In vitro human placental studies to support an adenovirusmediated VEGF-DNC maternal gene therapy for the treatment of severe early-onset fetal growth restriction

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In vitro human placental studies to support an adenovirus-mediated VEGF-D Δ N Δ C maternal gene therapy for the treatment of severe early-onset fetal growth restriction

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Reply to Reviewer's Comments

Reviewer: 1

Comments to the Author

It is a very novel and interesting study, which I believe would provide major support in answering some issues especially of safety aspect on fetal effect for clinical implementation in such EVERREST study. Regulations states that research without direct benefit to the mother of fetus should involve only minimal fetal risk. phase I trials often use a dose escalation design, where the starting dose may be so low that no beneficial effect is anticipated.

If I'm not mistaken, this study was addressed also to find optimal dose for delivering VEGF but unfortunately the dose stop at the level where an artefact leak should be avoided and it had caused FLAG tag for making gene transcription was negative. This is interesting because what we would have expected to see more if the correct dose was adequate were changes in vascular endothelial site, diameter of villous vessels, and wall thickness of the vessels. This would provide well powered data for saying that gene transcription process after the manipulation has been working well in adequate dose to produce protein that can cause good vascular remodelling process proven by histopathological findings. After this condition has been achieved and vector is not found in fetal venous perfusate, the conclusion would be solid.

And the one that concern me the most is that this study although has been designed and executed tremendously neat, almost near to perfection, but the immunological complex reaction were not particularly described. Immunological reaction aroused by viral penetration and gene synchronization process could lead either viral destruction by host or either host cell destruction. Although LDH release has been evaluated in this study but some parameters such as cytokines and other inflammatory agents should be evaluated also to make a sharp results. And also in contra-direction immunological reaction could also potentially diminish adenovirus, resulting no effect even in prompt dose rationally expected. Truthfully I do not now whether the immunological complex reaction could be provided by in vitro placenta set up for this study.

Nevertheless, I could say that this study is remarkable, took a lot of efforts to be done brilliantly.

It is well designed, well executed, and well written.

There is no reason at all not to accept this manuscript.

Some writing corrections:

- In page 16 line 38-36,

point numbers should be corrected in accordance with the order

(iv) placental endocrine release; (iv) placental structure and (iv) whether clinical grade....
should be corrected to (iv)....; (v) and (vi)

- Some line spacings are not consistent.

For example page 30 line 25-26 and line 39-40 compare to page 31 line 12-13

- Some margins are not justify consistently.

For example page 13 line 8-22.

We thank the reviewer for his / her careful review of our work and for the very generous complements given to our efforts.

We respectfully considered the comments made about the desire to investigate a dosage that might cause an equivalent of no- and lowest observed-adverse-effect levels (NOAELs/LOAELs), regarding placental function. Before embarking on this study we took detailed scientific advice from the Medicines Health Regulatory Authority and the Paul-Ehrlich-Institut as to the best reproductive toxicology studies to use to translate maternal VEGF gene therapy for fetal growth restriction into the clinic (EVERREST trial). Although the the human placenta models employed were not recognised reproductive toxicology models, they were considered to be worthwhile and informative pre-clinical trial test systems, particularly to test out the critical questions of whether exposure of the maternal side of the placental to the highest achievable vector dose would result in short term damage to the human placental barrier and whether the vector crossed over into the fetal-side placenta. So the stringency of experimental design was centred on surpassing the intended clinical dose for use in the EVERREST trial and not on determining the NOAELs/LOAELs. These have been investigated in a reproductive toxicology study in pregnant rabbits that has been conducted by the EVERREST consortium. Furthermore, the planned phase I EVERREST clinical trial will use a 3+3 dose escalation design as the reviewer suggests, with the starting dose may not produce any beneficial effect, but at the same time likely to have involve minimal risk to the fetus. To some extent, the unexplained marginally elevated maternal-side LDH release suggests that we might have begun to achieve an effect at a comparatively exaggerated villous exposure for both time and dose, compared to the intended *in vivo* application, albeit non-adverse with respect to other simultaneous outcome measures, like resistance and permeability. Importantly, the authors felt comfortable with the measured outcomes in both the explant and the perfusion model, subject to follow-on reproductive toxicology animal model testing, since we had exceeded the intended clinical dosage.

We agree with the reviewer that it would have been interesting to have studied changes in vascular endothelial site, diameter of villous vessels, and wall thickness of the vessels however the necessary short term nature of the human placental perfusion model (up to 5 hours) means that such analyses are not feasible. After this time there is breakdown of the placental barrier and cell death, rendering the model inadequate to properly address questions relating to the effect of gene transcription, transgenic protein production and vascular remodelling.

The immunological complex question that the reviewer raises is an interesting one. However, since viral penetration into the tissue was minimal and limited to extra-villous fibrin deposits found above the trophoblast basement membrane, we did not pursue this thought. Also, viral-trophoblast interaction was not found on staining in the placental explant study after a prolonged vector exposure. Coxsackie adenoviral receptors are absent in the human placental syncytiotrophoblast again suggesting this problem would be limited at the maternal blood–trophoblast interface. For these reasons we chose not to pursue questions of immunological effects.

The writing corrections have been made in the revised document “Desforbes-Main-document-post-review-PB AD -PB.doc”. The authors are grateful to the reviewer.

Reviewer: 2

Comments to the Author

Major comments:

The authors thank the reviewer for his / her appraisal of their work.

1. The authors state that “VEGF-D has a more favourable structural and functional profile than VEGF-A” (page 9). Add references and justify.

We have added the following referenced justifications for this statement to this part of the Introduction, on Page 8 of the revised document “Desforbes-Main-document-post-review-PB AD - PB.doc” :

“In HUVECs, in contrast to VEGF-A, VEGF-D weakly stimulated prostacyclin production and gene expression, had little effect on cell proliferation, and stimulated a smaller and more transient increase in intracellular $[Ca^{2+}]$. Nonetheless, VEGF-D was still biologically active, inducing a comparatively strong, but more transient phosphatidylinositol 3-kinase (PI3K)-mediated Akt activation compared to VEGF-A. VEGF-D increased PI3K-dependent endothelial nitric-oxide synthase phosphorylation compared to control conditions; and promoted cell survival, albeit more weakly than VEGF A {Jia, 2004}.”

2. Why was AdLacZ used and not an empty vector containing only a CMV promoter (AdCMV)? Expression of LacZ can induce immune reaction. Please justify the use.

Ad.LacZ was used in separate explant parallel cultures to indicate the potential location of the Ad. VEGF -D^{ΔNAC} vector in the human placental tissue. It does not replace or represent the potential of Ad.VEGF-D^{ΔNAC} to evoke responses within the tissue. Even considering the potential of Ad.LacZ to evoke an immune response, there was no observable changes to barrier structure following exposure.

3. Only part of patient demographics is presented in Suppl. Table 2. e.g. data of AdLacZ transduction of human placenta is not presented. Add all data and n/groups to all figure texts. How many placentas showed positivity for LacZ staining?

We have formed a new table (Supplementary Table 2a) to show the demographics of the explant investigation. The perfusion demographics are now in a renumbered "Supplementary Table 2b." Placental explants from all placentas showed some positive staining for β -galactosidase, but positive staining was sparse and as we point out in the paper, it was extremely rare to find incidences of viral vector access beyond the epithelial basement membrane within the villous stroma, and no staining could be detected at fetoplacental endothelium.

Show VEGF-D/FLAG immunohistostainings of AdVEGF-D treated placentas on day 5-7. There should be expression at this time point. How many placentas were tested? Did you do qPCR as well to detect VEGF-D levels? Additionally, stain vessels with CD31 as VEGF-D is angiogenic and should have an functional effect.

The study was designed following consultation with the medicines regulators (Medicines Health Regulatory Authority, MHRA and the Paul-Ehrlich-Institut, PEI). Ad.VEGF-D was not analysed within the explant tissues with qPCR as this arm of the study was to investigate potential morphological and endocrine changes within the tissue, and to determine the highest vector dose that we would best be able to apply for placental perfusion experiments. As we found no significant effects with the highest Ad.LacZ dose applied to the explant model we used the highest Ad.VEGF-D vector dose possible for the perfusion experiments. The critical question from the regulators (MHRA and PEI) was to use the *ex vivo* perfusion model to test for maternofetal transfer of the virus, within fetal venous perfusate.

4. Show VEGF-D levels in relation to hCG and LDH release (Figure 2).

As explained above, we do not have this data, as it was not considered appropriate to the toxicology testing scientific advice given.

5. LDH was 2-fold higher with VEGF-D in comparison to FB. Show the measurements with various AdLacZ VPs. Which LacZ VP amount was used in figure 2? Is this an effect of the transgene or adenovirus capsid i.e. immunity-related event?

The two fold elevation in LDH was only seen in the *ex vivo* dual perfusion model and not in the explant model (Figure 2 data). Ad.LacZ was not used in the *ex vivo* perfusion model, only the explant model. The explant model was a more chronic exposure model that would permit β -galactosidase expression. Interestingly, although Ad.LacZ was not tested in the perfusion model, any changes in transgenic protein expression would never have been detected within the time-frame of the perfusion experiments. Hence, LDH release in the perfusion experiments must have been consequential of a different mechanism, since its relative elevation became apparent at the two hour time point and was significant within a mere 3 hours.

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3 6. It is stated that LDH increase is a concern and the clinical trial will be performed with a lower dose
4 (p25). However in suppl. table 1 it is mentioned that 10^{11} VPs can be used in the trial. Please clarify
5 and discuss how this risk is taken into account in planning the trial.
6

7 From Table 1, the number of viral particles delivered and the dose of viral particles delivered in the
8 *ex vivo* model (5×10^{11} vp & 5×10^{10} vp / mL) exceeds that planned for the EVERREST trial (1×10^{11} vp &
9 2×10^{10} vp / mL). In fact, there is a typo in this latter value for the *in vivo* intervillous space
10 concentration, which should read 2×10^9 vp / mL. This makes the placental villous exposure dose
11 calculated for the EVERREST clinical trial to be 25 times lower than that of the *ex vivo* placental
12 model. We have added in this information to the manuscript. We have corrected Supplementary
13 Table 1, accordingly and apologise for the error.
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- 19 7. In figure 3 it is unclear how many samples were used per group. The variation is big therefore
20 making the statistics non-significant. Add the numbers/group to the figure text and discuss whether
21 the result is reliable in general or needs more replicates/group.
22

23 We have modified the legend of Figure 3 to state that this resistance and clearance markers relate to
24 N=3 formulation buffer experiments and N=7 Ad.VEGF^{ANAC} experiments. It is normal to see such
25 variation in the clearance of paracellular markers, which are membrane limited in their transfer
26 dynamics; and have a tendency to show variation across time due to the non-vascularised nature of
27 the intervillous space and the capacity for altered flow streams around the villi, where exchange
28 happens. On the whole it was reassuring to see that mean levels were unaltered following treatment
29 and retesting of clearance. It was important to have designed the experiment with a pre-treatment
30 test period for this reason.
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- 37 8. The manuscript contains sections “results and discussion” and “discussion and clinical impact”.
38 Modify and remove all materials&methods from the results section.
39

40 We have moved methods text from the “Results & Discussion” and placed it in the Supplementary
41 section. However, we cannot justify taking short methods statements sections from the “discussion
42 and clinical impact sections” as these reiterations are important in maintaining the context of the
43 discussion, and we feel, the understanding of the reader.
44
45

- 46 9. Future clinical trial is described in the “results and discussion” section. Additionally the human *ex*
47 *vivo* placenta model is compared to that of other animal models (3.5 pages). Although very
48 interesting, this makes the manuscript harder to read. Shorten and remove some of the text to
49 “discussion and clinical impact section”.
50

51 We have moved some of the text about the future clinical trial to Discussion and Clinical Impact
52 section as requested, but kept in the detail needed to allow the reader to appreciate the rationale
53 for the design of the reported human placenta studies. We have added in detail at the start of the
54 Discussion to remind the reader of the aim of the study.
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The sections on human explant culture and the human *ex vivo* dual placental perfusion model have now been moved from the Results & Discussion of the main document to the Methods section and therefore in the Supplement of the paper. We feel it is important to keep the full content of this section, as this study was the first to use this human perfusion model in ground-breaking gene-therapy toxicology testing. The detail was most pertinent in discussion with the regulators (MHRA and PEI) and so it is useful to have sufficient detail for future investigators, paving the way in the design of future gene therapy toxicology studies.

Minor comments:

1. Suppl. Table 1 presents a comparison of sheep and human models. However it seems that most of the data is missing from the sheep model. Please modify the table/title and add N/A.

Thank you for pointing out this omission. We have added in the relevant data and added N/A where appropriate.

2. Remove speculative texts “it is unlikely that significant gene transfer to the placenta will occur” (page 20) and “access the fetal circulation is only likely to be minimal” (page 26).

We feel that we have substantiated this speculation in the study, but have now prefixed these statements with the following additional text: “assuming transfer dynamics are comparable between the *ex vivo* model and the *in vivo* environment....” This text now appears on page 15 & 21, respectively.

3. Shorten the whole manuscript e.g. introduction (5.5 pages). Organize the tables and figures to follow the text. e.g. now the table/figure order is: Suppl. Table 2, Suppl. Table 1, Suppl. Fig 1, Suppl. Table 3 etc. Regroup supplemental figures together.

We have cut back the introduction where possible while keeping in important considerations about FGR, which we appreciate will be an unusual indication for a gene therapeutic approach.

4. Did not find figure texts for Suppl. Fig 5 and 6.

These have now been provided in the revised document “Figure Legends - Supplement”

***In vitro* human placental studies to support an adenovirus-mediated VEGF-D^{ΔNΔC} maternal gene therapy for the treatment of severe early-onset fetal growth restriction.**

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Short title: Human placental studies in VEGF-D^{ΔNΔC} gene therapy

Key words:

fetal growth restriction, adenovirus, vascular endothelial growth factor (VEGF), gene therapy, placental perfusion

Abbreviations:

ANTPTM: antennapedia gene from *Drosophila melanogaster*,

Ct: Threshold cycle,

CT: cytotrophoblast

DNA: Deoxyribonucleic acid,

FB: formulation buffer

gDNA: genomic DNA,

LacZ: β -galactosidase gene

LLOQ: Lowest limit of quantification,

NTC: No template control (negative control containing no reference analyte),

pfu: particle forming units

PCR: Polymerase chain reaction,

qPCR: quantitative polymerase chain reaction,

QC: Quality control,

RE%: Relative error (indicator of accuracy),

SCT: syncytiotrophoblast

VEGF^{ΔNΔC}: Pre-processed “short” form of Vascular Endothelial Growth Factor D isoform

Vp: viral particles,

Abstract (300 words max).

Severe fetal growth restriction (FGR) affects 1:500 pregnancies, is untreatable and causes serious neonatal morbidity and death. Reduced uterine blood flow (UBF) is one cause. Transduction of uterine arteries in normal and FGR animal models using an adenovirus (Ad) encoding VEGF isoforms, increases UBF and improves fetal growth *in utero*. Understanding potential adverse consequences of this therapy before first-in-woman clinical application is essential.

Objective: To determine whether Ad.VEGF-D^{ΔNAC} (i) transfers across the human placental barrier and (ii) affects human placental morphology, permeability and primary indicators of placental function and trophoblast integrity.

Methods: Villous explants from normal term human placentas were treated with Ad.VEGF-D^{ΔNAC} ($5 \times 10^{7-10}$ virus particles (vp)/mL), or virus formulation buffer (FB). Villous structural integrity (H&E staining) and tissue accessibility (LacZ immunostaining) was determined. Markers of endocrine function (hCG secretion) and cell death (LDH release) were assayed. Lobules from normal and FGR pregnancies underwent *ex vivo* dual-perfusion with exposure to 5×10^{10} vp/mL Ad.VEGF-D^{ΔNAC} or FB. Perfusion resistance, paracellular permeability, and hCG, alkaline phosphatase and LDH release were measured. Ad.VEGF-D^{ΔNAC} transfer across the placental barrier was assessed by qPCR in DNA extracted from fetal-side venous perfusate, and by immunohistochemistry in fixed tissue.

Results: Villous explant structural integrity and hCG secretion was maintained at all Ad.VEGF-D^{ΔNAC} doses. Ad.VEGF-D^{ΔNAC} perfusion revealed no effect on placental permeability, fetoplacental vascular resistance, hCG secretion, or alkaline phosphatase release but there was a minor elevation in maternal-side LDH release. Viral vector tissue access in both explant and perfused models was minimal, and the vector was rarely detected in the fetal venous perfusate, and at low titre.

Conclusion: Ad.VEGF-D^{ΔNAC} did not markedly affect human placental integrity and function *in vitro*. There was limited tissue access and transfer of vector across the placental barrier.

Except for a minor elevation in LDH release, these test data did not reveal any toxic effects of Ad.VEGF-D^{ΔNAC} on the human placenta.

Introduction.

Fetal Growth Restriction (FGR) is a major obstetric problem globally that affects up to 8% of all pregnancies. In this condition, the fetus fails to achieve its genetically determined growth potential and is born far smaller than anticipated. Most commonly placental insufficiency is the cause, whereby abnormally low uterine blood flow (UBF) restricts delivery of nutrients to the fetus resulting in slowing or even cessation of fetal growth. In approximately 10% of cases FGR is due to fetal structural abnormalities, aneuploidy, congenital viral infection or maternal medical disorders.

FGR is an important cause of perinatal mortality. It is implicated as the cause of death in over 50% of antepartum stillbirths, amounting to approximately 1.46 million fetuses worldwide/year ¹. Cohort studies suggest that the probability of encountering true FGR becomes most likely amongst fetuses that have an EFW below the 5th centile ²⁻⁴.

In the most severe cases of FGR (approx 1:500 fetuses), fetal growth slows in the middle third of pregnancy, termed severe early onset FGR, and delivery or fetal death occurs before 28 weeks of gestation. Such fetuses are usually detected first at the detailed ultrasound scan performed in mid-pregnancy (20-24 weeks of gestation) that is offered to all women in middle and high income countries. Commonly a notched uterine artery Doppler waveform is detected; this is a hallmark of reduced UBF and placental insufficiency. Subsequent scans over 2 – 4 weeks can reveal that fetal growth velocity is severely reduced and even ceases. Signs of fetal compromise develop, with progressive fetal Doppler abnormalities before fetal death ensues ^{5,6}.

There are no proven screening methods for FGR and, following its identification, there are no therapies in current obstetric practice that improve fetal growth *in utero*. Thus, management

is to deliver the fetus before death occurs *in utero* with the aim of minimising irreversible organ damage, particularly to the brain. This adds additional risks to the baby from extremely preterm birth, with its own attendant short and long-term complications ⁷. When the EFW is <500g, many condition the fetus to be non-viable. From cohort studies and local data ⁸, we estimate that the cumulative stillbirth/morbidity/neonatal mortality rate in pregnancies affected by severe early onset FGR is between 75-80% ^{5, 9, 10}.

For severe early-onset FGR the only management options currently available to parents are (i) to terminate the pregnancy, (ii) to await inevitable fetal death, or, (iii) to deliver the fetus by caesarean section if it achieves an EFW between 450 and 500 g, in the knowledge that there is a high chance of postnatal death or disability ¹¹. Survival studies suggest that even modest increases in birthweight (e.g from 500 to 600g) and gestation at delivery (e.g from 26 to 27 weeks) are associated with significant improvements in mortality and morbidity ¹². The risk benefit ratio is such that an antenatal therapy that led to even a small increase in fetal growth, gestation at delivery, or survival *in utero* is likely to improve outcome, with major health and economic benefits ¹³.

The central role of UBF providing adequate nutrients and oxygen to the conceptus appears key to the treatment of FGR. During human pregnancy, uterine perfusion normally increases dramatically from 50 mL/min in week ten to 500mL/min by mid-gestation and up to 1300 mL/min by full term (40 weeks gestation) ¹⁴. This occurs through trophoblast replacement of the endothelium and medial smooth cells of the uterine spiral arteries, changes that are enhanced by the placental production of vasoactive substances, such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF). VEGF and, to a lesser extent, PIGF cause angiogenesis and activate endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) and hence vasodilatation. A failure of uterine artery remodelling during

pregnancy leads to impaired blood flow to the placenta, restricting oxygen and nutrient delivery, therefore compromising fetal growth¹⁵. In pregnancies complicated by FGR, sonographically measured total uterine blood flow is reduced by 36.7% by 24 weeks until the end of pregnancy, as compared with normal pregnancy¹⁶.

Manipulation of angiogenic factors is one therapeutic option in FGR¹⁷. VEGF is essential for vasculogenesis and angiogenesis during development¹⁷; it vasodilates and improves blood flow in diverse animal blood vessels and vascular beds¹⁸⁻²¹, effects mediated partly through its stimulation of endothelial production of nitric oxide²⁰⁻²² and prostacyclin, mediated via its major signalling receptor KDR (VEGFR2)^{22, 23}. Local application of VEGF gene or protein therapy may prove therapeutically useful by stimulating vascular protective endothelial functions^{23, 24}. The major VEGF-A isoform, VEGF-A₁₆₅, is the predominant and most potent form in humans and it binds to VEGF Receptors 1 and 2. VEGF-D acts via VEGF Receptors 2 and 3. Both are angiogenic, but VEGF-D appears to have a more favourable structural and functional profile than that of VEGF-A₁₆₅, eliciting a more restricted range of biological responses, and may therefore have fewer side effects, making it the agent of choice for therapeutic angiogenesis. In HUVECs, in contrast to VEGF-A, VEGF-D weakly stimulated prostacyclin production and gene expression, had little effect on cell proliferation, and stimulated a smaller and more transient increase in intracellular [Ca(2+)]. Nonetheless, VEGF-D was still biologically active, inducing a comparatively strong, but more transient phosphatidylinositol 3-kinase (PI3K)-mediated Akt activation compared to VEGF-A. VEGF-D increased PI3K-dependent endothelial nitric-oxide synthase phosphorylation compared to control conditions; and promoted cell survival, albeit more weakly than VEGF A²⁵.

In a series of pre-clinical experiments, we have now shown in sheep and guinea pig models that local VEGF gene transfer to the uteroplacental circulation using adenovirus (Ad) vectors injected into the uterine arteries, leads to increased uterine blood flow, attenuated uterine

artery constriction but increased vascular relaxation. In transduced uterine arteries there is perivascular adventitial angiogenesis where VEGF expression is detected, and increased expression of eNOS, inducible NOS, and VEGF receptor^{26, 27}. These changes are associated with improved fetal growth in sheep and guinea pig FGR pregnancies without detectable spread of the vector across the placenta to the fetus, or any effect on maternal or fetal haemodynamic measures²⁷⁻³². Local maternal uterine artery Ad.VEGF gene therapy therefore seems a promising therapeutic for severe early onset FGR.

In the clinical context, vector delivery into the uterine arteries could be achieved through interventional radiology, which is supported by the Royal College of Obstetricians and Gynaecologists as a prophylactic measure before delivery in women at high risk of postpartum haemorrhage^{33, 34}. While this technique is more invasive than administering oral medication, it has the potential advantage of targeting vasoactive changes to the maternal uteroplacental circulation. The EVERREST Project aims to carry out a phase I/IIa clinical trial to assess the safety and efficacy of maternal uterine artery Ad.VEGF-D^{ΔNΔC} gene therapy for severe early-onset FGR. The project, funded by the European Union (EVERREST, www.everrest-fp7.eu), involves a multinational, multidisciplinary consortium, including experts in bioethics, fetal medicine, fetal therapy, obstetrics, and neonatology^{35, 36}. As well as performing a formal reproductive toxicology evaluation in pregnant rabbits, it was agreed in consultation with the relevant regulatory authorities that we would determine whether maternal administration of Ad.VEGF-D^{ΔNΔC} had any adverse effects in the human placenta. The two issues identified in scientific advice were firstly whether the Ad vector would damage the structure and function of the human placenta, and secondly how much, if any, of the Ad vector would cross the placental barrier between the mother and the fetus. Direct delivery of gene therapy to the fetus is currently deemed unacceptable because of concerns

about the associated risks^{24, 37}. For severe early onset FGR, the proposal is to deliver gene therapy to the maternal circulation, and not to directly target the fetus.

The human placenta is responsible for the transfer of substances between maternal and fetal circulations including oxygen and all the necessary nutrients for fetal growth^{38, 39}. It consists of a fetal blood-facing endothelial cell layer on a basement membrane and a maternal blood-facing syncytialised epithelial layer, the syncytiotrophoblast (SCT). Maternal blood is directed into the placental IVS from the uterine arteries where it then bathes the fetal-derived branched chorionic villous trees, allowing oxygen diffusion and nutrient transport. In human placenta, the SCT is renewed during pregnancy by a process of cellular turnover involving proliferation of underlying cytotrophoblast (CT) cells followed by their differentiation, fusion, and incorporation of nuclei into the SCT^{40, 41}. SCT renewal from the CT is essential to preserve its function as a solute transporting epithelium and endocrine/paracrine organ, maintaining nutrient delivery to the fetus and producing hormones that sustain pregnancy.

Here we have performed *in vitro* and *ex vivo* studies to investigate the effect of Ad.VEGF-D^{ΔNAC} on markers of human placental function and tissue integrity, and have examined the risk of Ad vector transfer into and across the human placenta, using two well recognised human placental models, the explant culture model and the *ex vivo* placenta perfusion model.

Results and Discussion.

1. Clinical Trial.

The clinical trial that these pre-clinical studies intend to support, will be an uncontrolled, open-label, dose finding safety and tolerability study (to mother and neonate) conducted in up to four EU member states. The entry criteria are designed to identify the most severe early onset FGR fetuses that have the highest perinatal mortality and morbidity, as described in the Discussion and Clinical Impact section. The study drug will be a replication deficient Ad. vector expressing short form vascular endothelial growth factor D, Ad.VEGF-D^{ΔNAC} manufactured to GMP. Following informed consent Ad.VEGF-D^{ΔNAC} will be delivered by intra-arterial balloon catheter to both uterine arteries sequentially, using X-ray-guidance⁴². During vector instillation blood flow within the uterine artery will be interrupted for up to 5 minutes using an occlusive balloon, a technique used previously in pre-clinical sheep studies²⁷⁻³⁰. Detailed maternal and fetal assessment will be made before and at intervals after vector instillation including changes to haematological, biochemical and hepatic blood parameters. Timing of delivery will be based on fetal Dopplers and growth, and/or maternal condition according to current clinical practice. Dose escalation will follow a 3+3 expansion design⁴³.

2. Objectives and Study Design.

The goal of this pre-clinical study was to investigate the effect on the human placenta of maternal-side exposure to Ad.VEGF-D^{ΔNAC}, including measures and markers of placental function, tissue integrity, gene transfer to the trophoblast, and vector cross-over to the fetal circulation. Pre-clinical studies of safety and efficacy have already been conducted in the pregnant sheep and guinea pig^{27-32, 44}, and reproductive toxicology studies are ongoing in the pregnant rabbit. However, placentation differs structurally and functionally between species and each has its strengths and weaknesses in terms of relevance to the human³³. The sheep is well established as a model in fetal physiology but is of limited value for placental research

because the ovine placenta is epitheliochorial, there is no trophoblast invasion of uterine vessels, and pregnancy immunology is very different. FGR can be induced in sheep by maternal overnutrition³⁰, or decreasing UBF by midgestation, which reduces term fetal size by 35-40%. It is worthwhile noting that the spiral arteries of sheep remain intact over the course of ovine pregnancy, retaining vasomotor control⁴⁵. Thus pregnant sheep offer some important similarities to the human situation observed in FGR pregnancies in which the spiral arteries are also not transformed and placental blood flow is impeded.

Amongst rodents, guinea pig placentation is the most similar to human placentation with a haemochorial type of placenta, a homologous process of trophoblast invasion^{45, 46} and trophoblast cell proliferation⁴⁷. Periconceptual maternal nutrient restriction is a well-established guinea pig model for the study of placental transfer and FGR, creating a reduction in fetal weight of approximately 35% and brain sparing, as in human FGR⁴⁸. However there is a significant variation in the gestational length of guinea pig pregnancy (63-72 days) and there is limited reproductive toxicology historical data available.

Non-human primates were considered as a possible safety model to support clinical translation. However the number of offspring is low, the gestational period is long, and there is a high rate of natural fetal loss, which means that the data obtained from such studies would be less useful. The pregnant rabbit is the most appropriate animal model for human pregnancy during the second trimester because it has the same haemodichorial placentation. There is also considerable historical rabbit data in the literature for comparative purposes, making it the animal model of choice for the planned reproductive toxicology study.

Because of the innovative nature of the proposed treatment, we felt it important to provide further safety analysis in a system as close to *in vivo* human pregnancy as possible. Using the human placenta after birth provided just this opportunity, and a further advantage is that we

could test out the effect of Ad.VEGF-D^{ΔNAC} in placentas from FGR affected pregnancies.

Two different human placental model systems have been used: *in vitro* human placental villous explant culture and *ex vivo* dual perfusion of the human placenta.

Human placenta explant culture involves maintaining dissected placental villous fragments in culture for 7 days^{51, 52} and recapitulates the normal event of *in vivo* SCT denudation and repair that is ongoing in the human placenta. In the first 2 days following villous dissection, the SCT layer is shed but by day 4-5 it has regenerated by processes of cellular turnover resembling those *in vivo*⁵¹. This new SCT layer is morphologically indistinguishable from the original. Therefore, this model allows for the effects of interventions on the newly fused SCT to be investigated^{52, 53}. Details of the experimental design in the employment of this model can be found in the Methods Supplement.

Human *ex vivo* dual placental perfusion has the advantage of introducing the viral vector to the SCT under conditions of simulated haemodynamics within the IVS and preserves villous volume density⁵⁴. The model involves independent perfusion of fetal vascularised and maternal non-vascular circulatory systems in one lobule of the placenta at a low resistance to flow, with a physiological buffer at supraphysiological soluble oxygen concentrations to compensate for the absence of haemoglobin⁵⁵. The model developed over 40 years ago has been used extensively to study transplacental transfer of biological agents, xenobiotics, water, nutrients and other endogenous substances including studies of placental toxicity, e.g. the acute effects of cadmium on placental zinc transport⁴⁹. The transfer of hydrophilic molecules across the placenta occurs through water-filled routes of a finite radius, which restricts the movement of larger molecules such as VEGF⁵⁷. This is supported by data from *ex vivo* human placental experiments, where the placenta exhibits an asymmetric release of synthesised free VEGF-A²⁰. Nevertheless, we considered it pertinent to study potential

changes to the paracellular route to capture any VEGF elicited permeability changes in the placental barrier. Evaluations of the model by various investigators have shown that the tissue architecture and metabolism remains intact, including after short ischaemic episodes which occur following delivery prior to ex vivo perfusion; and also after more prolonged controlled ischaemic experimental episodes^{58, 59}. Details of the experimental design in the employment of this model can be found in the Methods Supplement.

Selection of study participants

Procedures were followed in accordance with institutional guidelines and conformed to the standards set by the latest revision of the Declaration of Helsinki⁵⁰. All tissue was acquired with written consent from fully informed women attending St Mary’s Hospital, Manchester for their antenatal care under ethical approval 08/H1010/05 (North West Haydock Park Research Ethics Committee) and the University of Manchester Ethics Committee. Cases of fetal structural and genetic abnormalities, known infection, maternal renal disease, cardiovascular disease and diabetes were excluded. Placentas from both normal vaginal and caesarean section (emergency and elective) delivery were used. In experiments using the placental explant culture model, only placentas from pregnancies with an individualized birthweight centile (IBC) within the normal growth range (i.e. IBC 10-90) were used. In the *ex vivo* dual perfusion study, placentas from pregnancies with a full range of IBCs were used, including those from the normal IBC range (>10th centile), SGA cases (6th-10th centile) and those with a high likelihood of FGR (<6th centile). Patient demographics are described in

Supplementary Table 2.

3. Summary of Data.

Ad.VEGF-D^{ΔNAC} dose-ranging in human placental explants

Ad.VEGF-D^{ΔNAC} did not have any detrimental effect on tissue structure and integrity (**Figure 1a**). Ad.LacZ vector control exposure followed by LacZ gene product localisation showed that the virus had a limited capability for accessing the SCT; this occurrence was rare. It was also extremely rare to find incidences of viral vector access beyond the epithelial basement membrane within the villous stroma; and no staining could be detected at fetoplacental endothelium (**Figure 1b**).

These data are consistent with previous work showing that the human placenta is an effective barrier to the maternofetal transfer of viruses. Expression of the Coxsackie and adenovirus receptors on trophoblast cells varies with gestational age and trophoblast phenotype. The receptors, that are required for adenovirus infection, are continuously expressed in invasive CT but not in the SCT thus rendering the SCT resistant to maternal Ad infection and limiting transplacental transmission^{51, 52}. Adenovirus vector transduction of trophoblasts *in vitro* is determined by trophoblast differentiation, with efficient gene transfer to the inner CT, but reduced efficiency after it terminally differentiates into the multinucleated SCT^{53, 54}. Thus during the late second trimester, when we propose to expose pregnant women to Ad. vectors, assuming transfer dynamics are comparable between the *ex vivo* model and the *in vivo* environment, it is unlikely that significant gene transfer to the placenta will occur.

The explants study also showed that there was no detectable effect of the vector on hCG secretion (**Figure 2a**) and LDH release (**Figure 2b**), measures of placental endocrine function and cell death respectively. The lack of any toxic effect of Ad.VEGF-D^{ΔNAC} in the explant work directed us to evaluate the effect of the highest possible dose (i.e. 5×10^{10} vp/ml) of the Ad.VEGF-D^{ΔNAC} in the *ex vivo* dual perfusion model of the human placental lobule.

Placental perfusion with Ad.VEGF-D^{ΔNAC}

It was apparent from inspection of data that there was no difference in outcome of perfusion between normal and SGA/FGR groups (see **Supplementary Table 7**), so a decision was taken to merge all data, leaving two groups related to the administration of (1) Ad.VEGF-D^{ΔNAC} vector and (2) the FB. The perfusion outcome data are summarised as follows:

- (i) Effects on homeostatic regulation of placental villous microvascular resistance: Fetal-side inflow hydrostatic pressure, used as a measure of villous microvascular resistance to flow, did not change across time and between groups (**Figure 3a**).
- (ii) Effects on the transfer of paracellular markers, as an indication of altered transplacental permeability: There was no acute effect of high dose maternal side Ad. vector exposure on placental permeability of the membrane-limited paracellular markers, creatinine and FITC-inulin (**Figure 3b** and **3c**). The steady state maternofetal clearance values (K_{mf}) in the presence of Ad.VEGF-D^{ΔNAC} and FB were 18.0 ± 2.8 and 18.7 ± 2.1 $\mu\text{l}/\text{min}/\text{g}$, respectively, for creatinine and 1.7 ± 0.3 and 1.5 ± 0.6 $\mu\text{l}/\text{min}/\text{g}$, respectively, for FITC-inulin (mean $\pm$ SE). Neither Ad.VEGF-D^{ΔNAC} nor the FB had any effect on clearance when data was compared to pre-administrative K_{mf} values (Mann Whitney U-tests).
- (iii) Effects on placental barrier integrity: There was no significant development of a fetomaternal perfusate leak across time and no corresponding difference in fetal-side venous flow recovery between treatment groups (FB: 40 ± 30 $\mu\text{l}/\text{min}$, Ad.VEGF-D^{ΔNAC}: 110 ± 70 $\mu\text{l}/\text{min}$, mean $\pm$ SE averaged across T=3hr:31min – T=4hr:11min; 2-way ANOVA: NS; **Supplementary Figure 2**). Alkaline phosphatase release into the maternal circulation was unaltered following Ad. VEGF-D^{ΔNAC} vector administration (**Figure 4a**). However, LDH release into the maternal circulation was higher with the Ad.VEGF-D^{ΔNAC} vector than with the FB (**Figure 4b**).

(iv) Effect on placental endocrine release of hCG: hCG release from the trophoblast layer into the maternal placental venous perfusate was not altered following Ad.VEGF-D^{ΔNΔC} or FB administration (**Figure 4c**).

(v) Histological analysis (H&E staining) was performed on sections obtained from unperfused control placentas and treated placentas that were perfused with either Ad.VEGF-D^{ΔNΔC} or FB. Placentas were analysed blind to treatment. There were no differences observed in placentas perfused with Ad.VEGF-D^{ΔNΔC} or FB. Placental integrity was maintained in perfused placentas; the only difference was that perfused placentas were devoid of blood, compared to unperfused control placentas (**Figure 5**). Immunofluorescence to detect expression of the VEGF-D^{ΔNΔC} FLAG tag was negative. This is probably due to the short time allowed for perfusion, which fell within the time required for potential FLAG marker gene transcription. Total perfusion time was restricted in this model to maintain QC standards in the prevention of an artefact leak, which develops with time.

(vi) qPCR analysis of Ad.VEGF-D^{ΔNΔC} DNA in fetal venous perfusates was optimised, by first spiking control fetal venous perfusates with known quantities of Ad.VEGF-D^{ΔNΔC} DNA and subsequently deciding on the best method to retrieve 100% of this spiked DNA. A number of methods to extract and quantify Ad. vector DNA from perfusate, including standard DNA extraction kits and direct quantification (without extraction), were tested in attempts to achieve 100% recovery. Good extraction efficiency was obtained only when using DNA extraction kits based on anion exchange chemistry rather than silica-based chemistry, with 100% recovery of spiked Ad.VEGF-D^{ΔNΔC} DNA, without significant contamination by PCR inhibitors, in volumes of perfusate between 330 μL and 700 μL (see **Supplementary Materials**). Extracted Ad.VEGF-D^{ΔNΔC} DNA was quantified using a TaqMan qPCR method. The range of application of the assay was found to be between 5 and 50000 viral particles (vp)/μL of extracted DNA, with a linear correlation coefficient greater

than 0.99. The limit of detection was 2.5 vp/ μ L of extracted DNA (see **Supplementary Materials**).

vii) Fetal venous perfusates were tested for Ad.VEGF-D ^{Δ NA^C} DNA to determine vector spread across the placental barrier. All the sentinel controls returned no detectable quantity of Ad.VEGF-D ^{Δ NA^C} DNA. All the environmental controls returned no detectable quantity of Ad.VEGF-D ^{Δ NA^C} DNA, showing the absence of cross-contamination during sample collection. Among the 120 in-study samples tested, Ad.VEGF-D ^{Δ NA^C} DNA was not detected in 111 samples and was not quantifiable in one sample (**Table 1**). After re-extraction of the remaining eight samples, Ad.VEGF-D ^{Δ NA^C} DNA was not detected in four of these samples (Exp4 F22, Exp4 F23, Exp5 F16 and Exp5 F20) but a qPCR inhibition was still detected in these samples, even after re-extraction (RE% ranging from -64.17 to -50.23, **Table 1**). No conclusion could be made regarding the presence of Ad.VEGF-D ^{Δ NA^C} DNA in these samples, It is unlikely however that a large quantity of vector was present as the PCR inhibition was minimal and vector levels, if any, would be close to the LLOQ of the PCR assay. Ad.VEGF-D ^{Δ NA^C} DNA was detected and quantified in a further four samples, all from experiments 8 and 9 (Exp8 F14, Exp8 F22, Exp9 F13 and Exp9 F17) with quantities varying between 23.3 and 2.93×10^3 vp/well corresponding to 2.10×10^3 to 2.66×10^5 vp/mL of placental perfusates (**Table 1**). No qPCR inhibition was detected in these four samples. In summary, of the 116 qPCR non-inhibited placental perfusate samples, Ad.VEGF-D ^{Δ NA^C} DNA was detected in only 4.

Discussion and clinical impact

In this article we describe the rationale and studies in the *ex vivo* human placenta that will be used to support a first-in-human study (EVERREST) of maternal gene therapy, Ad.VEGF-D^{ΔNAC} vector, to treat severe early onset FGR. The clinical trial entry criteria are designed to identify the most severe early onset FGR fetuses that have the highest perinatal mortality and morbidity as follows: (1) EFW <3rd centile for gestational age⁵⁵, singleton fetus with (2) confirmed gestational age >22 weeks and <26+0 weeks at Ad.VEGF-D^{ΔNAC} vector administration and (3) evidence of placental insufficiency defined as raised mean uterine artery doppler Pulsatility Index and (4) raised umbilical artery Pulsatility Index. Data from the currently recruiting EVERREST prospective observational study⁸ and other cohorts^{5, 9, 10} give a cumulative perinatal mortality and morbidity rate in these pregnancies of 75-80%. Pregnant women with evidence of congenital infection, abnormal fetal karyotype and/or abnormal detailed anomaly scan which may cause severe early onset FGR will be excluded. The primary outcome measure for the clinical trial is safety and tolerability of the mother, fetus and neonate to the intervention (administration and product), with secondary exploratory outcomes relating to maternal, fetal and neonatal outcomes.

The aim of our studies was to investigate the effect of maternal side infusion of Ad.VEGF-D^{ΔNAC} on the integrity and function of the human placental barrier, and to identify if the vector could transfer to the fetal side of the placenta. We first performed dose ranging experiments with Ad.VEGF-D^{ΔNAC} vector manufactured to GMP in *in vitro* human placental explant culture, to inform on the appropriateness of single concentration dosing for a second phase of testing in *ex vivo* human dual placental perfusion. The latter was designed to temporally mimic the planned *in utero* flow-stasis-flow haemodynamics of vector administration to the uterine artery during the planned EVERREST clinical trial.

Human explant culture studies revealed limited access of the Ad.VEGF-D^{ΔNAC} vector into placental tissue and no effects on hCG secretion or LDH release, indicating that a maximal vector dose administration should be utilised in the perfusion model. During *ex vivo* perfusion, the maternal blood compartment of the placenta, the IVS, was estimated to carry a vector concentration which was at least twice as high as that expected for a second trimester placenta during *in utero* administration of the therapy (5×10^{10} and 2×10^{10} VP/mL, respectively). The design also built in over-exposure, ensuring that placental villi, as opposed to the uterine artery endothelium in the planned clinical trials, received this peak dose during the 5 minute matched stasis of perfusion.

During dual circuit perfusion, FIHP was used as a measure of villous microvascular resistance to flow and neither changed across time nor was different between Ad.VEGF-D^{ΔNAC} and FB groups. This finding is in agreement with the normal Doppler ultrasound observations of umbilical artery perfusion and resistance indices in sheep given Ad.VEGF-A₁₆₅ vector in mid-gestation and assessed at the end of gestation²⁷. The absence of any effect in FIHP was also in keeping with the absence of a fetomaternal fluid shift in either *ex vivo* human placental perfusion groups. A fetomaternal bulk-flow fluid shift is thought to be the consequence of altered villous microvascular resistance affecting the transmural capillary pressure differential at the placental exchange barrier⁵⁶⁻⁵⁸. We found that there was no effect of Ad.VEGF-D^{ΔNAC}, or FB on placental permeability, investigated through the maternofetal clearance (K_{mf}) of two inert paracellular markers, creatinine and FITC-inulin. The steady state K_{mf} values for these molecules were similar to those previously found *in vivo* and *ex vivo* for the normal human placenta^{59, 60} and suggest that nutrient transfer across the placenta via paracellular routes would be unaffected by Ad.VEGF-D^{ΔNAC} treatment, at least within the first 3 hours of exposure.

There was no evidence that the Ad. vector, or its FB buffer affected alkaline phosphatase release into the maternal perfusate. This marker appears to signify SCT microvillous shedding. In a previous study using this perfusion model, increasing intervillous flow 3 fold to create aberrant haemodynamics led to enhanced release of alkaline phosphatase from the maternal-facing trophoblast cell layer, probably due to syncytial damage⁶¹. As with the placental explant culture data, there was no effect on hCG release into the maternal perfusate. However, the steady state release of LDH was 2 fold higher following Ad.VEGF-D^{ΔNΔC} treatment, compared to the FB control. Taken together these data suggest that, at very high concentrations, Ad.VEGF-D^{ΔNΔC} may induce a small loss of SCT integrity, without a significant release of microvillous membrane into the maternal circulation. Whilst this increase in LDH release might be of some concern, it is important to consider that the concentration of vector to be used clinically in humans will be far lower than reported here for *ex vivo* perfusion calculated to be around 25 times lower (Table 1). In addition the peak dose exposure of the placental villi to the Ad. vector will occur for a shorter duration during the clinical trial (up to 5 minutes).

In the perfusates of eight placentae (5 normal, 3 FGR or FGR/SGA), each sampled across 15 timepoints over a period of 3 hours, only four samples tested positive for Ad.VEGF-D^{ΔNΔC} by PCR, and then at levels five to six orders of magnitude less than was administered to the maternal side. These positive sample results were not recorded consistently over the time course of perfusion in an individual placenta, as would be expected of receptor-mediated placental transfer of vector. Nevertheless, occasional passage, or transfer of vector across the placenta was detected in two out of eight human placentas. This finding therefore demonstrates that the Ad.VEGF-D^{ΔNΔC} vector cannot easily cross the human placenta and that, assuming transfer dynamics are comparable between the *ex vivo* model and the *in vivo* environment, access the fetal circulation is only likely to be minimal.

In one previous study of human placental transfer of Ad. vectors, term human placentas were perfused *ex vivo* for at least 9 hours in a dual perfusion chamber after injection of Ad.LacZ vector (5×10^9 particle forming units) into the maternal intervillous space ⁶². Adenovirus-mediated gene transfer to placental trophoblast cells increased as perfusion time extended. It is unclear however which trophoblast cells, whether the SCT or CT, were transduced in this study. Importantly it is known that the integrity of the placental barrier in the dual perfused human placenta becomes compromised after approximately 5 hours of perfusion, which was confirmed by the authors as leaking of perfusate across the barrier.

The extraction and quantification of Ad. vector from the fetal human placental perfusates was an important element in the evaluation of the exposure and safety of the Ad.VEGF-D^{ΔNAC} ahead of any clinical trial. For nucleic acid-based therapeutics, quantitative PCR provides an accurate and sensitive analytical approach. Our validation is based on the guidelines set out by regulatory authorities for bioanalytical methods ⁶³. We have shown that very low extraction efficiency of DNA from human fetal placental perfusates is achieved with commonly used methods. Only by extracting the DNA using anion exchange columns were we able to recover approximately 100% of the spiked Ad.VEGF-D^{ΔNAC} DNA. Indeed, the preliminary results with standard protocols suggested that little or no Ad. Vector DNA could be recovered from perfusates, even though a substantial amount was present. Studies utilising PCR to measure transplacental transfer of biological agents in placental perfusates are fairly novel. One other study reports the use of a guanidine-phenanthroline method to isolate *Trypanosoma cruzi* DNA from human placental perfusates prior to PCR analysis ^{64, 65}. On the whole, quantitative transfer of other substances tends to be done by colorimetric and fluorimetric assay of chromophores and fluorophores, scintillation counting of radio-labelled substance and high pressure liquid chromatography of other molecular species.

Difficulties to obtain consistent recovery of DNA from biological matrices with efficient removal of inhibitory substances have been previously reported in a few studies ⁶⁶. Our findings may therefore benefit other therapeutic projects and help investigators in the detection and quantification of low levels of DNA in matrices that contain PCR inhibitors. Collectively, our results meet the criteria for validation of a quantitative PCR analytical method according to current international guidelines. The method described here can be used to quantify Ad.VEGF-D^{ΔNAC} DNA in GLP-compliant regulatory studies and permits a rigorous evaluation of the fetal exposure to Ad.VEGF-D^{ΔNAC} through the placenta during therapeutic use.

Ethical considerations of a maternal gene therapy are important for clinical translation. The EVERREST consortium has evaluated the ethical and social acceptability of a clinical trial using maternal uterine artery VEGF gene therapy clinical trial to treat severe early-onset FGR in pregnant women. A review of the ethical, legal and regulatory issues raised by the planned EVERREST clinical trial was conducted ⁶⁷. This generated issues that were then explored in semistructured, qualitative interviews carried out in four European countries, with 34 key stakeholders and 24 women/couples who had experienced a pregnancy affected by severe early-onset FGR ⁶⁸. No fundamental or insurmountable objections to a trial of maternal gene therapy were identified.

In conclusion, our toxicology studies using the *in vitro* and *ex vivo* human placenta show that whilst the human placenta has a very low capacity to take up Ad.VEGF-D^{ΔNAC}, access by the viral vector is mainly limited to the maternal-facing epithelium and transfer to the fetoplacental circulation is unlikely to occur. There was no apparent endocrine disruption in response to Ad.VEGF-D^{ΔNAC} exposure across 48 hours in the explant culture model, or more acutely in the perfusion model. Although no changes to placental tissue structure were noted

in experiments using villous explants, perfusion studies indicate there is a small risk that trophoblast integrity might be compromised by a high dose of the clinical grade Ad.VEGF-D^{ΔNAC} in the presence of blood flow. Low levels of vector were only detected in 4 out of 116 fetal perfusate samples and at vector concentrations of at most a factor of six less than was administered to the maternal side, indicating that vector transfer to the fetal side of the placenta is likely to be at low levels in any clinical trial. This warrants further monitoring in future clinical trials but is unlikely to be a concern considering the proposed dosage during clinical drug administration.

Supplementary Information

1. Materials and Methods.

Culture of human placental villous explants

In an initial series of dose-ranging experiments designed to investigate the toxic dose of Ad vector particles on placental tissue, villous fragment explants from six normal pregnancies were exposed to a dose range of Ad.VEGF-D^{ΔNΔC} vector ($5 \times 10^{7-10}$ virus particles/ml), Ad.LacZ vector control (5×10^{10} vp/ml), or virus FB (glycerol). Placental villous tissue was randomly sampled and maintained in culture, as previously described⁶⁹. Explants of villous tissue (~2.5 mm³) were washed in PBS to remove maternal blood and transferred to Netwell™ permeable supports (70μm mesh; Corning Lifesciences) in 12-well culture dishes. 3x explants per Netwell were maintained at the liquid/air interface in 1.5 ml culture medium (100ml/L 10xCMRL 1066, 2.2g/L NaHCO₃, 100μg/ml streptomycin sulphate, 100IU/ml penicillin G, 0.1μg/ml hydrocortisone, 1μg/ml insulin, 0.1μg/ml retinol acetate, 100mg/L L-glutamine, 5% fetal bovine serum, pH 7.2) at 37°C (5% CO₂/air) for 7 days. Incubation of explants at the liquid/air interface of culture medium allows for adequate oxygenation of the tissue. Medium was collected daily and immediately frozen at -20°C for later measurement of human chorionic gonadotrophin (hCG) secretion (used as endocrine marker of S SCT regeneration), and lactate dehydrogenase (LDH) release (used as a marker of cell death). Details of these assays are provided in **Supplementary Materials**. Ad.VEGF-D^{ΔNΔC} was exposed to the explants on day 5 of culture for 1 hour. At day 7, fragments were harvested and lysed in 0.3M NaOH for determination of protein content using a BioRad protein assay (Ad.VEGF-D^{ΔNΔC} exposed explants), or fixed for β-galactosidase localisation and H&E staining (Ad.LacZ and Ad.VEGF-D^{ΔNΔC} exposed explants). Detailed methods for these procedures are provided in **Supplementary Materials**.

As shown in **Supplementary Table 1**, the highest dose exposed to the trophoblast exchange barrier (5×10^{10} vp/mL with 60 minutes with stasis) is estimated to be at least 5x higher than the vector concentration within the maternal blood entering the severely growth restricted placenta from the uterine artery during therapy. Furthermore, as the maternal blood distributes around its non-vascularised compartment (the IVS), a further 10 fold dilution of the vector is likely to occur rapidly, further reducing the concentration surrounding the maternal-facing side of the placental barrier.

Ex vivo human dual placental perfusion

The use of the *ex vivo* human dual placental perfusion model was to investigate possible effects on: (i) the homeostasis of resistance to flow within the villous microvasculature; (ii) the transfer of paracellular markers, as an indication of altered transplacental permeability; (iii) cell barrier integrity; (iv) placental endocrine release; (v) placental structure and (vi) whether clinical grade Ad.VEGF-D^{ΔNAC} vector accessed and crossed the placental barrier after maternal administration.

Supplementary Figure 1 is a schematic representation providing an overview of the experimental perfusion regimen. Perfusion was carried out in dual open circuit throughout⁷⁰ for an initial period to attain physiological steady state (30 minutes). At the beginning of this period (T = 6-7 minutes), a 60 second sampling of fetal venous perfusate was performed to check on fetal venous side flow efficacy as a percentage of calibrated fetal-side inflow. Only placental preparations achieving an initial 100% fetal venous recovery, and continuing to do so until T=1:11 when Ad.VEGF- D^{ΔNAC}, or FB buffer was administered, were accepted into the study (**Supplementary Table 2**). **Supplementary Table 3** shows the schedule for the *ex vivo* human dual placental perfusion experiments. At T=30 minute, a 40 minute trial was undertaken to assess normal paracellular permeability through the maternofetal clearance

(K_{mf}) of two inert hydrophilic markers, creatinine and FITC-inulin (maternal reservoir concentrations were 500mg/L and 200mg/L, respectively; **Supplementary Figure 3**)^{20, 59}.

Paracellular marker assays were performed on stored thawed samples after the experiments were completed (see **Supplementary Materials** for detailed methods). During experimentation, preparations were checked to ensure that fetal-side inflow hydrostatic pressure (FIHP) remained steady and fetomaternal leaks were absent, before (quality control (QC) purpose only) and after (an assessment of experimental effect) Ad.VEGF-D^{ΔNΔC}, or FB (glycerol control) administration occurred. The period before administration also acted as an internal control period for the subsequent assays to measure release of biomarkers for endocrine function (hCG), SCT shedding (alkaline phosphatase) and cell death (LDH) (see **Supplementary Materials**). In addition, two environmental control samples were taken at the fetal venous perfusate cannula ("C1 & C2"; see **Supplementary Figure 3**), at T=1:10:00–1:12:00 and T=1:12:00 – 1:15:30 (hr:min:sec) and were used to determine potential background air-borne contamination of all subsequent samples, by assessment of detectable quantitative transplacental transfer of Ad.VEGF-D^{ΔNΔC} DNA (see below). At T=1:11, a bolus injection of either Ad.VEGF-D^{ΔNΔC} (2ml 2.67×10^{11} vp/mL) or FB control was made into the maternal-side perfusate inflow line via a delivery port. After a 2 second pulse of flow, optimised for bolus delivery and villous exposure by way of time-lapse dye injection pilot experiments to the villous tissue (see **Supplementary Figure 4**), this was followed by a 5 minute period of maternal-side perfusion stasis of flow, to enhance exposure of the trophoblast epithelium to the viral vector or FB. The estimated compartment volume for the IVS of an average 35g perfused lobule is 10 ml, based on volume density estimations from a previous study⁵⁹. Hence, perfusion into this IVS causes bolus dilution, meaning that the true SCT dosage during the 5 minute exposure was 5.33×10^{10} vp/mL. The decidua basalis remained intact in the perfusion model, maintaining the IVS as a quasi-compartmentalised

space for exposure of the virus to the trophoblast, a phenomenon explored in a pilot study by quantitatively assessing the temporal elution of a bromophenol blue dye, with the inclusion of a matched period of perfusion stasis (see **Supplementary Materials and Supplementary Figure 4**). In the period one minute prior to bolus administration and during maternal-side perfusion stasis (T=1h:10min to 1hr:15min:30sec), two environmental control fetal-side venous samples (“C1” and “C2”; **Supplementary Figure 3 and Supplementary Table 3**) were collected to assay later and to set a baseline for potential air-borne artefact contamination of all subsequent fetal venous samples.

Treatment exposure was followed by the recommencement of maternal-side flow for another three hours. During the last 40 minutes of this period (T=3h:31min to T=4h:11min; **Supplementary Figure 3**), maternofetal hydrophilic marker clearance was again determined, as above, to assess any change in porosity in response to the treatment. This was followed by a final ten minute period of perfusion fixation from the IVS with 4% neutral buffered formalin (NBF). During fixation, FIHP was closely monitored to ensure that resistance increase in the placental villous microcirculation was avoided, which might have induced fetomaternal bulk flow shifts, potentially evoking vacuolation artefacts at the trophoblast layer⁵⁶. Fixed lobule was then excised and weighed. A wide vertical section (approximately: 4mm x 20mm x 20mm, retaining the decidua basalis and chorionic plate) was cut out and mounted within a histological cassette and immersion fixed before processing. This tissue section was then further processed for immunohistochemistry/immunofluorescence (see **Supplementary Materials**). At intervals during perfusion, fetal and maternal venous perfusate samples were taken from ice and centrifuged at 2000 x g x 10 min at 4°C and the supernatants recovered, aliquoted and stored at -80°C, prior to use in the aforementioned assays. In addition, for Ad.VEGF-D^{ΔNAC} treated lobules only, fetal venous environmental control samples (C1& C2) and subsequent post-treatment fetal-venous samples, were assayed

by PCR for the artefact environmental transfer and maternofetal transplacental transfer of viral vector, respectively (see **Supplementary Materials**).

The study design infers that the viral concentration within the IVS during *ex vivo* perfusion was 25 times greater than that which will occur within the IVS *in utero* following gene therapy exposure to the uterine artery lumen, as is planned in a first-in-woman clinical trial (**Supplementary Table 1**). Furthermore, the *ex vivo* investigation permitted exposure of this vector concentration to the SCT for a much longer period than is likely during the clinical trial intervention, when the placental bolus passage is transient with a short blood perfusion stasis of up to 5 minutes.

Vector Manufacture

The Ad.VEGF-D^{ΔNΔC} viral vector is a first generation recombinant replication-deficient adenovirus (Ad5 serotype) containing E1- and partial E3- deletions, and an expression cassette for the human VEGF-D. The expression cassette contains the human cytomegalovirus (CMV) enhancer and promoter elements, an intron and a SV40 polyadenylation signal. The transgene consists of the interleukin-3 (IL-3) signal peptide, FLAG octopeptide and VEGF-D gene nucleotides 277 – 603. The IL-3 signal peptide facilitates VEGF-D^{ΔNΔC} secretion from the expressing cell, and the signal peptide itself is cleaved from the secreted protein. The FLAG-tag resides on the N terminus of secreted VEGF-D^{ΔNΔC}. The nucleotides 277 – 603 of VEGF-D encode amino acids 93 – 201 which correspond to the VEGF homology domain, the most active form of mature VEGF-D. Ad.VEGF-D^{ΔNΔC} (HPLC- titer 2.7×10^{12} vp/mL) was manufactured by Ark Therapeutics Oy, Kuopio, Finland as previously described⁷¹. The final formulation of material was 5 mM HEPES, 20% glycerol buffer, pH 7.8. The genetic map is presented in **Supplementary Figure 5**. Ad.LacZ (HPLC- titer 1.5×10^{12} vp/mL) was prepared by AIV Institute, Kuopio,

Finland as previously described ⁷¹. The final formulation of the material was 5 mM HEPES, 20% glycerol buffer, pH 7.8.

Assessment of placental endocrine function and cell death

Samples of explant culture medium and perfusate were assayed for markers of placental endocrine function (hCG) and cell death (LDH). Samples of perfusate were also assayed for alkaline phosphatase activity. Alkaline phosphatase is an enzyme present at the microvillous membrane of the SCT. Detectable alkaline phosphatase activity in placental perfusate is indicative of microvillous membrane shedding. All samples collected for the purpose of these assays were frozen immediately after collection and care was taken to avoid thaw-refreeze cycles.

hCG secretion and LDH release by explants was expressed per mg protein per hour. Explant protein content (mg) was determined using a commercial kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) after lysing in 0.3M NaOH. hCG secretion, LDH, and alkaline phosphatase release into the maternal perfusate was expressed as a percentage change from release at the 67.5 minute time point, prior to administration of the vector or FB. This is an important standardisation step to overcome differences in the released quantities of analyte attributed to variance in perfused tissue mass between experiments.

i) Measurement of hCG

hCG secretion from the placenta directly correlates with trophoblast health and regeneration ⁶⁹. hCG secretion was assayed by sandwich ELISA (DRG Diagnostics, DRG International, Marburg, Germany) according to the manufacturer's instructions. The range of the assay is between 5-1000 mIU/ml. The intra and inter assay variability is reported as 2.2-4.7% and 3.3-4.3% respectively.

ii) Measurement of LDH

Basal LDH release occurs normally in the explant model, even during SCT regeneration and occurs at basal levels into the IVS during dual perfusion of the human placenta. In the human placenta LDH release reflects cellular integrity⁶⁹. In the explant model SCT degeneration at day one of culture is approximately 4-fold of that seen when a healthy new syncytium has formed at day five⁶⁹. LDH release was quantified using the Cytotoxicity Detection kit (Roche Applied Science, Indianapolis, USA) according to manufacturer's instructions. The LDH activity is determined in an enzymatic test: In the first step NAD⁺ is reduced to NADH/H⁺ by the LDH-catalysed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H/H⁺ from NADH/H⁺ to the tetrazolium salt INT, which is reduce to formazan and measured spectrophotometrically at 500nm. Values obtained are arbitrary, achieved by calculating the difference in absorbance measured at 490nm and 620nm.

iii) Measurement of alkaline phosphatase

Alkaline phosphatase activity assay utilises the kinetics of the enzyme with the substrate p-nitrophenylphosphate, leading to the phosphorylation of diethanolamine over a 2 minute period which is measured by spectrophotometry at 410nm. The coefficient of variation (CV) for serum alkaline phosphatase at normal levels is 2% with a standard deviation of 0.04 Units/ml (Sigma Diagnostics, Poole, UK).

Assessment of transplacental permeability

The concentration of two inert hydrophilic markers, creatinine and FITC-inulin, were measured in samples of perfusate to assess maternofetal transplacental permeability, principally relating to the mean pore radius, the number of pores of the paracellular route and the length of pathway between the fetal and maternal circulations.

Transfer of creatinine and FITC-inulin was calculated in terms of unidirectional maternofetal clearance (Km-f):

$$Km-f = ([\text{marker}]_{\text{fetal side}} \cdot Q) / ([\text{marker}]_{\text{maternal side}} \cdot W)$$

Units are: $\mu\text{l}/\text{min}/\text{g}$ perfused placental tissue.

where: $[\text{marker}]_{\text{fetal side}}$ and $[\text{marker}]_{\text{maternal side}}$ represent the concentration of the marker substance in the fetal venous sample and maternal perfusate reservoir, respectively; Q is the measured flow rate in the fetal circulation and W is the wet weight of the perfused cotyledon. FITC-inulin has a molecular radius just under the point of steric hindrance for placental transfer and is therefore more susceptible than creatinine (with a much smaller molecular radius) to changes in pore size. Fick's Law of diffusion infers that an increase in marker clearance would reflect increases in permeability through wider or more numerous paracellular pores, or a reduction in the length of pathway between the circulatory systems, provided that perfusion is in a steady state and the surface available for exchange is unaltered, according to study design. Decreases in marker clearance relate to a reduction in pore size, or number, particularly affecting FITC-inulin transfer; but might also reflect cellular morphology changes, since this might increase the length of the pathway and hence the transfer of both hydrophilic solute between the SCT and the fetal endothelium.

i) Creatinine maternofetal transplacental permeability

Creatinine concentration was measured using a diagnostic kit ('Infinity' Reagent and standard from Thermo Electron Corporation, via Alpha Laboratories, UK). The principle of this assay is based on the kinetic dissociation of the yellow creatinine-picric acid chromogen under acidic conditions, resulting in a discoloration. This discoloration is measured spectrophotometrically using a plate reader (Versamax; measured at 500nm at 60, 120 and 180 seconds following reagent addition). A single creatinine standard concentration was also read alongside the samples ($5.0 \text{ mg} / \text{dl} = 440 \text{ }\mu\text{M}$; purchased with reagent). Linearity has

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3 been predetermined to hold between 0 and 1.8 mM (0–200 mg/L). The maternal inflow
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5 reservoir sample was diluted 1 in 5 in EBB to fit this range and all fetal venous samples fell
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7 below the 200 mg/L).

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10 ii) FITC-inulin maternofoetal transplacental permeability

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12 FITC-inulin was measured by fluorescence spectrophotometry (excitation = 494 nm;
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14 emission range = 515-519 nm. The maternal perfusate reservoir sample was diluted 1 in 50 in
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16 EBB. A FITC-inulin standard range was prepared (0.1 – 10 µg/mL; EBB diluent) and
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18 measured alongside the maternal reservoir perfusate samples and fetal-side venous samples.
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20 A standard linear regression plot was produced (GraphPad Prism software) for the samples
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22 and all experimental sample FITC-inulin concentrations were interpolated from their
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24 fluorescence values.
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30 *Histological analysis of placental tissue*

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32 Routine haematoxylin and eosin staining was performed on 5µm sections of formalin-fixed
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34 wax-embedded explant tissue samples before imaging using Leitz Dialux22 and Olympus
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36 Bx41 light microscopes. The villous SCT sheds during days 1-3 in explant culture and over
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38 days 4-7 a new SCT regenerates⁶⁹. Captured images were examined by two independent
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40 blinded observers to assess structural integrity of the villous tissue and confirm SCT
41
42 regeneration. Perfused fixed human placental tissues were examined by a perinatal
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44 pathologist with almost 20 years of experience in placental histological analysis and who was
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46 blinding to treatment group (NJS). For this purpose Haematoxylin and Eosin stained sections
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48 were subjectively evaluated using standard diagnostic histological criteria including villous
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50 architecture, villous viability, vascularity, and presence or absence of described patterns of
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52 histological changes reported in association with a range of placental disorders⁷². Cases were
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54 categorised as normal or abnormal according to these criteria, after accounting for the effects
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of perfusion on normal appearance of blood filled vessels and intervillous space. No morphometric analysis was performed.

Immunohistochemistry analysis of human placental tissues

Immunohistochemical and immunofluorescence techniques were performed on sections of fixed placental explants and perfused placenta respectively to determine whether Ad. vector gains access to the tissue. Immunohistochemistry (IHC) to detect LacZ gene product was performed on explants treated with Ad.LacZ. Immunofluorescence to detect VEGF-D FLAG-tag was performed on sections of perfused placenta treated with Ad.VEGF-D^{ANAC}.

i) LacZ immunohistochemical staining

Placental explants were fixed on Day 7 in 10% neutral buffered formalin overnight at 4°C before thorough washing in Tris buffered saline (TBS) and paraffin embedding. Sections of 5 µm were de-waxed and rehydrated. Non-specific staining was prevented by quenching endogenous peroxidase activity with 3% aqueous H₂O₂ for 30 minutes at room temperature. Sections were washed in TBS followed by a trypsin digest (0.1% trypsin in 0.1% CaCl₂) for 10 minutes at 37°C. Sections were washed in H₂O₂ followed by 30min incubation with non-immune block (10% normal goat serum, 0.1% Tween-20 in TBS). Primary antibody (mouse monoclonal anti-LacZ, Promega Cat # Z3781) was diluted 1:1000 in non-immune block and applied to sections overnight at 4°C. For negative controls, mouse IgG replaced primary antibody. Following repeated washes with TBS containing 0.6% Tween-20, antibody binding was detected by sequential application of biotinylated goat anti-mouse IgG (Dako: Ely, UK; 1:200 dilution) and avidin-peroxidase (20 ng/ml), with the chromogen diaminobenzidine, to produce a brown precipitate. Sections were counterstained with Harris' haematoxylin, dehydrated and mounted with DPX for imaging using Leitz Dialux22 and Olympus Bx41 light microscopes.

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3 ii) Immunohistochemical analysis for VEGF-D^{ΔNΔC} FLAG tag
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5 Perfused human placental samples were fixed immediately after the end of the perfusion
6 experiment in 4% neutral buffered formalin overnight at 4°C before thorough washing in Tris
7 buffered saline (TBS) and paraffin embedding. Sections of 8 μm were de-waxed and
8 rehydrated. Placental sections were permeabilized in phosphate buffered saline (PBS) with
9 Triton 0.1 % (Life Technologies, UK), and saturated in goat serum 10 % in PBS (Life
10 Technologies, UK). Sections were incubated over night with mouse anti-FLAG antibody
11 (1:1000; Sigma, UK) in bovine serum albumin (Sigma, UK) and 0.5% Tween (Sigma, UK).
12 Subsequently, the placental tissue was incubated with Alexa Fluor 488 anti mouse secondary
13 antibody (1:3000; Life Technologies, UK) for 1 hour at room temperature, DAPI staining for
14 nuclei was performed and slides were mounted in aqueous mounting medium. The imaging
15 analysis was performed on a Leica DMI8 fluorescence direct microscope (Leica, UK).
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32 *PCR analysis of fetal perfusates*
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34 Transplacental Ad. vector transfer was assessed in fetal perfusates using PCR.
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Statistical methods.

A calculated p value of <0.05 was considered statistically significant using GraphPad Prism version 6.

2. Quality assurance.

Placental explant studies

Over the first 2 days of explant culture, SCT sheds and thereafter regenerates to form a new SCT that is morphologically indistinguishable from normal by day 7⁶⁹. This process is accompanied by temporal changes in hCG secretion which is low on day 2 of culture and increases with regeneration of SCT on days 5-7^{69, 73}. This well characterised profile of hCG secretion is therefore indicative of healthy explant tissue and can be used as a QC measure, along with examination of H&E stained tissue sections for visual confirmation of explant tissue integrity. In addition, explants are examined daily for any evidence of infection. The number of explant experiments performed in total was 7 but 1 had to be excluded due to infection.

Placental perfusion studies

Following establishment of open circuit perfusion⁷⁰ an initial period of physiological steady state perfusion for 30 minutes was included. At the beginning of this period, at T=7-8 minutes a 60 second collection of fetal venous perfusate was sampled into a pre-weighed tube. Following collection the tube was re-weighed and the fetal-side venous recovery was calculated as a percentage of calibrated inflow (specific gravity of EBB perfusate at ambient temperature is 1.013 g/mL). Only preparations achieving a 100% fetal venous recovery at T=6-7 minutes were accepted into the study. As a further QC measure during this period, fetal-side inflow hydrostatic pressure (FIHP), as a surrogate for net downstream fetal-side

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3 placental vascular resistance to flow, must reduce on the elution of blood to a basal steady
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5 level between 30-60 mmHg. This must occur concomitantly with the absence of a
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7 fetomaternal leak known as 'bulk flow' (the fetomaternal shifting of perfusate across
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9 placental barriers in response to a heightened fetomaternal hydrostatic pressure gradient).
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11 Aberrant FIHP values with a poor resistance resting state occurs when the time from
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13 parturition to the fetal-side venous flushing with heparin-containing perfusate is in excess of
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15 20-30 minutes. FGR placentas do have an inherently higher resistance to blood and perfusate
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17 flow, *in vivo* and *ex vivo*, respectively ⁷⁴. However, this was tolerated for experimental
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19 purposes, provided fetal-side venous perfusate recovery was 100%. The number of collected
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21 placentas for perfusion was 107; based on initial inspection for tissue breakages, the number
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23 of attempted perfusions was 43; the number of QC failed experiments after initial evaluation
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25 was 29, with an additional 1 failing on fetal-side venous recovery prior to IVS administration
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27 of vector; and the number of QC passed experimental perfusions was 14, with 4 used in pilot
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29 investigations (data not included) and 10 used for the study.
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33 In perfusion design planning, there was a clear need to optimise FB and Ad.VEGF- D^{ΔNAC}
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35 bolus retention within the intervillous space during the 5 minute stasis of flow, emulating
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37 prolonged placental *in vivo* exposure during hemostasis. To do this we carried out pilot
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39 perfusions with bromophenol blue dye boluses and used time-lapse photography to inform of
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41 the optimal timings for maternal pump "ON/OFF" switching, to be used following *ex vivo*
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43 bolus administration of FB, or Ad.VEGF- D^{ΔNAC} in the data yielding experiments (see
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45 **Supplementary Figure 4**).
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51 52 *DNA extraction from fetal perfusates*

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54 At the start of the study no information could be found relating to nucleic acid extraction
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56 from placental perfusates. The extraction of nucleic acids from biological matrices presents
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several challenges arising from the complex composition of the tissue or fluid, including proteins, exosomes, cellular DNA and RNA, and the presence of RNAses, DNAses and other degradatory enzymes present in the matrix. Furthermore, the placental perfusates contain not only the perfusion buffer (phosphate-based buffer), but also hormones, sugars, organic acids and salts released into the perfusate during the experimental procedure ⁷⁰. During the extraction process, chemicals and proteins may be co-extracted or co-eluted with the target DNA and these materials may interact directly with the target DNA or inhibit the PCR polymerases and thus interfere with quantification ⁷⁵. Examples of such materials include inorganic salts (such as KCl, NaCl and bile salts), ionic detergents and ethanol.

A number of nucleic acid extraction kits are commercially available and have been used successfully with different tissues and fluids. We investigated various extraction techniques based on different principles of extraction. Once a suitable extraction method had been identified, a qPCR-based method was established for quantification of the Ad.VEGF-D^{ΔNAC} vector DNA. The quantification method was validated to meet current international guidelines, addressing accuracy, precision, sensitivity, specificity and dynamic range, as well as matrix effects, PCR inhibition and vector stability in human placental perfusates.

Control human fetal venous placental perfusate samples from women untreated with Ad.VEGF-D^{ΔNAC} were used for optimization of the extraction protocols after spiking with known quantities of the Ad.VEGF-D^{ΔNAC} vector. The following approaches were evaluated (the manufacturers recommended extraction protocols were followed):

- QIAamp DNA Blood Midi kit (Qiagen)
- QIAamp DNA Mini kit (Qiagen)
- TRIzol (Thermo Fisher) in combination with QIAamp Blood Midi kit (Qiagen)
- TRIzol (Thermo Fisher) and chloroform extraction method
- NucleoSpin Tissue kit (Macherey Nagel)

- NucleoSpin Tissue kit (Macherey Nagel) with heating step
- DNA concentration using sodium acetate/ethanol
- DNA concentration using a SpeedVac (Thermo Scientific)
- qPCR detection without DNA extraction
- qPCR detection without DNA extraction with heating step
- qPCR detection without DNA extraction with 10-fold dilution
- qPCR detection without DNA extraction with 10-fold dilution and heating step
- NucleoBond extraction kit (Macherey Nagel): based on a different (anionic) binding chemistry

DNA from each extracted perfusate sample was quantified using a NanoDrop ND-8000 instrument (Labtech, France). The Ad.VEGF-D^{ΔNAC} DNA was quantified using a qPCR calibration curve (see below), and extraction efficiency (recovery yield) was calculated for each method.

DNA was extracted from six fetal venous perfusate samples spiked with 10⁶ vp of Ad.VEGF-D^{ΔNAC} (three 330 μL samples volume and three 500 μL samples). PCR inhibition was determined in each sample. The mean DNA extraction yield was defined as the ratio between the measured and the nominal numbers of vp in the spiked Ad.VEGF-D^{ΔNAC}. For the final optimized DNA extraction protocol, a 700 μL sample of perfusate was spiked with 10⁶ vp.

Extraction efficiency of Ad.VEGF-D^{ΔNAC} DNA from fetal perfusates

Supplementary Table 4 demonstrates the extraction efficiency of Ad.VEGF-D^{ΔNAC} DNA from fetal perfusates. Spiking of 10⁶ copies of Ad.VEGF-D^{ΔNAC} DNA into 700 μL perfusate samples, followed by DNA extraction with the QIAamp Blood Midi kit, failed to recover any of the spiked material; all samples showed total DNA concentrations below the limit of quantification using the NanoDrop (Thermo Scientific) and below the qPCR detection limit

for Ad.VEGF-D^{ΔNAC} DNA. A trial of QIAamp DNA Mini kit was also made, but produced only 1.32% extraction efficiency. In another extraction attempt, spiked perfusate samples were mixed first with TRIzol to isolate the nucleic acids before extraction with the QIAamp Blood Midi kit, but this approach also failed to yield any DNA. A kit from a different manufacturer, NucleoSpin® was then tested under similar conditions and less than 10% of the spiked Ad.VEGF-D^{ΔNAC} DNA was recovered. Adding a heating step after the extraction did not improve extraction recovery. Different protocols were then tested to quantify the spiked Ad.VEGF-D^{ΔNAC} DNA, including directly quantifying the perfusate in qPCR experiments without DNA extraction, precipitation of the DNA from perfusate with acetate/ethanol and qPCR quantification, and evaporation of the perfusate with a SpeedVac, but all methods failed to yield any DNA or resulted in complete inhibition of qPCR assay. In total, thirteen different methods were tested, but most were found to be ineffective, mostly due to co-purification of PCR inhibitors.

An extraction kit from Macherey Nagel (NucleoBond® kit), which is based on a different DNA extraction chemistry to the methods previously used, was then tested. NucleoSpin® columns use traditional silica-membrane technology with chaotropic salt binding, whilst the NucleoBond® kit uses anion-exchange chromatography from solid phase extraction. Spiked Ad.VEGF-D^{ΔNAC} was recovered with 100% efficiency when using the NucleoBond® kit (**Supplementary Table 4**). With a mean RE% of -45.18% of the Ad.VEGF-D^{ΔNAC}, no significant matrix effect was found in the extracted samples from human placental perfusates. Three volumes of perfusate used for DNA extraction (330, 500 and 700 μL), corresponding to one third of the total volume, were tested to assess the DNA extraction yield, which ranged from 53.77 to 137.48%, without significant PCR inhibition (**Supplementary Table 5**). Interestingly, despite the greater number of columns, extraction efficiency decreased with the

placental perfusate volume, and the mean extraction yield ranged from 113% for 330 μ L to 75% for 700 μ L.

Real-time qPCR amplification of Ad.VEGF-D^{ΔNAC} DNA

Specific primers and probes for the detection of Ad.VEGF-D^{ΔNAC} DNA by qPCR were designed. Primers and probe sequences were as follows:

- forward primer: Fw-VEGF-D: 5'-TCTTCCACCTGGGACTCCAA-3',
- reverse primer: Rev-VEGF-D: 5'-CACTGAGTTCTTTGCCATTCTTCAT-3',
- internal Taqman® probe: Probe-VEGF-D: 5'-TGACATTGAAACACTAAAAG-3'.

The probe was 5'-labeled with 6-FAM reporter dye and 3'-conjugated with MGB quencher.

The primers and probe used were obtained from Applied Biosystems. The final concentrations of primers and probe were set at 400 nM and 200 nM, respectively. Analyses by qPCR were performed using an ABI 7900 HT instrument (Applied Biosystems), with the following previously optimized conditions: 50°C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min for 40 cycles.

Two calibration curves were prepared with calibration points diluted in RNase/DNase-free water or in 0.4 μ g of herring sperm DNA using 1, 5, 10, 50, 100, 500, 10³ and 10⁴ vp per well of the reference analyte. As the calibration curve was similar in both matrices, herring sperm DNA was chosen for all subsequent preparations with the reference analyte (5, 10, 31.8, 100, 317, 10³, 10⁴ and 10⁵ vp per well).

Acceptance criteria for each qPCR plate were as follows:

- the 6-carboxy-X-rhodamine (ROX) signal must be constant,
- the Cycle threshold (Ct) for the negative control (NTC) sample must be higher than the Ct for the lowest limit of quantification (LLOQ) of the plate,
- the calibration curve correlation coefficient should be higher than 0.98,
- the calibration curve efficiency should be between 80% and 110%,

- For calibration points, QC samples and in-study samples, the intra-replicate Ct difference (ΔCt) should be lower than 0.70,
- for calibration points and QC samples, the RE% between back calculated and nominal number of copies should be lower than 50% (65% at the anticipated LLOQ),
- 75% of the calibration points should be accepted to validate the calibration curve,
- In at least 4 of 6 QC samples the RE% between nominal and back-calculated copy numbers should be lower than 50%.

Validation parameters of Real-time qPCR of Ad.VEGF-D^{ΔNAC}

The dynamic range of the assay was determined from four calibration curves performed on separate occasions (over at least 2 days), and the determined limits of quantification (samples with highest and lowest numbers of vp precisely and accurately quantified with a $\Delta Ct < 0.7$). Specificity of the qPCR assay was determined using DNA extracted from five fetal venous perfusate samples assayed in duplicate in parallel with the determination of PCR inhibition. The qPCR assay was considered as specific if all DNA extracts returned an undetermined Ct or a Ct higher than the LLOQ.

PCR inhibition

Potential qPCR inhibition in samples was assessed by spiking and quantifying an exogenous DNA from a plasmid containing Drosophila melanogaster ANTPTM ORF cDNA. The primers (forward primer ANTP-F and reverse primer ANTP-R) and probe (internal Taqman[®] probe ANTP-P) were developed. For each assay, 10⁴ copies of ANTPTM (exogenous DNA) were spiked into the DNA samples and inhibition was revealed as a recovery percentage lower than -50%.

Validation of the qPCR primers and probe performance

In an absolute quantification, a calibration curve was performed in parallel to assess the quantity of Ad.VEGF-D^{ΔNAC} vector vector in in-study samples. The PCR efficiency and

correlation coefficient were assessed during the validation, and found to have a slope at -3.85, corresponding to an efficiency of 82%, and a correlation coefficient greater than 0.99. The qPCR calibration curve met the acceptance criteria for an absolute quantification of the adenovirus vector DNA (**Supplementary Figure 6**).

Validation of the qPCR assay

The reproducibility and robustness of the method were assessed during the analytical phase validation. The assay was found to be highly sensitive, with a dynamic range from 5 to 50000 vp/ μ L of extracted DNA and a very low detection limit of 2.5 vp/ μ L extracted DNA. Thus, this analytical method is able to detect and quantify as few as 300 vp in the total extraction volume (**Supplementary Table 6**). The qPCR assay for Ad.VEGF-D ^{Δ NA^C} DNA quantification was found to be specific, with no product detected ($C_t > 40$) in unspiked fetal venous perfusate DNA.

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Author Disclosure Statement

No competing financial interests exist for MD, AR, NP, CR, EO, RF, ML, NJS, SLG, CPS, PB. The authors are employees of their respective organisations.

ALD is consultant for Magnus Growth, for which she receives 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 FGR.

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Figure 1

Figure 1 (a) Haematoxylin & eosin staining of explant tissue fixed in 4% neutral buffered formalin on day 7 of culture (syncytiotrophoblast, STB; fetal capillary, FC; intervillous space, IVS; villous stroma, VS). Evidence of syncytiotrophoblast regeneration, comparable to untreated controls, in tissue treated on day 5 with virus formulation buffer (FB) or 5x10⁷-10vp/ml Ad.VEGF-DΔNΔC. Images also confirm villous structural integrity was maintained throughout the 7 day culture period, regardless of treatment and dose. Representative images from N=6 placentas, duplicate observations for each. (b) Detection of β-gal protein (brown staining) in Ad.LacZ-treated tissue (ii-v) using IHC. β-gal was observed in only a minority of villi and was mainly confined to the syncytiotrophoblast with occasional staining of villous stromal cells ("S" arrow, v) and was absent from the fetal vascular endothelium. β-gal staining was absent from untreated controls (i) and a negative control is shown, where mouse IgG was substituted for the primary antibody during IHC protocol (vi).

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Figure 2

Figure 2 (a) hCG secretion and (b) LDH release by placental explants on day 6 (closed bars) and day 7 (open bars) of culture expressed as a % of levels on day 5 (i.e. prior to any treatment).

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Figure 3

Figure 3 Fetal-side resistance and maternofoetal paracellular marker clearance in ex vivo perfused human placental lobules treated with either formulation buffer (N=3), or Ad.VEGF-D Δ N Δ C (N=7). Fetal-side inflow hydrostatic pressure (a, FIHP), reflecting resistance in the villous microcirculation in formulation buffer (circles) and Ad.VEGF- D Δ N Δ C (squares) treated lobules. Data are expressed as the mean $\pm$ SE percentage change from the pre-treatment T=32.5 minute value (100%). There was no difference in clearance between treatment groups and no effect of time (two-way ANOVA). Effect of formulation buffer (open bars) and Ad.VEGF-D Δ N Δ C (closed bars) on steady state maternofoetal creatinine (b) and FITC-inulin clearance (c); there was no difference between treatment groups (Mann-Whitney test, both markers; data is represented as the medians & interquartile ranges, expressed as the percentage change from the pre-treatment clearance period).

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Figure 4

Figure 4 Alkaline phosphatase (AP; a), lactate dehydrogenase (LDH; b) and human chorionic gonadotropin (hCG; c) release into the maternal circulation of the *ex vivo* dually perfused human placental lobule following treatment with formulation buffer (open circle; N=3) and Ad.VEGF-D^{ANAC} (open square; N=7). AP and hCG release were not significantly different between treatment groups, but their release significantly reduced with time (two-way ANOVA; $P < 0.001$ & $P < 0.01$, respectively). LDH release was significantly higher in the Ad.VEGF-D^{ANAC} group (two-way ANOVA; $P < 0.0001$; Sidak's multiple comparison test: $*P < 0.05$, $**P < 0.01$) and LDH release reduced with time ($P < 0.01$). Data are represented as mean $\pm$ SE.

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Figure 5

Figure 5 Photomicrographs demonstrating normal third trimester placental tissue (top left) and third trimester placenta with infarction (bottom left) which have been immersion fixed and routinely processed versus cases of similar gestational age placentas which have undergone perfusion (right column; all haematoxylin and eosin, original magnification x100). Perfusion results in apparent 'clearing' of the intervillous space, an empty appearance of the villous vessels and slight villous enlargement.

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Table 1. Detection and quantification of Ad.VEGF-D^{ΔNAC} in fetal side human placental perfusates using qPCR. Numbers indicate virus particles per ml of Ad.VEGF-D^{ΔNAC} vector detectable in the sample.

IBC definition Placenta study no.	Normal	Normal	FGR/SGA	Normal	Normal	FGR/SGA	Normal	FGR
	4	5	6	8	9	11	12	13
1:16 - 1:31	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
1:31 - 1:51	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
1:51 - 2:11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2:11 - 2:31	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2:31 - 2:51	-ve	-ve	-ve	-ve	8.78x10 ³	-ve	-ve	-ve
2:51 - 3:11	-ve	-ve	-ve	3.59x10 ⁵	-ve	-ve	-ve	-ve
3:11 - 3:31	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3:31 - 3:36	-ve	Inhibited	-ve	-ve	-ve	-ve	-ve	-ve
3:36 - 3:41	-ve	BLQ	-ve	-ve	1.51x10 ⁵	-ve	-ve	-ve
3:41 - 3:46	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3:46 - 3:51	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3:51 - 3:56	-ve	Inhibited	-ve	-ve	-ve	-ve	-ve	-ve
3:56 - 4:01	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4:01 - 4:06	Inhibited	-ve	-ve	2.83x10 ³	-ve	-ve	-ve	-ve
4:06 - 4:11	Inhibited	-ve	-ve	-ve	-ve	-ve	-ve	-ve

IBC: Individualised Birthweight Centile; FGR/SGA: IBC<10th centile >5th centile; FGR: IBC<5th centile; -ve: no Ad.VEGF-D^{ΔNAC} detectable and no inhibitors detected; BLQ: below limit of detection; Inhibited: inhibitors detected despite multiple testing of sample.

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

KEY

Perfusate reservoirs:

Supplemented EBB (glucose / albumin / heparin / L-Arg; both sides in open circuit perfusion).

Km-f paracellular marker periods: the maternal inflow perfusate is "supplemented EBB" with paracellular markers (500mg/l creatinine, 200 mg/l FITC-inulin). 5 minute continuous sampling of fetal venous perfusate. Mid-point sampling of maternal inflow perfusate reservoir for clearance calculation. Both sides in open circuit.

"Supplemented EBB" with VEGF transfecting adenovirus or formulation buffer ($2\text{ml } 2.67 \times 10^{11} \text{ vp/mL}$) followed by 5 minute maternal-side stasis of flow, permitting trophoblast exposure.

Perfusion fixation of placental lobule from the maternal-side (intervillous space) with 4% neutral buffered formalin

Sampling:

"C1" & "C2" Environmental control samples. Two fetal venous samples are taken during stasis of maternal side flow (C1: between T = 1:10:00 and 1:12:00; C2: between T = 1:12:00 and 1:15:30 (hr:min:sec)).

Continuous fetal-side venous sampling (for evaluation of: (i) maternofetal viral transfer; (ii) monitoring of fetomaternal fluid shift effect).

Maternal venous perfusate sampled intermittently for 60 sec (for monitoring of: (i) fetomaternal fluid shift effect; (ii) hCG, LDH and alkaline phosphatase release (T=57min, 1hr:52min, 2hr:52min & 4hr:08min)).

Supplementary Figure 4

Supplementary Figure 5

Supplementary Figure 6

Supplementary Table 1. Placental barrier exposure to Ad.VEGF-D^{ANAC}. Experimental model comparison in pre-clinical sheep studies and *ex vivo* human placenta studies with proposed human uterine treatment procedure.

Model / Body	Viral volume / conc. in starting stock (mL; vp/mL)	Initial stock dilution factor	Viral volume / conc. used of diluents (mL; vp/mL)	Intervillous space / incubation volume	Maximum viral conc. surrounding placental villi (vp/mL)	Total maximum delivered viral particles (vp)
Human <i>in vivo</i> placenta: proposed clinical trial				50 mL [‡]	2 x 10 ⁹	1 x 10 ¹¹
Sheep <i>in vivo</i> placenta: pre-clinical studies	10 mL; 1x10 ¹⁰	N/A	N/A	N/A	N/A	1 x 10 ¹¹
Human placenta <i>in vitro</i> explants	1 mL; 2.67x10 ¹²	N/A	N/A	1.5 mL	5x10 ¹⁰	7.5x10 ¹⁰
Human placenta <i>ex vivo</i> perfusion	1 mL; 2.67x10 ¹²	10	2 mL; 2.67x10 ¹¹	10 mL ^α (normal and FGR)	5x10 ¹⁰	5x10 ¹¹

^α Based on approximately 35% volume density for the intervillous space in the perfusion fixed lobule of an *ex vivo* perfused normal term placenta⁵⁴, interpolated to a mean fresh wet perfused mass of approximately 30g (equating to a total perfused tissue volume of 30 mL). [‡] Assumes an estimated human placenta mass of 150 g (approximately 150mL) in the severe FGR case at approximately 25 weeks of gestation, where the intervillous space volume density is extrapolated to 35% based on evidence from ^α.

Supplementary Table 2.

a. Patient demographics for placentas used in explant culture.

IBC definition	GA Median (25 th & 75 th centiles)	IBC	Systolic / diastolic blood pressure at booking (≠ maximum BP when taken)	Mode of delivery
Normal	283 (271, 286)	42.5 (10.25, 79.00)	110/70 (100/54, 115/75)	3 x NVD 3 x ELCS

Data is shown as median (25th, 75th centiles).

b. Patient demographics for placentas used in dual perfusion experiments.

IBC definition	Vector Treatment group	GA at delivery (weeks + days)	IBC	Systolic / diastolic blood pressure at booking (≠ maximum BP when taken)	Mode of delivery	Perfused placenta wet weight (g)
Normal	FB	39 + 0	51	106 / 60 (*110 / 70)	ELCS	56
Normal	Ad.VEGF ^{ANAC}	39 + 6	98	120 / 70	NVD	69
FGR	Ad.VEGF ^{ANAC}	36 + 2	2	100 / 60 (*110 / 58)	ELCS	64
FGR	FB	39 + 6	5	120 / 70	ELCS	45
SGA	Ad.VEGF ^{ANAC}	39 + 0	8	100 / 60	ELCS	41
SGA	Ad.VEGF ^{ANAC}	39 + 1	9	120 / 62	ELCS	53
Normal	FB	39 + 3	40	102 / 64	ELCS	60
FGR	Ad.VEGF ^{ANAC}	39 + 2	1	100 / 60	NVD	27
FGR	Ad.VEGF ^{ANAC}	39 + 4	4	86 / 60 (*100 / 60)	ELCS	36
Normal	Ad.VEGF ^{ANAC}	39 + 0	33	116 / 68 (*120 / 80)	ELCS	66

Abbreviations: FGR, fetal growth restriction, where the individualised birthweight centile (IBC) was ≤ 5.0⁴; SGA, small for gestational age, where the IBC was ≤ 10 and > 5.0; GA: gestational age; Ad.VEGF-D^{ANAC}, adenoviral vector carrying the vascular endothelial growth factor D pre-processed short form gene; FB, formulation buffer; BP, blood pressure; ELCS, elective Caesarean section; NVD, normal vaginal delivery.

Supplementary Table 3 *Ex vivo* human dual placental perfusion operations schedule. Ad.VEGF-D: Adenovirus vector containing VEGF-D^{ANΔC}; FB: formulation buffer.

Fetal-Side			Fetal-Side Samples to Aliquot					Maternal-Side			Maternal-Side Aliquots			
sample time (hr:min:sec)	sample number	collection tube (ml)	No. of Eppendorfs	for creatinine FITC-inulin (@-20oC)	for #viral transfer (@-80oC)	for LDH/ nitrite (@-20oC)	spare (@-20oC)	sample time (hr:min)	sample number	collection tube (ml)	No. of Eppendorfs	for alk. phos. / hCG LDH / nitrite (@-20oC)	spare (@-20oC)	
0:00:00 fetal side of lobule - connect & start flow hydrostatic pressure - record														
0:07 - 0:08 6ml venous volume return check (60 sec)														
Ad.VEGF-D / FB - to microbiological safety cabinet paracellular markers - thaw in water bath maternal-side of lobule - cannulation & perfusion commences Ad.VEGF-D / FB - 1:10 dilution in EBB - into 5 mL syringe - syringe to fridge														
00:29	maternal reservoir - switch to paracellular markers													
0:30 - 0:35	F1	50ml	4	2 x 1ml			2 x 1ml	0:32 - 0:33	M1	50ml	6	4 x 1ml	2 x 1ml	
0:35 - 0:40	F2	50ml	4	2 x 1ml			2 x 1ml	0:37 - 0:38	M2	50ml				
0:40 - 0:45	F3	50ml	4	2 x 1ml			2 x 1ml	0:42 - 0:43	M3	50ml				
0:45 - 0:50	F4	50ml	4	2 x 1ml			2 x 1ml	0:47 - 0:48	M4	50ml				
0:50 - 0:55	F5	50ml	4	2 x 1ml			2 x 1ml	0:52 - 0:53	M5	50ml				
0:55 - 1:00	F6	50ml	4	2 x 1ml			2 x 1ml	0:57 - 0:58	M6	50ml				
1:00 - 1:05	F7	50ml	4	2 x 1ml			2 x 1ml	1:02 - 1:03	M7	50ml				
01:08 syringe - to perfusion cabinet														
1:05 - 1:10	F8	50ml	4	2 x 1ml			2 x 1ml	1:06 - 1:07	M8	50ml	5 x 0.5mL reservoir samples also taken			
01:10:00 control sample pot # 1 - to fetal venous cannula maternal inflow 3-way tap - attach administration syringe maternal buffer - normal EBB maternal inflow 3-way tap - open 3 ways														
01:10:50 maternalpump - switch off														
01:11:00 maternal inflow 3-way tap - Viral / FB admin: 5 seconds plunge, close luer lock) followed by 2 second of MATERNAL flow maternal inflow 3-way tap - close to syringe maternal pump - 2 second pulse of flow														
01:11:08 maternal pump - is now off														
01:12:00 control sample pot # 1 - replace with CONTROL SAMPLE POT # 2														
01:15:30 control sample pot # 2 - cap & remove														
01:16:00 maternal pump - on														
1:16 - 1:31	F9	120ml	2	2 x 1ml				1:17 - 1:18	M9	50ml	3 x 0.5mL reservoir samples also taken			
1:31 - 1:51	F10	120ml	2	2 x 1ml				1:32 - 1:33	M10	50ml	6	4 x 1ml	2 x 1ml	
1:51 - 2:11	F11	120ml	6	2 x 1ml	2 x 1ml	2 x 1ml		1:52 - 1:53	M11	50ml				
2:11 - 2:31	F12	120ml	2	2 x 1ml				2:12 - 2:13	M12	50ml				
2:31 - 2:51	F13	120ml	2	2 x 1ml				2:32 - 2:33	M13	50ml	6	4 x 1ml	2 x 1ml	
2:51 - 3:11	F14	120ml	6	2 x 1ml	2 x 1ml	2 x 1ml		2:52 - 2:53	M14	50ml				
3:11 - 3:31	F15	120ml	2	2 x 1ml				3:12 - 3:13	M15	50ml				
03:30 maternal reservoir - switch to paracellular markers														
3:31 - 3:36	F16	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:33 - 3:34	M16	50ml	5 x 0.5mL reservoir samples also taken			
3:36 - 3:41	F17	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:38 - 3:39	M17	50ml				
3:41 - 3:46	F18	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:43 - 3:44	M18	50ml				
3:46 - 3:51	F19	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:48 - 3:49	M19	50ml				
3:51 - 3:56	F20	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:53 - 3:54	M20	50ml				
3:56 - 4:01	F21	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	3:58 - 3:59	M21	50ml				
4:01 - 4:06	F22	50ml	6	2 x 1ml	2 x 1ml		2 x 1ml	4:03 - 4:04	M22	50ml				
4:06 - 4:11	F23	50ml	8	2 x 1ml	2 x 1ml	2 x 1ml	2 x 1ml	4:08 - 4:09	M23	50ml	6	4 x 1ml	2 x 1ml	
04:11 - 4:21 fetal perfusate - remains as normal EBB maternal perfusate - 4% neutral buffered formalin hydrosattic pressure - monitor that it does not exceed +3-4mmHg and stop after 10 min, or before if necessary.														

Supplementary Table 4: Recovery of spiked Ad.VEGF-D^{ΔNΔC} DNA from fetal perfusate samples with different DNA extraction protocols

Method used	Extraction efficiency (%)
QIAamp DNA Blood Midi kit	No detection
QIAamp DNA Mini kit	1.32
TRIzol in combination with QIAamp Blood Midi kit	No detection
TRIzol and chloroform extraction method	No detection
NucleoSpin Tissue kit	8.83
NucleoSpin Tissue kit with heating step	6.70
DNA concentration using the sodium acetate/ethanol	No detection
DNA concentration using the Speedvac	No detection
qPCR detection without DNA extraction	13.34
qPCR detection without DNA extraction with heating step	4.69
qPCR detection without DNA extraction with 10-fold dilution	13.56
qPCR detection without DNA extraction with 10-fold dilution and heating step	No detection
NucleoBond kit	90.82

Supplementary Table 5: Recovery of spiked Ad.VEGF-D^{ΔNAC} DNA from different volumes of fetal perfusates using the NucleoBond® extraction kit.

Sample ID	Placental perfusate volume (μL)	Ad.VEGF-D ^{ΔNAC}					ANTP detection						
		Nominal concentration (vp/well)	Mean Ct	ΔCt	Back-calculated concentration (vp/well)	Extraction yield	Nominal concentration (copies/well)	Mean Ct	ΔCt	Ct Status	Back-calculated concentration (copies/well)	RE%	Inhibition?
330-1	330	3.33E+04	24.26	0.18	3.33E+04	99.82	1.00E+04	29.51	0.23	ACCEPTED	1.37E+03	14.36	No
330-2	330	3.33E+04	24.42	0.36	3.00E+04	90.14	1.00E+04	29.58	0.39	ACCEPTED	1.32E+03	10.1	No
330-3	330	3.33E+04	23.97	0.01	3.95E+04	118.55	1.00E+04	29.01	0.04	ACCEPTED	1.86E+03	55.6	No
500-1	500	3.33E+04	24.11	0.16	3.64E+04	109.27	1.00E+04	29.13	0.07	ACCEPTED	1.73E+03	44.19	No
500-2	500	3.33E+04	24.39	0.46	3.06E+04	91.78	1.00E+04	29.48	0.35	ACCEPTED	1.40E+03	16.62	No
500-3	500	3.33E+04	23.73	0.06	4.58E+04	137.48	1.00E+04	28.92	0.04	ACCEPTED	1.96E+03	63.51	No
700-1	700	2.5 E+04	25.96	0.1	2.85E+04	113.91	1.00E+04	30.62	0.08	ACCEPTED	9.44E+03	2.36	No
700-2	700	2.5 E+04	27.26	0.03	1.34E+04	53.77	1.00E+04	30.42	0.21	ACCEPTED	1.07E+04	16.37	No
700-3	700	2.5 E+04	27.12	0.08	1.46E+04	58.23	1.00E+04	30.94	0.11	ACCEPTED	7.62E+03	-17.33	No

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Supplementary Table 6: Ad.VEGF-D^{ΔNΔC} qPCR analytical validation parameters

Ad.VEGF-D^{ΔNΔC} analytical validation parameters	
Dynamic Range	5 to 5x10 ⁴ vp/μL of DNA
Limit of detection	2.5 vp/μL of DNA
Accuracy (%RE)	-7.97 to 28.28%
Precision (%CV)	-9.75 to 26.66%
Specificity	Highly specific

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Supplementary Table 7 A comparison of analyte measurements between the normal and FGR groups following Ad.VEGF-D^{ΔNAC} administration to the *ex vivo* dually perfused human placental cotyledon.

Analyte	Ad.VEGF-D ^{ΔNAC} treated normal group (mean ± SE; N=4)	Ad.VEGF-D ^{ΔNAC} treated FGR group (mean ± SE; N=3)
materno-fetal creatinine steady state clearance (of 35-40 minute sample) %change of post- vs pre Ad.VEGF-D ^{ΔNAC} exposure	122.4 ± 10.6	133.6 ± 35.8
materno-fetal FITC-inulin steady state clearance (of 35-40 minute sample) %change of post- vs pre Ad.VEGF-D ^{ΔNAC} exposure	129.2 ± 23.9	80.6 ± 46.4
LDH _{maternal venous} (at 258.5 minutes) % change from 57.5 minute value	95.4 ± 10.8	66.1 ± 20.8
hCG _{maternal venous} (at 258.5 minutes) % change from 57.5 minute value	62.3 ± 3.1	65.5 ± 13.4
Alkaline phosphatase _{maternal venous} (at 258.5 minutes) % change from 57.5 minute value	39.6 ± 8.6	55.0 ± 17.1

Figure 1 (a) Haematoxylin & eosin staining of explant tissue fixed in 4% neutral buffered formalin on day 7 of culture (syncytiotrophoblast, STB; fetal capillary, FC; intervillous space, IVS; villous stroma, VS). Evidence of syncytiotrophoblast regeneration, comparable to untreated controls, in tissue treated on day 5 with virus formulation buffer (FB) or $5 \times 10^{7-10}$ vp/ml Ad.VEGF-D^{ΔNAC}. Images also confirm villous structural integrity was maintained throughout the 7 day culture period, regardless of treatment and dose. Representative images from N=6 placentas, duplicate observations for each. **(b)** Detection of β-gal protein (brown staining) in Ad.LacZ-treated tissue (ii-v) using IHC. β-gal was observed in only a minority of villi and was mainly confined to the syncytiotrophoblast with occasional staining of villous stromal cells (“S” arrow, v) and was absent from the fetal vascular endothelium. β-gal staining was absent from untreated controls (i) and a negative control is shown, where mouse IgG was substituted for the primary antibody during IHC protocol (vi).

Figure 2 (a) hCG secretion and **(b)** LDH release by placental explants on day 6 (closed bars) and day 7 (open bars) of culture expressed as a % of levels on day 5 (i.e. prior to any treatment).

Figure 3 Fetal-side resistance and maternofetal paracellular marker clearance in *ex vivo* perfused human placental lobules treated with either formulation buffer (N=3), or Ad.VEGF-D^{ΔNAC} (N=7). Fetal-side inflow hydrostatic pressure (**a**, FIHP), reflecting resistance in the villous microcirculation in formulation buffer (circles) and Ad.VEGF-D^{ΔNAC} (squares) treated lobules. Data are expressed as the mean ± SE percentage change from the pre-treatment T=32.5 minute value (100%). There was no difference in clearance between treatment groups and no effect of time (two-way ANOVA). Effect of formulation buffer (open

bars) and Ad.VEGF-D^{ΔNΔC} (closed bars) on steady state maternofetal creatinine (**b**) and FITC-inulin clearance (**c**); there was no difference between treatment groups (Mann-Whitney test, both markers; data is represented as the medians & interquartile ranges, expressed as the percentage change from the pre-treatment clearance period).

Figure 4 Alkaline phosphatase (AP; **a**), lactate dehydrogenase (LDH; **b**) and human chorionic gonadotrophin (hCG; **c**) release into the maternal circulation of the ex vivo dually perfused human placental lobule following treatment with formulation buffer (open circle; N=3) and Ad.VEGF-D^{ΔNΔC} (open square; N=7). AP and hCG release were not significantly different between treatment groups, but their release significantly reduced with time (two-way ANOVA; $P < 0.001$ & $P < 0.01$, respectively). LDH release was significantly higher in the Ad.VEGF-D^{ΔNΔC} group (two-way ANOVA; $P < 0.0001$; Sidak's multiple comparison test: $*P < 0.05$, $**P < 0.01$) and LDH release reduced with time ($P < 0.01$). Data are represented as mean $\pm$ SE.

Figure 5 Photomicrographs demonstrating normal third trimester placental tissue (top left) and third trimester placenta with infarction (bottom left) which have been immersion fixed and routinely processed versus cases of similar gestational age placentas which have undergone perfusion (right column; all haematoxylin and eosin, original magnification x100). Perfusion results in apparent 'clearing' of the intervillous space, an empty appearance of the villous vessels and slight villous enlargement.

Supplementary Figure 1 *Ex vivo* human placental perfusion apparatus, depicting the assembled cannulated human placental lobule, perfused by peristaltic roller pumps. The lobule was maintained within a heated perfusion chamber and held within a placental clamp platform (Hemotek, Accrington, UK). The fetal chorionic plate, serving the intact lobule has been cannulated along with the corresponding fetal chorionic plate vein and inflow arterial hydrostatic pressure is recorded by way of a hydrostatic pressure transducer, processed by a Nanologger coupled to a pc and displayed by software (Gaeltec, Isle of Skye, UK). The maternal perfusate was pumped into the intervillous space via 5 cannulae, emanating from a perfusion manifold (Harvard Apparatus, UK), with the option of introducing the Ad. VEGF- $D^{\Delta NAC}$, or formulation buffer, from a 5 mL syringe coupled to a three way-tap afferent of the manifold. Maternal-side ‘venous’ and fetal-side venous perfusate was periodically collected into pre-weighted universal tubes for 60 seconds, reweighed, centrifugated, aliquoted and the supernatants stored frozen prior to assay. The perfusate timed collection and weight determination permitted a calculation of the development of potential fetomaternal leakage by bulk flow (specific gravity of perfusate is 1.013 at RT).

Supplementary Figure 2 Fetal-side venous recovery expressed as a percentage of fetal arterial inflow was not different between FB (n=3) and Ad.VEGF- $D^{\Delta NAC}$ (n=7) treatment groups and there was no effect of time on the development of a loss in fetal venous recovery (two-way ANOVA).

Supplementary Figure 3 Schematic of experimental design for *ex vivo* dual perfusion of the human placental lobule. Maternal- and fetal-side perfusate compositions are represented in a series of continuous rectangles against a time-line and sampling points for various outcome

measures are symbolised and explained in the Key. Quality assurance (QA) measures are tested within the 70 minutes of perfusion, ensuring fetal side inflow hydrostatic pressure, representing resistance to flow in the villous microcirculation is within reasonable limits (< 60 mmHg) and that a fetomaternal perfusate “leak” is absent.

Supplementary Figure 4 Time lapse photography following the administration of bromophenol blue dye to maternal intervillous space of the *ex vivo* dually perfused human placental lobule. Following an initial period of dual perfusion the maternal pump was stopped and a 2mL injection of the dye was made from a syringe via the 3 way tap and the maternal-side pump was switched back on (T=0 seconds) to recommence flow at 14 mL/min. Dye compartmentalisation status at T=4 seconds (**a**) and T=6 seconds (**b**). This pilot investigation allowed an understanding of the optimal timings for maternal pump “ON/OFF” switching, to maximise FB and Ad.VEGF- D^{ΔΔC} bolus retention within the intervillous space during the 5 minute stasis of flow.

Supplementary Figure 5 The genetic map for Ad.VEGF-D^{ΔΔC}.

Supplementary Figure 6 The qPCR calibration curve permitting an absolute quantification of the adenovirus vector DNA.