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PERINATAL AND LONG TERM EFFECTS OF MATERNAL UTERINE ARTERY ADENOVIRAL VEGF-A$_{165}$ GENE THERAPY IN THE GROWTH RESTRICTED GUINEA PIG FETUS

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Running head: Long term outcomes of uterine artery VEGF gene therapy

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ABSTRACT

Uterine artery application of adenoviral vascular endothelial growth factor gene therapy (Ad.VEGF-A$_{165}$) increases uterine blood flow and fetal growth in experimental animals with fetal growth restriction (FGR). Whether Ad.VEGF-A$_{165}$ reduces lifelong cardiovascular disease risk imposed by FGR remains unknown. Here, pregnant guinea pigs fed 70% normal food intake to induce FGR received Ad.VEGF-A$_{165}$ (1×10^{10} viral particles, n=15) or vehicle (n=10), delivered to the external surface of the uterine arteries, in mid-pregnancy. *Ad libitum* fed controls received vehicle only (n=14). Litter size, gestation length, and perinatal mortality were similar in control, untreated FGR and FGR+Ad.VEGF-A$_{165}$ animals. Compared to controls, birth weight was lower in male but higher in female pups following maternal nutrient restriction, whilst both male and female FGR+Ad.VEGF-A$_{165}$ pups were heavier than untreated FGR pups (P<0.05 ANOVA). Postnatal weight gain was 10-20% greater in female FGR+Ad.VEGF-A$_{165}$ than untreated FGR pups, depending on age, although neither group differed from controls. Maternal nutrient restriction reduced heart weight in adult female offspring, irrespective of Ad.VEGF-A$_{165}$ treatment, but did not alter ventricular wall thickness. In males, postnatal weight gain and heart morphology were not affected by maternal treatment. Neither systolic, diastolic nor mean arterial pressure, adrenal weight, basal or challenged plasma cortisol were affected by maternal undernutrition or Ad.VEGF-A$_{165}$ in either sex. Therefore, increased fetal growth conferred by maternal uterine artery Ad.VEGF-A$_{165}$ is sustained postnatally in FGR female guinea pigs. In this study we did not find evidence for an effect of maternal nutrient restriction or Ad.VEGF-A$_{165}$ therapy on adult offspring blood pressure.

Key words: vascular endothelial growth factor, developmental programming, placenta, growth, blood pressure

INTRODUCTION

FGR is a common obstetric complication, affecting up to 10% of all pregnancies, whereby the fetus does not reach its genetic growth potential. In the most severe cases, preterm delivery to assure the immediate survival of the fetus is the only available treatment. Both FGR itself and preterm delivery carry a significant burden of morbidity and mortality in the neonatal period but also have lifelong health consequences. Neonates born growth restricted may exhibit a failure to thrive and often grow poorly in infancy, in terms of weight gain and head growth, such that they remain at a deficit in stature and are at increased risk of neurodevelopmental defects relative to normally grown neonates (6, 29, 31). FGR also increases the risk for later life cardiovascular disease, such that adults who were born with FGR have higher resting blood pressure and a greater prevalence of hypertension than those who were born at the same gestational age with a normal birth weight (2, 18, 23, 61). In part, this may be due to a programmed increase in hypothalamic-pituitary adrenal axis activity in adults born with FGR, particularly in subjects that were born preterm (38). Similarly, when fetal growth restriction is experimentally induced in pregnant animals, the offspring have increased blood pressure and abnormal endocrine function in adulthood (5, 8, 41, 45, 58).

FGR is commonly associated with impaired uterine perfusion and placental insufficiency. In healthy pregnancies, uterine blood flow rises with advancing gestational age, through increased maternal cardiac output and conversion of the uterine muscular spiral arteries into distended, thin-walled flaccid vessels, to support the oxygen and nutrient requirements of the growing fetus (9). Thus, in both humans and experimental animals, fetal weight at term is proportional to uterine artery volume flow (25, 37, 44, 47, 51, 55). In humans, the process of spiral artery conversion is dependent upon the invasion of placental extravillous trophoblast (EVT) cells, which become incorporated into the spiral artery wall as intramural trophoblast while the endothelium, vascular smooth muscle, and elastic lamina are destroyed and replaced by fibrinoid. Failure to transform uteroplacental spiral arteries underpins FGR (46). Reduced uterine artery volume flow rate and notching of the uterine artery waveform are evident from the second trimester in pregnancies with FGR (42). Moreover, abnormalities of uterine artery Doppler velocimetry in the third trimester, such as elevated pulsatility index and diastolic notching, correlate with poor perinatal outcome, even when umbilical artery Doppler indices are normal (27). Uteroplacental vascular resistance falls during gestation in part due to EVT secretion of paracrine signals including vascular endothelial growth factor (VEGF), which is pro-angiogenic and vasodilatory in a nitric oxide dependent manner (14). In FGR, maternal

serum total concentration of VEGF is significantly lower than in normal pregnancy (4, 57) and the concentration of its soluble receptor is higher, effectively lowering the circulating concentration of free VEGF (16). Reduced VEGF vasodilator activity and uterine artery blood flow therefore appear to be contributory factors in FGR in humans. Consistent with this, fetal growth is restricted in experimental animals when uterine blood flow is limited, for example through surgical or nutritional manipulations, or when maternal nitric oxide dependent vasodilatation is reduced genetically (63).

Local overexpression of VEGF in the uterine arteries may be an effective therapy to improve uterine blood flow, angiogenesis and hence fetal growth in severe FGR pregnancies. In pregnant sheep, transduction of the uterine arteries with *VEGF-A₁₆₅* by intravascular delivery of an adenoviral vector (Ad.) results in transient local overexpression of the transgenic protein, and a sustained increase in uterine blood flow measured in vivo either by Doppler sonography or indwelling transit-time flow probe (17, 49). The haemodynamic response to Ad.VEGF-A$_{165}$ therapy in the ewe is associated with functional and morphological adaptations in the uterine artery *ex vivo*, including reduced maximal phenylephrine-mediated contractility, increased maximal nitric oxide-dependent relaxation, greater local endothelial nitric oxide synthase (eNOS) abundance and increased neointimal vessel formation (17, 49). When uterine artery Ad.VEGF-A$_{165}$ gene therapy is administered to ewes bearing experimentally growth restricted fetuses, fetal size near term is increased and the incidence of severely growth restricted neonates is reduced compared to control treated FGR pregnancies (12). Lambs born to mothers that received mid-gestation uterine artery Ad.VEGF-A$_{165}$ gene therapy also have lower neonatal morbidity, increased postnatal growth and improved glucose tolerance in adulthood (11). We recently sought to determine the effects of uterine artery Ad.VEGF-A$_{165}$ gene therapy in the guinea pig, which has a haemochorial placenta, invasive cytotrophoblast and spiral artery remodelling closely resembling that of the human (13, 15, 70, 72). When the external surface of the guinea pig uterine artery is transduced with Ad.VEGF-A$_{165}$ in a thermolabile pluronic gel there are increases in uterine artery relaxation, eNOS abundance and adventitial vessel growth similar to those that occur in the sheep given Ad.VEGF-A$_{165}$ intravenously (65). Maternal uterine artery Ad.VEGF-A$_{165}$ treatment also increases fetal weight and ultrasound indices of intrauterine growth near term in growth restricted guinea pig fetuses (50, 65). However, the postnatal effects of maternal Ad.VEGF-A$_{165}$ treatment in the guinea pig remain unknown. Understanding the postnatal effects of maternal uterine artery Ad.VEGF-A$_{165}$ therapy in the guinea pig are important for establishing the safety and efficacy of the treatment.

125   When fetal growth is restricted by maternal undernutrition in the guinea pig, the offspring

126   exhibit hypertension, abnormal glucose homeostasis and altered hypothalamic-pituitary-

127   adrenal function in adulthood (5, 39, 41, 56, 64). Moderate maternal dietary restriction ($\leq$30%

128   caloric reduction) before and during pregnancy recapitulates many of the pre- and post-natal

129   aspects of human FGR, including placental vascular insufficiency, reduced placental weight

130   and surface area, fetal brain-sparing, offspring hypertension and glucose intolerance (56, 60)

131   (19, 39, 41, 64). Often in this model, the severity of fetal growth restriction and the postnatal

132   phenotype are more pronounced in the male offspring of dietary restricted dams (19, 39, 41).

133   Therefore, this study determined the effects of maternal uterine artery Ad.VEGF-A$_{165}$ therapy

134   on postnatal growth and adult blood pressure in guinea pigs growth restricted by moderate

135   maternal undernutrition, relative to untreated FGR animals and normally grown controls. We

136   hypothesised that Ad.VEGF-A$_{165}$ therapy would normalise growth and postnatal phenotype in

137   FGR fetuses, in a manner dependent on fetal sex.

METHODS

Animals

All procedures were conducted under the Animals (Scientific Procedures) Act 1986. Dunkin-Hartley guinea pigs (B&K Universal, Hull, UK) were housed under 12hr dark-light cycle conditions and maintained on water, hay and pelleted chow (10% fat, 25% protein, 65% carbohydrate; Standard FDI (P), Special Diets Services, Witham, UK). Nulliparous sows ($\geq$750g, n=39) were singly-housed throughout the study, except during oestrus, when they were mated with a stud male for 3-5 days. Females were identified as pregnant by ultrasound scan ~20 days after mating and gestational age was counted from the middle day of oestrus, which was designated day 0 of pregnancy (term $\approx$ 67 days). A subgroup of 25 sows were randomly assigned to receive a restricted diet for $\geq$30 days before conception to induce placental insufficiency and FGR in pregnancy (39, 56, 64). Daily chow intake was restricted to 70% of normal until day 30 of pregnancy, when it was increased to 90% of normal until term (39). Preliminary studies established that ad libitum food intake in pregnant guinea pigs was 0.6g/100g body weight/day (64). Nutrient restricted dams were therefore weighed daily and fed 0.42g/100g body weight (70%), rising to 0.54g/100g body weight (90%) at 0900 each day. The remaining 14 control females were fed *ad libitum* throughout the experiment.

Uterine artery Ad.VEGF-A$_{165}$ gene therapy

At mid-gestation (mean 34 days, range 29-37 days), after an overnight fast, sows were pre-medicated (0.05 mg/kg atropine *s.c.* and 2.5 mg/kg diazepam *i.m.*) then anaesthetised (40 mg/kg ketamine *i.m.* and 2% isofluorane in O_2 inhaled) and the uterus exposed via mid-line laparotomy. Either Ad.VEGF-A$_{165}$ (1×10^{10} viral particles) in combination with vehicle (1ml Pluronic F-127, 30% w/v in sterile water, n=15 sows), or vehicle alone (1ml, n=10 sows) was applied directly to the external surface of the uterine and radial arteries of nutrient restricted sows for 5 minutes, as described previously (50, 65). All control fed sows received vehicle only (1ml, n=14). The rectus sheath was closed with continuous 2-0 Vicryl with tapercut needle (Ethicon, Sint-Stevens-Woluwe, Belgium) to prevent herniation of the abdominal contents. The subcutaneous fat was closed with continuous 2-0 Vicryl with tapercut needle (Ethicon) and continuous subcuticular 2-0 Vicryl with cutting needle (Ethicon) was used to close the skin. A 10% solution of lidocaine hydrochloride (0.5 mL) was administered subcutaneously just before closing the skin to provide local anaesthesia. The sow was recovered from anaesthesia and returned to her home cage. Analgesia was administered on the

170   day of surgery (carprofen, 4 mg/kg *s.c.* and buprenorphine 0.05 mg/kg *i.m*) and for 3 days

171   thereafter (4 mg/kg carprofen *s.c.* daily). All animals were monitored daily for the remainder

172   of pregnancy, and advice taken from a veterinary surgeon when there was evidence of vaginal

173   bleeding, pain or wound dehiscence/hernia. There was a low threshold to cull sows that

174   developed a wound dehiscence or hernia as there was concern that sows delivering pups with

175   these complications might experience labour dystocia and severe pain. Fates of individual

176   animals are given in Table 1. For minor wound dehiscence without obvious animal distress and

177   under advice from the veterinary surgeon, subcuticular skin closure under local anaesthetic was

178   performed where possible. Sows were allowed to labour naturally. Pups were weighed on the

179   day of birth and every two days thereafter, up to weaning at ~3 weeks of age. Following

180   weaning, pups were housed in same-sex groups of 2-5 individuals, with *ad libitum* access to

181   food and water, and weighed weekly. Fractional growth rate over each four week period after

182   birth was determined as (final weight – initial weight)/(initial weight x 28 days). Sows were

183   killed (200 mg/kg Na pentobarbitone *i.p.*, Euthatal, Merial Animal Health, Harlow, UK) and

184   samples of uterine and radial artery, uterus, ovary, liver, spleen, lung, heart, adrenal, kidney

185   and thymus were fixed in 4% paraformaldehyde and processed for standard histopathological

186   analysis (N.S.)

Adult offspring blood pressure and cardiovascular phenotype

188   Adult pups (aged 3-5 months, n=57) were catheterised under general anaesthesia, using the

189   same anaesthetic regimen as described above but without overnight fasting. A polyurethane

190   catheter was inserted into the carotid artery (AT-RCAC-0612A, Access Technologies, IL,

191   USA), exposed via a ventral neck incision. In a subgroup of animals (n=23), the left jugular

192   vein was also catheterised (AT-RJVC-0612A, Access Technologies, IL, USA). Catheters were

193   tunnelled under the skin and exteriorised at the nape of the neck. Following closure of the

194   incision (4-0 coated Vicryl, Ethicon, NJ, USA), catheters were flushed (10 IU/ml heparin

195   sodium in 0.9% saline w/v), locked (500 IU/ml heparin sodium in 50% glycerol, Cath-Loc

196   HGS, Sandown Scientific, Hampton, UK) and sealed with a steel pin. Analgesia was

197   administered on the day following surgery (4mg/kg carprofen *s.c.*) and for 3 further days (0.5

198   mg/kg meloxicam, *p.a.*), together with supplementary fluids (3.5 ml saline 0.9% w/v *i.v.*).

199   Animals were recovered in their home cage and catheters were flushed every 2-3 days to

200   maintain patency. Fates of individual catheterised offspring are given in Table 1.

201 After at least 4 days post-operative recovery, carotid arterial blood pressure was measured in
202 catheterised pups in the morning, beginning between 09.00 and 10.00. Animals were moved to
203 an individual cage but maintained visual contact with their normal cage mates. A fluid-filled
204 extension line was connected to the carotid catheter such that the animal was able to move
205 freely during the measurement period. Carotid blood pressure was recorded continuously over
206 ~2 hours using a calibrated piezo-resistive transducer calibrated to two external set points, quad
207 bridge amplifier and data acquisition system (all ADInstruments, Oxford, UK). Average
208 systolic, diastolic, pulse and mean arterial pressures, and heart rate were calculated using
209 automated cycle detection software (LabChart, ADInstruments, Oxford, UK). In animals with
210 a patent jugular venous catheter, adrenocorticotrophic hormone (ACTH) challenge studies
211 were also conducted at least 48 hours after blood pressure measurement. On the day of the
212 ACTH challenge, blood samples (~200µl) for measurement of basal cortisol concentration
213 were collected from the carotid arterial catheter prior to infusion of an ACTH bolus into the
214 jugular vein (1.25 µg/kg in 2.5ml). Blood was subsequently collected 15, 30, 60, 90 and 120
215 minutes after the ACTH infusion. All samples were centrifuged (3000 rpm, 5 min) and the
216 separated plasma stored at -80°C for later dilution (400-fold) and determination of cortisol
217 concentration using a commercially available ELISA (ADI-900-071, Enzo Life Sciences,
218 Exeter, UK). Linearity of the assay in the dilution range 100-fold to 400-fold was 93% and
219 recovery of a known concentration of cortisol from guinea pig plasma was 86%. Intra- and
220 inter-assay coefficients of variability were 5% and 4%, respectively. At the end of the
221 experiment, all pups were killed (200 mg/kg Na pentobarbitone *i.v.*) and a cardiac blood sample
222 was collected for measurement of standard haematological and biochemical parameters (total
223 protein, albumin, globulin, Na^+, K^+, Cl^-, Ca^+, PO_4^{3-}, urea, creatine, glucose, cholesterol,
224 bilirubin, triglycerides, aspartate transaminase, creatine kinase, glutamate dehydrogenase,
225 Diagnostic Laboratories, Royal Veterinary College, Herts, UK). Correct catheter placement
226 was also confirmed post-mortem. The carcass and major organs were dissected and samples
227 processed for histopathological analysis.

Statistics

229 Results are presented as mean ± SEM. Overall effects of maternal treatment on pregnancy
230 outcome, birth weight, postnatal growth rate, blood pressure and cardiac morphology were
231 determined by one-way ANOVA. Offspring postnatal weight gain was assessed by general
232 linear model with maternal treatment and pup age as independent factors, and birth weight as
233 a covariate. Plasma cortisol response to ACTH challenge was assessed by repeated measures

234 two-way ANOVA with time from ACTH administration and maternal treatment as
235 independent factors. When main effects were significant by ANOVA, multiplicity corrected
236 post-hoc comparisons of control, FGR and FGR+Ad.VEGF-A165 groups were made using the
237 Holm-Sidak method. Statistical analyses were conducted separately for male and female
238 offspring except for ACTH challenge data. In all cases effects were considered significant
239 when $P<0.05$.

240

RESULTS

Pregnancy outcome

In total, nineteen control, FGR and Ad.VEGF-A$_{165}$ treated FGR sows delivered spontaneously at term (Table 1). A further fifteen sows were euthanised under veterinary advice $\leq$ 22 days after surgery (Table 1). Four sows miscarried before day 60 of pregnancy. There was no difference between maternal treatment groups in the rate of pre-term pregnancy loss (17% overall, P=0.777, Chi-squared test). Maternal weight did not differ with treatment group either at conception, surgery or term (Table 2). Neither was there a difference in gestational age at delivery or number of pups per litter (Table 2). There were no maternal complications at delivery. Six FGR pups of undernourished sows were stillborn whilst one pup was runted, failed to suckle and was subsequently culled on postnatal day 6 (Table 1). There was no difference between maternal treatment groups in the rate of neonatal death (11%, P=0.149, Chi-squared test). All remaining pups survived until catheterisation at 139±5 days. Sows experienced no behavioural abnormalities or complications up to the time of weaning when they were euthanized. There were no post-mortem histological abnormalities observed in the sows given Ad.VEGF-A$_{165}$ or control treatment.

Birth weight and postnatal growth

There was an overall effect of maternal treatment on birth weight in both male and female pups (Table 2). On average, birth weight tended to be greater in both male and female FGR+Ad.VEGF-A$_{165}$ than untreated FGR pups and was significantly higher in female, but not male, FGR+Ad.VEGF-A$_{165}$ pups compared to the offspring of *ad libitum* fed control dams. However, neither male nor female untreated FGR pups differed significantly in weight from *ad libitum* fed control offspring. Moreover, when litter size was used as a covariate in the general linear model comparing control, untreated FGR and FGR+Ad.VEGF-A$_{165}$ pups, there was no significant effect of maternal treatment on birth weight in either male (P=0.178) or female pups (P=0.146).

There was a significant interaction effect between pup age and maternal treatment on net postnatal weight gain in female pups (Fig. 1B). The interaction effect remained significant when postnatal weight gain was treated as a repeated measure in the general linear model used to determine the effect of treatment on weight gain up to 11 weeks, when data was available for all pups (P<0.001, effect of age P<0.05, effect of treatment P>0.05). Net weight gain was significantly greater in female FGR+Ad.VEGF-A$_{165}$ than untreated FGR pups from 7 weeks of

age until the end of the experiment at 19 weeks (Fig. 1B). Weight gain was also greater in female FGR+Ad.VEGF-A$_{165}$ pups compared to controls between 10 and 14 weeks of life, although there was no difference between the two groups in subsequent weeks (Fig. 1 B). There was no significant difference in postnatal weight gain between control and untreated FGR female offspring at any age (Fig 1B). Neither maternal undernutrition nor Ad.VEGF-A$_{165}$ therapy affected postnatal weight gain in male pups (Fig. 1A). When fractional growth rate was calculated as mg gained per g body weight per day, over each four week period after birth, there was no significant effect of maternal treatment in either male or female pups (Table 1, $P > 0.05$ all cases). Moreover, absolute body weight did not differ between treatment groups of either sex at postnatal age ~20 weeks, when blood pressure was measured (Table 3).

Adult offspring blood pressure and cardiovascular phenotype

Neither mean, systolic nor diastolic carotid arterial pressure differed with maternal treatment group in adult male or female offspring catheterised at ~20 weeks of age (Table 3). Heart rate was also similar in all treatment groups, irrespective of offspring sex. Pulse pressure was greater in untreated FGR females and both FGR and FGR+Ad.VEGF-A$_{165}$ males, compared to their respective controls, but was <4mmHg in all groups (Table 3) and markedly lower than previously reported values in this species (17-27mmHg) (32, 41). In females, maternal nutrient restriction, irrespective of Ad.VEGF-A$_{165}$ treatment, decreased heart weight in adulthood, when expressed either as an absolute value or as a percentage of total body weight. However, heart weight did not differ between control, FGR and FGR+Ad.VEGF-A$_{165}$ male offspring (Table 3). Left ventricular, right ventricular and septal thicknesses did not differ with maternal treatment in either males or females (Table 3).

When male and female offspring were combined, basal plasma cortisol concentration before the ACTH challenge was similar in control, FGR and FGR+Ad.VEGF-A$_{165}$ groups ($P > 0.05$, Figure 2A). Following ACTH infusion, plasma cortisol concentration increased with time in all three groups but the response did not differ with maternal treatment ($P > 0.05$, Figure 2B). Neither was there an effect of maternal treatment on the area under the ACTH challenge curve (Figure 2C), or the absolute or relative adrenal weights of the offspring (Table 2, $P > 0.05$ all cases).

Standard clinical analyses of haematology, blood biochemistry and post-mortem histology did not detect any difference from normal parameters in the offspring of Ad.VEGF-A$_{165}$ treated sows or other treated pup groups.

DISCUSSION

This study is the first to determine the post-natal effects of maternal uterine artery Ad.VEGF-A_{165} treatment for fetal growth restriction in a nutrient-restricted guinea pig model, with haemochorial placentation. The results show that the treatment tends to increase birth weight in the offspring of nutrient restricted dams, although the effect of maternal nutrient restriction, compared to *ad libitum* feeding, was mild and sex-dependent. Ad.VEGF-A_{165} also increased post-natal weight gain in female pups of nutrient restricted dams, compared to their untreated counterparts. The results did not show an effect of maternal nutrient restriction or uterine artery Ad.VEGF-A_{165} treatment on mean arterial blood pressure in the adult offspring. There were no adverse postnatal effects associated with prenatal Ad.VEGF-A_{165} gene therapy, in terms of dam or pup histology, haematology and blood biochemistry.

The effect of maternal Ad.VEGF-A_{165} gene therapy on birth weight in FGR pups was small, although on average both male and female pups of Ad.VEGF-A_{165} treated dams tended to be ~3% heavier than their untreated FGR counterparts, consistent with the increase in fetal weight demonstrated previously in Ad.VEGF-A_{165} treated guinea pigs near term (65). The therapeutic effect in individual fetuses may have been larger, but we did not track the size of individual fetuses within each litter from treatment through to delivery and therefore have no indication of the effect of Ad.VEGF-A_{165} on intrauterine growth rate that accounts for the initial size of the fetus. Certainly, uterine artery Ad.VEGF-A_{165} treatment increases ultrasound-measured fetal growth velocity in the sheep fetus without significantly affecting birth weight (11, 12). Mechanistically, greater birth weight in the offspring of Ad.VEGF-A_{165} pregnancies is likely underlain by enhanced uterine blood flow with a concomitant increase in the fetal supply of oxygen and nutrients (17, 49), although the effect of local Ad.VEGF-A_{165} administration on uterine artery volume flow has not been determined in the guinea pig, to date. The quantitatively small therapeutic effect of Ad.VEGF-A_{165} on birth weight in this study may relate to the route of vector delivery, which was extravascular, rather than intravascular as in previous ovine studies (11, 12, 17, 49). Nonetheless, extravascular Ad.VEGF-A_{165} administration in pluronic gel has been shown to produce efficient uterine artery gene transfer (50) and vessel remodelling (65) similar to that in the sheep.

The small effect of maternal Ad.VEGF-A_{165} therapy on mean birth weight may also be attributable to the varying efficacy of the maternal dietary restriction in producing a phenotype of FGR. Previous studies suggest that only ~60% of pups of dietary restricted dams are growth restricted below the 10% centile of control weights (19). Due to poor post-surgical recovery of

338 all dams in this study, insufficient litters of pups were delivered to reliably determine which

339 fetuses were growth restricted on a weight percentile basis and therefore whether Ad.VEGF-

340 A$_{165}$ therapy reduced the incidence of FGR, an effect also demonstrated previously in the sheep

341 (12). In turn, the small effect of the dietary manipulation on mean birth weight may relate to

342 the tendency for nutrient restriction in the pregnant guinea pig to reduce mean litter size at

343 term, which reduces competition between litter mates for maternal resources and therefore

344 alleviates fetal growth constraints (60). Certainly, the overall effect of maternal treatment on

345 birth weight in both male and female pups was abolished when litter size was taken into account

346 as a covariate in the general linear model used in the present study. Alternatively, birth weight

347 variability within control and FGR animals may be related to differences in fetal position in

348 the uterus or to maternal body composition and pre-pregnancy fuel reserves, which are known

349 to influence uteroplacental blood flow and the incidence of both small and large fetuses in

350 polytocous species (43, 51). The effect of the maternal undernutrition on birth weight depended

351 on offspring sex, tending to reduce the weight of male pups but increase the weight of female

352 pups, in line with the demonstrated gender specificity of this model (39), The less severe effect

353 of nutrient restriction on female pups may relate to compensatory increases in pro-angiogenic

354 and erythropoietic signals specific to the female placenta under hypoxia (20). Overall, although

355 Ad.VEGF-A$_{165}$ does not completely correct FGR in pre-clinical models, incremental

356 improvements in intrauterine growth in human fetuses might be expected to improve clinical

357 outcome by delaying the requirement for iatrogenic preterm delivery (3).

358 Maternal Ad.VEGF-A$_{165}$ therapy increased net postnatal weight gain only in FGR female pups

359 from the 7th week of life. Maternal uterine artery Ad.VEGF-A$_{165}$ therapy similarly increases

360 postnatal weight in lambs growth restricted *in utero* when they are aged between 7 and 12

361 weeks (11). Increased postnatal growth rate is unlikely be a direct effect of Ad.VEGF-A$_{165}$

362 gene therapy, which does not cross the placenta or spread to fetal tissues (65). Neither is there

363 likely to be any effect of the therapy on lactation or milk quality as maternal VEGF transgenic

364 protein expression is short term, lasting only up to 1 week in the guinea pig, and has no maternal

365 physiological effect pre- or post-partum in sheep (11). Improved nutrient and oxygen supply

366 as a consequence of the uterine artery VEGF transgenic protein expression during fetal

367 development may therefore program persisting improvements in growth, for example through

368 epigenetic modifications to key growth and metabolism regulating genes (11). However, since

369 postnatal growth rate did not differ between female Ad.VEGF-A$_{165}$ treated and untreated FGR

370 pups when determined as the number of mg gained per g body weight per day, the greater net

gain in FGR+Ad.VEGF-A$_{16}$ pups may alternatively reflect their relative size advantage conferred at birth and magnified with increasing postnatal age. Combined with the absence of a difference in cumulative postnatal weight gain between female Ad.VEGF-A$_{165}$ treated pups and normal control animals, this reassuringly suggests that Ad.VEGF-A$_{165}$ does not promote postnatal catch-up growth, which is independently associated with increased cardiovascular disease risk in humans (21), but instead may have a beneficial effect in combating failure to thrive (6, 29, 31). The lack of difference in postnatal weight gain or fractional growth rate between control and untreated FGR pups of either sex is consistent with previous data, showing that postnatal weight in the offspring of nutrient restricted and *ad libitum* fed guinea pigs is proportional to birth weight but not related to any difference in postnatal growth rate *per se* (41). The sex dependency of the Ad.VEGF-A$_{165}$ treatment effect, whereby female but not male Ad.VEGF-A$_{165}$ treated offspring exhibited improved postnatal weight gain, may reflect alterations in body composition, in terms of lean and fat mass, which differ between male and female guinea pigs (22) and are known to be affected by both prenatal growth restriction (34, 40) and Ad.VEGF-A$_{165}$ therapy (11). However, fewer male than female offspring were studied in this cohort, because treatments were randomly allocated at the time of maternal surgery and not stratified by fetal sex. Collectively, these observations suggest that the beneficial effects of maternal uterine artery Ad.VEGF-A$_{165}$ therapy on body size are sustained postnatally but do not persistently increase growth rate.

The present study did not associate maternal nutrient restriction with increased blood pressure in the adult offspring, in contrast with the majority of studies conducted in growth restricted human infants (2) and experimental animals exposed to maternal undernutrition *in utero* (48, 69). Although pulse pressures were higher in the offspring of nutrient restricted compared to *ad libitum* fed dams, they were substantially lower than those measured previously in this species by indwelling catheter (41) or radiotelemetry (32). These low pulse pressure values are likely to be an artefact of suboptimal catheter patency or overdamping in the fluid-filled extension catheter used during blood pressure measurement (62) and therefore cannot be confidently attributed to an effect of the maternal treatment. In humans, it is well recognised that adults born after growth restriction have a high risk of cardiovascular disease and hypertension as described by the Barker hypothesis, which links both fetal growth and lifelong health to the prenatal environment (2). Certainly experimental manipulations in pregnant rodents, such as maternal calorie or protein restriction, or uterine artery ligation generally reduce birth weight and accelerate the age-dependent increase in systolic blood pressure in the

offspring (48, 69). Moreover, biomechanical and histological indicators of passive arterial stiffness are widely increased in rodents that are growth restricted by maternal undernutrition, uterine artery ligation or environmental hypoxia (10, 28, 53, 54, 67). The increases in blood pressure induced in F1 offspring rats by maternal protein restriction are furthermore transmitted to the F2 generation through the maternal line, in the absence of any further F1 insult, suggesting that an epigenetic mechanism underlies the abnormal cardiovascular phenotype (68). However, intrauterine growth restriction does not always foreshadow adult hypertension, commonly in animal models where maternal dietary restriction or uterine artery ligation occurs only in the latter half of pregnancy (33, 36). The mechanisms determining birth weight and programmed cardiovascular phenotype are therefore distinct. The discrepancy in blood pressure effect between this and previous studies may relate to the relatively young age at which the offspring are studied, less than 150 days, when they are still juvenile. Comparison with reference data for this guinea pig breed indicates that the animals were approximately 75% of full-grown adult body weight at the time of catheterisation (26). Although the maternal dietary restriction has been shown previously to increase systolic blood pressure in male offspring at this age, the increase is relatively small (<7mmHg) and is not accompanied by a change in mean arterial pressure (41). Such a change in systolic blood pressure following maternal dietary restriction may therefore have been sex specific and not statistically apparent using the relatively small number of pups studied here. The lack of measurable hypertension in the adult guinea pigs born to nutrient restricted dams is also consistent with the absence any alteration in basal activity or sensitivity of the adrenal glands, which are thought to contribute to developmentally programmed hypertension (30). The reduction in heart weight in the FGR female pups was consistent with smaller fetal cardiomyocyte number reported in other animal models of growth restriction (7, 52, 71), although there was no evidence of relative compensatory hypertrophy or ventricular thickening in this study, in line with the absence of offspring blood pressure changes. Overall, the results of this study do not indicate that maternal nutrient restriction programs offspring hypertension in the guinea pig, and therefore cannot provide evidence for uterine artery Ad.VEGF-A$_{165}$ therapy modifying the cardiovascular sequelae of FGR. Nonetheless, protective effects on postnatal cardiac and vascular function have been demonstrated in response to other therapeutic interventions that increase uteroplacental function, including intra-placental insulin-like growth factor adenovirus gene therapy (1) and pharmacological vasodilators such as sildenafil (35).

There are several limitations to this study, as already highlighted, that must be taken into account in the interpretation of the results and evaluation of the therapeutic efficacy of uterine artery extravascular Ad.VEGF-A$_{165}$ therapy for fetal growth restriction. Notably, we have not directly measured uterine artery blood flow or longitudinal fetal growth dynamics in response to the gene therapy in this study. The technically challenging maternal surgical intervention also reduced the number of pups available and therefore the power of the study to detect inter-group differences in birth weight, growth and cardiovascular phenotype, particularly against the background of litter size variability and sexual dimorphism. Nevertheless, taken together with the absence of any indication of abnormal histological, haematological or biochemical change in the adult offspring, the results of this study support maternal uterine artery Ad.VEGF-A$_{165}$ gene therapy as safe and suggest that it ameliorates the fetal growth responses to maternal nutrient restriction in a manner that is sustained postnatally.

PERSPECTIVES AND SIGNIFICANCE

Translation of maternal gene therapy into the clinic is complex. The European Commisssion funded EVERREST Project aims to carry out a phase I/IIa clinical trial to assess the safety and efficacy of maternal uterine artery Ad.VEGF gene therapy for severe early-onset FGR (24). No ethical or legal objections to the intervention, or to a trial of this intervention have been identified, and a potential trial of maternal gene therapy to treat severe early-onset FGR was found to be ethically acceptable to many key stakeholders and to women who had had a previous affected pregnancy (59). In patients, vector delivery into the uterine arteries could be achieved through interventional radiology, a techniques that has been used to prevent postpartum haemorrhage in placental attachment disorders (66). While this is more invasive than administering oral medication, it has the potential advantage of targeting vasoactive changes to the maternal uteroplacental circulation and reducing systemic effects. The data presented here adds to the preclinical evidence underpinning such a trial, suggesting that maternal VEGF gene therapy for FGR may improve growth in offspring and supports going forward to the clinic.

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685   **Table 1 Fate of experimental animals**

	Control	FGR	FGR+Ad.VEGF-A$_{165}$	Total
Sows				
Operated	14	10	15	39
Culled[#]	6	3	6	15
Miscarried	2	1	1	4
Delivered	6	6	8	20
Pup delivery outcomes				
Delivered	20	22	22	64
Neonatal death[†]	0	3	4	7
Pup surgical outcomes				
Operated	20	19	18	57
Culled[§]	6	6	6	18

686   Values represent number of individuals. [#]Culled on veterinary surgeon advice due to abdominal wound dehiscence (n=9), abdominal herniation (n=1) or
687   evidence of postoperative pain (n=5). [†]Stillborn (n=6), or runted and failing to suckle, culled on postnatal day 6 (n=1). [§]Culled due to poor recovery from
688   anaesthesia (n=5), complication during catheterisation surgery (n=6) or postoperative pain or complication (n=7).

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Table 2 Mean ± SEM (median, interquartile range) maternal weight, gestational age at delivery, litter size, birth weight and postnatal growth rate of live-born pups in untreated control sows and those undernourished to restrict fetal growth, and administered with vehicle or Ad.VEGF-A$_{165}$ gene therapy in mid-gestation.

	Control	FGR	FGR+Ad.VEGF-A$_{165}$	P value
Dams	n=6	n=6	n=8	
Body weight (g)				
Conception	824 ± 39 (823, 742-906)	832 ± 6 (831, 824-841)	802 ± 30 (788, 733-861)	0.751
Surgery	879 ± 44 (862, 797-983)	891 ± 9 (890, 873-909)	855 ± 33 (817, 799-940)	0.720
Term	1194 ± 60 (1235, 1048-1319)	1266 ± 33 (1240, 1198-1342)	1191 ± 44 (1220, 1101-1283)	0.475
Gestational age at term (days)	68.7 ± 0.8 (67.5, 67.0-69.0)	67.2 ± 1.2 (68.0, 66.5-68.0)	67.2 ± 2.2 (68.0, 65.5-69.75)	0.361
Litter size (pups)	3.7 ± 0.2 (4.0, 3.0-5.0)	3.7 ± 0.1 (4.0, 3.0-4.0)	3.4 ± 0.2 (4.0, 3.0-4.0)	0.608$^\$$
Male pups	n=7	n=6	n=8	
Birth weight (g)	**109 ± 4 (101, 100-122)**	**96 ± 2 (96, 92-100)**	**99 ± 3 (98, 93-107)**	0.050
Fractional growth rate (mg g^{-1} day^{-1})				
Weeks 1-4	78 ± 3 (80, 71-84)	86 ± 6 (80, 77-97)	83 ± 6 (83, 70-97)	0.588
Weeks 5-9	24 ± 3 (25, 18-29)	27 ± 3 (27, 21-33)	25 ± 3 (27, 19-30)	0.765
Weeks 9-12	12 ± 2 (12, 10-15)	12 ± 1 (12, 11-13)	13 ± 1 (13, 10-15)	0.940
Weeks 13-16	5 ± 1 (5, 4-7)	4 ± 1 (4, 3-5)	5 ± 1 (4, 4-7)	0.548
Female pups	n=13	n=13	n=10	
Birth weight (g)	**85 ± 3 (84, 78-92)**	**95 ± 4 (97, 78-103)**	**98 ± 4 (100, 89-105)***	0.032
Fractional growth rate (mg g^{-1} day^{-1})				
Weeks 1-4	82 ± 4 (86, 71-94)	74 ± 2 (78, 65-81)	81 ± 5 (81, 69-95)	0.274
Weeks 5-9	23 ± 1 (24, 19-25)	20 ± 2 (19, 17-23)	22 ± 1 (21, 20-25)	0.328
Weeks 9-12	11 ± 1 (11, 10-11)	11 ± 1 (10, 10-13)	12 ± 1 (12, 11-13)	0.261
Weeks 13-16	4 ±1 (5, 4-5)	5 ± 1 (5, 4-5)	5 ±1 (5, 4-6)	0.682

694   Values in bold indicate significant overall effect by one-way ANOVA or $^\$$Kruskal-Wallis test. *, P<0.05 versus control (Holm-Sidak post-hoc test).

Table 3 Mean ± SEM blood pressure, heart rate, cardiac morphometry and body weight in male and female adult offspring of untreated control sows and those undernourished to restrict fetal growth, and administered with vehicle or Ad.VEGF-A$_{165}$ gene therapy in mid-gestation.

	Control	FGR	FGR+Ad.VEGF-A$_{165}$	P (ANOVA)
Male pups	n=4	n=4	n=5	
Carotid arterial pressure (mmHg)				
Systolic	73.0 ± 1.4 (73.5, 70.2-75.3)	74.2 ± 4.5 (71.6, 67.6-83.4)	67.5 ± 3.0 (70.5, 60.4-73.3)	0.318
Diastolic	72.0 ± 1.1 (72.4, 69.8-73.9)	70.8 ± 4.3 (69.0, 63.8-79.6)	64.8 ± 3.0 (67.6, 57.7-70.5)	0.238
Mean	72.5 ± 1.2 (73.0, 70.0-74.4)	72.6 ± 4.4 (70.3, 65.9-81.7)	66.4 ± 3.0 (69.3, 59.2-72.0)	0.297
Pulse	**0.9 ± 0.4 (0.9, 0.24-1.63)**	**3.4 ± 0.7 (3.8, 2.0-4.4)***	**2.7 ± 0.3 (2.83, 2.2-3.1)***	0.009
Heart rate (bpm)	221 ± 44 (222, 144-296)	239 ± 26 (249, 185-283)	305 ± 18 (295, 272-342)	0.113
Heart weight (g)	3.6 ± 0.4 (3.6, 2.9-4.3)	3.9 ± 0.7 (3.5, 3.0-5.2)	3.3 ± 0.3 (3.4, 2.3-3.9)	0.591
% body weight	37 ± 4 (38, 29-44)	38 ± 3 (38, 35-41)	34 ± 4 (37, 25-39)	0.751
Left ventricular wall thickness (mm)	3.6 ± 0.3 (3.5, 3.2-4.2)	3.2 ± 0.2 (3.3, 2.9-3.4)	3.2 ± 0.2 (3.3, 2.8-3.5)	0.350
Right ventricular wall thickness (mm)	1.5 ± 0.1 (1.4, 1.4-1.6)	1.5 ± 0.2 (1.5, 1.1-1.8)	1.9 ± 0.1 (1.9, 1.7-2.1)	0.076
Septal thickness (mm)	3.2 ± 0.2 (2.9, 2.9-3.7)	3.4 ± 0.2 (3.4, 3.1-3.8)	3.3 ± 0.2 (3.4, 2.9-3.5)	0.625
Adrenal weight (g)	0.28 ± 0.03 (0.28, 0.22-0.32)	0.23 ± 0.02 (0.22, 0.19-0.28)	0.22 ± 0.03 (0.20, 0.15-0.31)	0.345
% body weight, x1000	55 ± 6 (55, 43-68)	45 ± 5 (45, 40-50)	47 ± 5 (40, 40-60)	0.573
Body weight (g)	902 ± 101 (895, 715-1092)	951 ± 34 (972, 882-1000)	911 ± 71 (910, 758-1066)	0.888
Female pups	n=10	n=9	n=7	
Carotid arterial pressure (mmHg)				
Systolic	70.5 ± 1.6 (70.7, 67.8-75.2)	70.2 ± 1.8 (71.6, 64.2-74.7)	70.9 ± 1.9 (70.7, 66.7-74.6)	0.969
Diastolic	68.7 ± 1.5 (69.2, 66.2-71.8)	67.1 ± 1.6 (68.8, 62.3-69.9)	68.4 ± 1.9 (68.1, 64.4-71.5)	0.257
Mean	69.6 ± 1.6 (69.9, 66.9-74.2)	68.8 ± 1.7 (70.9, 63.4-72.2)	69.8 ± 1.9 (69.5, 65.7-73.2)	0.922
Pulse	**1.4 ± 0.2 (1.5, 1.2-1.7)**	**3.1 ± 0.6 (2.1, 1.8-4.7)***	**2.5 ± 0.2 (2.6, 2.3-2.7)**	0.010
Heart rate (bpm)	210 ± 27 (253, 141-271)	271 ± 11 (279, 239-301)	264 ± 13 (264, 234-293)	0.069
Heart weight (g)	**3.5 ± 0.3 (3.3, 3.0-4.0)**	**2.7 ± 0.2 (2.8, 2.4-3.2)**	**2.8 ± 0.2 (2.9, 2.4-3.2)**	0.048
% body weight	**44 ± 4 (42, 40-47)**	**37 ± 3 (34, 31-44)**	**32 ± 2 (33, 27-37)***	0.042
Left ventricular wall thickness (mm)	3.5 ± 0.3 (3.7, 3.0-3.9)	3.5 ± 0.1 (3.5, 3.3-3.7)	2.9 ± 0.3 (2.9, 2.6-3.1)	0.124
Right ventricular wall thickness (mm)	1.7 ± 0.1 (1.6, 1.6-1.9)	1.7 ± 0.1 (1.7, 1.4-1.9)	1.8 ± 0.1 (1.8, 1.7-1.9)	0.844
Septal thickness (mm)	3.6 ± 0.2 (3.6, 3.2-4.1)	3.1 ± 0.2 (3.1, 2.8-3.5)	2.8 ± 0.1 (2.8, 2.7-2.8)	0.124
Adrenal weight (g)	0.22 ± 0.02 (0.21, 0.18-0.26)	0.24 ± 0.02 (0.24, 0.18-0.29)	0.22 ± 0.02 (0.21, 0.19-0.25)	0.756
%body weight, x1000	52 ± 5 (50, 40-63)	62 ± 5 (60, 55-70)	58 ± 4 (60, 45-70)	0.229
Body weight (g)	718 ± 52 (692, 609-778)	717 ± 42 (753, 570-835)	852 ± 12 (861, 841-864)	0.095

697   Values in bold indicate significant overall effect by one-way ANOVA. *, P<0.05 vs control by Holm-Sidak post-hoc test.

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FIGURE LEGENDS

Figure 1

Mean ± SEM net postnatal weight gain determined every 3 days from birth to weaning and weekly thereafter in (A) male and (B) female offspring of sham treated control sows (open circles, n=7 male pups, n=13 female pups), offspring of sows undernourished to induce FGR (black circles, n=6 male pups, n=13 female pups), and offspring of sows undernourished and given Ad.VEGF-A$_{165}$ gene therapy (grey circles, n=8 male pups, n=10 female pups). The effects of age (P$_{age}$), prenatal treatment and the interaction of the two (P$_{age*treatment}$) were determined by general linear model, accounting for birth weight as a covariate, and are given in the figure when significant. * P<0.05 vs controls, [†]P<0.05 vs untreated FGR at same age (Holm-Sidak post-hoc test).

Figure 2

Mean ± SEM (A) basal plasma cortisol concentration (B) plasma cortisol response to *i.v.* adrenocorticotrophic hormone challenge (1.25 µg kg^{-1}) and (C) area under curve of cortisol response to challenge in combined male and female adult offspring of untreated control sows (open circles/bars, n=5), those undernourished induce FGR (black circles/bars, n=10), and those undernourished and given Ad.VEGF-A$_{165}$ gene therapy (grey circles/bars, n=8). The effects of prenatal treatment and time from ACTH bolus (P$_{time}$) were determined by one-way ANOVA (A and C) or repeated measures two-way ANOVA (B) and are given in the figure when significant.
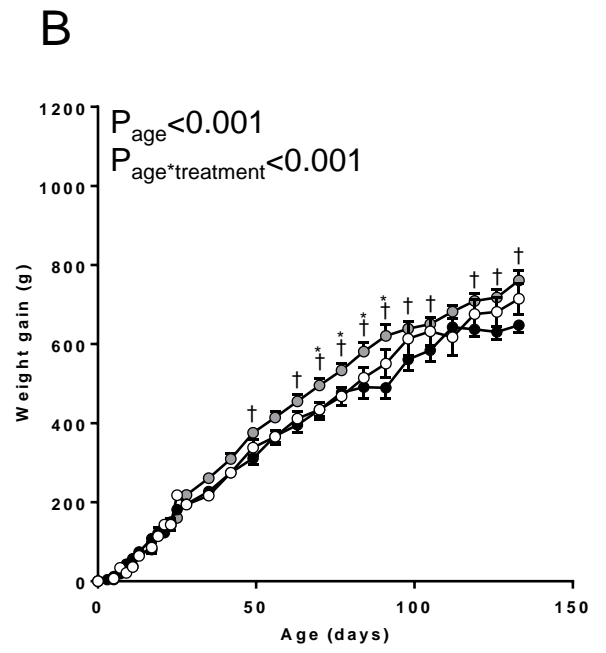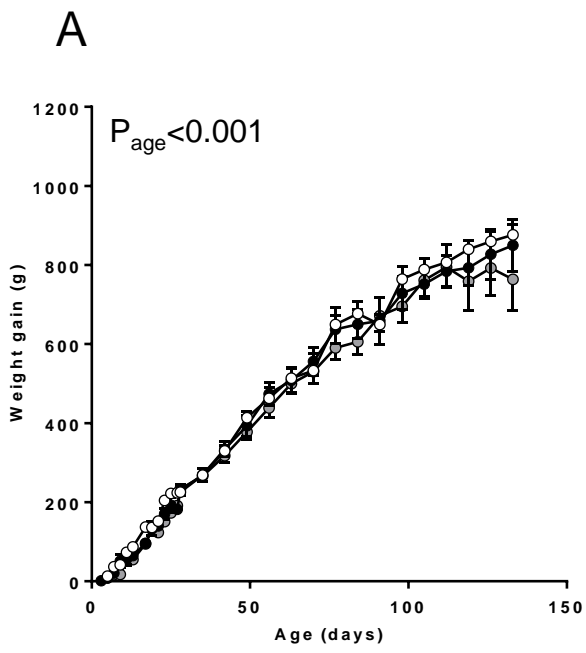
Figure 1

A

$P_{age}<0.001$

B

$P_{age}<0.001$
$P_{age*treatment}<0.001$

Figure 2