Comparison of efficiency and function of VEGF adenovirus vectors in endothelial cells for gene therapy of placental insufficiency*

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Short title: In vitro comparison of adenovirus VEGF vectors

Abstract:

Introduction: Severe fetal growth restriction (FGR) affects 1:500 pregnancies, is untreatable and causes serious neonatal morbidity and death. Reduced uterine blood flow (UBF) and lack of bioavailable VEGF due to placental insufficiency is a major cause. Transduction of uterine arteries in normal or FGR sheep and guinea pigs using an adenovirus (Ad) encoding VEGF isoforms A (Ad.VEGF-A_{165}) and a FLAG-tagged pre-processed short form D ($D^{\Delta N \Delta C}$, Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG) increases endothelial nitric oxide expression, enhances relaxation and reduces constriction of the uterine arteries and their branches. UBF and angiogenesis are increased long term, improving fetal growth *in utero*. For clinical trial development we compared Ad.VEGF vector transduction efficiency and function in endothelial cells (ECs) derived from different species.

Objective: To compare the transduction efficiency and function of the pre-clinical study Ad. constructs (Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG) with the intended clinical trial construct (Ad.VEGF-$D^{\Delta N \Delta C}$) where the FLAG tag is removed.

Methods: We infected ECs from human umbilical vein, pregnant sheep uterine artery, pregnant guinea pig aorta and non-pregnant rabbit aorta, with increasing multiplicity of infection (MOI) for 24 or 48 hours of three Ad.VEGF vectors, compared to control Ad. containing the LacZ gene (Ad.LacZ). VEGF supernatant expression was analysed by ELISA. Functional assessment used tube formation assay and Erk-Akt phosphorylation by ELISA.

Results: VEGF expression was higher after Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N \Delta C}$ transduction compared to Ad.VEGF-A_{165} in all EC types (*$p<0.001$). Tube formation was higher in ECs transduced with Ad.VEGF-$D^{\Delta N \Delta C}$ in all species compared to other constructs (***$p<0.001$, *$p<0.05$ with rabbit aortic ECs). Phospho-Erk and phospho-Akt assays displayed no differences between the three vector constructs, whose effect was, as in other experiments, higher than Ad.LacZ (***$p<0.001$).

Conclusion: We observed high transduction efficiency and functional effects of Ad.VEGF-D$^{\Delta N \Delta C}$ vector with comparability in major pathway activation to constructs used in pre-clinical studies, supporting its use in a clinical trial.

Introduction:

Placental insufficiency is a severe obstetric condition in which placental development is impaired, and as a result, there is an adequate supply of nutrients and oxygen to the developing fetus. The pathology starts with impaired trophoblast invasion of the spiral arteries in the first and early second trimester of pregnancy [1] leading to suboptimal increase in maternal blood flow to the uterus in the first half of pregnancy and hypoxia related abnormalities of placental development, including failed development of the fetoplacental circulation [2]. This "insufficient" placental function results in suboptimal fetal growth or fetal growth restriction (FGR) and is a common cause of stillbirth [3]. There is currently no treatment and prophylaxis is often delivered too late or is insufficient to prevent the disease [4,5].An International consensus definition of FGR defines FGR as early or late according to gestational age at onset [10]. The European Medicines Agency Committee for Orphan Medicinal Products (EMA COMP) granted orphan status in 2015, with an estimated annual incidence of 3.33 per 10,000 EU population (95% CI 3.07 to 3.60) [11,12.]

Normal vasculogenesis, the formation of new blood vessels, and angiogenesis or blood vessel growth are vital for functional development of the placenta and healthy pregnancy outcome. Members of the Vascular Endothelial Growth Factor (VEGF) family and their receptors are key regulators in trophoblast invasion and in angiogenesis of the placenta and maternal decidua [13,14]. The soluble form of VEGFR-1, soluble fms-like tyrosine kinase 1 (sFlt-1), binds VEGF-A and PlGF, inhibiting their actions. In FGR and placental insufficiency however the normal balance of angiogenic factors is perturbed towards anti-angiogenesis, with an increase in sFlt1 and a reduction in the maternal circulating VEGF-A and PlGF observed in FGR and the related condition pre-eclampsia[18,19,20,21,22], whilst PlGF is over-expressed in hypoxic ischaemic placental lesions that are pathological hallmarks of FGR [16]. Therapies based on the manipulation of VEGF and related angiogenic factors are therefore likely to be effective for FGR[4]. The pro-angiogenic transgene is not required to integrate in the genome as short-term expression is sufficient. Given the angiogenic and vasodilatory actions of VEGF, it may be

preferable to target increased VEGF availability to the maternal uteroplacental circulation using locally delivered gene therapy, rather than increase systemic maternal VEGF levels, which may have generalised haemodynamic side effects. Long-term administration of a proangiogenic peptide into the uteroplacental circulation is not feasible due to the risk of haemorrhage and infection leading to perinatal loss. As an alternative we are exploring therapeutic angiogenesis via a local gene therapy approach that has been applied in many cardiovascular diseases such as severe cardiac and peripheral ischemia, heart failure and vein graft failure [26,27].

Studies assessing the entire VEGF family for angiogenic potency as gene therapies demonstrated that VEGF-A_{165} and VEGF-$D^{\Delta N \Delta C}$ induced the strongest effects [28]. In pre-clinical models we targeted an adenovirus vector (Ad.) carrying the pro-angiogenic transgene VEGF-A_{165} to the uterine circulation at maternal laparotomy, with direct intravascular injection in pregnant sheep under direct vision, distal to a 5 minute digital occlusion. For guinea pig experiments, Ad. vectors were applied externally to the uterine arteries and their branches in combination with a thermolabile pluronic gel [29]. Experiments were performed in normal pregnant sheep,[30,31,32], FGR pregnant sheep [33,34] and FGR pregnant guinea pigs [29,35,36]. We chose pregnant sheep in which to first assess the effect of maternal gene therapy on uterine blood flow and fetal growth because many aspects of sheep fetal and vascular physiology are similar to the human and because the ovine fetus is similar in size to the human. Moreover, ultrasound quantification of uterine blood flow, fetal growth and wellbeing are validated techniques for monitoring normal and FGR sheep pregnancy, and which allows comparison with human FGR [51,52]. Guinea pigs were chosen as the optimal preclinical animal model in which to test the effectiveness of maternal VEGF gene therapy to increase fetal growth, as guinea pig placentation is the most similar to human placentation with a haemochorial type of placenta, a homologous process of trophoblast invasion and trophoblast cell proliferation [53]. In addition, the long gestational age of 60 days in the guinea pig allows time for gene therapy to be administered in mid or late gestation and for an effect on fetal growth to be realised.

Ad.VEGF-A_{165} consistently showed a potential therapeutic effect with increased VEGF protein concentration locally in tissues and in the blood, and in FGR animals this improved fetal growth velocity, fetal size at the end of gestation and postnatal growth. Vector delivery also increased endothelial nitric oxide expression, and enhanced relaxation and reduced constriction of the uterine arteries and their branches, with increased uterine blood flow and angiogenesis long term. There was no evidence of vector in a wide variety of fetal tissues[30,31,32,35,36].

The Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG vector has been developed for the treatment of refractory angina patients using percutaneous endocardial vector administration [37,38]. The marker FLAG sequence on the amino-terminus of the expressed transgene product is a standard peptide motif that is included to facilitate experimental methodologies, with sequence DYKDDDDK. Ad. vectors containing the VEGF-$D^{\Delta N\Delta C}$-FLAG and VEGF-$D^{\Delta N\Delta C}$ can bind receptors VEGFR2 and VEGFR3 with greatly increased affinity, compared with unprocessed or partially processed forms of VEGF-D [39,28]. In the mid-gestation pregnant sheep, local uterine artery Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG administration had similar short and long term effects to Ad.VEGF-A_{165}, with VEGF-D protein detected locally in tissues and in the blood, increased endothelial nitric oxide expression and enhanced relaxation and reduced constriction of the uterine arteries and their branches, with significant periadventitial angiogenesis long term [40]. Ad. VEGF-$D^{\Delta N\Delta C}$-FLAG however elicited lower vascular permeability and a lack of inflammatory cell infiltration compared to Ad.VEGF-A_{165}, which lead us to propose using the VEGF-$D^{\Delta N\Delta C}$ isoform for a first-in-human clinical trial in pregnant women with severe FGR due to placental insufficiency [41]. Ad.VEGF-$D^{\Delta N\Delta C}$ was further tested in an *ex vivo* human placenta perfusion study to assess vector spread[42]. In the dual perfused human placenta Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG did not markedly affect human placental integrity and function *in vitro* and there was limited tissue access and transfer of vector across the placental barrier. Except for a minor elevation in lactate dehydrogenase (LDH) release, there were no toxic effects of Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG. Scientific advice from the regulator advised the removal of the FLAG sequence from the intended clinical vector construct as it has no therapeutic role.

The definitive GLP-compliant reproductive toxicology study discussed with the MHRA, has been conducted in the pregnant New Zealand white rabbits, as its haemodichorial placentation is the most appropriate animal model for human pregnancy during the second trimester. In addition, rabbits provide a sufficient number of offspring for meaningful data for interpretation, there is considerable historical normal range data, and Ad. vectors can be injected into the uterine arteries via angiographically placed catheters inserted into the carotid artery, with an acceptable rate of miscarriage [54]. Using this technique we observed that the Ad.VEGF-D$^{\Delta N\Delta C}$ vector did not appear to adversely affect rabbit dam or pup survival and there was no evidence of vector spread across the placenta to the pups [44].

The EC funded EVERREST project proposed a first-in-woman uncontrolled, open-label, dose finding safety and tolerability (to mother and neonate) clinical trial using a GMP manufactured replication deficient Ad.VEGF-D$^{\Delta N\Delta C}$ vector[43]. Entry criteria would be designed to identify the most severe early onset FGR fetuses with the highest perinatal mortality and morbidity: (1) estimated fetal weight (EFW) <3rd centile for gestational age [45], singleton fetus with (2) confirmed gestational age >22 weeks and <26+0 weeks at vector administration and (3) evidence of placental insufficiency defined as raised mean uterine artery doppler Pulsatility Index and (4) raised umbilical artery Pulsatility Index. Pregnant women with other causes of FGR such as congenital infection, abnormal fetal karyotype and/or abnormal detailed anomaly scan would be excluded. Ad.VEGF-D$^{\Delta N\Delta C}$ vector would be delivered to pregnant women using X-ray guidance by intra-arterial balloon catheter to both uterine arteries sequentially [50] with a short 2-5 minute cessation of the blood flow within the uterine artery during vector instillation. Vascular occlusion has been used previously in the pre-clinical rabbit and sheep studies to increase local uterine artery delivery [30,31,32–34]. Detailed maternal, fetal and neonatal assessment for *in vivo* toxicity and biodistribution would be made before and at intervals after vector instillation with birth timed according to fetal Doppler examination and growth, and/or maternal condition according to current clinical practice. Primary and secondary exploratory outcomes would be compared to outcomes from the contemporaneous EVERREST

prospective observational study where currently the cumulative perinatal mortality and severe morbidity rate in these pregnancies is 75-80% [46].

As the preclinical package for this programme comprises studies of Ad.VEGF-$D^{\Delta N \Delta C}$, Ad.VEGF-$D^{\Delta N \Delta C}$ with a FLAG tag (Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG) with supporting data from Ad.VEGF-A_{165} we performed bridging studies to demonstrate functional and mechanistic equivalence of the vectors. The constructs were tested using a panel of assays following infection of endothelial cells from four species (sheep, guinea pig, rabbit and human), that have been involved in the preclinical experiments, the reproductive toxicology and the future clinical trial.

Results and Discussion:

Objectives and Study Design.

We compared the *in vitro* transduction efficiency and function of three VEGF adenovirus vectors (Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N \Delta C}$) involved in preclinical studies that are being used to support a first-in-woman clinical trial of a maternal gene therapy for the treatment of severe early onset FGR caused by placental insufficiency. The study was required by the European Medicines Agency (EMA) in order to compare the infectivity and expression of the transgene, and major cellular pathway activation across the three preclinical species used. We chose to test different multiplicity of infection (MOI) of the three VEGF adenoviral vectors in a relevant cell model for uterine artery endothelium, the target cell population for the clinical trial; a fourth Ad. vector of the same serotype but carrying the LacZ transgene was used as control.

Four endothelial cell sources were used for these experiments, from the three species that had been used for preclinical efficacy (sheep, guinea pig) and reproductive toxicology (rabbit) experiments, as well as a primary human cell line. For this study endothelial cells were isolated from the uterine arteries of two pregnant sheep at a comparable gestational age to the planned clinical trial intervention (0.6 gestation, see Material and Methods). As there is no commercially available primary cell line, endothelial cells have been isolated from the guinea pig abdominal aorta (see Materials and Methods) as it was not possible to isolate endothelial cells from the uterine artery, due to its small size (200⌠m diameter) and relatively short length. We used commercially available primary endothelial cell lines isolated from rabbit aorta and human Umbilical Vein Endothelial Cells (HUVECs) [55,56]. For each of the 4 endothelial cell sources, we studied VEGF expression, tube formation assay as an assessment of VEGF function, and major signalling pathway activation after application of the 4 different Ad. Vectors (Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N \Delta C}$, Ad.VEGF-$D^{\Delta N \Delta C}$-FLAG and Ad.LacZ, Figure 1).

Summary of Data

VEGF expression

The first series of experiments quantified VEGF expression in cell supernatants after *in vitro* infection of the four endothelial cell lines with the three VEGF Ad. vectors, after 24 and 48 hours of infection, in comparison to the control Ad.LacZ. Cell supernatants were analysed by ELISA assays specific for the VEGF isoforms expressed by the three adenoviruses (VEGF-A_{165} and VEGF-$D^{\Delta N\Delta C}$). Experiments were performed in triplicate. Among all MOI applied at 24 and 48 hours, there was no difference in VEGF expression at an MOI of 1 when compared to the control Ad.LacZ vector, suggesting that this MOI was too low to achieve VEGF expression. An MOI of 10000 however was found to generate a cytopathic effect, leading to cell death and detachment from the cell culture plate surface. There were no differences between the various endothelial cell types and the different vectors (data not shown). After an initial screening, with MOI between 1 and 10000, we excluded results from 1 MOI and and 10000 MOI due to no effect and cell death respectively.

At the middle range of MOI (10-1000 MOI) VEGF expression displayed similar results both at 24 hours and 48 hours (lower expression after 24 hours of incubation). We present as indicative results the data obtained with 100 MOI after 48 hours of incubation in **Figures 2-4**. All the results are expressed as fold change, considering that a basal VEGF expression, when cells are infected with Ad.LacZ, is valued as 1. VEGF expression in psUAECs (**Figure 2** top left) was comparable between Ad.VEGF-A_{165} and Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG transduced cells, but higher in Ad.VEGF-$D^{\Delta N\Delta C}$ transduced cells (fold change 243 vs 247 vs 303 respectively, $p<0.001$). VEGF expression in gpAECs (**Figure 2**, top right) was not significantly different between the three VEGF Ad. vectors. VEGF expression in rAECs (**Figure 2**, bottom left) was higher with Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$, compared to Ad.VEGF-A_{165} (fold change 465 vs 478 vs 283 respectively, $p<0.001$). In HUVECs (**Figure 2** bottom right) VEGF expression was higher with Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$, compared to Ad.VEGF-A_{165} (fold change 498 vs 507 vs 303 respectively, $p<0.001$). All three Ad. vectors led

to higher VEGF expression when compared to Ad.LacZ control ($p<0.001$). These data altogether suggest that Ad.VEGF-$D^{\Delta N\Delta C}$ and Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG provide a higher functional VEGF expression, when compared to Ad.VEGF-A_{165}, in the endothelial cells tested.

Tube formation assay

In order to compare the pro-angiogenic action of the three adenoviruses *in vitro* in relevant models of endothelium, a functional assay is required (**Figure 3**) [57]. Tube formation assay, as *in vitro* formation of new vessels due to endothelial cell differentiation, is commonly used and it was performed using a standard and widely accepted protocol (see Material and Methods and **Supplementary Figure 2**). Tube formation was quantified as fold change, compared to the basal endothelial differentiation that occurred when cells were infected with Ad.LacZ vector. Experiments were performed in triplicate. For an MOI of 100 after 48 hours of incubation, tube formation assay performed on psUAECs (**Figure 3,** top left) showed no difference between the three VEGF Ad. vectors, all producing a higher number of neovessels when compared to Ad.LacZ (***$p<0.001$). When the assay was performed on gpAECs (**Figure 3,** top right), the number of neovessels obtained with Ad.VEGF-$D^{\Delta N\Delta C}$ was higher, when compared with Ad.VEGF-A_{165} and Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG (fold change 1.7 vs 1.3 vs 1.4 respectively, $p<0.001$); Ad.VEGF-A_{165} and Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG induced neovessel formation in a higher extent when compared to control Ad.LacZ transduction (*$p<0.05$). Tube formation assay in rAECs (**Figure 3**, bottom left) was not different between Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$ vectors, both inducing higher neovessel formation when compared to Ad.VEGF-A_{165} vector (fold change 1.8 vs 1.9 vs 1.4 respectively, $p<0.05$) and when compared to the control Ad.LacZ vector (**$p<0.01$). Finally, the tube formation test was performed on HUVECs (**Figure 3,** bottom right), in order to obtain an indication of the pro-vascular effect of the three VEGF adenoviruses on an *in vitro* model of human endothelium. Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$ transduction resulted in the highest extent of neovascularization when compared to Ad.VEGF-A_{165} (fold change 2.6 vs 2.3 vs 1.8 respectively, $p<0.02$) and when compared to control Ad.LacZ ($p<0.001$). Altogether, these

results suggest that all three vectors when tested in endothelial cell preparations from different species, provide an angiogenic effect higher than the control Ad.LacZ vector, with Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$ being more efficient compared to Ad.VEGF-A_{165} in rAECs and in particular HUVECs. The latter is important data suggesting higher efficiency of Ad.VEGF-$D^{\Delta N\Delta C}$ in human therapy.

Major cellular pathway activation

The aim of the final set of experiments was to underline the effect of the three VEGF Ad. vectors in major cellular pathway activation. The focus was on the MAPK/ERK and PI3K/AKT/mTor pathways as they are highly involved in angiogenesis and endothelium development. The MAPK/ERK pathway cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression [58]. The MAPK/ERK pathway is extremely complex, as there are multiple members of the kinase, transcription factor, apoptotic regulator and caspase executioner families, which can be activated or inactivated by protein phosphorylation; the pathway plays an important role in angiogenesis, as it represents one of the checkpoints leading to gene expression for neovascularisation[59]. Concerning PI3K/AKT/mTor pathway, it has a great involvement in the process of angiogenesis. This pathway, in fact, is known to play a key role in numerous cellular functions including proliferation, adhesion, migration, invasion, metabolism, survival and angiogenesis[59]. AKT controls protein synthesis and cell growth by leading to the phosphorylation of mammalian target of rapamycin (mTOR) [60]. Phosphorylation of such proteins is considered an event in the cellular machinery leading to gene expression and functional formation of new endothelium. Therefore we chose this process for analysis as a measure of the effect of endothelial cell transduction with the three VEGF Ad. vectors on expression of VEGF and cellular major pathways activation (**Figure 4**). All the measurements have been performed with specific ELISA assays (see Materials and Methods). Experiments were performed in triplicate. For ERK and AKT phosphorylation, in all endothelial cell sources, all of the three VEGF Ad. vectors resulted in higher ERK and AKT phosphorylation, when compared to

Ad.LacZ control ($p<0.001$, **Figure 4**). For psUAECs, rAEC, and gpAECs there were no significant differences in ERK and AKT phosphorylation between the three VEGF Ad. vectors. In the case of HUVECs (**Figure 4** bottom right), pAKT fold change was higher in Ad.VEGF-$D^{\Delta N\Delta C}$ and Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG compared to with Ad.VEGF-A_{165} transduced cells (fold change 9.8 vs 9.6 vs 7.2 respectively, $p<0.05$). In all cases, there were higher phosphorylation levels, when compared to the control Ad.LacZ vector (***$p<0.001$). These data altogether suggest no differences among the three vectors tested in all four species, suggesting that all the vectors promote activation of major intracellular signalling pathways.

Conclusions

This study describes a comparison of the effect of three Ad. vectors with the same serotype and promoters, but carrying transgenes coding with different VEGF isoforms (VEGF-A_{165}, VEGF-$D^{\Delta N\Delta C}$-FLAG and VEGF-$D^{\Delta N\Delta C}$) on endothelial cells from four different species. Three of the four species (sheep, guinea pig and rabbit) have been used for pre-clinical efficacy and safety assessments to support clinical translation of a novel maternal Ad.VEGF vector gene therapy to treat FGR due to placental insufficiency. It was advised before final clinical translation of our chosen Ad.VEGF-$D^{\Delta N\Delta C}$ vector, that we study Ad. vector function in endothelial cells from these three species to compare them to functional effects on human endothelial cells. We analysed transgenic VEGF protein expression after endothelial cell transduction, and consequent neovascularization from immature endothelium, and activation of major cellular pathways.

The data obtained in this study suggest comparability in the effect on the four endothelial cell types (sheep, guinea pig, rabbit and human) between Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$ in activating major cellular pathways (MAPK/ERK and PI3K/AKT/mTor). A marginally stronger effect in VEGF expression and tube formation was generally observed after Ad.VEGF-$D^{\Delta N\Delta C}$ transduction, in particular in HUVECs, a human *in vitro* model of endothelium.

The data show that removal of the FLAG-TAG moiety from the Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG as advised by regulatory authorities does not compromise the functional effect of the VEGF-D transgene in endothelial cells. Indeed, in human cells it may even improve it. For the first-in-woman clinical trial we aim to treat women with severe early onset FGR between 20 -26 weeks of gestation, with a high rate of perinatal loss (40-50%) where there is balance of risks and benefits. Experiments in unaffected pregnant women would not be ethical as there would be no potential benefit to their baby. A bioethical review and stakeholder survey concluded that there were no ethical or legal objections to maternal gene therapy, or to a clinical trial of this intervention and respondents viewed the proposed trial in positive terms [61]. Women previously

affected by severe early onset FGR were generally interested in participating in clinical trials that conferred a potential benefit to their unborn child. It would be beneficial however if there was the potential to reach a therapeutic effect in the phase I trial, thus the findings from these experiments are encouraging.

This is the first time that the Ad.VEGF-D$^{\Delta N\Delta C}$ construct without the FLAG moiety has been tested out in sheep, guinea pig and human cells. The FLAG-TAG Ad.VEGF-D$^{\Delta N\Delta C}$ construct has been extensively studied in rabbits and pigs to support clinical translation of local therapeutic angiogenesis for severe cardiac and peripheral ischemia. All studies have shown VEGF-D to increase both capillary size and perfusion in target muscles and no major side effects were detected with clinically relevant doses [28,62–64]. In humans the FLAG-TAG Ad.VEGF-D$^{\Delta N\Delta C}$ construct has been used in a phase I/IIa trial of patients with refractory angina. In this study a method for targeting the gene transfer to the hibernating and ischemic tissue was developed [65]. In addition, the treatment was well tolerated and there was a significant increase in myocardial perfusion reserve 3 months and 12 months after the gene transfer [37]. Studies of adenovirus vectors in guinea pigs have been mainly limited to cochlear gene transfer or for preclinical toxicology for hepatocellular carcinoma [66,67] as they are not a commonly used animal model of disease.

In conclusion we observed high transduction efficiency and functional effects of Ad.VEGF-D$^{\Delta N\Delta C}$ vector in endothelial cells from four species, with comparability in major pathway activation responses to Ad.VEGF-A_{165}, Ad.VEGF-D$^{\Delta N\Delta C}$-FLAG constructs used in pre-clinical studies, supporting its use in a clinical trial. This result supports the use of Ad.VEGF-D$^{\Delta N\Delta C}$ in a human clinical trial for FGR caused by placental insufficiency.

Supplementary Data

Material and Methods:

Adenovirus vector production

Ad. vectors carrying VEGF genes or LacZ gene were used in this study: Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$, Ad.VEGF-A_{165} and Ad.LacZ (FinVector Vision Therapies Oy, Finland). The Ad. viral vectors are a first-generation recombinant replication-deficient adenovirus (Ad5 serotype) containing E1- and partial E3- deletions, and an expression cassette for the human VEGF-$D^{\Delta N\Delta C}$, VEGF-A_{165} or LacZ. The expression cassette contains the human cytomegalovirus (CMV) enhancer and promoter elements, an intron and a SV40 polyadenylation signal. The VEGF-$D^{\Delta N\Delta C}$ transgene consists of the interleukin-3 (IL-3) signal peptide, VEGF-D gene nucleotides 277 – 603. The VEGF-$D^{\Delta N\Delta C}$-FLAG transgene has an additional FLAG octopeptide which resides on the N terminus of secreted VEGF-$D^{\Delta N\Delta C}$. The IL-3 signal peptide facilitates VEGF-$D^{\Delta N\Delta C}$ secretion from the expressing cell, and the signal peptide itself is cleaved from the secreted protein. 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. VEGF-A_{165} is the predominant variant of differently spliced VEGF-A isoforms and is a secreted protein with heparin-binding properties. The LacZ transgene has an additional signal sequence for nuclear localization of the produced protein.

The genetic maps are presented in **Supplementary Figure 1**.

Adenovirus vector expansion, isolation and titration

All Ad. vectors were expanded in 293T cells for adenoviral packaging (Clontech, USA) according to the following protocol: 5 T-75 flask per adenovirus (Corning, UK), containing 293T cells in DMEM medium (Life Technologies, UK), supplemented with 10% FBS (Sigma, UK) at 80% confluence, were infected with 100 multiplicity of infection (MOI). Cells were maintained at 37°C, 5% CO_2, in DMEM medium, 2% FBS for 2-5 days until cytopathic effect. Cells were harvested through pipetting and subjected to three freeze/thaw cycles, in 37°C water bath and

ethanol/dry ice bath. Subsequent Ad. vector purification was performed with Adenopure®Adenovirus Purification Kit (Puresyn, USA), according to manufacturer's instructions. Purified Ad. Vector was quantified with Quicktiter™ Adenovirus Titer ELISA Kit (Cell Biolabs Inc., USA). The MOI in the experiments are defined as PFU, and the viral titer per each preparation was derived from a standard curve from Ad-β gal positive control titrations, as per manufacturer's instructions. The final formulation of the material was 5 mM HEPES, 20% glycerol buffer, pH 7.8. One preparation of each adenovirus vector has been used in the experiments.

Endothelial cell culture

Endothelial cells were obtained from the uterine arteries of pregnant sheep and the aorta of guinea pig aorta under terminal anaesthesia. All animal procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and by local ethics committee review.

Human umbilical vein endothelial cells (HUVECs): HUVECs used in this study are commercially available (Lonza, UK). For their expansion, HUVECs were cultured in Endothelial Growth Medium (EGM, Lonza, UK) in T-75 flasks (VWR, UK), pre-coated with Attachment Factor (AF) 1X (Like Technologies, UK) were used. Cells were detached with Trypsin 0.25% (Life Technologies, UK) and seeded in 6-well plates (Corning, UK), at a density of $10x10^5$ per well and in 24-well plates (for tube formation assay, Corning, UK), precoated with AF 1X. Experiments were performed at passage 3 of culture. One cell culture batch has been used for all the experiments.

Pregnant sheep uterine artery endothelial cells (psUAECs): To extract pregnant sheep uterine artery endothelial cells, the uterine arteries from mid-gestation pregnant sheep (n=2, 90 days of gestation, term = 145 days) were carefully dissected distally from the serosal surface of the uterus back to their origin from the internal iliac artery under terminal anaesthesia as described [32]. The ewe was then put down with an overdose of intravenous pentobarbitone (40ml,

Euthatal, Merieux, UK). The uterine arteries were ligated at the two ends using 1-0 silk ties, removed as a single piece and transferred in 50 ml Falcon tubes (Corning, UK) containing Endothelial Cell Basal Medium (EBM, Lonza, USA) supplemented with 1.5µg/ml Fungizone (Life Technologies, UK). The uterine arteries were placed in a petri-dish under sterile laminar flow hood and cleared of surrounding connective tissue and blood clots. The vessels were secured distally with a 23 gauge butterfly needle and flushed with M-199 (50ml, Life Technologies, UK) to remove blood clots. The distal end of the vessel was then tied off and inflated with EBM containing 5mg/ml collagenase (Roche Diagnostics, Germany) and 0.5% bovine serum albumin (BSA, Sigma Aldrich, UK) to dissociate endothelial cells. Inflated vessels were placed in the 37°C incubator for 15 minutes. The distal tie was cut and the contents of the vessel flushed into a Falcon tube with Endothelial Cell Growth Medium (EGM, Lonza, UK) and centrifuged at 1000 rpm for eight minutes twice to concentrate the endothelial cells. After each cycle, the supernatant was removed and the pellet was re-suspended in EGM to remove debris. Freshly isolated cells were considered to be passage 0 and plated in 4 wells of a 6-well plate (Corning, UK) in EGM containing 10% Fetal bovine serum, 1% penicillin-streptomycin (Life Technologies, UK). Plate surfaces were treated with gelatin (Sigma Aldrich, UK) to enhance cell adhesion. Cells were grown for approximately 6 days and passaged (passage 1) to T-25 flasks (Corning, UK). Cells were trypsinized, seeded and grown to 70% confluence in T-25 flasks and then passaged (passage 2) to T-75 flasks (Corning, UK). Cells were again grown to approximately 70% confluence and passaged once more (passage 3).

We have previously confirmed endothelial cell identity using this isolation technique by microscopy observation of their typical cobblestone morphology in culture. The psUAECs also stained positively with fluorescently tagged Ac-LDL, anti-vWF, anti-VE cadherin and anti β-catenin, confirming their endothelial identity [32]. One cell culture batch has been used for all the experiments.

Rabbit aortic endothelial cells (rAECs): rAECs used in this study are commercially available (Cell Biologics Inc., USA). For their expansion, cells were cultured with endothelial growth

medium (EGM, Lonza, UK) in T-75 flasks (VWR, UK), pre-coated with Attachment Factor (AF) 1X (Like Technologies, UK). Cells were detached with Trypsin 0.25% (Life Technologies, UK) and seeded in 6-well plates (Corning, UK), at a density of 10^5 per well and in 24-well plates (for tube formation assay, Corning, UK), precoated with AF 1X. Experiments were performed at passage 3 of culture. One cell culture batch has been used for all the experiments.

Guinea pig aortic endothelial cells (gpAECs): Isolation and culture was based on a previously published study [68]. To obtain these cells, under terminal anaesthesia the abdominal cavity of normal non-pregnant female Dunkin-Hartley guinea pigs (n=2) was opened. The abdominal aorta was carefully dissected between the diaphragm and bifurcation (4-5 cm) and the animal was then put down with an overdose of intravenous pentobarbitone (5ml, Euthatal, Merieux, UK). The abdominal aorta was ligated at the two ends using 1-0 silk ties, removed as a single piece and transferred in a 50 ml Falcon tube (Corning, UK) containing Endothelial Cell Basal Medium (EBM, Lonza, USA) supplemented with 1.5µg/ml Fungizone (Life Technologies, UK). The vessels were placed in a petri-dish under sterile laminar flow hood. At the proximal end a 26 gauge butterfly needle was introduced and secured with a haemostat and M-199 (5ml, Life Technologies, UK) was flushed through the vessel to remove blood clots. The vessel was then tied off distally and inflated with EBM containing 500 µl (5 mg/ml) collagenase Roche Diagnostics, Germany) and 0.5% bovine serum albumin (BSA, Sigma Aldrich, UK). The inflated vessel was placed at 37°C for 10 minutes, following which the distal tie was then cut. The vessel was flushed into a falcon tube using Endothelial Cell Growth Medium (EGM, Lonza, UK) and the contents were centrifuged twice at 1000rpm for eight minutes to concentrate endothelial cells. The supernatant was removed after each cycle and the pellet was re-suspended in EGM to remove debris. Freshly isolated cells were plated in 2 wells of a 6-well plate (Corning, UK) in EGM containing 10% Fetal bovine serum, 1% penicillin-streptomycin (Life Technologies, UK). All surfaces on which endothelial cells were cultured were treated with gelatin (Sigma Aldrich, UK) to enhance adhesion. Cells were grown for approximately 6 days and passaged (passage 1) to T-25 flasks (Corning, UK). Cells were then trypsinized (Life

Technologies, UK) and kept in incubator for 2 minutes. Cells were seeded and grown to 70% confluence in T-25 flasks and then passaged (passage 2) to T-75 flasks (Corning, UK). Cells were grown to approximately 70% confluence and passaged once more (passage 3).

To confirm endothelial cell isolation, aliquots of 10000 cells were on glass slides in 24-well plates and stained in immunofluorescence for CD31, VE-cadherin and von Willebrand factor. Analyses confirmed that >98% of cells were positive for the three markers (data not shown). One cell culture batch has been used for all the experiments.

Endothelial cell transduction and processing

Endothelial cells from the four different species selected were infected with 1, 10, 100, 1000 and 10000 MOI in 6-well plates for 48 hours. After 48 hours of Ad. infection, cell supernatants were collected for analysis.

ELISA assays for VEGF quantification, phospho-Erk and phospho-Akt

Commercially available ELISA kits were used to quantify VEGF expression in cell supernatants according to manufacturer's instructions as follows: VEGF-A expression: R&D Systems Quantikine ELISA kit DVE00; VEGF-D: R&D Systems Quantikine ELISA kit DVED00. For the measurement of phospho-Erk and phospho-Akt, analyses were performed on cell lysates, with [pThr202/Tyr204]Erk1/2 ELISA kit (Enzo, UK) and Phospho-Akt (S473) Pan Specific Cell-Based ELISA (R&D Systems, USA) respectively, according to manufacturer's instructions. Protein content was quantified with Bradford reagent (Sigma, UK); the amount loaded per well in ELISA kits was 50 µg. Validation of the assay and information on sensitivity and specificity of all assays is according to the manufacturer's instructions. All results are expressed as fold-change compared to control.

Tube formation assay

In order to determine neovascularization after Ad. vector infection, endothelial cells from the four different species were infected with the Ad. vectors, detached with Trypsin 0.25% (Life

Technologies, UK) and seeded in well of 24-well plates (Corning, UK), pre-coated with 500 µl Matrigel® (Life Technologies, UK), at a density of 10^5 cells/well in EBM medium (Lonza, UK), supplemented with 0.5% FBS (Sigma, UK). Cells were then incubated overnight at 37°C, 5% CO_2 and the next day newly formed tubes were quantified under light microscope (Zeiss, Germany). For the quantification, 5 random fields per well under 10X magnification were selected.

Statistical analyses

ANOVA test has been performed in all data sets. To assess normal distribution of data, as prerequisite for ANOVA, D'Agostino-Pearson normality test was performed, which confirmed the data was normally distributed. To determine homogeneity of variance for the data we applied Bartlett's test which confirmed homoscedasticity. For the comparisons between group pairs, two-tailed t-test analyses were performed ($p<0.05$); we did not perform corrections for multiple comparisons. GraphPad Prism has been used for all analyses.

Quality Assurance

Vector quality assurance

To ensure vector quality, adenoviral vector has been expanded in 293T cells from a tested batch. Isolation procedure has been performed with Adenopure®Adenovirus Purification Kit (Puresyn, USA), according to manufacturer's instructions in sterile environment. Multiple filtration processes ensure absence of contaminants. After expansion, adenoviruses have been quantified with Quicktiter™ Adenovirus Titer ELISA Kit (Cell Biolabs Inc., USA), according to manufacturer's instructions, in order to ensure that a standard efficiency is maintained among different isolation processes. In addition, absence of mycoplasma, RCA and other impurities, sterility and expression of trans gene were measured from all virus batches.

Cell quality assurance

To ensure cell quality, we sourced endothelial cells either commercially (human, HUVECs and rabbit, rAECs) where available or from the animal species directly and confirmed endothelial cell identity using a variety of techniques described above (sheep and guinea pig). For commercially available endothelial cell sources, cultured cells were verified through performing staining for CD31, VE-cadherin and von Willebrand factor in order to confirm the endothelial nature of the cells. Similarly, for endothelial cells derived 'in house', cultured cells were verified through performing staining for CD31, VE-cadherin and von Willebrand factor in order to confirm the endothelial nature of the cells. Species specificity of the cells was confirmed through the above antibodies, as they either covered a range of species in which the relevant species was included, or they were matched to the relevant species (Guinea Pig).

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Author Disclosure statement:

ALD, JM and DMP own shares in Magnus Growth, a company which is aiming to take to market a novel treatment for fetal growth restriction.

CR, ML, VM, TH, IZ, RS, RS, MK, SYH: No competing financial interests exist

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Figure legends:

Figure 1 Study Plan: Two types of experimental studies were performed with three Ad. vector types in four endothelial cell sources. The experimental studies were (1) ELISA for expression of VEGF-D or VEGF-A protein, ERK or AKT phosphorylation, and (2) tube formation assay. These were performed with Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG or Ad.VEGF-$D^{\Delta N\Delta C}$ vectors in pregnant sheep uterine artery endothelial cells (psUAECs), guinea pig aortic endothelial cells (gpAECs), rabbit aortic endothelial cells (rAECs) or human umbilical vein endothelial cells (HUVECs).

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Figure 4: Functional assessment of VEGF pathway major signalling pathway activation by quantification of ERK and AKT phosphorylation. Phospho-ERK and -AKT expression was measured by ELISA in the supernatant of cultured endothelial cells after infection with Ad.VEGF-A_{165}, Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG and Ad.VEGF-$D^{\Delta N\Delta C}$ vectors, presented as fold change in comparison to Ad.LacZ vector transduction results. Vectors were applied to pregnant sheep uterine artery endothelial cells (psUAECs), Guinea Pig aortic endothelial cells (gpAECs), rabbit aortic endothelial cells (rAECs) or HUVECS and analysed at 24 and 48 hours after transduction. Data is presented at 48 hours after gene transfer as mean +-SEM, (*$p<0.05$; ***$p<0.001$, N=3 per each experiment).

Supplementary Figure 1: Genetic maps of Ad.VEGF-$D^{\Delta N\Delta C}$-FLAG vector, Ad.VEGF-$D^{\Delta N\Delta C}$ vector, Ad.VEGF-A_{165} vector and Ad.LacZ vector (FinVector Vision Therapies Oy, Finland).

Supplementary Figure 2: Top Panel: Tube formation in a sample experiment in which HUVECs have been infected with 100 MOI Ad.VEGF-$A_{165.}$ (a) shows cultured uninfected HUVECS, (b) shows tube like formation seen 24 hours after transduction with Ad.VEGF-A_{165} vector. Bottom Panel: Comparison between tube formation by HUVECs either (c) untreated control, or 48 hours after infection with (d) 100 MOI Ad.LacZ or (e) 100 MOI Ad.VEGF-A_{165}.

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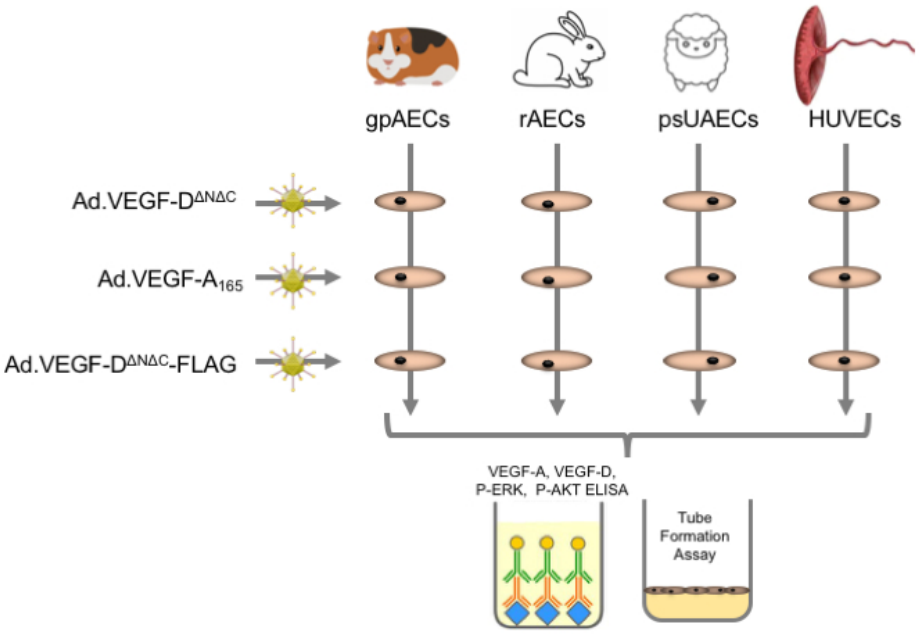

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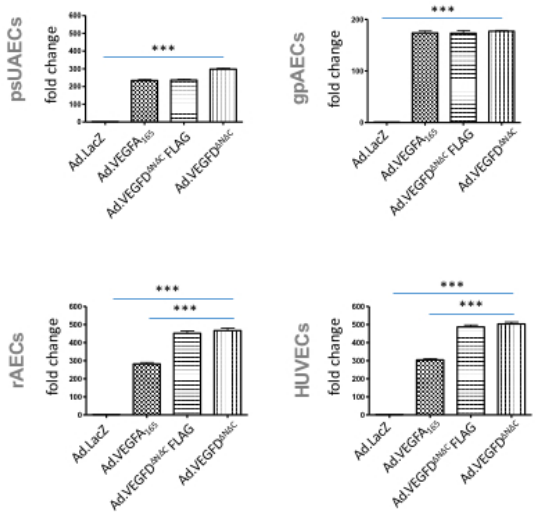
Figure 2



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338x190mm (54 x 54 DPI)

Figure 3

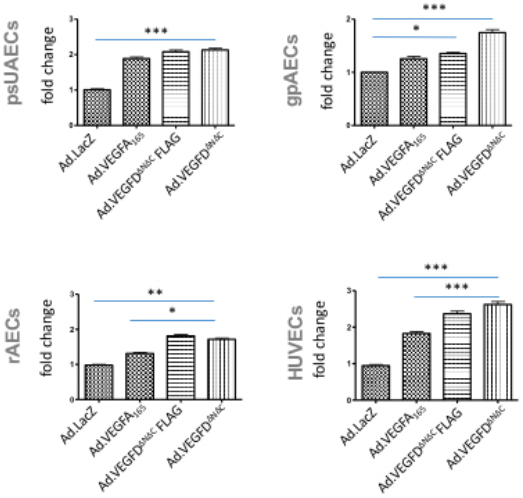

Figure 3: Functional assessment of vector transduction using Tube formation assay. Tube formation was measured by counting newly formed tubes under light microscopy after overnight incubation of transduced endothelial cells cultured in Matrigel® (Life Technologies, UK). For the quantification, 5 random fields per well under 10X magnification were selected. Endothelial cells from 4 species (pregnant sheep uterine artery endothelial cells, psUAECs; Guinea Pig aortic endothelial cells, gpAECs; rabbit aortic endothelial cells, rAECs; or HUVECS) were incubated with Ad.VEGF-A165, Ad.VEGF-DΔNΔC-FLAG, Ad.VEGF-DΔNΔC or Ad.LacZ vectors and analysed at 24 or 48 hours after transduction. Results are presented as fold change in comparison to Ad.LacZ vector tube formation results. Data is presented at 48 hours after gene transfer as mean +-SEM, (*p<0.05; **p<0.01; ***p<0.001).

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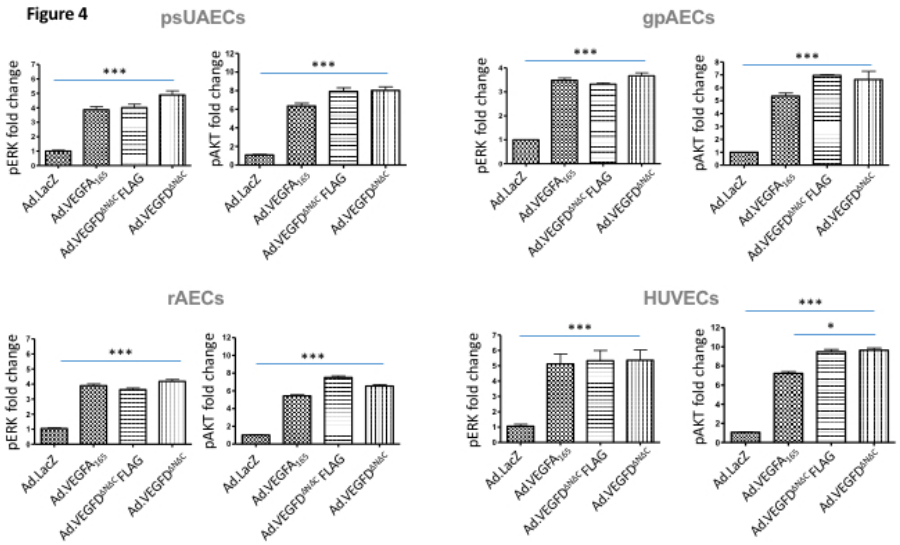

Figure 4: Functional assessment of VEGF pathway major signalling pathway activation by quantification of ERK and AKT phosphorylation. Phospho-ERK and -AKT expression was measured by ELISA in the supernatant of cultured endothelial cells after infection with Ad.VEGF-A165, Ad.VEGF-DΔNΔC-FLAG and Ad.VEGF-DΔNΔC vectors, presented as fold change in comparison to Ad.LacZ vector transduction results. Vectors were applied to pregnant sheep uterine artery endothelial cells (psUAECs), Guinea Pig aortic endothelial cells (gpAECs), rabbit aortic endothelial cells (rAECs) or HUVECS and analysed at 24 and 48 hours after transduction. Data is presented at 48 hours after gene transfer as mean +-SEM, (*p<0.05; ***p<0.001).

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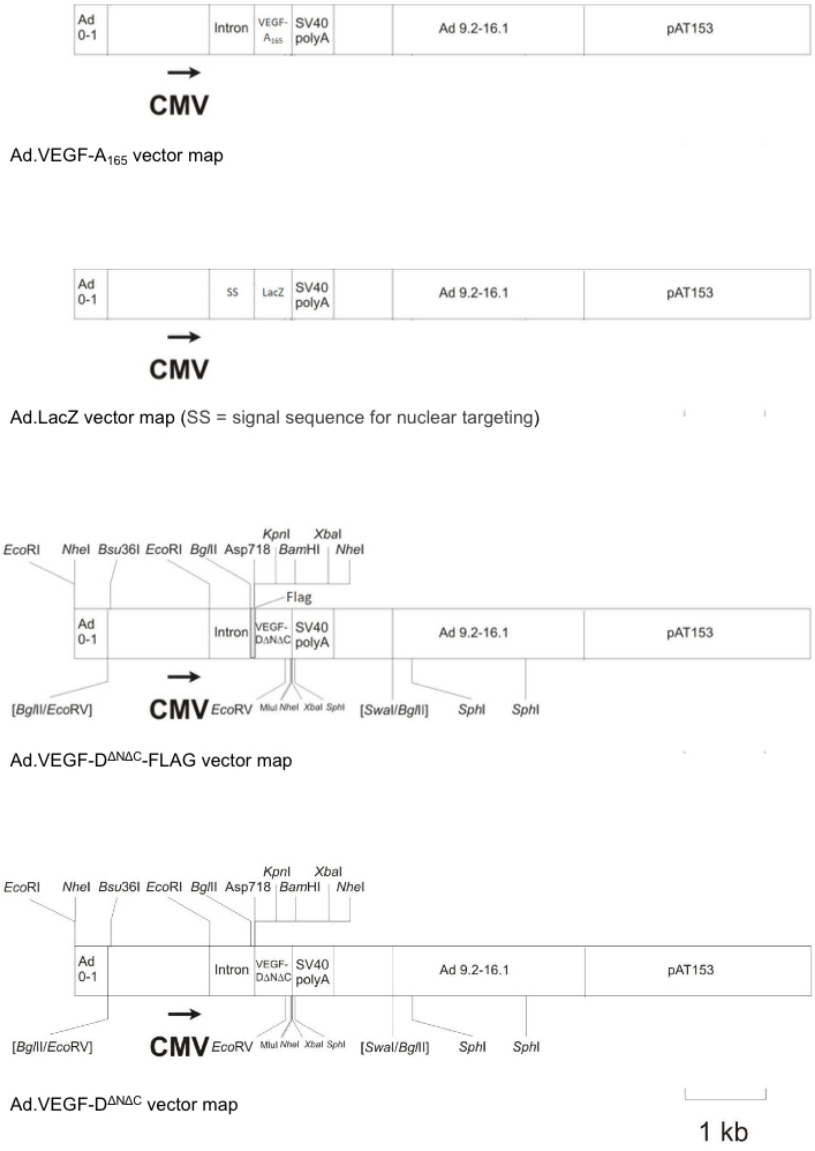
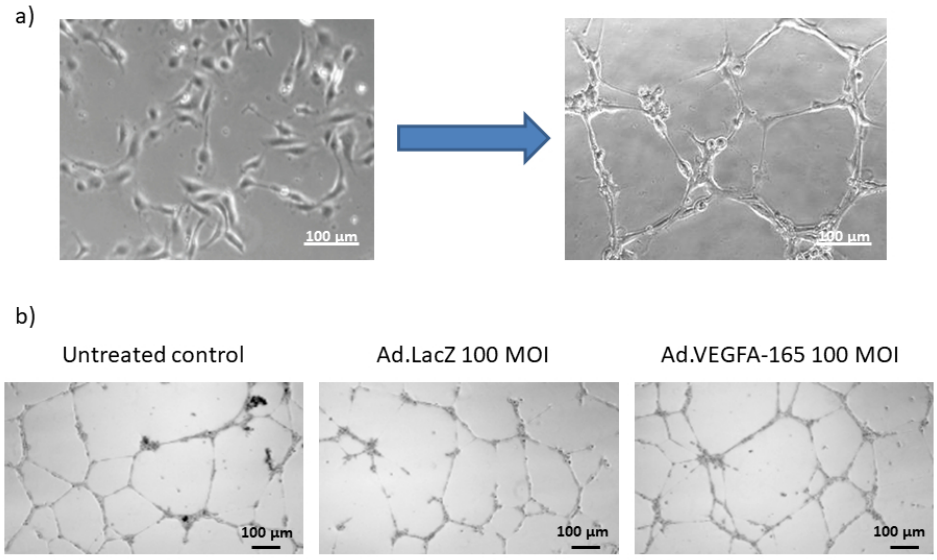


Supplementary Figure 1: Genetic maps of Ad.VEGF-DΔNΔC-FLAG vector, Ad.VEGF-DΔNΔC vector, Ad.VEGF-A165 vector and Ad.LacZ vector (FinVector Vision Therapies Oy, Finland).

190x275mm (96 x 96 DPI)



Supplementary Figure 2: Top Panel: Tube formation in a sample experiment in which HUVECs have been infected with 100 MOI Ad.VEGF-A165. (a) shows cultured uninfected HUVECS, (b) shows tube like formation seen 24 hours after transduction with Ad.VEGF-A165 vector. Bottom Panel: Comparison between tube formation by HUVECs either (c) untreated control, or 48 hours after infection with (d) 100 MOI Ad.LacZ or (e) 100 MOI Ad.VEGF-A165.

254x190mm (96 x 96 DPI)