Abstract

Background: Overexpression of sFlt-1 or modulation of FKBPL, key anti-angiogenic proteins, are important in the pathogenesis of preeclampsia.

Materials and Methods: A newly developed non-viral gene delivery system, RALA, capable of overexpressing sFlt-1 (e15a isoform) was delivered *in vivo* in transgenic haploinsufficient (*Fkbpl*$^{+/-}$) mice. RALA was also used *in vitro* to deliver human Flt1 (hFlt1) in trophoblast cells.

Results: Serum-stable and non-toxic RALA/DNA-based nanoparticles induced an increase in sFlt-1 protein levels in blood and total protein in the urine; the effect was more pronounced in *Fkbpl*$^{+/-}$ mice. *In vitro*, RALA-hFlt nanoparticles significantly reduced secretion of sFlt-1 in trophoblasts.

Conclusion: The RALA-based genetic nanodelivery system can be safely and effectively applied to emulate preeclampsia-like features or reduce sFlt-1 levels *in vitro*.

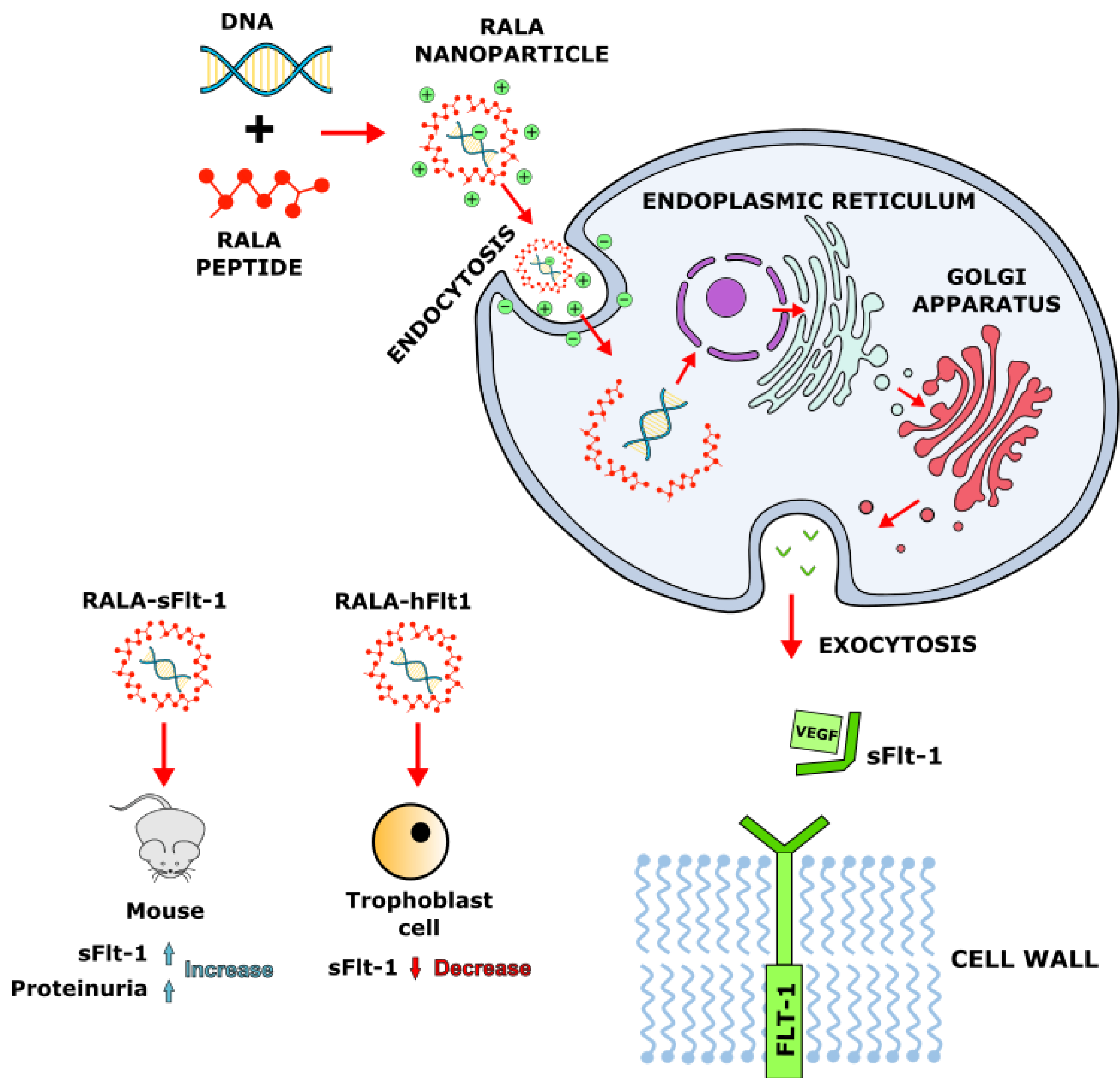
Lay abstract: In this study, we utilized a safe and effective approach to modulate ~~sFlt-1~~ ~~(~~an important circulating protein in pregnancy, sFlt-1,~~)~~ ~~levels~~ that is one of the key mechanisms leading to ~~a~~ the pregnancy complication, preeclampsia. Preeclampsia is a complex ~~multifactorial~~ disease caused by multiple factors and a leading cause of death in pregnancy with no current effective treatment strategies. This is likely due to a lack of reliable pre-clinical models. Here we demonstrate the feasibility of a new model of preeclampsia that is based on dysfunction of two key vascular proteins, sFlt-1 and FKBPL (an important protein involved in the development of new blood vessels), that could be utilized in the future for testing and development of new treatments targeting these important mechanisms in preeclampsia~~sFlt-1~~.

Tweetable abstract: Development of a new model of #preeclampsia ~~in #pregnancy~~ using serum stable and safe non-viral gene delivery system, #RALA, to modulate #sFlt-1 secretion in #FKBPL knockdown mice *in vivo* or #trophoblasts *in vitro*.

Summary points:

- Overexpression of sFlt-1 is associated with the development of preeclampsia symptoms including hypertension and proteinuria.
- RALA is an emerging non-viral gene delivery system capable of efficiently and safely delivering different types of DNA *in vivo*.
- This is the first study demonstrating that administration of RALA-sFlt-1 nanoparticles can lead to overexpression of sFlt-1 *in vivo.*
- *In Fkbpl* knockdown transgenic mice, preeclampsia-like features including the increase in sFlt-1 plasma concentration and proteinuria appear more prounouced.
- RALA was also effective at reducing sFlt-1 in trophoblast cells *in vitro* by overexpressing its antagonist, hFlt1.
- This nanomedicine system can be safely and effectively applied to emulate preeclampsia-like features *in vivo* or reduce sFlt-1 levels *in vitro.*
- RALA could be utilized in the future for testing and development of new treatments.

Graphical Abstract



Keywords: sFlt-1, RALA, preeclampsia, FKBPL, gene delivery, trophoblast cells

Introduction

Preeclampsia is a multifactorial disease of unknown aetiology, affecting 5-7% of pregnancies that can be stratified into several different phenotypes, including early- or late-onset preeclampsia[1]. Currently, there are no reliable early predictive markers, preventative measures, or effective treatments. Evidence suggests that impaired angiogenesis and endothelial dysfunction are key biological processes implicated in the progression of this condition, wherein a "two-stage model" has been proposed that involves: i) inadequate trophoblast invasion early in gestation resulting in the incomplete remodelling of the spiral uterine arteries leading to a poorly perfused and ischaemic placenta, and ii) the release of angiogenesis-related factors from the ischaemic placenta resulting in the clinical manifestations of preeclampsia[2,3]. Different phenotypes of preeclampsia are characterised by some distinct but also overlapping features of preeclampsia[4].

An anti-angiogenic protein, soluble fms-like tyrosine kinase 1 (sFlt-1), is a vascular endothelial growth factor (VEGF) splice variant that lacks the cytoplasmic domain of the VEGFR1 receptor. Through binding free VEGF-A and placental growth factor (PlGF), sFlt-1 functions as an antagonist to VEGFR1 or Flt1[5]. It is now well-established that the systemic concentration of sFlt-1 increases in women with preeclampsia and subsequently decreases postpartum, likely contributing to endothelial dysfunction, hypertension, and proteinuria in preeclampsia[6]. The levels of circulating sFlt-1 increase approximately 1.5 fold several weeks before the onset of preeclampsia, in conjunction with lower PlGF levels, compared to healthy pregnancies[7,8]. The sFlt-1:PlGF ratio is now used as a predictive clinical tool for preeclampsia[9]. Furthermore, targeting sFlt-1 overexpression has been shown to abrogate preeclampsia features[10]. There are a number of sFlt-1 isoforms, with the most dominant one, e15a, being highly expressed in the placenta and increased significantly during preeclampsia, compared to healthy controls[11]. Additionally, the e15a transcript is more abundantly expressed within the syncytiotrophoblast and cytotrophoblast cells of the placenta[12].

Angiogenesis-related factors and associated receptors play an important role in the vascular development of the placenta, and many have been examined as potential biomarkers or therapeutic targets in preeclampsia[13]. The anti-angiogenic activity of FK506 binding protein like (FKBPL) was first described in 2011[14], and this protein was later shown to be critical in both developmental and pathological angiogenesis[15–20]. An important role of FKBPL has been demonstrated in preeclampsia where low FKBPL and high CD44 (its target protein), were

predictive of preeclampsia[21]. FKBPL is also a determinant of cardiovascular disease[22], and women who suffered from preeclampsia have an increased risk of future cardiovascular diseases[23]. Despite this, the pathogenesis of preeclampsia remains somewhat elusive, and as such, further methods to study the progression of this condition are highly desirable. To date, the current pre-clinical models of preeclampsia are all induced[24] with viral delivery vectors designed to overexpress anti-angiogenic factors, most commonly sFlt-1[25]. However, viral vectors have low packing capacity and present safety considerations due to unsought genetic mutations and immunogenicity. Viral vectors are also poorly suited for large-scale fabrication for clinical translation. In contrast, non-viral, nanoparticle-based vectors are often non-immunogenic and permit facile bulk production[26,27].

In this study, we spontaneously assembled nanoparticles between DNA and a short peptide (namely RALA) owing to the electrostatic interaction between the anionic DNA and cationic peptide. This nanoparticle-based non-viral gene delivery vehicle, RALA, is efficient at condensing and delivering DNA intercellularly, enabling the escape of the particle from endosomal vesicles and eliciting gene expression in both *in vitro* and *in vivo* studies[28,29].

For the first time, we show that RALA can be used to generate nanoparticles capable of overexpressing the sFlt-1 e15a variant in FKBPL transgenic knockdown mice to develop the non-viral generated animal models of preeclampsia *in vivo*. Furthermore, we demonstrate that RALA-Flt1 nanoparticles could be utilised to reduce sFlt-1 *in vitro*, which could have therapeutic applications in the future for treatment of preeclampsia. Finally, we investigate the interplay between overexpressing sFlt-1 and downregulating FKBPL and whether a deficiency in this critical anti-angiogenic factor, *Fkbpl,* may have any further influence on features of preeclampsia and sFlt-1 levels. Taken together, our RALA delivery system provides safe gene delivery to create pre-clinical models of preeclampsia by modulating sFlt-1 secretion. The nanodelivery system can be useful for application in other diseases.

Materials and Methods

Plasmids

Human VEGF (hFLT1)[30], sFlt-1 splice variant (sFlt-1-e15a V5)[12] were a gift from Christie Thomas (Addgene, Massachusetts, USA) and propagated in competent bacterial cells. Glycerol stocks containing plasmid stock were prepared and extracted using a PureLink™ HiPure Plasmid DNA Purification Kit (Thermo Fisher Scientific, UK) as detailed according to the manufacturer's instructions. Plasmid quality and DNA concentration was determined using the Nanodrop ONE (Thermo Fisher Scientific, UK). DNA (1µg/µl) having a 260/280 ratio ~1.8 was used for nanoparticle formation.

Cell lines

BeWo and JAR cell lines were obtained from the American Type Culture Collection (ATCC) and cultivated in DMEMF12 or RPMI (Thermo Fisher Scientific, UK), respectively, supplemented with 10% FBS (Thermo Fisher Scientific, UK). Cells were cultured at 37°C in a humidified atmosphere of 21% O_2/5% CO_2, unless otherwise stated. Cells were routinely verified to be mycoplasma free with the MycoAlert detection kit (Lonza, Switzerland) and the PCR Mycoplasma Test Kit I/C (Promokine, Germany). Cells obtained were authenticated by the ATCC with karyotyping, polymerase chain reaction (PCR), including COI analysis, and short tandem repeat profiling for cell authentication. Cell culture morphology was assessed visually during each assay to further confirm the identity of these lines.

In vivo experiment

In vivo experiments were carried out in female C57BL/6 mice, with one copy of FKBPL knocked down (*Fkbpl*$^{+/-}$), and littermate wild-type controls (*Fkbpl*$^{+/+}$) as previously described[15]. Animals were bred in an open facility at 21°C and 50% humidity with food and water *ad libitum*. The experimental protocols were compliant with the UK Scientific Act of 1986 and ARRIVE guidelines and were completed under the Project License Number 2787, and Personal License Number 1855.

Preparation of RALA-DNA & RALA-siRNA nanoparticles

The molar ratios of positively charged nitrogen atoms in the peptide to negatively charged phosphates in the plasmid DNA backbone (N:P ratios) were generated, ranging from 1 to 20, by adding appropriate volumes of RALA peptide solution to 1 µg plasmid DNA as detailed in

McCarthy *et al.* (2014)[28]. Complex mixtures were incubated at room temperature for 30 min to form nanoparticles and used immediately.

Complex size & zeta potential analysis

RALA-DNA complexes were prepared at N:P ratios as previously described and diluted to 1 ml with DNase/RNase free H_2O before measuring the particle size and zeta potential with a Nano ZS Zetasizer and DTS software (Malvern Instruments, UK). The mean hydrodynamic size of the complexes was determined by dynamic light scattering on a Malvern Nano ZS (Malvern Instruments, UK).

Electrophoretic mobility shift assay

Nanoparticles were prepared at a range of N:P ratios as per the standard protocol as previously described[31]. Samples were electrophoresed through a 1 % agarose gel (1.5 g agarose in 150 ml TAE 1X buffer). For SybrSafe (Invitrogen, United States) stain gels, 10 µl stain was added to 150 ml gel. Molten gel mix was poured into the cassette and allowed to set, then placed into the electrophoresis tank with TAE 1x buffer. Loading buffer (2 µl) was added to nanoparticle sample (18 µl) and pipetted into each well before the gel was run at 100 V for 60 min and imaged under UV light with an AutoChemi System (UVP Bioimaging Systems, United States).

Serum stability assay

RALA-DNA nanoparticles were prepared at an N:P ratio of 10. The nanoparticle complexes were incubated for 1, 2, 3, 4, 5, or 6 h at 37 °C ± 10 % foetal calf serum. Each hour 5 µl serum (FCS) was added to appropriate tubes (added to 6 h tube first, then 5 h, then 4 h, etc.). Following this, samples were run on an agarose gel. Nanoparticle samples were prepared in four groups (6 samples for 4 groups; 24 samples x 45 µl). Following incubation, sodium dodecyl sulfate (SDS, 10%) (Sigma-Aldrich, UK) was added to break the pDNA from RALA, and after 10 min samples were electrophoresed through a 1 % agarose gel at 100 V for 60 min and imaged with an AutoChemi System (UVP Bioimaging Systems, United States).

Scanning electron microscopy

RALA-sFlt1-e15a and RALA-hFlt1 complexes were prepared at N:P ratio 10. Each sample (10µl) was deposited on a silicon wafer (0.5x0.5cm) and incubated overnight to air dry. Samples were sputter-coated with Gold/palladium with a thickness of 10nm using Leica EM ACE600 Sputtering and Carbon Thread Coater. The coated wafers were imaged with Zeiss Supra 55VP at an accelerating voltage of 3kV at a magnification of 40K.

MTS cytotoxicity assay

JAR (1.5x10^4) or BeWo (4x10^4) cells were seeded in 96-well plates in triplicates and allowed to adhere overnight at 37°C in a humidified atmosphere of 21% O_2/5% CO_2. Two hours before transfection, media was changed to ~~serum-free~~reduced serum OptiMEM before the RALA-DNA complex was added to the cells for 6 h, and OptiMEM replaced with serum-supplemented media. An MTS assay (Promega, United States) was carried out 24 and 48 h after the addition of RALA to assess cell viability in a 96-well plate. MTS reagent (10 µl) was added to each well and incubated at 37°C (in the dark, covered with tin foil) for 2-4 h. Absorbance was measured at 490 nm using a FLUOstar Omega plate reader (BMG labtech, Germany), and the percentage of viable cells was calculated relative to untreated control cells.

Transfection of cells in vitro

BeWo (5x10^5) or JAR (3x10^5) cells were seeded in a 60 mm tissue culture dish in triplicates and allowed to adhere overnight at 37 °C in a humidified atmosphere of 21% O_2/5% CO_2. Two hours before transfection, media was changed to reduced serum~~serum-free~~ OptiMEM (Thermofisher, UK) before the RALA complex was added to the cells for 6 h and OptiMEM replaced with ~~serum-supplemented~~normal cultivation media.

Delivery of RALA-pDNA complexes in vivo

RALA-DNA nanoparticles were administered to 25 pregnant female mice as follows: Seven *Fkbpl*$^{+/+}$ mice and four *Fkbpl*$^{+/-}$ mice received empty vector, whilst nine *Fkbpl*$^{+/+}$mice and five *Fkbpl*$^{+/-}$ mice received nanoparticle-containing sFlt-1 (e15a) overexpression plasmid and RALA. Pregnancy was determined by the technical staff at the Biological Services Unit at Queen's University Belfast. The experimental plan is presented below.

Preparation of RALA complexes for *in vivo injection*

RALA-DNA nanoparticle complexes (5 µg sFlt-1-e15a or 5 µg empty vector) were prepared to a ratio of N:P 10 on the morning of injection in 100 µl aliquots. RALA-DNA complex (sFlt-1-e15a) was administered to groups of mice by tail-vein injection.

Collection of excreted mouse urine

Individual mouse urine was collected using a disposable plastic container before the end point of the study, between day 15-18. The mouse was held by tail, gently pressed on its lower back, and excreted urine was collected in an Eppendorf via micropipette and stored at -80 °C. Protein concentration was assessed using BCA assay (Thermofisher, UK) according to the manufacturer's instructions.

sFlt-1 ELISA

Following the transfection of cells *in vitro* the Human VEGFR1/Flt-1 Quantikine ELISA Kit DVR100C (R&D Systems, UK) was used to assess sFlt-1 levels in the supernatants. Samples and standards were plated in duplicate, and assay was performed as detailed according to the manufacturer's instructions. Cell lysate samples were diluted ten-fold prior to use in this assay, and a four-parameter logistic regression curve was used to determine results.

Terminal blood collection was performed post-mortem on gestational day 18, and the plasma fraction was separated. The mouse VEGFR1/Flt-1 Quantikine ELISA Kit MVR100 (R&D Systems, UK) was used to assess sFlt-1 level in plasma according to manufacturer's instructions. Plasma samples were diluted two-fold prior to assay, and a four-parameter logistic regression curve was used to determine results.

Results

Characterisation of RALA-psFlt-1 nanoparticle complex

RALA is a non-viral DNA delivery peptide, which consists of 30 amino acids (NWEARLARALARALARHLARALARALRACEA-C), whose hydrophilic arginine (R)

domain facilitates nucleic acid binding while its hydrophobic leucine (L) region interacts with lipid membranes. These two regions are separated by alanine (A) rich regions providing the peptide with its amphipathic properties[28].

Overexpression of sFlt-1 in pregnant mice has previously been shown to induce hypertension[32], supporting the role of sFlt in the development of preeclampsia. Additionally, the induction of the most abundant isoform, e15a, has effectively induced preeclampsia in mice[33]. Therefore, we generated a stable sFlt-1 containing nanoparticle complex using RALA as a novel non-viral delivery system and characterised these nanoparticles before use *in vivo* to determine efficacy and safety. Optimisation of the RALA-DNA complex was initially established by assessing N:P ratios, with N:P 8 (Fig. 1. A; 144 ± 40; n=4) and 10 (Fig. 1. A; 88 ± 7; n=4) demonstrating ideal size and charge characteristics for cellular entry. Dynamic light scattering (DLS) confirmed the size distribution (Supplementary Fig. 1). Representative images from scanning electron microscopy indicated that these complexes are spherical, measured at a size similar to that detected by the DLS, and show no evidence of aggregation (Fig 1. D).

An electrophoretic shift assay was performed on all N:P ratios. With N:P ratio of above 2, the plasmid DNA was fully immobilised in the wells of the agarose gel (Fig. 1. B), indicating nanoparticle stability. At the ratios of N:P of 4 and higher, there is reduced visibility of DNA retained in the wells indicative of a tighter condensation. To ensure the RALA-sFlt-1 nanoparticle complex is stable in the presence of serum, important for remaining intact in the *in vivo* setting, a serum stability assay was therefore performed to assess the stability of the RALA-DNA (sFlt-1-e15a) complex. When incubated in serum for up to 6 h, RALA-DNA particles remained complexed in the wells of the agarose gel (Fig. 1. C). Upon addition of SDS, the plasmid DNA from the decomplexed particles were shown to be intact in comparison to that of control plasmid DNA (Fig 1. C), indicating that they had remained stable over the incubation period.

Cytotoxicity of RALA-psFlt-1 nanoparticle complex

Based on RALA-DNA (sFlt-1-e15a) nanoparticle characterisation, N:P 8 and 10 were deemed the optimal complexes to take forward for further study. The cytotoxicity of these complexes was assessed *in vitro* using trophoblast cells (BeWo and JAR). Two trophoblast cell lines were

used due to different FKBPL protein expression; BeWo cells expressed half the amount of FKBPL compared to JAR (Supplementary Fig. 2). The MTS assay is commonly used to assess cell proliferation, viability, and cytotoxicity. At the N:P ratio of 8 and 10, there were no observed toxic effects in BeWo cells, as indicated by cell viability at both 24 h (Fig. 2. A) and 48 h (Fig. 2. B). Likewise, there was no toxicity in JAR cells at either 24 h (Fig. 2. C) or 48 h (Fig. 2. D), compared to control.

Inducing sFlt-1-e15a overexpression *in vivo* in FKBPL transgenic mice and wild type controls

Overexpression of sFlt-1 (e15a variant), an antagonist of VEGF, has been previously shown to induce preeclampsia features when delivered using the adenovirus delivery method *in vivo*[33]. Furthermore, *Fkbpl* haploinsufficient mice (*Fkbpl*$^{+/-}$) were utilised for the study, given published data to suggest that low FKBPL protein levels are associated with increased preeclampsia risk, as an anti-angiogenic factor[21]. We, therefore, evaluated the ability of RALA-mediated delivery of sFlt-1 (e15a variant) to induce preeclampsia-like features/characteristics *in vivo* using *Fkbpl*$^{+/-}$ and *Fkbpl*$^{+/+}$ mice; based on initial safety and suitability testing of nanoparticles (Fig. 1&2) and previous reports showing effective and safe delivery of various biomolecules *in vivo* with RALA[29,31,34,35]. Both *Fkbpl*$^{+/-}$ and *Fkbpl*$^{+/+}$ female mice were mated with *Fkbpl*$^{+/+}$ male mice, and both non-pregnant and pregnant mice proceeded to receive RALA-based nanoparticles on day 8, as described in Fig. 3.

Importantly, proteinuria is commonly associated with preeclampsia in pregnant women[36], and has also been shown to develop in several animal models of preeclampsia[37,38]. In our study, urine was collected from mice towards the end of the experiment and protein levels were quantified using a BCA assay. We could detect an increase in urine protein concentration in the RALA-sFlt-1 (e15a) overexpression group compared to the empty vector group irrespective of *Fkbpl* genotype (Fig. 4. A, 32,912 ± 4,317 µg/ml vs. 22,708 ± 3,921 µg/ml), however it did not reach statistical significance (p=0.07). Although we attempted to measure blood pressure using tail cuff plethysmography, we were unable to obtain reliable results because conditioning of the mice to enter the restrainer did not reduce stress levels hence producing high variable and inconsistent data. This is one of the limitations of our study and in the future, radio telemetry ~~should~~will be used for this purpose.

Mice were then divided into four groups based on the genotype or plasmid treatment, and urine protein concentration was compared. Compared to *Fkbpl*$^{+/+}$ (wild-type, WT) and empty vector group, *Fkbpl*$^{+/-}$ sFlt-1-e15a overexpression showed statistically significant increase in urine protein concentration (Fig. 4. B; 23,204 ± 5,891 µg/ml vs 45,473 ± 10,437 µg/ml; p=0.02), indicating the presence of a typical preeclampsia feature.

Furthermore, we measured plasma sFlt-1 concentration using mouse ELISA to determine overexpression of sFlt-1 in both genotypes of *Fkbpl* mice, which is also often elevated in preeclampsia. When all mice were grouped together, irrespective of the *Fkbpl* genotype, there was~~ a trend towards~~ an increase in sFlt-1 plasma concentration in the RALA-sFlt-1 overexpression group (Fig. 5 A; 37,688 ± 4,105 pg/ml vs. 20~~3~~,682~~549~~ ± 4,44~~9~~8~~4~~ pg/ml; p=0.02~~7~~, n≥10~~1~~ in each group).

When divided into sub-groups based on the genotype and plasmid treatment, an increase in sFlt-1 plasma concentration was only observed in *Fkbpl*$^{+/-}$ mice that were administered the overexpression vector compared to *Fkbpl*$^{+/+}$ empty vector mice (Fig. 5 B; 4~~,~~3,425 ± 4,722 pg/ml vs. 15,578~~7~~ ± 3,271 pg/ml; p=0.03). There was a trend towards an increase in the *Fkbpl*$^{+/+}$ sFlt-1 overexpression group compared to *Fkbpl*$^{+/+}$ empty vector controls (Fig. 5 B; 34,819 ± 6,529 pg/ml vs. 15,577 ± 3,271 pg/ml; p=0.08), although it did not reach statistical significance.

The effect of RALA-DNA human VEGFR1 overexpression on sFlt-1 levels in trophoblast cells

VEGFR1 or Flt1 is a membrane-bound Flt1 receptor and an established inducer of angiogenesis, which specifically promotes endothelial cell proliferation, migration, and new vessel formation with opposite effects from sFlt-1[39]. Increased circulating levels of sFlt-1 observed in preeclampsia prevent VEGF from binding to the VEGFR1 receptor, and are thus implicated in disease pathogenesis, in particular, development of endothelial dysfunction, hypertension, and proteinuria[6]. The RALA-Flt1 complex formed individual nanoparticles without aggregates (Fig. 6A) and nanoparticles were of optimal size (Supplementary Fig. 1). Trophoblast cells were transfected with RALA-human Flt1 plasmid (phFLT1) to investigate whether overexpression of this protein could reduce sFlt-1 secretion by BeWo that express half the amount of FKBPL compared to JAR cells~~ or JAR cells~~, testing its early potential as a novel treatment of preeclampsia. Transfection with 1 µg of phFLT1 had no significant effect on sFlt-

1 levels in the supernatant of cultured BeWo (Fig. 6. B; 100.5 ± 18.1 pg/ml vs 139.0 ± 22.7 pg/ml; n=3) cells versus controls. However, when BeWo cells were transfected with 5 μg phFLT1 there was a significant decrease in detectable sFlt-1 protein in the supernatant in comparison to empty vector controls (Fig. 6 C; 43.1 ± 4.8 pg/ml vs 244.0 ± 29.4 pg/ml; n=3; p= 0.002).

Discussion

The lack of effective treatments for preeclampsia, a leading cause of maternal morbidity and mortality worldwide, highlights the importance of developing low-risk and reliable animal models to emulate this condition. Animal models of disease enable us to identify novel disease mechanisms, test the effectiveness and safety of treatments, and determine action mechanisms. Preclinical models of preeclampsia differ from spontaneous development in the human as it must be induced by either surgical, environmental, pharmacological, immunological or genetic interventions[24]. One commonly used method to generate a preeclampsia model is the exogenous administration of an adenovirus to overexpress sFlt-1, which has been shown to induce preeclampsia symptoms and features in rats[40]. Multiple studies have explored overexpression of various anti-angiogenic factors or downregulation of pro-angiogenic factors with adenovirus[41] or lentivirus[42] vectors. In an adenoviral model overexpressing sFlt-1 through AdvsFlt-1, there was a notable increase in proteinuria, blood pressure, and glomerular damage in Balb/c mice. However, the co-administration of AdvsFlt-1 with AdvVEGF alleviated preeclampsia-like symptoms through a 70% reduction in free sFlt-1[43]. While these models do present with pre-eclamptic phenotypes, adenovirus can be detrimental due to the induction of immune response to the adenoviral vector, leading to severe complications and obstruct gene delivery[44]. It is especially risky to administer the virus in pregnancy. There is also known to be a high degree of variability with various adenoviral preparations. Additionally, the direct toxic effects of adenoviruses in intravenous delivery can induce acute liver injury in animal models[45].

In this study, we demonstrated a proof of concept of overexpressing sFlt-1 *in vivo* to induce ~~a~~ preeclampsia-like features using a safe and effective non-viral gene delivery system, RALA. This is the first study to have shown the feasibility of using RALA to overexpress sFlt-1 *in vivo* and induce proteinuria, typical of human preeclampsia. It appears that downregulation of *Fkbpl in vivo*, which is characterised by pro-angiogenic phenotype but with vascular dysfunction led

to more pronounced proteinuria and overexpression of sFlt-1, suggesting a synergistic effect of sFlt-1 and FKBPL modulation. Nevertheless, RALA was capable of overexpressing sFlt-1 in mice irrespective of the *Fkbpl* genotype. The advantage of having two key angiogenesis-related pathways modulated in an *in vivo* model of preeclampsia, is that it reflects the multifactorial nature of preeclampsia. Recently, the FKBPL pathway has emerged as a critical mechanism in the pathogenesis of preeclampsia independently of VEGF-sFlt1, where low systemic plasma levels of FKBPL early in pregnancy were associated with increased risk of preeclampsia [21]. This is the first study utilising *Fkbpl*$^{+/-}$ transgenic mice in combination with sFlt-1 overexpression to model preeclampsia-like features *in vivo*. -The development of animal models with a non-viral gene delivery system represents an important and beneficial advance for future studies. Biophysical characterisation of RALA nanoparticles revealed excellent parameters for the use of this vector *in vivo*, in addition to no observable cytotoxicity, confirming similar findings in the literature[35,46] and thereby demonstrating its biocompatibility *in vivo*. Notably, RALA nanoparticles were previously reported to preferentially localise to highly vascularised regions *in vivo*[28], so their suitability as means of inducing an experimental preeclampsia model is of clear interest, given that placenta is a highly vascularised tissue.

Furthermore, in this study we investigated a potential of RALA-hFlt1 nanocomplex in downregulating sFlt-1 *in vitro*. Given that there are no effective treatments for preeclampsia, and a number of pre-clinical[47] and proof of concept clinical studies[10] have shown that downregulation of sFlt-1 can resolve preeclampsia, this is an area of great importance. The lack of specific knowledge about the molecular regulation of preeclampsia's pathogenesis has impeded the development of successful preventative or therapeutic strategies. The limited range of medicines that can be safely used in pregnancy has hindered the development of specific preeclampsia therapies. Thus, a therapy that selectively targets the placenta without systemic effects is valuable. As the placenta is highly vascularised, it is likely that nanoparticles will be widely distributed throughout the tissue. Given sFlt-1's apparent role in the pathogenesis of the condition, it is evident that treatments, which could downregulate sFlt-1 in pregnancy complicated by preeclampsia, are an attractive target for nanoparticle-based delivery. Efficient and safe approaches to reducing sFlt-1 such as the one demonstrated here using non-viral gene delivery system, RALA-Flt1 nanoparticles, could be promising as new

preeclampsia treatments. Here we show its early feasibility *in vitro* in placental trophoblast cells. This will need to be validated *in vivo* using reliable pre-clinical models of preeclampsia.

As this is the first study of this kind, further validation using larger numbers of mice per group and detailed characterisation of the effects and mechanisms of RALA-sFlt1 nanoparticles should be carried out in the future. This should include the effect on blood pressure, intrauterine growth restriction and placental sFlt-1 expression. Further testing of higher plasmid doses and extended duration of expression should also be considered in the future.

Conclusion

The findings presented in this study demonstrate that sFlt-1-overexpressing RALA nanoparticles can efficiently condense and protect plasmid DNA cargo. Our results show that nanoparticles could successfully induce sFlt-1 overexpression *in vivo* while exhibiting low toxicity. The induction of preeclampsia-like fearures was more pronounced in *Fkbpl* haploinsufficient mice suggesting that the combination of overexpressing RALA-sFlt1 nanoparticle administration and FKBPL deficiency is potentially more effective as a model of preeclampsia and representative of its multifactorial nature. Importantly, overexpression of Flt1 or VEGFR1 via RALA nanoparticles reduced the detectable level of sFlt-1 *in vitro* and thus showed potential as a future therapeutic option for preeclampsia that should be validated in the future *in vivo*. A reduction in sFlt-1 has been linked to a diminishing preeclampsia phenotype. Notwithstanding the emerging heterogeneity of preeclampsia and the lack of suitable animal models, the results reported in this study could have an important impact in the field of preeclampsia in the future.

Future perspectives: There is a great need for safe and reliable pre-clinical models of preeclampsia representing a multifactorial nature of the disease *in vivo*. The non-viral RALA-sFlt-1 overexpression *in vivo* model in *Fkbpl* deficient mice developed and characterised in this study represents a promising future platform for pre-clinical testing of targeted therapies for these key anti-angiogenic pathways in preeclampsia. Given the there are currently no effective treatments for this condition, this is an area of unmet clinical need that needs further research and specialised preclinical models to expedite treatment development for preeclampsia.

Summary points:

In this novel study describing a new non-viral gene delivery approach to targeting sFlt-1 secretion *in vivo* and *in vitro* relevant to preeclampsia, we demonstrated:

- **Excellent formulation and serum stability**: Physiological stability and blood longevity are critical for designing a gene nano-delivery system. This nanoparticle-based non-viral gene delivery vehicle was efficient at condensing and delivering DNA intercellularly, enabling the escape of the particle from endosomal vesicles and eliciting gene expression.
- **Excellent genetic overexpression to develop animal models**: The development of animal models with a non-viral gene delivery system represents an important and beneficial advance for future research. We showed that by using the RALA-mediated nano-delivery system, we could overexpress sFlt-1 *in vivo* and induce proteinuria, typical of preeclampsia.
- **Novel and safe approach to downregulating sFlt-1 in trophoblast cells**: The many different phenotypes of preeclampsua and the lack of specific knowledge about the molecular regulation of pathogenesis of preeclampsia has impeded the development of personalised preventative or therapeutic strategies. We demonstrated that our non-viral nanoparticle-based delivery can decrease sFlt-1 secretion *in vitro* in trophoblast cells that are key in placental development and, in the future, could be developed as a new treatment for preeclampsia.

Figure 1. Biophysical characterisation of RALA/psFlt1 complexes. (A) Hydrodynamic size and zeta potential of nanoparticles (bars = size, lines = charge), n=4. Data points are mean ± SEM, N:P ratio 1-20. **(B)** Electrophoretic mobility shift assay of sFlt1 overexpression plasmid (psFlt1) DNA and RALA peptide complexes of N:P ratio 1-20. **(C)** Serum stability of RALA/psFlt1 complexes at N:P ratio of 10 incubated for 1-6 hours at 37°C. Following incubation SDS was added to decomplex RALA/psFlt1 to verify integrity of psFlt1. Top row = no serum. Bottom row = serum. **(D)** Scanning electron microscope image of RALA-psFlt1 nanoparticles formed complexes at N:P 10. Average size of these particles were approximately 60 nm.

Figure 2. Nanoparticle complex cytotoxicity in trophoblast cells. Percentage viability of **(A, B)** BeWo, or **(C, D)** JAR cells at 24h **(A, C)** or 48h **(B, D)** post transfection with RALA/psFlt1 complexes (N:P ratios 8, 10) was determined using MTS assay. Data points are mean ± SEM, n=3; one-way ANOVA.

Figure 3. Schematic of the *in vivo* delivery of RALA/psFlt1 nanoparticles. Mating pairs were established and after eight days of gestation, nanoparticles containing either an empty vector, or sFlt-1 overexpression plasmid (psFlt1) were injected via tail vein. Urine collection was repeated at day 15-18. Blood was collected at end point after sacrifice of mice. GD: Days of gestation.

Figure 4. Protein concentration in the urine of mice receiving either empty vector or RALA-sFlt-1 nanoparticle. Individual mouse urine was collected between day 15-18. Urinary protein concentration was assessed via BCA assay and compared between groups as follows: **(A)** RALA-sFlt-1-e15a (n=13) or RALA-empty vector (n=11), nanoparticle independent on the *Fkbpl* genotype; **(B)** *fkbpl*$^{+/+}$ RALA-empty vector (n=7), *fkbpl*$^{+/-}$ RALA-empty vector (n=4), *fkbpl*$^{+/+}$ RALA-sFlt-1-e15a (n=9), and *fkbpl*$^{+/-}$ RALA-sFlt-1-e15a (n=4). Data are mean ± SEM; unpaired t-test (A), or one-way ANOVA with Sidak's multiple comparisons test compared to WT Empty Vector (B). *Fkbpl*$^{+/+}$ *= Wild type (WT), Fkbpl*$^{+/-}$ *= FKBPL knockdown. * p<0.05.*

Figure 5. sFlt-1 concentration in EDTA plasma of mice receiving either empty vector or sFlt-1 overexpression plasmid. Individual mouse blood was collected on day 18. sFlt-1 level was assessed by ELISA and compared between groups as follows: **(A)** RALA-sFlt-1-e15a (n=12) or RALA-empty vector (n=10~~1~~), nanoparticles independent of *Fkbpl* genotype; **(B)** *fkbpl*$^{+/+}$ RALA-empty vector (n=6), *fkbpl*$^{+/-}$ RALA-empty vector (n=4), *fkbpl*$^{+/+}$ RALA-sFlt-1-e15a (n=8), and *fkbpl*$^{+/-}$ RALA-sFlt-1-e15a (n=4). sFlt-1 ELISA was used to determine the plasma levels in both pregnant and non-pregnant mice. Data are mean ± SEM; Mann-Whitney test (A), or one-way ANOVA (C-D). *Fkbpl*$^{+/+}$ *= Wild type (WT), Fkbpl*$^{+/-}$ *= FKBPL knockdown. * p<0.05.*

Figure 6. *In vitro* efficacy of RALA as a transfection agent. Effect of RALA/human VEGFR1 plasmid (phFLT1) transfection on sFlt-1 level in trophoblast cells. **(A)** Scanning electron microscope image of RALA-hFlt1 pDNA complexes at N:P 10. **(B, C)** sFlt-1 level in BeWo cells post transfection with RALA/pDNA complexes (N:P 10) was determined via ELISA assay. **(B)** 1 µg or **(C)** 5µg RALA/DNA complex. RALA supernatant was collected after 48 h for analysis by ELISA, n=3. Data points are mean ± SEM; ** p <0.01; unpaired Student's t-test.

Supplementary Figure 1: Dynamic Light Scattering of RALA- sFlt1-e15a (red) and RALA-hFlt1 (blue) at N:P 10 with a PDI less than 0.6.

Supplementary Figure 2: Differential expression of FKBPL in trophoblast cell lines. $5x10^5$ cells were seeded and incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2 for 24 h before protein lysates were collected and western blotting performed. Data points are mean ± SEM; n=3; one-way ANOVA with Tukey's post-hoc test.

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