnatal age.

Gene[a]	F-saline (n = 8)	M-saline (n = 7)	F-Ad.VEGF-A$_{165}$ (n = 8)	M-Ad.VEGF-A$_{165}$ (n = 8)	P values[b]		
					Treatment	Gender	Interaction
INS	87.3 ± 0.84	88.9 ± 0.26	86.8 ± 0.65	88.5 ± 0.36	0.53	**<0.01**	0.87
IGF1	84.8 ± 0.51	84.5 ± 0.30	84.8 ± 0.14	84.1 ± 0.15	0.44	**0.05**	0.64
IGF2	22.0 ± 1.05	21.2 ± 0.67	21.5 ± 0.65	20.7 ± 0.56	0.41	0.37	0.84
H19	40.2 ± 1.41	40.2 ± 0.48	40.1 ± 0.48	39.4 ± 0.67	0.53	0.86	0.66
GHR	2.53 ± 0.42	2.45 ± 0.29	2.28 ± 0.21	3.41 ± 0.81	0.45	0.27	0.21
IGF1R	43.4 ± 2.30	42.0 ± 1.38	40.5 ± 0.96	42.4 ± 0.94	0.20	0.54	0.13
IGF2R	47.4 ± 0.47	47.7 ± 0.78	48.8 ± 1.16	48.8 ± 1.16	0.69	0.53	0.87
INSR	79.6 ± 1.28	79.2 ± 0.61	79.0 ± 0.87	78.6 ± 0.43	0.23	0.28	0.57

[a] *IGF(R)* = IGF (receptor); *INS* = insulin; *INSR* = insulin receptor; *GHR* = growth hormone receptor.
[b] P values below the threshold for formal significance (P < 0.05) are indicated in bold.

TABLE 5. Relative mRNA expression of *IGF1* in lamb hepatic tissue at 83 days of age.

Gene[a]	F-saline (n = 8)	M-saline (n = 7)	F-Ad.VEGF-A$_{165}$ (n = 8)	M-Ad.VEGF-A$_{165}$ (n = 8)	P values[b]		
					Treatment	Gender	Interaction
IGF1[c]	1.06 ± 0.024	1.08 ± 0.026	1.05 ± 0.018	1.10 ± 0.025	0.87	**<0.05**	0.66
ACTB	20.7 ± 0.43	21.2 ± 0.68	22.3 ± 0.71	20.2 ± 0.60	0.37	0.85	0.17
GAPDH	20.7 ± 0.66	22.6 ± 0.92	22.9 ± 0.80	22.0 ± 0.74	0.76	0.20	0.22

[a] *ACTB* = beta-actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *IGF(R)* = IGF (receptor).
[b] P values below the threshold for formal significance ($P < 0.05$) are indicated in bold.
[c] Expressed relative to geometric mean of *ACTB* + *GAPDH*.

as the significance levels for the birth weight differences did not change substantially after mathematical correction for gestation length ($P = 0.08$ each). Although the absolute difference in gestation length was only 2 days, this is on a background of 5 days prematurity for the genotype in this model of FGR, represented by the saline group (mean gestational age at delivery, 140 days), which is precisely known in view of timed conception. The proportion of lambs delivering prior to 140 days gestation appeared to be reduced in the Ad.VEGF-A$_{165}$ group relative to the saline group (five versus one), suggesting a targeted effect in the most severely affected pregnancies. This is in agreement with our previous observation that Ad.VEGF-A$_{165}$ reduced the proportion of markedly FGR pregnancies, implying selective benefits in compromised pregnancies and limited effects on the 50% of fetuses that are growing normally despite maternal overnutrition [6]. Regardless, as sheep tolerate prematurity poorly, even small changes in gestation length can have relatively large effects on morbidity and mortality rates during lambing, and a 2-day difference would be considered clinically significant from a veterinary perspective. If this were to be directly extrapolated to human pregnancy, an extension of ~4 days may seem less relevant for healthy term pregnancies, but, in cases of FGR near to the threshold of viability, this might well confer some clinical benefit on neonatal outcome.

Interestingly, at necropsy there was a reduction in the relative adrenal weight in the Ad.VEGF-A$_{165}$ group relative to the saline group. Relative adrenal weight is increased in the late-gestation fetuses of overnourished adolescent dams [9, 17], and may reflect the accelerated maturation of the adrenal gland that is known to occur in several animal models of FGR, presumably in response to increasingly poor transplacental nutrient supply to the fetus. Increased relative adrenal weights have also been documented in ovine models of FGR induced by carunclectomy [37], placental embolization [38], and single umbilical artery ligation [39]. In the present study, there was a negative correlation between the relative adrenal weight and gestation length in the saline group, implying the greater the relative size of the adrenal, the earlier the onset of labor. Moreover, there appeared to be a dissociation of this relationship following Ad.VEGF-A$_{165}$ treatment, in keeping with the slight increase in gestation length seen in this group. These observations support the concept of a functional relationship between premature activation of the fetal hypothalamic-pituitary-adrenal axis and the initiation of labor in this model. However, it is a limitation of the present study that we were unable to assess fetal adrenal function immediately prior to parturition, for example, by measuring fetal cortisol levels.

Lambs born following Ad.VEGF-A$_{165}$ treatment continued to grow at a faster rate during the first 12 wk of life, and were significantly larger than lambs born to saline-treated ewes from 6 wk onward. Increased postnatal growth was apparent when expressed in absolute terms, but not as a fraction of birth weight, implying that there had been no disassociation of the normal inverse relationship between lamb birth weight and fractional growth rate. Previously, when the postnatal growth trajectories of small lambs born to overnourished mothers were compared with those of normally grown offspring from control-intake pregnancies, the absolute growth rates to weaning were not significantly different (368 ± 13.4 vs. 359 ± 10.9 g/day, $P > 0.05$), indicative of the phenomenon known as "catch up" growth [11, 13]. In the present study, mean fractional growth rates in the Ad.VEGF-A$_{165}$ group (10.0% per day) were similar in magnitude to those observed in lambs born to (untreated) overnourished dams (10.1% per day), suggesting that the increased absolute postnatal growth rates after Ad.VEGF-A$_{165}$ treatment were not excessive when compared to baseline for this cohort of offspring, but, rather, reflected the relative size advantage of these lambs at birth, magnified with advancing postnatal age. These lambs appeared to be thriving following prenatal treatment, as they remained in good health and were growing at an appropriate rate. The lack of any significant differences between groups in DNA methylation of the various somatotropic axis genes examined herein, or in expression of *IGF1* at the mRNA or protein level, further supports this assumption. However, we cannot say for certain that these differences were not due to an intrauterine programming effect that remained undetected by the assays utilized.

Despite the lack of any impact of FGR on the epigenetic status of a panel of eight genes or mRNA/protein expression of *IGF1*, there was evidence of sexual dimorphism with respect to the *IGF1* and *INS* genes. *IGF1* mRNA expression was higher and DNA methylation tended to be lower in male versus female lambs, which is in keeping with the higher circulating concentrations of IGF1 in males from early postnatal life, and has been reported previously in our sheep model of FGR [36]. A more novel observation was the finding that *INS* DNA methylation was lower in females versus males, and correlated inversely with fasting plasma insulin (irrespective of gender). This relationship was in the expected direction, as higher degrees of methylation would hypothetically be associated with less transcription of the precursor protein preproinsulin, although the physiological sequence of events thereafter is complex and was not examined in any further detail by the present study.

There were no sex differences in any other metabolic parameters; however, lambs born following prenatal Ad.-VEGF-A$_{165}$ gene therapy demonstrated a significant increase in insulin secretion (AUC) during the i.v. glucose tolerance test compared to lambs whose mothers were treated with saline. Reassuringly, however, glucose disposition (AUC) did not differ between the groups, suggesting normal responsiveness to insulin. Furthermore, there were no significant differences in any markers of insulin sensitivity, such as insulin-to-glucose or glucose-to-insulin AUC ratios. This implies that enhanced insulin secretion did not reflect worsening of insulin resistance, but, rather, an increased capacity to secrete insulin in the absence of major alterations in peripheral insulin action. There were also no significant differences in fasting levels of glucose or insulin,

suggesting that Ad.VEGF-A$_{165}$ treatment did not impact basal metabolic function in either a positive or negative way.

It was initially reassuring that, despite the increased growth rates following Ad.VEGF-A$_{165}$, there were no differences in fat mass or percentage body fat between the the Ad.VEGF-A$_{165}$ and the saline group when examined by DEXA at 11 wk postnatal age, but, rather, a tendency towards increased BMD, BMC, and lean tissue mass. However, at the point of necropsy at 12 wk postnatal age, the relative weight of the perirenal fat depot (g/kg lamb) tended to be increased in the Ad.VEGF-A$_{165}$ group relative to the saline group with a significant gender $\times$ treatment interaction, such that the greatest degree of adiposity was observed in Ad.VEGF-A$_{165}$-treated female lambs. This observation indicates a possible intrauterine programming effect, and may suggest that females are more sensitive to putatively enhanced nutrient supply in utero than males, although there were no gender differences in ultrasonographic fetal growth or lamb birth weight in the present study. Of note, we were most likely underpowered to detect sex differences in the present study, and had no control over lamb gender, as recruitment of ewes into the study occurred at midgestation. By contrast, many purely postnatal studies begin at birth and feature stratification by gender. Regardless, there was no evidence that female and/or Ad.VEGF-A$_{165}$-treated lambs had a worse metabolic profile than male and/or saline-treated lambs. Ultimately, the perirenal fat is only one of several major fat depots in the body, and assessment of total body fat stores by DEXA did not suggest any differences in the overall degree of adiposity between Ad.VEGF-A$_{165}$ and saline-treated lambs. It is also noteworthy that residual carcass weights at necropsy were greater following Ad.VEGF-A$_{165}$ treatment, indicating enhanced lean tissue accretion.

The present study complements and extends the findings of our previous investigation [6], which had its endpoint in late gestation, in several respects. First, it has closely replicated our earlier finding of increased fetal growth velocity at 3 and 4 wk following maternal UtA Ad.VEGF-A$_{165}$ treatment. The absolute change in fetal AC measurements relative to baseline at these two time points was remarkably similar to that demonstrated previously (75.8 $\pm$ 2.16 vs. 75.6 $\pm$ 3.62 mm and 93.1 $\pm$ 2.57 vs. 92.1 $\pm$ 4.31 mm at 3 and 4 wk, respectively), which adds both precision and robustness to the estimate of effect size. Relative to the change in fetal AC for the saline-treated pregnancies, this corresponds to an additional 18% increase in fetal growth, which was mirrored by the differential in birthweight of 681 g (19.8% greater than saline-treated FGR pregnancies). Although this difference did not reach statistical significance ($P = 0.08$), in retrospect, this is likely to reflect the much greater birthweight variability encountered in this study compared to previous studies (SD, 1212 vs. 607 g), meaning we were relatively underpowered to detect any difference attributable to Ad.VEGF-A$_{165}$ on this background. This was largely due to the inclusion of one unusually large fetus in the saline-treated group (born weighing 6050 g), which greatly impacted the variability, but was not excluded as an outlier. Moreover, an anticipated birthweight increase of 20% may, in retrospect, have been slightly overambitious, given that the size of the effect of Ad.VEGF-A$_{165}$ on fetal weight at 131 days gestation in our previous study was a (nonsignificant) 242 g (5.8%) compared to saline-treated pregnancies of overnourished dams, despite significant effects on ultrasonographic fetal growth velocity and the proportion of fetuses demonstrating marked FGR [6]. Unfortunately, however, we did not have these data available at the time that we commenced the present study.

Regardless, a 20% improvement in fetal growth, as demonstrated by ultrasound, is likely to be clinically significant in human pregnancies affected by FGR due to uteroplacental

insufficiency, as relatively small increases in growth (and/or time spent in utero), particularly at the threshold of viability, can translate into relatively large improvements in the rates of overall and intact survival [40]. Second, there was a tendency toward increased placental efficiency following Ad.VEGF-A$_{165}$ treatment, in agreement with our previous study, in which fetal-to-placental weight ratios were greatest in the Ad.VEGF-A$_{165}$-treated pregnancies and there was upregulation of caruncular mRNA expression of VEGF receptors 1 and 2. Collectively, these observations might reflect increased placental vascularity and/or nutrient transport capacity. The latter is implied by the trend toward increased plasma glucose levels observed in the Ad.VEGF-A$_{165}$-treated lambs at birth, which suggests enhanced transplacental transfer. Finally, the current investigation adds further to the safety profile of maternal Ad.VEGF-A$_{165}$ therapy to treat uteroplacental FGR, for which there is currently no effective treatment. There were no adverse responses of either the mother or the fetus to the injection or the occlusion procedure, and all Ad.VEGF-A$_{165}$-treated pregnancies continued until term, with no antenatal or intrapartum complications. There were no acute concerns during the perinatal period, and no significant differences in the level of neonatal care required to ensure lamb survival. Moreover, Ad.VEGF-A$_{165}$ did not impact colostrum yield or composition, or the number or frequency of supplementary feeds, suggesting that its effects are localized to the pregnant uterus without any impact on mammary gland function. Neonatal serum biochemistry and liver function were unaltered, which is particularly reassuring, as adenoviral vectors can induce liver inflammation [41]. Previous work has shown no evidence of transfer to the fetus following administration via the maternal UtAs in sheep [29]. To investigate safety further, we are currently conducting full reproductive toxicology experiments as part of a program of work aiming to translate prenatal maternal Ad.VEGF gene therapy into the clinic (European Union Framework Programme 7 project EVERREST [does vascular endothelial growth factor gene therapy safely improve outcome in severe early-onset fetal growth restriction?]).

This study has some clear limitations. First, the greater-than-expected variability in birthweight is likely to have left us underpowered to detect differences in our primary outcome, despite clear and statistically significant differences in various ultrasonographic markers of fetal growth, which are arguably more sensitive at picking up subtle shifts in the prenatal growth trajectory [27, 28]. Second, while the lack of any significant differences in DNA methylation of key somatotropic axis genes is reassuring, it does not completely exclude an intrauterine programming effect of prenatal Ad.VEGF-A$_{165}$ gene therapy, particularly given the observation that female Ad.VEGF-treated fetuses had increased perirenal fat. Although the pyrosequencing technique (following bisulphite conversion) interrogate CpG methylation at specific loci with very high resolution, ultimately, relatively few sites can be examined in a given assay, which limits the extent of the assessment, particularly compared to the genome-wide methylation arrays that are currently available for other species, including humans and rodents (but not the sheep). Although we used a validated approach [36] to target sites of presumed biological significance, it remains possible that significant changes in DNA methylation in other genes may have been overlooked. In addition, we only examined a single tissue at a single time point. Although liver is an excellent candidate for epigenetic analyses, given its central role in metabolism and postnatal growth via the GH-IGF1 axis [42], it should be borne in mind that methylation changes (or any lack thereof) observed in one organ cannot automatically be extrapolated to another [43]. Given the apparent effects of Ad.VEGF-A$_{165}$ on perirenal fat deposition in female fetuses,

analysis of adipose tissue would be the appropriate next step to further evaluate for potential programming effects.

In summary, midgestation delivery of Ad.VEGF-A$_{165}$ gene therapy in FGR pregnancies induced by overnourishing adolescent sheep dams significantly increased fetal growth velocity at 3 and 4 wk posttreatment compared to a saline control. The resultant lambs tended to be heavier at birth, and there were no differences between groups in the level of neonatal care required to ensure survival. Ad.VEGF-A$_{165}$-treated lambs demonstrated accelerated absolute postnatal growth rates (with evidence of enhanced lean tissue accretion) and increased insulin secretion following a glucose challenge (in the absence of any changes in insulin sensitivity), which likely represent a greater anabolic drive in the Ad.VEGF-A$_{165}$-treated animals. There were no differences in DNA methylation of the key genes of the somatotropic axis, or in mRNA or protein expression of *IGF1*, suggesting that these postnatal effects were a reflection of the lambs' relative size advantage at birth, rather than an intrauterine programming effect.

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