Human Gene Therapy

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Title

Maternal therapy with Ad.VEGF-A_{165} increases fetal weight at term in a guinea pig model of fetal growth restriction

Short title

Ad.VEGF-A_{165} therapy in fetal growth restriction model.

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Maternal therapy; adenovirus; VEGF; fetal growth restriction.

Abstract

Objectives

In a model of growth restricted sheep pregnancy, we previously demonstrated that transient uterine artery VEGF overexpression can improve fetal growth. We tested this approach in guinea pig pregnancies, where placental physiology is more similar to human.

Method: Fetal growth restriction (FGR) has been obtained through peri-conceptual nutrient restriction in virgin guinea pigs. Ad.VEGF-A_{165} or Ad.LacZ ($1x10^{10}$vp) were applied at mid-gestation in laparotomy, delivered externally to the uterine circulation via thermosensitive gel. At short-term (3-8 days post-surgery) or at term gestation, pups have been weighed and tissues sampled for vector spread analysis, VEGF expression and its downstream effects.

Results:

Fetal weight at term was increased (88.01±13.36g, n=26) in Ad.VEGF-A_{165}-treated animals, compared to Ad.LacZ treatment (85.52±13.00g, n=19, p=0.028). Brain, liver, lung weight and crown rump length were significantly larger in short-term analyses, as well as VEGF expression in transduced tissues. At term, molecular analyses confirmed the presence of VEGF transgene in target tissues, but not in fetal samples. Tissue histology analysis and blood biochemistry/haematological examination were comparable with controls. Uterine artery relaxation in Ad.VEGF-A_{165}-treated dams was higher compared to Ad.LacZ-treated dams.

Conclusions: Maternal uterine artery Ad.VEGF-A_{165} increases fetal growth velocity and term fetal weight in growth restricted guinea pig pregnancy.

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Introduction

Fetal growth restriction (FGR), where a fetus fails to achieve its genetic growth potential, affects up to 8% of all pregnancies and is a leading cause of perinatal death (1). Affected fetuses are also at increased risk for neonatal death, birth asphyxia and major long term morbidity (1). There is currently no treatment (2). Even for normally grown fetuses born very premature, small increases in fetal growth and gestation at birth are associated with major improvements in survival and morbidity (3,4). Therapeutic interventions for severe FGR might therefore be successful by improving outcome if they lead to even small increases in fetal growth, or later gestation at delivery.

More than half of FGR occurs secondary to placental insufficiency, which is characterized by reduced uterine blood flow (UBF). During pregnancy UBF increases tremendously as a result of increased maternal cardiac output, increased uterine artery diameter and trophoblast driven modification of the uterine spiral arteries (5)(6). Reducing UBF is known to cause FGR in animals such as the pregnant rat, guinea pig or sheep, emphasizing that fetal growth is dependent on UBF (7).

We have explored the therapeutic use of vascular endothelial growth factor (VEGF), a potent vascular endothelial cell mitogen, with powerful vasodilatory and pro-angioenic effects. These are mediated in part through the ability of VEGF to stimulate endothelial production of nitric oxide and prostacyclin via its major signalling receptor KDR (VEGF Receptor 2) (8). VEGF plays an important role in uteroplacental development, angiogenesis and blood flow, and in FGR, maternal serum levels of VEGF are significantly lower than in normal pregnancy (9)(10). We have developed a targeted approach using an adenovirus vector (Ad) to transiently over-express VEGF locally in the uteroplacental circulation. We have demonstrated that this increases UBF (11)(12)(13) and is associated with improved fetal growth velocity in a sheep paradigm of FGR (14). The placental physiology of sheep differs

substantially from humans (7). We selected the guinea pig for this study for a variety of reasons including its haemomonochorial placentation, that which is most similar to third trimester human placentation (15), homologous process of trophoblast invasion (15) and trophoblast cell proliferation (16), and a longer gestation than other rodents, making it thus more suited for therapeutic evaluation (7). Finally, periconceptual nutrient deprivation in the guinea pig impairs placental functional development, reduces the placental exchange and trophoblastic surface, increases the thickness of the exchange barrier and causes a 40% reduction in fetal weight with brain sparing (17)(18). Ad.VEGF-A_{165} induced reversal of FGR in this model, where there is a general impairment of placental function, would make these studies relevant to human FGR where the lesion is rarely simply due to reduced UBF. The primary aim of our study was to determine the effect of short term overexpression of VEGF-A_{165} applied locally to the uteroplacental site on fetal growth velocity and weight at term. Secondary aims were to assess safety aspects and possible mechanisms of action of the therapy.

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Materials and Methods

Animals

To induce FGR, female virgin Dunkin Hartley guinea pigs received 70% normal nutrient intake for 4 weeks before time-mating (18). Pregnancy was confirmed using ultrasound (18). 70% nutrient restriction was continued until day 35 of gestation, when it was increased to 90% until term at 60-65 days. Dams were given unlimited access to food during recovery from surgery. All procedures on animals were conducted in accordance with UK Home Office regulations and the Guidance for the Operation of Animals (Scientific Procedures) Act (1986), under Home Office Project Licence.

Animal surgery and vector injection

At mid-gestation (range 29-39 gestational days), under a general anaesthetic (ketamine, 44mg/kg and xylazine, 5 mg/kg IM, maintained by isoflurane inhalation, 1.5-2.0%) fetal measurements were taken (18) and a laparotomy was performed. Vector ($1x10^{10}$ particles per animal), either control (Ad.LacZ) or treatment (Ad.VEGF-A_{165}), suspended in freshly made thermosensitive gel (F-127, Sigma) was applied under direct vision to exposed uterine and radial arteries supplying both uterine horns as described (19). Dams were randomly assigned to VEGF or control vector and subsequent analyses were performed blinded to study group assignation. After a short period (3-8 days), or at term (60-64 gestational days), animals were killed for post mortem examination. Maternal and fetal measurements were recorded using electronic callipers (biparietal diameter (BPD), occipital snout length (OSL), crown rump length (CRL), femur length (FL)) and measuring tape (abdominal circumference (AC)), and tissues were extensively sampled. Fresh tissues were flash frozen in liquid nitrogen and stored at -80°C. Tissue for histological and immunohistochemical studies were fixed in 4% paraformaldehyde before being embedded in paraffin wax.

ELISA

Quantitative determination of VEGF protein expression in uterine arteries (UtAs), radial arteries (RAs) and serum was performed using ELISA, as recommended by the manufacturer (DVE00, R&D Systems, Abingdon, UK). Tissue samples were solubilized by homogenization in Radio ImmunoPrecipitation Assay (RIPA) Buffer (R0278, Sigma Aldrich). Protein content was determined using a Protein Assay Kit (500-0001, Bio-Rad,UK). Serum was assayed undiluted.

Molecular analyses

Ad.VEGF-A_{165} vector expression was assessed using semi-nested reverse transcription-PCR in maternal and fetal tissues, with method and target primers as described (12). Primers designed to amplify sequences within the guinea pig β-actin gene were used as a positive control (20). The reaction conditions for both rounds were initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 60 secs, annealing for 64°C for 60 sec and extension for 72°C for 60 secs, and a final extension period of 72°C for 10 mins. PCR products were analysed on a 1.2% agarose gel using GelRed fluorescent nucleic acid gel stains (VWR).

Biochemistry, haematology and histological analysis

Blood was collected from dams and pups at necropsy. Standard small mammal biochemistry and haematology panels were performed by Royal Veterinary College Clinical Services Diagnostic Laboratories. Control blood was from *ad libitum (ad lib)* fed dams from the same animal colony. Stained hematoxylin & eosin (H&E) maternal, fetal and placental tissue sections were reviewed by an expert perinatal and placental pathologist who was blinded to experimental group. Trophoblast invasion was scored from zero (no invasion) to three (maximal invasion), in uterine and radial arteries.

Intima to media ratios

H&E stained sections of the uterine arteries were imaged using a digital slide scanner (Nanozoomer, Hamamatsu). The relative thicknesses of the intimal and medial layers were determined with NDP.view software and used to calculate the intima:media ratios.

Immunohistochemistry

To evaluate neovascularisation around the uterine and radial arteries, artery sections were immunostained with anti-von Willebrand factor (1:200, A0082, Dako) using an ABC kit (Vector Laboratories, Peterborough, UK); 3,30- diaminobenzidine (Sigma Aldrich, Gillingham, UK) and a hematoxylin counterstain. Slides were imaged as above and positively stained adventitial blood vessels were counted. To assess apoptosis and proliferation, placental sections were stained with anti-cleaved caspase 3 (1:800, 9664S, Cell Signalling) or anti-Ki-67(1:125, M724029-2, Dako) respectively. Stained placental sections were imaged using a digital slide scanner, as above. To obtain cell counts, each image was divided into six sections, and a field of view at 20x magnification randomly captured from each section. Total positive cells per field of view were counted in the ImageJ program (National Institutes of Health, USA) using the Cell Counter function.

Western blot

Protein extracts from the snap-frozen uterine artery (UtA) tissues were used to estimate eNOS, iNOS and phosph-eNOS levels by western blotting. Tissue was lysed by sonication in RIPA buffer (R0278, Sigma, Dorset, UK) and the resulting lysates were analysed by SDS-PAGE on 4-20%% Tris-glycine gels (Novex EC6025BOX, Invitrogen, Paisley, UK), followed by electrotransfer onto nitrocellulose membranes (LC2001, Invitrogen, Paisley, UK). Membranes were blocked with 5% (w/v) bovine serum albumin (BSA, A9418, Sigma, Dorset, UK) in phosphate-buffered saline (PBS-T) for 1 h at room temperature, before being probed with the primary antibody by overnight incubation at 4°C, followed by incubation for

1 h at room temperature with a horseradish peroxidase (HRP)-linked secondary antibody (1:8000, Santa-Cruz Biotechnology) and detection using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Little Chalfont, UK), following the manufacturer's protocol. Immunoblots were quantified by scanning of films with a calibration strip and analysis by densitometry using ImageJ (National Institutes of Health). The primary antibodies used were monoclonal mouse anti-eNOS/iNOS Type III antibody (1:3000, 610296, BD Transduction Laboratories, Oxford, UK), monoclonal mouse anti-iNOS/NOS Type II antibody (1:3000, 610328, BD Transduction Laboratories , Oxford, UK) GAPDH was probed using a goat polyclonal, GAPDH antibody (V-18)-HRP conjugate (1:1000, 20357, Santa Cruz Biotechnology, Heidelberg, Germany).

M**yography**

Cleared uterine artery (UtA) segments 1-2 mm in length were mounted in a two-chambered wire myograph (410A, Danish Myotechnology, Aarhus, Denmark) run using Myodaq.2.vi software, and maintained according to standard protocols (21). Concentration response curves to phenylephrine (PE) and acetylcholine (ACh) were constructed for each vessel.

Statistics

Sample size calculations were based on the primary outcome of fetal birthweight, and a sample data set of birth weight data was obtained from 29 normal dams and 89 offspring. A Generalized Linear Mixed Models (GLMM) approach was used for statistical analysis as described (22), with dam and treatment (Ad.LacZ or Ad.VEGF-A_{165}) set as a factor, with fetus sex, litter size and gestational age (gestational days) at post mortem as covariates. Fetus sex was found to have no effect and was removed as covariate. GLMM statistical analysis was needed for our set of data as the model is predicting random effects in addition to the usual fixed effects, with non-normal data. Statistical significance was considered achieved if $P<0.05$, and p-values are reported.

Results

Seven dams treated with Ad.LacZ delivered of 19 pups, and 8 dams treated with Ad.VEGF-A_{165} delivered of 26 pups were included in the study. A further 10 females underwent surgery but were excluded from the study (Supplementary Table 1). Eight of those dams had immediate postoperative surgical complications: four miscarried 1, 5 and 6 days post-surgery, two sustained leg wounds during operative handling and were culled, one died secondary to aspiration pneumonia and one developed an abdominal infection and was culled. Two further animals were culled at later stages in consultation with veterinarians, one after exhibiting signs of distress and weight loss likely resulting from pregnancy toxaemia at day 5 postoperatively, and one with a wound hernia at day 7 postoperatively. The average litter size was 2.7±1.7 for controls and 3.25±1.8 for Ad.VEGF-A_{165} animals. There was no effect of treatment on litter size (p=0.50; 2 tailed T test).

Fetal Measurements

There was no significant difference between the groups in any fetal measurements taken at mid gestation by detailed fetal ultrasound before Ad vector treatment. Treatment with Ad.VEGF-A_{165} resulted in a significant increase in fetal weight at term (88.01±13.36g) compared to control Ad.LacZ treated fetuses (85.52±13.00g, p=0.028, Generalized Linear Mixed Models, Figure 1 and Table 1). There was no effect of treatment on placental weight or diameter, although placental depth was increased (Table 1a, Figure 1). Placental efficiency, as indicated by the placental/fetal weight ratio, was not significantly different (Table 1a). Other fetal growth measurements were significantly increased by Ad.VEGF-A_{165} treatment (Table 1b), including brain, liver, lung weight and crown rump length (CRL). Kidney weight, heart weight and biparietal diameter (BPD) were not affected. Ad.VEGF-A_{165} increased the fractional growth rate of the BPD and occipital snout length (OSL) head

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measurements and the brain to fetal weight ratio at term compared to Ad.LacZ treatment (Table 1b). Fractional growth rate of the abdominal circumference (AC), CRL, femur length (FL), placental diameter and thickness was not different between the groups (data not shown).

VEGF protein expression

VEGF expression following treatment with Ad.VEGF-A_{165} was confirmed by ELISA in collected samples. Protein was detected in maternal and fetal serum, with higher levels detected in fetal serum from dams treated with Ad.VEGF-A_{165} (maternal n=6, fetal n=5) than those treated with Ad.LacZ (maternal n=3, fetal n=4) (Figure 2a). VEGF protein was also detected in uterine artery samples (Ad.VEGF-A_{165} n=9 samples from 6 animals) but levels were not significantly higher compared to Ad.LacZ samples (n=3 samples from 6 animals, Figure 2b). Results were similar in the radial artery samples (Ad.VEGF-A_{165} n=7 samples from 6 animals, Ad.LacZ n=4 samples from 3 animals, Figure 2c).

Immunohistochemical analysis for VEGF expression, also at 3-8 days post therapy, showed specific staining of VEGF around the target vessels in both the Ad.LacZ- and Ad.VEGF-A_{165}-treated animals, with a visible increase in Ad.VEGF-A_{165} treated uterine arteries (Figure 3 a, d). We found no detectable difference in VEGF receptor 1 or 2 expression in the uterine arteries of Ad.LacZ- and Ad.VEGF-A_{165}-treated animals (Figure 3b, c, e, f). Overall immunohistochemical staining was more intense for all three proteins in radial arteries, with no clear difference between Ad.LacZ- and Ad.VEGF-A_{165}-treated animals (data not shown).

Vector spread

RT-PCR analysis showed the presence of the transgene in the target uterine and radial arteries of three of four Ad.VEGF-A_{165} treated animals (Figure 4). All other tissues tested (Maternal:

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uterine arteries, radial arteries, uterus, lung, heart, ovary, liver, adrenal gland, kidney, spleen, brain, retina, thymus: and Fetal: placenta, membranes, umbilical cord, lung, thymus, heart, spleen, liver, gonads, adrenal gland, kidney, muscle, skin, brain and bone marrow) were negative, with the exception of one maternal ovary sample.

Histological, haematological and biochemical evaluations

A standard small mammal haematology panel returned similar values for all term maternal serum submitted (VEGF n=3, LacZ n=1, *ad lib* n=3), within the standard range for the panel (Pathology department, Royal Veterinary College, Hawkshead, UK) and comparable to published guinea pig values (23). Fetal values differed as expected from maternal values, but there were no differences between values from fetuses of *ad lib* (n=3 from 3 dams), Ad.VEGF-A_{165} (n= 7 from 4 dams) and Ad.LacZ (n=2 from 1 dam) subjects, with the exception of mean corpuscular volume (MCV), which was slightly elevated in the Ad.VEGF-A_{165} treated fetuses (93.1±8.0fL) compared to Ad.LacZ treated and *ad lib* fetuses (82.0±6.8fL). In biochemistry tests, there were decreased levels of total protein and globulin and increased levels of potassium, compared with the standard ranges for small mammals and guinea pigs as before (23). However, these differences were preserved across all groups (Ad.VEGF-A_{165} n=5, Ad.LacZ n=1, *ad lib* n=5). As with the haematology, there were no difference between values from fetuses of *ad lib* (n=4 from 4 dams), Ad.VEGF-A_{165} (n= 8 from 5 dams) and Ad.LacZ (n=2 from 1 dam) subjects.

Microscopic histological examination of dams (n=2 each group), fetuses (n=2 each group) and placentas (control n=6, Ad.VEGF-A_{165} n=7) did not reveal any pathological observations. The proportion of animals with moderate or maximum (scored 2 or 3) trophoblast invasion in the uterine spiral arteries did not differ between dams treated with Ad.VEGF-A_{165} (4 of 10 dams) and Ad.LacZ (2 of 6 dams, P=1.00, Fisher's exact test).

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Neither was there a difference between treatment groups in the proportion of dams with moderate or maximal trophoblast invasion in the radial arteries (Ad.LacZ, 4 of 4 dams, Ad.VEGF-A_{165} , 3 of 5 dams, P=0.44, Fisher's exact test).

Mechanism of action

Myography experiments on uterine artery (UtA) segments sampled at post mortem examination 38 days ±5 post vector application showed no change in the mean contractile response to phenylephrine in the Ad.VEGF-A_{165} (n=7) transduced vessels when compared with Ad.LacZ vessels (n=5) (Figure 5a). Neither was there an overall change in the relaxation of vessels in response to acetylcholine as analysed by 2-way ANOVA with repeated measures (Figure 5b). However, at high concentrations (ACh= $3x10^{-8}$, $1x10^{-9}$M) prior to saturation, there was significantly more relaxation, with E_{max} values 72.7±5.8 for Ad.VEGF-A_{165} vs. 50.5±3.5 for Ad.LacZ (p=0.050, two-way ANOVA).

We observed no difference in intima/media ratios in uterine arteries between Ad.LacZ (n=7 arteries from 4 animals) and Ad.VEGF-A_{165} (n=11 arteries from 6 animals) treated animals, measured on haematoxylin and eosin stained sections (data not shown). The finding was the same in the radial arteries, where no differences between Ad.LacZ (n=24 arteries from 4 animals) and Ad.VEGF-A_{165} (n=15 arteries from 4 animals) treated animals were seen (data not shown).

We detected an increase in the levels of eNOS and iNOS between the Ad.LacZ (n=3) and Ad.VEGF-A_{165} (n=3) transduced uterine arteries by western blotting 3-8 days after gene transfer (Supplementary Figure 1). eNOS and iNOS expression was significantly higher in uterine arteries treated with Ad.VEGF-A_{165} compared to Ad.LacZ (eNOS: 1.58 ± 0.2 (Ad.VEGFA_{165}) vs. 0.69 ± 0.18 (Ad.LacZ), p<0.001; iNOS: 2.9 ± 0.59 (Ad.VEGFA_{165}) vs. 0.81 ± 0.1 (Ad.LacZ), p<0.001).

Ad.VEGF-A_{165} transduction increased the average number of von Willebrand factor-stained adventitial blood vessels around the uterine arteries (77.5±38.6, n=12 arteries from 8 animals) compared with Ad.LacZ transduction (32.5±14.2, n=8 arteries from 5 animals, p=0.006, t test) (Figure 6a). In the radial arteries the difference in number of adventitial vessels failed to reach statistical significance (Ad.VEGF-A_{165}: 26.4±19.2, n=28 arteries from 8 animals, Ad.LacZ: 16.4±13.5, n=20 arteries from 6 animals, p=0.051, t test). The number of vessels per mm^2 of adventitial tissue was lower in radial arteries compared to the uterine arteries (Figure 6b).

The restricted diet had a significant effect on both proliferation and apoptosis in the placenta. Proliferation was reduced in Ad.LacZ (19.8±20.4 cells/field of view, n=5 placentas from 5 dams) and Ad.VEGF-A_{165} treated animals (37.3±34.2, n=4 placentas from 4 animals) compared to *ad lib* animals (70.1±25.0, n=6 placentas from 3 dams), as measured by positive Ki-67 staining (p=0.001, Mann Whitney U test) (Figure 6c). Similarly, apoptosis was reduced in Ad.LacZ (90.7±128.5 cells/field of view, n=5 placentas from 5 dams) nutrient restricted animals compared to *ad lib* animals (342.8±45.4, n=6 placentas from 3 dams), as measured by positive cleaved caspase-3 staining (p=0.001, Mann Whitney U test) (Figure 6d). Following Ad.VEGF-A_{165} treatment however, there was a significant increase in the number of cleaved caspase-3 stained cells (287±114.8, n=4 placentas from 4 animals, p=0.025, Mann Whitney U test), such that there was no difference between *ad lib* and Ad.VEGF-A_{165} treated groups (p=0.179, Mann Whitney U test).

Discussion

In this study we provide further evidence, in a guinea pig model of fetal growth restriction that mid-gestation maternal gene therapy giving transient local over-expression of VEGF in the uteroplacental circulation improves fetal growth rate and fetal weight at term. We compared the results of animals treated with Ad.VEGF-A_{165} Ad.LacZ vector with two contemporaneous groups of (1) normally fed non-FGR guinea pigs and (2) untreated maternal nutrient restricted FGR guinea pigs as reported previously (18). We confirmed short term VEGF expression in fetal and maternal blood and in transduced maternal uterine vessels without detectable vector transmission to the fetus. There was upregulation in eNOS and iNOS detected in transduced vessels, and enhanced vascular relaxation short term. Guinea pigs have a haemomonochorial placentation, which is the most similar to human placentation in an animal model, with the exception of some non-human primates. In contrast to our work in sheep, this model is also closer to the human condition, in that the FGR is early rather than late onset, and therapy is applied mid-gestation at approximately the same stage as would be feasible in humans (therapy 29-39 gestational days, term 65 gestational days vs therapy 20-26 weeks gestation, term 40 weeks gestation). Thus, experimental results obtained from pregnant guinea pigs are likely to hold great relevance for clinical translation.

Although the mean weight increase is a modest 3%, there was nevertheless a significant improvement in fetal growth rate in fetuses receiving Ad.VEGF-A_{165}. Even for normally grown fetuses born very premature, small increases in fetal growth and gestation at birth are associated with major improvements in survival and morbidity (3,4). Therapeutic interventions for severe fetal growth restriction might therefore be successful by improving outcome if they lead to even small increases in fetal growth, or later gestation at delivery. Several other fetal measurements were significantly increased in response to Ad.VEGF-A_{165}

gene therapy, with treated fetuses having an increased crown rump length, brain and liver weight, although no change in the abdominal circumference was detected. This is in contrast to ultrasound measurement findings from our previous research in sheep (14) and from other groups studying the FGR mouse (22). Placental weight, diameter and area were not affected by Ad.VEGF-A_{165} treatment, with only placental depth being increased. The gain in fetal weight did not appear therefore to be mediated through an increase in placental dimensions.

Delivery of Ad.VEGF-A_{165} to the uterine and radial artery increased expression of VEGF shortly after vector delivery, showing efficient transduction of the vector, and high concentration of VEGF as detected in fetal blood serum, as well as a mild increase in maternal blood serum. We propose that released VEGF-A_{165} protein promoted an increase of eNOS and iNOS levels, via functional activation of VEGFR-2, potentially being the factor leading to angiogenesis and hypotension in the target vessels (24). We cannot speculate at this stage about the mechanism by which Ad.VEGF-A_{165} induces neovascularization in the uterine and radial artery system, as it will require further studies. Immunohistochemistry of the guinea pig uterine arteries (UtA) and radial arteries (RA) showed increased VEGF staining in Ad.VEGF-A_{165} treated animals, supporting the ELISA data. There was also positive staining for VEGR-1 and VEGFR-2, thus either or both of these receptor could have been involved in mediating the effects seen following Ad.VEGF-A_{165} treatment.

Uterine arteries from Ad.VEGF-A_{165} treated dams demonstrated a greater relaxation in response to acetylcholine when examined in a myograph. This complements our previous work in the sheep, where the relaxation response in uterine arteries collected at short term time points was significantly increased in Ad.VEGF-A_{165}-transduced vessels (11). Others have observed *in vitro* that VEGF-A_{165} is a powerful vasodilator of the fetoplacental

vasculature (25), and adult vascular tone is regulated via VEGF-A induced vasodilation (26). ACh-induced relaxation of guinea-pig uterine artery is dependent on the release of nitric oxide (NO) from vascular endothelial cells (27).

We also observed enhanced uterine artery vascularization via immunohistochemical staining of new blood vessels in the adventitia. In animal experiments, VEGF gene transfer is capable of inducing therapeutic angiogenesis in diverse tissues and organ systems. Overexpression of VEGF using viral vectors can stimulate significant neovascularization in skeletal muscles and myocardium (reviewed in (28)). Our own work has suggested that an increase in UBF is likely to be mediated in the longer term through neovascularisation within the perivascular adventitia (12,13).

Safety will be an important consideration for translation into the clinic. Reassuringly there was no transgenic protein expression in any fetal tissues, as determined by RT-PCR. Only one maternal sample of non-target tissue tested positive, from an ovary sample, likely due to an inadvertent positioning of the gel/vector mixture proximal to the ovary during surgery. Pluronic gel has been used previously as a delivery vehicle for adenoviral vectors to the carotid artery of rats (29)(30)(31). We have demonstrated that external vascular application of Ad. vector combined with pluronic gel achieves very high levels of localized gene transfer and expression specifically at the site of administration in the uterine arteries in guinea pigs when compared to direct intravascular injection (19). The relatively smaller size of the uterine artery in guinea pigs compared to sheep limited the feasibility of intravascular injection. In pregnant women however, transfemoral uterine artery catheterization with temporary balloon occlusion of the vessel lumen could be used as a minimally invasive technique.

FGR in sheep and humans is associated with smaller placentas and reduced cellular proliferation (32)(33)(34). We found similarly a significant reduction of proliferation in the placenta of the nutrient restricted guinea pigs. Treatment with Ad.VEGF-A_{165} did not affect the level of proliferation compared with Ad.LacZ-treated animals, as both remained lower than *ad lib* fed animals. FGR in the human is associated with increased placental apoptosis. In our study, however we found the opposite effect. Diet restricted animals treated with Ad.LacZ had significantly less apoptosis in the placenta than animals fed *ad lib*. However, treatment with Ad.VEGF-A_{165} restored apoptosis back to *ad lib* level. Whether this is a phenomenon confined to the guinea pig is unknown. It is unlikely to be a product of the Ad. treatment, as no evidence of vector was found in any placental samples.

The degree of rescue of FGR seen in this study in nutrient restricted FGR guinea pigs did not appear to be as much as in our previous in sheep where FGR was created in the over-nourished adolescent ewe (14, 35). There are differences in the animal models and experimental techniques that may explain this. The overnourished FGR sheep is a highly manipulated and controlled experimental model where exclusively singleton pregnancies with maximum genetic homogeneity are created by *in vitro* fertilization using eggs from superovulated donor ewes inseminated by a single sire with embryo transfer into adolescent ewes that are subsequently over fed. In contrast the nutrient restricted FGR guinea pig conceives over a 3-5 day period, carries between 1-5 pups, and is genetically heterogeneous (7, 18), resulting in a wider range of offspring weights and gestational ages than is found in the FGR sheep. The vector was applied externally to the uterine and radial arteries of the guinea pig circulation by necessity because of the small vessel size, using a thermolabile pluronic gel technique (19) rather than a direct uterine artery injection with stasis of blood

flow used in the sheep study, which delivers the total dose concentrated to the vessel. In addition the vector dose was double in the sheep experiments: $1x10^{10}$vp per guinea pig ($1x10^{10}$ per kg assuming a dam weight of 1kg), compared to $1x10^{11}$ vp per sheep ($2x10^{10}$ per kg assuming a ewe weight of 50kg). To ensure the optimum gene transfer, in the clinical trial we plan to use an intravascular injection technique with doses comparable to those used in the sheep study.

This study has confirmed that maternal gene therapy with Ad.VEGF-A_{165} is able to increase the fetal growth rate and term weight of fetuses in an animal model with placentation relevant to human FGR. Transduction with Ad.VEGF-A_{165} appears to achieve this outcome by increasing UtA vasorelaxation and promoting adventitial angiogenesis, with virtually no vector spread outside target tissues. Further work in this model to examine the long term effects of Ad.VEGF-A_{165} therapy in treated offspring will lend weight to applications for translation to the clinic.

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Author Disclosure Statement: ALD and DMP are consultants for Magnus Growth, for which they receive a token consultancy payment and shareholding in the company. ALD is

director of Magnus Growth, part of Magnus Life Science, which is aiming to take to market a novel treatment for fetal growth restriction. IZ is a consultant for Magnus Life, part of Magnus Life Science for which he receives a token consultancy payment and shareholding in the company.

Maternal therapy with Ad.VEGF-A165 increases fetal weight at term in a guinea pig model of fetal growth restriction (doi: 10.1089/hum.2016.046)
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Figures and tables legends

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Table 1

a) Fetal weight and placental measurements:

Maternal uterine artery treatment	Ad.LacZ n=19[a]	Ad.VEGF-A_{165} n=26[b]	p-value
fetal weight	85.52 ± 13	88.01 ± 13.36*	0.028
placental weight	5.33 ± 1.04	4.93 ± 0.18	0.220
placental diameter	27.96 ± 2.54	26.92 ± 4.95	0.228
placental depth	8.66 ± 1.83	8.96 ± 1.62**	0.001
fetal/placental weight ratio	0.061±0.01	0.058±0.01	0.282

b) Fetal size characteristics and fractional growth rate:

Maternal uterine artery treatment	Ad.LacZ n=19[a]	Ad.VEGF-A_{165} n=26[b]	p-value
lung weight (g)	1.62 ± 0.37	1.73 ± 0.29**	0.002
liver weight (g)	4.38 ± 1.41	4.76 ± 1.41*	0.027
kidney weight (g)	0.43 ± 0.12	0.40 ± 0.09	0.205
heart weight (g)	0.49 ± 0.12	0.49 ± 0.06	0.413
brain weight (g)	2.24 ± 0.35	2.48 ± 0.18*	0.039
biparietal diameter (BPD) (mm)	20.03 ± 1.18	20.30 ± 1.54	0.348
BPD fractional growth rate (mm/day)	0.309 ± 0.093	0.32 ± 0.081**	0.008
crown rump length (CRL) (mm)	108.92 ± 8.62	113.61 ± 7.26*	0.020
occipital snout length (OSL) (mm)	40.18 ± 4.63	43.2 ± 2.20*	0.033
OSL fractional growth rate (mm/day)	0.863 ± 0.166	0.88 ± 0.081*	0.040
abdominal circumference (AC) (mm)	94.82 ± 5.67	94.71 ± 6.60	0.991
brain/fetal weight ratio	0.026 ± 0.004	0.029 ± 0.005*	0.036

Table 1: Fetal weight and placental measurements of FGR pregnancies following mid-gestation treatment with Ad.LacZ or Ad.VEGF-A_{165} (a); Fetal size characteristics and fractional growth rate in pregnancies affected by FGR following mid-gestation maternal uterine artery treatment with Ad.LacZ or Ad.VEGF-A_{165} (b). [a]n=19 pups from 7 dams; [b]n=26 pups from 8 dams. All data are means ± standard deviation. Data were analysed by Generalized Linear Mixed Models. Significant difference between treatment groups, * $P<0.05$, ** $P<0.01$

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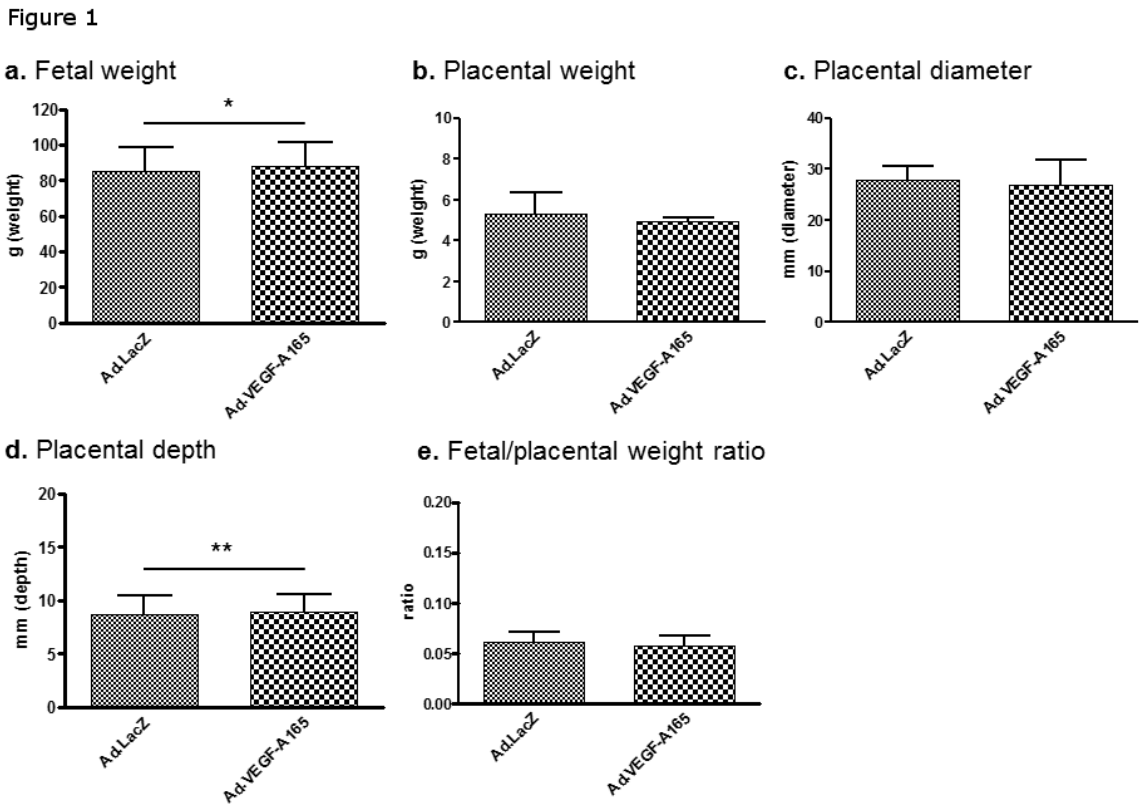

Figure 1: Fetal weight and placental measurements of FGR pregnancies following mid-gestation treatment with Ad.LacZ (n=19 pups from 7 dams) or Ad.VEGF-A_{165} (n=26 pups from 8 dams). All data are means ± standard deviation. Data were analysed by Generalized Linear Mixed Models. Significant difference between treatment groups, * $P<0.05$, ** $P<0.01$.

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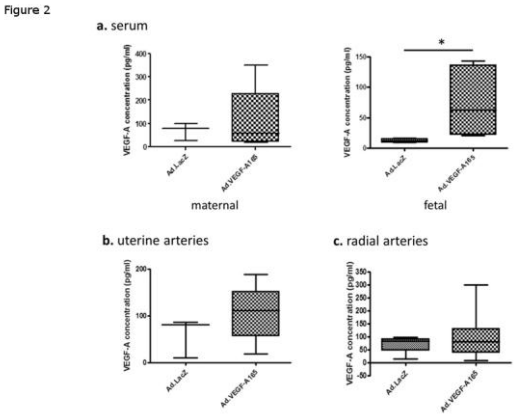

Figure 2: ELISA measurement of VEGF-A in the serum (a), uterine arteries (b), and radial arteries (c) following treatment with Ad.LacZ or Ad.VEGF-A_{165}. Samples were collected 3-8 days following vector application.

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Figure 3

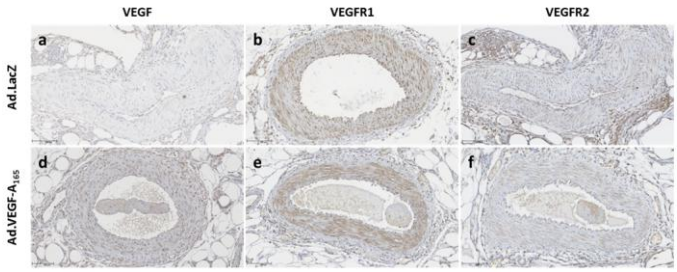

Figure 3: Immunohistochemistry for localisation of expression of VEGF (a, d), VEGFR1 (b, e) and VEGFR2, (c, f) in the uterine arteries following gene therapy with Ad.LacZ or Ad.VEGF-$A_{165.}$ Samples were collected 3-8 days following adenoviral transduction.

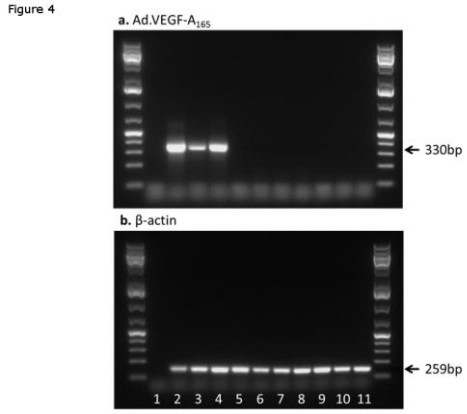

Figure 4: RT-PCR to examine vector spread. (a) A semi-nested reverse transcription (RT)-PCR with primers specific for Ad.VEGF-A_{165} in guinea pig maternal and fetal samples was positive in the Ad.VEGF-A_{165}-transduced uterine and radial arteries, but negative in other tissues. (b) RT-PCR with primers specific for the β-actin gene is positive in all guinea pig samples. Lane 1, no template control; 2, positive control of human umbilical vein endothelial cells infected in vitro with Ad.VEGF-A_{165}; 3, uterine artery; 4, radial artery; 5, maternal heart; 6, maternal lung; 7, placenta; 8, umbilical cord; 9, fetal heart; 10, fetal lung; 11, fetal gonad. A 1kb DNA ladder was used for sizing.

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Figure 5

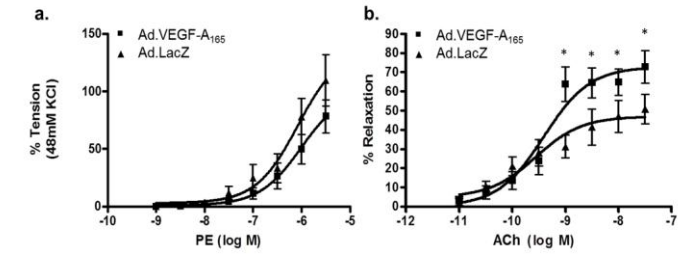

Figure 5: Vascular reactivity of uterine arteries 3-8 days after vector administration, measured by wire myography. (a) Logarithmic dose-response curve to L-phenylephrine (PE). The contractility of the vessel is expressed as a percentage of the response to KCl. (b) Logarithmic dose-response curve to acetylcholine (ACh). The relaxation is expressed as a percentage of inhibition of PE-induced contractions. Error bars denote standard deviation. *Relaxation $p<0.05$, tension non-significant.

Figure 6

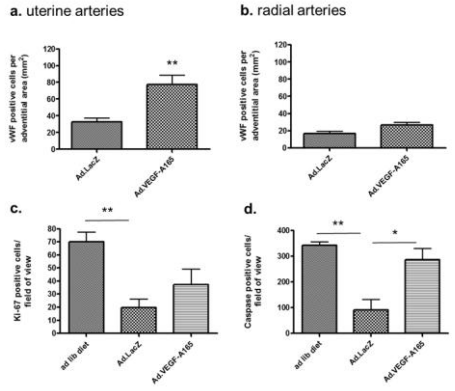

Figure 6: Immunohistochemistry analysis of endothelial cell proliferation and placental turnover. Von Willebrand Factor staining for vessels in the adventitia of uterine (a) and radial (b) arteries showed an increase in neovascularisation around uterine, but not radial, arteries in Ad.VEGF-A_{165}-treated animals. Diet restriction reduced both proliferation (c) and apoptosis (d) in the placenta. Ad.VEGF-A_{165} treatment partially restored apoptosis. Error bars denote standard deviation.

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Supplementary Table 1

Guinea Pig dams included in the study (n=15)

Number of dams	Treatment	Pups generated
7	Ad.LacZ	19
8	Ad.VEGF-A_{165}	26

Guinea Pig dams excluded from the study due to surgical complications (n=10)

Number of dams	Reason for exclusion
4	Miscarriage at 1, 5, and 6 days post-surgery
2	Leg wound during operative handling, culled
1	Aspiration pneumonia
1	Abdominal infection
1	Sign of distress and weight loss post-operation, culled
1	Wound hernia post-operation, culled

Supplementary Table 1: Detailed report on number of Guinea Pig dams included (a) and excluded from the study (b), with details that determined their exclusion.

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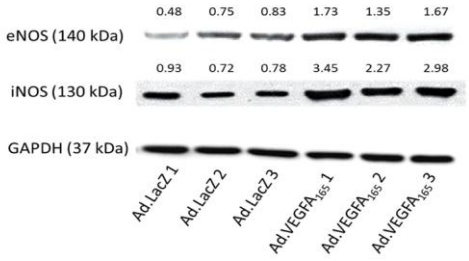

Supplementary Figure 1: Representative Western blot for eNOS/iNOS (1 of 3 performed, GAPDH as housekeeping). Quantification is determined in relation with GAPDH.