DEVELOPMENT OF NOVEL SURGICAL, IMAGING AND MOLECULAR METHODS FOR THE IN VIVO MODELING OF PREECLAMPSIA

PhD thesis

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1. INTRODUCTION

I.1. Preeclampsia is a 'Great Obstetrical Syndrome'

Preeclampsia is one of the 'Great Obstetrical Syndromes', complicates 3-5% of pregnancies, and is a major cause of maternal and perinatal morbidity and mortality. The clinical diagnosis is based on new-onset hypertension and proteinuria in normotensive women developing after the 20th week of gestation. Based on the time of the onset of the clinical symptoms, preeclampsia can be divided into early-onset (<34 weeks) and late-onset (>34 weeks) disease. The clinical outcomes are more benign among women with late-onset preeclampsia and more severe among women with early-onset preeclampsia. The severe complications may include generalized endothelial damage and multi-organ failure mostly affecting the central nervous system, kidneys and liver. Preeclampsia is characteristic to humans, since it has not been detected in any other species except some cases in anthropoid primates. The central role of the placenta in the pathogenesis is unquestionable, and the definitive treatment is still the delivery of the placenta.

1.2. Pathophysiology and molecular mechanisms

Preeclampsia is associated with placental histopathological lesions consistent with defective trophoblast invasion and the failure of physiologic transformation of spiral arteries by these trophoblasts into low resistance vessels, which impairs the continuous blood supply of the placenta, leading to hemorheological changes and oxidative stress. The injured placenta releases apoptotic-necrotic trophoblast microparticles, cytokines, and yet unknown factors. This terminal pathway of preeclampsia is characterized by increased placental expression and maternal systemic concentrations of anti-angiogenic molecules [e.g. soluble fms-like tyrosine kinase-1 (sFlt-1) and endoglin (sEng)]. Since sFlt-1 is a decoy receptor for PlGF and VEGF, the high maternal blood sFlt-1 concentrations decrease the bioavailability of angiogenic factors, leading to an anti-angiogenic state, generalized endothelial dysfunction, hypertension and proteinuria.

1.3. sFlt-1 isoforms

Soluble Flt-1 constitutes of four alternatively spliced isoforms of the transmembrane Flt-1 receptor. Flt-1 contains seven extracellular Ig-like domains and an intracellular tyrosine kinase domain. The first three Ig-like domains are essential for ligand-binding, while the 4-7th Ig-like domains for receptor dimerization. The first identified sFlt-1 isoform (sFlt-1-i13)

is encoded by the first 13 exons of *FLT1*, and is generated by skipped mRNA splicing and premature termination due to intron 13 polyadenylation. It contains the first six Ig-like domains along with a unique 31-amino-acid tail. It acts as a potent VEGF and PlGF antagonist and inhibitor of angiogenesis, and it maintains a barrier against extreme VEGF-signaling and vascular hyperpermeability in the placenta. The sFlt-1-e15a mRNA contains 14 exons of *FLT1* and an alternatively spliced exon (Exon 15a) within an AluSeq retrotransposon. This is primate specific, since AluSeq retrotransposons appeared in primates. SFlt-1-e15a contains a unique 28-amino-acid tail, it is predominantly expressed in the placenta in humans where it has a dominant abundance after the first trimester. Two additional sFlt-1 isoforms (hsFlt-1-e15b and hsFlt-1-i14) are alternatively spliced after exon 14, and contain 13 and 31-amino-acid unique C-termini. These four sFlt-1 isoforms account for 95% of *FLT1* transcripts in the placenta in healthy pregnancies. HsFlt-1-e15a expression is up-regulated in the trophoblast by hypoxia, and it is the most abundant isoform in the placenta in patients with preeclampsia. These suggest that hsFlt-1-e15a may have important functions in normal pregnancy; however, its overexpression may promote preeclampsia.

1.4. Animal models of preeclampsia

Humans have hemochorial placentation with deep trophoblast invasion, similar to chimpanzees and gorillas but not to other species. Because of the anatomical and physiological uniqueness of human placentation, it has been impossible to develop adequate animal models to mimic the placental stages of preeclampsia. However, various animal models could model the terminal pathway, either utilizing hypertensive strains or models of impaired uterine perfusion, nitric oxide synthase function, metabolic functions, oxidative and nitrosative stress, or altered renin-angiotensin system functions. Other models generated systemic maternal inflammation or utilized the overexpression of anti-angiogenic molecules. Among various species, mice turned to be good to study late-onset preeclampsia, since they have hemochorial placentation similar to humans. Although trophoblast invasion is limited in mice, placentation events can be somewhat similar to those in humans.

Most anti-angiogenic preeclampsia models utilized the overexpression of an artificially truncated sFlt-1 mutant [sFlt-1(1-3)], which is not expressed in any species, lacks the conserved sFlt-1 domains important in dimerization, bioavailability and yet unknown functions, and may induce a stronger preeclampsia

phenotype than the full-length sFlt-1-i13. However, no study previously investigated whether the overexpression of full-length hsFlt-1-e15a may have effects in inducing hypertension and proteinuria in mice similar to the truncated sFlt-1(1-3). In addition, many models of preeclampsia in rodents had several technical constraints, which limited follow-up during pregnancy and postpartum, including the: 1) lack of appropriate imaging techniques to determine pregnancy status in early gestation posed by the small size of these rodents; 2) lack of urine protein measurements due to difficulties with urine collection techniques; 3) limitations in continuous and/or non-stressed blood pressure monitoring; and 4) lack of postpartum monitoring.

2. OBJECTIVES

Because of the above listed limitations of earlier animal models of preeclampsia, we designed our studies to overcome these limitations. Accordingly, we set up our aims to:

1. develop a biologically more relevant anti-angiogenic mouse model of preeclampsia by overexpressing the most abundant human placental hsFlt-1-e15a isoform in preeclampsia in order to detect its presumed *in vivo* pathologic effects;
2. use high-frequency ultrasound imaging in early pregnancy determination;
3. utilize high-frequency ultrasound for the detection of telemetric catheter positioning in the aortic arch in order to promote more accurate blood pressure monitoring;
4. utilize non-stressed blood pressure monitoring during pregnancy and postpartum;
5. develop a novel survival cesarean section for enabling postpartum monitoring;
6. develop a novel method for cystocentesis with the use of high-frequency ultrasound to enable accurate urine protein analysis;
7. investigate the tissue distribution of hsFlt-1-e15a viral transgene expression and its relation to the induced clinical symptoms;
8. utilize histopathological, cell- and molecular biological as well as immunological methods for the *in vitro* investigations of the functional effects of hsFlt-1-e15a;
9. compare the biological effects of the full-length hsFlt-1-e15a with that of the truncated msFlt-1(1-3) on the development of preeclampsia in mice;
10. examine the biological effects of full-length hsFlt-1-e15a with that of the truncated msFlt-1(1-3) on the fetus and the placenta.

Aims 1 to 8 were addressed in *Study I*, while Aims 9 to 10 were addressed in *Study II*.

3. METHODS

3.1. Study I

3.1.1. Ethics statement

The study protocol (A#11-03-11) was approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University. Animal handling and care followed all standards in accordance with the recommendations in the *"Guide for the Care and Use of Laboratory Animals"* of the National Institutes of Health. Mice were euthanized in accordance with the *"Guidelines on Euthanasia"* of the American Veterinary Medical Association, and the IACUC guidelines at Wayne State University.

3.1.2. Animals and husbandry

Timed-pregnant CD-1 mice arrived from Charles River Laboratories on gestational day (GD) 5, and then were acclimated for two days. Mice were kept separately in cages and fed with *ad libitum* water and food. Constant temperature (24±1ºC) and humidity (50±5%) were maintained in the animal room with a daily regular 12:12 hour light-dark period. Mice were monitored daily for food and water intake, vital signs, activity, and behavior. Incision sites were examined daily to detect any signs of infection and/or inflammation, and genital regions for signs of vaginal discharge or preterm labor. Animals were excluded from the study in case of miscarriage, surgical complications, or any condition that a veterinarian deemed severe enough to warrant exclusion.

3.1.3. Determination of pregnancy status with ultrasound

Ultrasound scans were performed on GD6 (n=12) or GD7 (n=35) to determine pregnancy status. Anesthesia was induced by inhalation of 4-5% isoflurane (Baxter Healthcare Corp.) and 1-2 L/min of O_2. Anesthesia was maintained with a mixture of 2% isoflurane and 1-1.5 L/min of O_2. Body temperature was supported in the range of 37±1˚C. Respiratory and heart rates were monitored throughout the ultrasound scans with Vevo Imaging Station (Visual Sonics Inc.). The pregnancy status was evaluated with a 55MHz linear ultrasound probe while looking for signs of a gestational sac (GD6) as well as an embryo and an advanced endometrial reaction (GD7).

3.1.4. Implantation of the telemetric blood pressure monitoring system

Mice with confirmed pregnancies underwent telemetric blood pressure monitoring system implantation on GD7. Body temperature was controlled by a T/Pump warm-water

circulating blanket (Gaymar Industries Inc.). The incision site was scrubbed with Betadine (Purdue Pharma L.P.); and 2% lidocaine (0.5 mg/kg, Vedco Inc.) and 0.5% bupivacaine (1.5mg/kg, Hospira Inc.) were injected subcutaneously (s.c.) before the incision. An approximate 1.5cm midline incision was made on the neck, and the salivary glands were gently dissected and retracted laterally. An approximate space of 1cm of the left common carotid artery was exposed from the bifurcation in the direction of the heart. After carotid artery ligation at the level of bifurcation, arteriotomy and cannulation were prepared, and the blood pressure monitoring catheter (TA11PA-C10 or HD-X11, Data Sciences International) was positioned into the aortic arch. The catheter was fixed and the transmitter was placed in a subcutaneous pocket in the left flank, preformed with blind dissection. After repositioning the salivary glands over the catheter, the skin was closed. Postoperative pain was reduced with s.c. injection of carprofen (5 mg/kg/24h, Pfizer Inc.), and with the administration of lidocaine and bupivacaine adjacent to the surgical incision site. In order to avoid post-surgical dehydration, 0.5ml of 0.9% saline solution was s.c. injected.

3.1.5. Determination of the telemetry catheter position with high-frequency ultrasound

Transmitter catheter tip positions were examined with the 55MHz linear ultrasound probe during the routine GD13 ultrasound scans. The left carotid artery, aortic arch, and ascending aorta were visualized, and the position of the catheter tip was determined.

3.1.6. Telemetric blood pressure monitoring

Telemetry monitoring was started on GD10, three days after the catheter implantations, and was continued until postpartum day (PPD) 7 using the Dataquest A.R.T. 4.31 acquisition and analysis system (Data Sciences International). Blood pressures were recorded for 10s every five minutes for at least 8-12 hours a day during both the light and dark cycles.

3.1.7. Adenoviral gene delivery

Adenoviruses expressing enhanced green fluorescent protein (GFP) or hsFlt-1-e15a under the control of a cytomegalovirus promoter (Ad-CMV-GFP and Ad-CMV-hsFlt-1-e15a, respectively) were constructed by Vector BioLabs. Mice were divided into four groups [hsFlt-1-e15a 1x (n=6), hsFlt-1-e15a 2x (n=5), GFP 1x (n=4), and GFP 2x (n=5)]. All mice were injected with adenovirus constructs [$1x10^9$ plaque-forming units (PFU)] via the tail vein on GD8, and a subset of mice (GFP 2x and hsFlt-1-e15a 2x) was repeatedly injected with $1x10^9$ PFU adenoviral constructs on GD11.

3.1.8. Ultrasound-guided bladder puncture (cystocentesis)

Ultrasound-guided cystocentesis was performed on GD7, GD13, GD18, and PPD8 under isoflurane anesthesia. Urine samples were obtained using a micro-injection system and a linear 55 MHz high-frequency ultrasound probe. The transcutaneous bladder puncture was performed under continuous ultrasound guidance using the mechanical holder of the micro-injection system. Urine samples were evaluated for blood contamination using a highly sensitive Urine Chemstrip 5 OB (Roche Diagnostics), and then were stored at -80^{o}C.

3.1.9. Cesarean section

Mice underwent survival cesarean section on GD18. After a short (1-1.5cm) midline abdominal incision, a short segment of one uterine horn was exteriorized at once, and kept moisturized with sterile 0.9% saline. According to the number of pups, two to three exteriorizations and minimal (3-5mm) longitudinal midline hysterectomies were made on each horn, on the opposite side of the mesometrial arterial arcade, while keeping the residual parts of the uterine horn inside the abdominal cavity to avoid contamination. After delivering pups and placentas, the minimal incisions were closed and lavage was applied to the abdominal cavity with 0.9% sterile saline. The abdominal wall was closed with sutures and the skin with staples (Braintree Scientific Inc.). Body fluids were replenished by an injection of 0.5ml of 0.9% sterile saline sc. Postoperative pain was reduced with s.c. carprofen (Pfizer Inc.), and with the injection of lidocaine and bupivacaine adjacent to the incision line. Postoperative care was similar to that which followed telemetry implantation.

3.1.10. Tissue collection

Following cesarean section, each fetus was separated from the placenta and umbilical cord. All fetuses and placentas were weighed. The first placentas adjacent to the uterine cervix in both uterine horns were fixed in 4% paraformaldehyde (PFA) diluted with phosphate buffered saline (PBS, Life Technologies Corp.) for 24h, then dehydrated in 70% graded ethanol (Thermo Fisher Scientific Inc.), and embedded in paraffin for histopathological examinations. The second placentas adjacent to the uterine cervix were collected and homogenized in TRIzol (Life Technologies Corp.) and stored at -80˚C.

After euthanization, tissues from several organs (spleen, uterus, liver, kidney, and brain) were dissected and fixed in 4% PFA for 24h, then dehydrated in ethanol, and embedded in paraffin for histopathological examinations, or homogenized in TRIzol reagent and stored at

-80˚C until gene expression analyses. To evaluate the changes in uterine histology with time after the cesarean sections, additional untreated mice were euthanized on PPD38 (n=3), PPD50 (n=3), and PPD77 (n=3), respectively, and uteri were processed as those on PPD8.

3.1.11. Total RNA isolation, cDNA generation, quantitative real-time RT-PCR

Tissues were homogenized in TRIzol immediately after tissue collection. Total RNAs were isolated using the QIAshredder and RNeasy Mini Kit (Qiagen). cDNAs were generated with SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR assays were performed on the Biomark System (Fluidigm) using TaqMan assays (Life Technologies Corp) for *GFP* (Mr04097229_mr) and human *FLT1* (Hs01052961_m1).

3.1.12. Histopathological evaluation of tissues

Five-µm-thick sections of paraffin embedded placenta, kidney, and uterus tissue blocks were serially cut, mounted on silanized slides, deparaffinized, and rehydrated in ethanol. Selected levels of all tissues were stained with hematoxylin and eosin (H&E) to evaluate general morphology, and selected levels of kidneys were stained with periodic acid Schiff (PAS) reagent for the visualization of basement membranes of glomerular capillary loops and tubular epithelium. Histopathological examinations were performed on a BX50F light microscope (Olympus) by a pathologist. Kidney sections were evaluated for glomerular endotheliosis in at least 20 glomeruli in the inner cortex of one kidney in each animal.

3.1.13. Immunohistochemistry

Selected layers of uteri were immunostained for CD68 and smooth muscle actin (SMA). Immunostainings were performed using a rabbit anti-mouse SMA polyclonal antibody (1:300; Abcam Inc.) and the Bond Polymer Refine Detection Kit (Leica Microsystems) on a Bond Max automatic staining system (Leica Microsystems), or using a rabbit anti-mouse CD68 polyclonal antibody (1:150; Abcam Inc.) and the DAB Map Detection Kit on an automatic staining system (Ventana Medical Systems Inc.).

3.1.14. Aortic ring assays

Thoracic aortas were dissected and placed in a Petri dish containing DMEM + GlutaMAX low glucose medium (Life Technologies Corp.). The peri-adventitial fibro-adipose tissue was removed, then aortas were sectioned into 1mm-long rings, and incubated in 12-well plates at 37^{o}C in Opti-MEM+GlutaMAX reduced serum medium (Life Technologies Corp.) overnight for serum starvation. Then these were placed into 96-well tissue culture

plates pre-coated with 50µL of Growth Factor Reduced BD Matrigel Matrix (BD Biosciences). Aortic rings were covered with an additional 50µL of Matrigel and 100µL of Opti-MEM medium supplemented with 1% Penicillin/Streptomycin (P/S, Life Technologies Corp.), 2.5% fetal bovine serum (FBS; Atlanta Biologicals), and 30ng/mL of vascular endothelial growth factor (VEGF-A; ProSpec). Plates were incubated at 37^{o}C for six days with a change of medium every second day. Then, aortic rings were fixed with 4% PFA, and images were obtained with an Olympus 1X51 inverted microscope camera.

3.1.15. Albumin-creatinine immunoassays

Urine specimens were examined for albumin with the Albuwell kit (Exocell Inc.) and for creatinine with the Creatinine Companion assay (Exocell Inc.).

3.1.16. Adenoviral infection of BeWo cells

BeWo cells (American Type Culture Collection) were cultured with F12 medium supplemented with 10% FBS and 1% P/S (Life Technologies Corp.). Cells were plated on either 6-well plates or 35mm cell culture dishes, and infected with Ad-CMV-GFP or Ad-CMV-hsFlt-1-e15a. After 16h, supernatants were removed, and cells were washed with PBS and used for Western blotting or confocal imaging.

3.1.17. Protein isolation and Western blot

Total protein from BeWo cell samples was extracted with the RIPA lysis buffer (Sigma-Aldrich Corp.) containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Protein concentrations were determined with the Quick Start Bradford Protein Assay (Bio-Rad). Twenty micrograms of total protein were electrophoresed on 4-12% SDS-PAGE gels (Life Technologies Corp.), and electro-transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed with goat anti-human Flt-1 polyclonal antibody (AF321, 1:2000, R&D Systems Inc.) at 4C^{o} for 16h, and then with peroxidase-conjugated anti-goat IgG (1:5000, Vector Laboratories) at room temperature for 1h. Protein bands were developed using the ChemiGlow Western Blotting Detection Reagents (Protein Simple), then scanned and imaged with a Fujifilm LAS-4000 Image Reader (GE Healthcare).

3.1.18. Confocal microscopy

BeWo cells infected with adenovirus constructs were mounted with ProLong Gold Antifade Reagent and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) followed by confocal microscope imaging using a TCS SP5 spectral confocal system (Leica Microsystems).

3.1.19. Data and statistical analyses

Blood pressures: Mean arterial blood pressures (MAP) were calculated from systolic and diastolic blood pressures for each time point and animal, and then were averaged. The mean MAP values on GD10 were subtracted from all MAP values to obtain ΔMAP values. We fitted the MAP and the ΔMAP data with a Linear Mixed Effects (LME) models for the time intervals before and after cesarean delivery. These models included explanatory variables such as the treatment (GFP or hsFlt-1-e15a) or the dose (1x or 2x), and a continuous measure of time (gestational day or postpartum day) while allowing a random intercept for each animal. An interaction was allowed between the treatment and time, and therefore, we could test if the slope of the MAP or ΔMAP over time was different between the treatments. We relaxed the linear fixed effect patterns to quadratic for the analysis of time intervals after cesarean delivery. Since blood pressure has a circadian daily rhythm in mice, we also examined blood pressures in 12-hour light and dark cycles separately.

Urine albumin/creatinine ratios: Albumin/creatinine ratios between the hsFlt-1-e15a and GFP groups on different time points were compared with the Student's t-test.

Gene expression profiling: Relative gene expressions were quantified by averaging target (*FLT1* or *GFP*) and reference (*Gapdh*) gene Ct values over technical replicates, and then by subtracting mean target gene Ct values from mean reference gene Ct values within the same sample. The Student's t-test was used to compare gene expression levels between treatments in a given tissue. To examine the dose effect on gene expression for each tissue, we computed the percentage of samples expressing a given gene when over-expressed with a given dose of that gene. Statistical comparison on the percentages across all tissues between the two doses was performed with the one-tailed paired Student's t-test.

Aortic ring assays: A ruleset was developed using Definiens Developer XD2 (Definiens) to analyze images. A new image layer with enhanced local contrast was produced to distinguish the newly formed microvessel sprouting from the aortic ring. A series of segmentation and classification operations was performed to exclude the ring from the area measurements, and the total area of the objects determined to be "outgrowth" was reported. Data were averaged on the picture level for the same ring, then further averaged on the ring level for the same animal, followed by a Student's t-test for group comparisons.

Fetal survival rates, fetal and placental weights: The fetal survival rate for each mouse was computed, and the non-parametric Kruskal-Wallis test was used for multiple group

comparisons. Fetal weights, placental weights, and placental/fetal weight ratios were compared with the two-way ANOVA test and with a linear mixed effects model.

3.2. Study II.

3.2.1. Ethics statement

The mouse study was approved as part of the animal study protocol (A#11-03-11). Collection and utilization of human samples for research purposes were approved by the Institutional Review Boards of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NIH) and Wayne State University. Written informed consent was obtained from all pregnant women prior to the collection of clinical data and tissue samples. These specimens were coded, and data were stored anonymously.

3.2.2. Animals and husbandry

Timed-pregnant CD-1 mice (n=48) were shipped on gestational day 5 (GD5), and were housed, fed and monitored as in Study I. Exclusion criteria were the same as in Study I.

3.2.3. Evaluation of pregnancy status with high-frequency ultrasound

Scans were performed on GD7 to evaluate pregnancy status as described in Study I.

3.2.4. Telemetric blood pressure catheter implantation

Telemetric blood pressure catheter implantation was done as described in Study I.

3.2.5. Determination of the telemetry catheter position with high-frequency ultrasound

The telemetry catheter positions were determined as described in Study I.

3.2.6. Telemetric blood pressure monitoring

Telemetric blood pressure monitoring was performed as described in Study I.

3.2.7. Adenoviral gene delivery

We used three different viral vectors constructed from replication deficient adenovirus and an "RGD fiber-mutant" adenovirus with distinct tissue-tropism as well as two different gene promoters that differ in terms of tissue-specific promoter activity. Adenoviruses and fiber-mutant adenoviruses expressing the full-length hsFlt-1-e15a, the truncated msFlt-1(1-3) or GFP were constructed and titered by Vector BioLabs. HsFlt-1-e15a was overexpressed by 1) a wild-type adenovirus under the control of the cytomegalovirus promoter (Ad-CMV-hsFlt-1-e15a; n=6), 2) an RGD fiber-mutant adenovirus under the control of the cytomegalovirus promoter (Ad-RGD-CMV-hsFlt-1-e15a; n=6), or 3) an RGD fiber-mutant

adenovirus under the control of the human *CYP19A1* promoter (Ad-RGD-CYP-hsFlt-1-e15a; n=5). Truncated msFlt-1(1-3) was overexpressed by the RGD fiber-mutant adenovirus under the control of the cytomegalovirus promoter [Ad-RGD-CMV-msFlt-1(1-3); n=6]. GFP was overexpressed by 1) the RGD fiber-mutant adenovirus under the control of the cytomegalovirus promoter (Ad-RGD-CMV-GFP; n=12), or 2) by the RGD fiber-mutant adenovirus under the control of the human *CYP19A1* promoter (Ad-RGD-CYP-GFP; n=4). Mice in these treatment and control groups were injected via the tail vein with $2.5x10^9$ plaque-forming units (PFU) of adenovirus constructs on GD8 and then repeatedly with $2.5x10^9$ PFU adenoviral constructs or saline on GD11. A group of mice (n=9) that was used only for the expression profiling of endogenous mouse transmembrane Flt-1 and sFlt-1-i13 received only 100µl saline injection on GD8 and GD11 via the tail vein.

3.2.8. Minimal invasive survival cesarean section

Aseptic survival cesarean section was performed as described in Study I.

3.2.9. Tissue collection

Tissue collection was done as described in Study I.

3.2.10. Total RNA isolation, cDNA generation, quantitative real-time RT-PCR

Tissues were homogenized in TRIzol with QIAshredder and total RNA was isolated with RNeasy Mini Kit. Total RNA from primary human trophoblasts was isolated with TRIzol and RNeasy kit. Five hundred ng of total RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System. TaqMan assays for human *FLT1* (Hs01052961_m1), mouse *Flt1* (Mm01210866_m1: exon boundary 1-2; Mm00438980_m1: exon boundary 15-16), *GFP* (Mr04097229_mr), human *CYP19A1* (Hs00903411_m1), and the endogenous human and mouse controls [*RPLP0* (Hs99999902_m1); *Gapdh* (Mm99999915_g1)] were used for qRT-PCR on the Biomark System.

3.2.11. Histopathological evaluation

Four-µm-thick sections were cut from paraffin embedded kidney blocks, mounted on silanized slides, deparaffinized and rehydrated. Morphology was analyzed on selected tissue levels after staining with H&E. Selected kidney sections were stained with PAS reagent and with the Jones basement membrane reticulum stain (Dako North America Inc.) for the evaluation of the glomerular capillary loop basement membranes. Two pathologists blinded to the clinical outcome evaluated 10 glomeruli from each kidney for glomerular

endotheliosis and changes in the mesangium. Glomerular damage was scored as follows: 0 = no glomerular changes in 10 glomeruli examined; 1+ = 1 to 5 of 10 glomeruli examined with either segmental or diffuse endotheliosis; 2+ = 6 or more glomeruli with segmental or diffuse endotheliosis. Images were taken with an Olympus BX50F light microscope.

3.2.12. Immunohistochemistry

Placentas were immunostained with a rabbit anti-CD31 monoclonal antibody (1:50; Spring Bioscience) and Bond Polymer Refine Detection Kit on a Leica Bond Max autostainer.

3.2.13. Aortic ring assays

Aortic ring assays were performed as described in Study I.

3.2.14. Urine collection and albumin-creatinine immunoassays

Urine collection and albumin-creatinine assays were performed as described in Study I.

3.2.15. Primary human trophoblast isolation and cultures

Human placentas (n=4) were collected from normal pregnant women who delivered a healthy neonate at term. Villous tissues were cut into pieces, rinsed in PBS, and digested sequentially with Trypsin (0.25%; Life Technologies Corp.) and DNAse I (60U/ml; Sigma-Aldrich Corp.) for 90min at 37^{o}C. Dispersed cells were filtered through 100μm Falcon nylon mesh cell strainers (BD Biosciences), and then erythrocytes were lysed with NH_4Cl (Stemcell Technologies). Washed and resuspended cells were layered over Percoll gradients (20-50%) and centrifuged (20min, 1200g). The bands containing trophoblasts were collected; non-trophoblastic cells were excluded by negative selection with anti-CD9 (20μg/ml) and anti-CD14 (20μg/ml) mouse monoclonal antibodies (R&D Systems) and MACS anti-mouse IgG microbeads (Miltenyi Biotec). Trophoblasts were plated on collagen-coated plates (BD Biosciences) in triplicate and kept in Iscove's modified Dulbecco's medium (Life Technologies Corp.; supplemented with 10% FBS, 5% human serum and 1% P/S) for 7 days. Cells were harvested for total RNA isolation in every 24h.

3.2.16. Confocal microscopy

Five-μm-thick tissue sections were cut from frozen placentas, and were mounted on silanized slides. Tissue sections were fixed with -20˚C acetone for 10min, then rinsed three times in ice-cold PBS, mounted with ProLong Gold Antifade Reagent and 4',6-diamidino-2-phenylindole (DAPI; Life Technologies Corp.), and imaged by a Leica TCS SP5 spectral

confocal system. After fixation, aortic rings were also mounted with ProLong Gold antifade reagent with DAPI, and image stacks were acquired on the same confocal microscope.

3.2.17. Data and statistical analyses

Gene expression profiling: Relative gene expression levels were quantified by averaging target (human *FLT1*, mouse *Flt1* or *GFP*) and reference (*Gapdh*) gene Ct values over technical replicates, and then subtracting the mean target gene Ct values from the mean reference gene Ct values within each sample. The expression values across different arrays were further adjusted using calibration samples. The Student's t-test was used to compare gene expression levels between treatments in a given tissue. Mouse transmembrane Flt-1 expression was calculated from the data generated by the Mm00438980_m1 TaqMan assay, which detects only the full-length Flt-1 mRNA expression levels. MsFlt-1-i13 mRNA expression was calculated by subtracting full-length Flt-1 mRNA expression levels from the expression data generated by the Mm01210866_m1 TaqMan assay, which detects all full-length and alternatively spliced Flt-1 mRNA levels. We used a linear model to estimate the effect of the transgene and the vector on endogenous msFlt-1-i13 expression.

Blood pressure: MAP was calculated at each time point. MAP values for each mouse on a given day were averaged. Within the dataset of each mouse, the mean MAP value on GD11 was subtracted from all data to obtain a normalized blood pressure, ΔMAP. A separate LME model was fit the data for the time periods before and after cesarean delivery. The fixed effect terms in the model included the treatment group, polynomial terms of the gestation day, and their interaction terms. The random components in the mixed effects models included an intercept term and a quadratic term of gestational day for each animal. A likelihood ratio test comparing the fit quality of the model with and without interaction terms between the group and gestational day was used to test if the blood pressure profile over gestation was different between the groups. Blood pressure levels at specific gestational days were compared between the groups using a t-test.

Glomerular changes, urine albumin/creatinine ratios: Glomerular damage scores were evaluated using a logistic regression model. Albumin/creatinine ratios at different time points were compared with a Student’s t-test.

Aortic ring assays: A ruleset was developed using Definiens Developer XD2 to analyze 3D confocal images. A series of segmentation and classification operations was performed on the DAPI channel to exclude the ring from the volume measurements, and the total volume

of the objects determined to be "outgrowth" was summed for each image stack. Volume data were averaged for the same ring, and then further averaged over the multiple rings for the same animal. A t-test was used to compare the volume data between the groups.

Fetal survival rates, fetal and placental weights: The number of total and live fetuses, fetal survival rate, maternal weights, total fetal and placental weights, and the total placental/total fetal weight ratios were compared between the treatment and control groups using t-tests.

Microarray and qRT-PCR data visualization: Microarray gene expression profiles downloaded from the SymAtlas/BioGPS database, and expression data for 40 adult and fetal tissues was visualized via barplots using the R statistical environment. Primary human trophoblast *CYP19A1* expression data were normalized to the reference gene (*RPLP0*) obtained for each sample as $-\Delta Ct_{(gene)}=Ct_{(RPLPO)}-Ct_{(gene)}$ and displayed as a function of time.

4. RESULTS

4.1. Study I

4.1.1. Pregnancy status determination with high-resolution ultrasound

On GD6, only 1.9mm-2.7mm gestational sacs could be visualized. Since an advanced endometrial reaction and the embryo were already visible on GD7, we chose this day for the further examinations. In non-pregnant animals, none of these signs were visible. In the set of 35 mice scanned on GD7, pregnancy was diagnosed in 32 mice. Among the three mice diagnosed as non-pregnant, two were non-pregnant, and one delivered at term.

4.1.2. Implantation and evaluation of telemetry devices

Telemetry catheter implantations took place after ultrasound confirmed pregnancy. The length of the implantation surgeries varied between 20-35 minutes. The TA11PA-C10 device required shorter surgery than the HD-X11 transmitter. Ultrasound examinations on GD13 showed that all animals had correctly positioned telemetry catheter. The rate of uncomplicated telemetry system implantations was 79% (30/38): in 86% (18/21) of cases using the TA11PA-C10 transmitter and in 71% (12/17) of cases using the HD-X11 device.

4.1.3. Survival cesarean surgery

We developed a new survival cesarean surgery, in which only a short segment of one uterine horn is exteriorized at a time. In total, two or three short longitudinal hysterectomies were performed on each horn, in which pups and placentas could easily be delivered. Due

to the minimal invasiveness of this aseptic technique, administration of appropriate pain medication, and replenishment of lost body fluids, the eight-day survival rate of this new surgery was 100% (30/30). Histopathological examination of H&E-stained and SMA-immunostained uterine cross-sections showed granulation tissues on PPD8. The endometrium was completely healed on PPD77 in all examined cases, and the two-layered myometrium showed complete healing of the inner layer and focal disruption at suture sites in the outer layer. Suture granuloma was observed in the submucosa at the incision site.

4.1.4. Overexpression of human placental sFlt-1-e15a and GFP with adenoviral vectors

Human sFlt-1-e15a and GFP mRNA expression was highest in the liver among the six investigated tissues. The placental expression of hsFlt-1-e15a and GFP was not directly comparable with their expression in maternal tissues because of the difference in tissue sampling time points. Viral dose-effect ($1x10^9$ PFU vs. $2x10^9$ PFU*)* was not seen in GFP expression, since there was no difference between the percentage of tissues expressing GFP in the two control groups. On the contrary, there was a dose-effect in the expression of hsFlt-1-e15a, which had lower expression in all investigated tissues than GFP. For mice in the hsFlt-1-e15a 1x group ($1x10^9$ PFU), the average rate of tissue samples with detectable hsFlt-1-e15a expression was 46.7%, and 72.2% in the hsFlt-1-e15a 2x ($2x10^9$ PFU) group ($p=9.7x10^{-3}$). The hsFlt-1-e15a mRNA expression was 6.3-fold higher in the kidneys of hsFlt-1-e15a 2x mice than of hsFlt-1-e15a 1x mice ($p=5.3x10^{-2}$). The number of hsFlt-1-e15a expressing placenta, brain, and uterine tissues was low in the hsFlt-1-e15a 1x group.

To confirm the placental overexpression of hsFlt-1-e15a and GFP proteins in the trophoblast *in vitro*, we infected BeWo cells with Ad-CMV-GFP or Ad-CMV-hsFlt-1-e15a. Western blotting revealed that non-infected BeWo cells expressed low amounts of the 185kDa Flt-1 membrane receptor as well as 145kDa and 110kDa sFlt-1 variants. Ad-CMV-GFP-infected BeWo cells expressed these proteins to the same extent. Ad-CMV-hsFlt-1-e15a-infected BeWo cells overexpressed the 145kDa and 110kDa sFlt-1 variants. Confocal microscopy revealed cytoplasmic GFP expression in BeWo cells infected with Ad-CMV-GFP, indicating also the efficient transfection of these trophoblastic cells.

4.1.5. Blood pressure telemetry monitoring

Prior to parturition, there was no significant change in the blood pressure over time in control mice (ΔMAP slope=0.513 mmHg/day; p=0.187). However, in response to hsFlt-1-

e15a treatment, blood pressures increased over time (ΔMAP slope=2.05 mmHg/day; $p=8.09x10^{-8}$). The ΔMAP slope in the hsFlt-1-e15a group was higher compared to the controls (1.53 mmHg/day; p=0.0043), resembling late-onset preeclampsia in humans. The ΔMAP at parturition was 13.2 mmHg higher in hsFlt-1-e15a-treated mice than in the control mice (p=0.00107). A similar quadratic pattern of ΔMAP was found in both control and hsFlt-1-e15a-treated mice after cesarean delivery, suggesting a common pattern of drop of blood pressures. However, ΔMAP dropped below the baseline in control mice, while it remained above this in the postpartum period in hsFlt-1-e15a-treated mice. ΔMAP on PPD7 was 1.96 mmHg below the baseline in control mice (p=0.560), while it was 6.88 mmHg above the baseline in hsFlt-1-e15a-treated mice (p=0.0346). We did not observe any effect of the number of viral construct injections ($1x10^9$ vs. $2x10^9$ PFU*)* in hsFlt-1-e15a-treated or in control mice either before delivery or in the postpartum period.

The ΔMAP was 2.67 mmHg higher during the night cycles than during the day cycles ($p=2.5x10^{-3}$) before cesarean delivery, and ΔMAP was 4.37 mmHg higher during the night cycles than during the day cycles ($p=2.7x10^{-6}$) after cesarean delivery.

In addition to normalizing mean arterial blood pressures to obtain ΔMAPs, we also repeated the above analysis with mean arterial pressures (MAPs). Prior to parturition, there was no change in the blood pressures over time in control mice (MAP slope=0.426 mmHg/day; p=0.174). However, in response to hsFlt-1-e15a treatment, blood pressures increased over time (MAP slope=2.02, mmHg/day; $p=1.13x10^{-10}$). The difference in the MAP slope in the hsFlt-1-e15a groups was higher compared to the controls (1.59 mmHg/day; $p=2.6x10^{-4}$). Similarly as above, we did not observe any effect of the number of viral construct injections ($1x10^9$ vs. $2x10^9$ PFU) in hsFlt-1-e15a-treated or in control mice either before delivery or in the postpartum period.

4.1.6. Evaluation of endothelial and kidney functions

The mean microvessel outgrowth area of the aortic rings was 46% smaller in hsFlt-1-e15a overexpressing mice than in controls (p=0.012).

In the kidneys, focal glomerular changes, including swollen capillary endothelial cells and occlusion of glomerular capillaries, were found in mice overexpressing hsFlt-1-e15a. There were no significant morphological changes in the glomeruli of control mice.

Mean urine albumin/creatinine ratios did not change in control mice; however, these increased by GD18 and then dropped postpartum in hsFlt-1-e15a overexpressing mice. Mean urine albumin/creatinine ratio on GD18 was five times higher in hsFlt-1-e15a-treated (109.3±51.7μg/mg) than in control mice (19.3±5.6μg/mg; $p=4.4x10^{-2}$). The mean urine albumin/creatinine ratio on PPD8 was still marginally significantly higher in hsFlt-1-e15a-treated (36.6±9.0μg/mg) than in control mice (18.0±4.9μg/mg; p=0.06).

4.1.7. Evaluation of fetal survival rate, placental and fetal weights

Fetal survival rate was not affected by hsFlt-1-e15a treatment, as no differences (p=0.38) were found in the groups (GFP 1x: 100%; hsFlt-1-e15a 1x: 100%; GFP 2x: 96.43%; hsFlt-1-e15a 2x: 100%). Fetal weights, placental weights and placental/fetal weight ratios were not affected by hsFlt-1-e15a treatment.

4.1.8. Evaluation of a mouse with early-onset preeclampsia-like symptoms

One mouse in the hsFlt-1-e15a 2x group showed a different biological response despite receiving the same treatment as others in the group. This "EM35" mouse had a markedly higher and earlier (GD15) blood pressure peak than other hsFlt-1-e15a-treated mice. This was followed by the normalization of blood pressures and by a second blood pressure peak on PPD1. The urinary albumin/creatinine ratio on GD18 was higher in the "EM35" mouse than in other hsFlt-1-e15a-treated mice. HsFlt-1-e15a expression in the liver was lower in the "EM35" mouse than in other hsFlt-1-e15a-treated mice. The "EM35" mouse had multiple cystic biliary hyperplasia and recent and remote thrombotic infarcts in the hepatic parenchyma. The lowest aortic ring endothelial outgrowth area was in the "EM35" mouse among hsFlt-1-e15a-treated mice. Mean fetal weights (0.749±0.029g) and placental weights (0.080±0.006g) of the "EM35" mouse were lower than in other hsFlt-1-e15a-treated mice (fetal weight: 1.06±0.023g, $p=2.017x10^{-15}$, placental weight: 0.111±0.004g, $p=2.05x10^{-6}$). In addition, the "EM35" mouse placenta had multiple thrombi in the decidual vessels.

4.2. Study II

4.2.1. The development of various transgene delivery systems

To compare the effects of the full-length hsFlt-1-e15a with that of the truncated msFlt-1(1-3), viral constructs containing these two transgenes were constructed**.** We used an "RGD fiber-mutant" adenovirus with a tissue tropism distinct from the replication deficient adenovirus to investigate the effect of varying tissue expression profiles of hsFlt-1-e15a on

its biological effects. Since the 501bp placenta-specific promoter of human *CYP19A1* is able to drive placenta-specific gene expression in transgenic mice, we also tested the effect of this 501bp *CYP19A1* promoter besides the CMV promoter in our viral constructs.

4.2.2. Unique placental expression of msFlt-1

The expression of the endogenous transmembrane mFlt-1 mRNA was the highest in placentas among the six tissues of mice without virus injection; msFlt-1-i13 mRNA expression was solely detected in the placenta of these animals. In msFlt-1(1-3)-treated mice, msFlt-1 mRNA expression appeared in the liver. Placental transcript levels of msFlt-1-i13 mRNA were not increased in animals with msFlt-1(1-3) treatment compared to saline-treated mice, suggesting that the endogenous placental msFlt-1-i13 mRNA expression is higher than that of the transgene expressed msFlt-1(1-3) mRNA.

4.2.3. Expression patterns of various viral transgenes

HsFlt-1-e15a and GFP mRNA expression varied according to the viral constructs and promoters. The fiber-mutant adenovirus supported a higher hsFlt-1-e15a mRNA expression in the kidney and liver compared to the adenovirus, while the CYP promoter restricted hsFlt-1-e15a mRNA expression in the liver compared to the CMV promoter. The CYP promoter restricted GFP mRNA expression in the liver (49.8-fold down-regulation, p=0.005), kidney (9.3-fold down-regulation, p=0.02) and spleen (13.5-fold down-regulation, p=0.01), leading to the highest GFP mRNA expression in the placenta. GFP expression was mainly restricted to the labyrinth zone of the placenta irrespective of the promoter in RGD fiber-mutant virus injected mice. We did not find any effect of either the transgene or the vector on the endogenous expression of msFlt-1-i13.

4.2.4. Blood pressure telemetry monitoring

The blood pressure profile over gestation was different in msFlt-1(1-3)-treated mice from that in GFP-treated mice ($p=3.7x10^{-5}$) prior to cesarean delivery. The ΔMAP at GD15 was 11.1mmHg higher (p=0.0008) in msFlt-1(1-3)-treated mice than in control mice, and this difference was even larger on GD18 (ΔMAP: 12.8mmHg, p=0.005). One msFlt-1(1-3)-treated mouse had a very high ΔMAP and a blood pressure of 175/135mmHg on GD18. In contrast to other mice, this animal had constantly increasing blood pressure until PPD7 with a peak of 182/146mmHg, resembling chronic hypertension following preeclampsia.

The blood pressure profile over gestation in hsFlt-1-e15a-treated mice (all subgroups combined) was different from that in GFP-treated controls ($p=4.3x10^{-4}$) prior to cesarean delivery. The ΔMAP at GD15 was 8.4mmHg higher (p=0.0005) in hsFlt-1-e15a-treated mice than in control mice, and it was 7.8mmHg higher on GD18 (p=0.009). Among the three sub-groups, those that received Ad-CMV-hsFlt-1-e15a and Ad-RGD-CMV-hsFlt-1-e15a had the highest increase in ΔMAP on GD15 (Ad-CMV-hsFlt-1-e15a: 11.3mmHg, p=0.0007; Ad-RGD-CMV-hsFlt-1-e15a: 8.3mmHg, p=0.009) and on GD18 (Ad-CMV-hsFlt-1-e15a: 7.4mmHg, p=0.09; Ad-RGD-CMV-hsFlt-1-e15a: 9.3mmHg, p=0.04) compared to controls. The blood pressure was 5mmHg (GD15) and 6.6mmHg (GD18) higher in Ad-RGD-CYP-hsFlt-1-e15a-treated mice than in control mice; however, p-values did not reach statistical significance (0.14 and 0.16, respectively).

4.2.5. Morphological and functional changes in the kidneys in sFlt-1 treated mice

The kidneys from GFP-treated mice showed widely open capillary loops with thin delicate walls, and no mesangial thickening or hypercellularity was noted. These were confirmed using the Jones basement membrane reticulum stain. The most consistent histopathological changes in the kidneys of mice overexpressing hsFlt-1-e15a or msFlt-1(1-3) were focal and segmental, with swollen capillary endothelial cells, occlusion of glomerular capillaries, and focal mesangial thickening. Scattered glomeruli appeared sclerotic. Glomerular capillary changes were further confirmed by PAS staining and Jones basement membrane reticulum stain, which showed thickened capillary loops and focal expansion of the mesangium.

The dam in the msFlt-1(1-3)-treatment group with the constantly increasing blood pressure had dramatic changes in kidney histology, with extensive glomerular lesions seen in all glomeruli examined. These glomeruli appeared to be somewhat enlarged with marked thickening and expansion of the mesangium, and marked occlusion of capillaries and thickened capillary loops. In this mouse, Jones basement membrane reticulum stain showed marked thickening and reduplication of the capillary loop basement membranes.

The glomerular damage score was significantly higher in all treatment groups compared to the combined control group. Mice treated with msFlt-1(1-3) had an odds ratio (OR) of 2.4 for glomerular damage (p=0.01). The OR for glomerular damage was 3.1 in hsFlt-1-e15a-treated mice ($p=2.8x10^{-5}$). Among hsFlt-1-e15a-treated mice, mice in the Ad-CMV-hsFlt-1-e15a group had the largest OR (3.9, $p=4.8x10^{-5}$) for glomerular damage.

Urine albumin/creatinine ratios were higher in hsFlt-1-e15a-treated mice (GD18, 1.9-fold, p=0.04; PPD8, 1.7-fold, p=0.03) than in controls. The albumin/creatinine ratio was markedly elevated in msFlt-1(1-3)-treated mice postpartum (17-fold, $p=4x10^{-5}$). The dam in the msFlt-1(1-3)-treatment group with the constantly increasing blood pressure had extreme proteinuria with albumin/creatinine ratios of 3,070µg/mg on GD18 and 15,401µg/mg on PPD8. Subgroup analysis showed that Ad-CMV-hsFlt-1-e15a led to an increase in albumin/creatinine ratio on PPD8 (3-fold, p=0.003); Ad-RGD-CMV-hsFlt-1-e15a led to an increase in albumin/creatinine ratio on GD18 (2.4-fold, p=0.04); while Ad-RGD-CYP-hsFlt-1-e15a led to a marginally significant increase in albumin/creatinine ratio on GD18 (2.4-fold, p=0.056) and an increase on PPD8 (1.8-fold, p=0.04). In summary, msFlt-1(1-3) had a stronger effect than hsFlt-1-e15a, and hsFlt-1-e15a expressed by the fiber-mutant adenovirus led to an earlier proteinuria than hsFlt-1-e15a expressed by the adenovirus.

4.2.6. Aortic endothelial dysfunction caused by hsFlt-1-e15a and msFlt-1

The mean microvessel outgrowth volume was 77% reduced in hsFlt-1-e15a overexpressing mice compared to controls (p=0.007), while the outgrowth volume was decreased by 66% in msFlt-1(1-3) overexpressing mice compared to controls (p=0.02) In the msFlt-1(1-3)-treated dam with the constantly increasing blood pressure, the microvessel outgrowth volume was only 53% of the mean microvessel outgrowth volume in other mice in this group, showing a strongly dysfunctional endothelium.

4.2.7. Human sFlt-1-e15a but not msFlt-1(1-3) increases litter sizes

Fetal survival rate, average fetal weights, placental weights, and placental/fetal weight ratios were not affected by either hsFlt-1-e15a or msFlt-1(1-3) treatments. Controls (n=17) and msFlt-1(1-3)-treated mice (n=6) had a litter size consistent with the average published by the vendor (n=11.5). The number of pups (13.8±0.4, p=0.046) and living pups (13.6±0.45, p=0.05) were higher in hsFlt-1-e15a-treated mice (n=18) than in controls. The total weight of living pups (14.2±0.56g, p=0.04) and maternal weights (56.3±1.1g, p=0.04) were higher in hsFlt-1-e15a-treated mice than in controls. MsFlt-1(1-3)-treated mice did not differ in any parameters from the controls. The total weights of living pups was higher in the Ad-RGD-CYP-hsFlt-1-e15a treatment group than in controls (15±0.48g, p=0.043). The number of pups (14.3±0.42, p=0.047) and the number of living pups (14.1±0.46, p=0.039) was higher in the Ad-RGD-CMV-hsFlt-1-e15a-treated mice than in controls.

5. CONCLUSIONS

The principal developments and findings of our two studies included the followings:

1. A biologically relevant anti-angiogenic mouse model of preeclampsia was developed;
2. High-frequency ultrasound predicted pregnancy in 97% of the cases on GD7;
3. High-frequency ultrasound determined telemetry catheter positions in all cases;
4. Telemetry enabled non-stressed blood pressure monitoring in pregnancy and postpartum;
5. The survival rate of the newly developed survival cesarean section was 100%;
6. Ultrasound-guided cystocentesis was developed that enabled urine protein analysis;
7. The hsFlt-1-e15a transgene was mainly overexpressed in the liver by the adenovirus;
8. HsFlt-1-e15a treatment increased mean arterial blood pressure on GD18;
9. HsFlt-1-e15a treatment induced albuminuria on GD18;
10. Glomerular changes were found in the kidneys of hsFlt-1-e15a-treated mice;
11. Aortic ring microvessel outgrowth was inhibited in hsFlt-1-e15a-treated mice;
12. There was no effect of hsFlt-1-e15a on placental and fetal weights;
13. One hsFlt-1-e15a-treated mouse had severe, early-onset preeclampsia-like symptoms associated with fetal growth restriction and multi-organ involvement;
14. The in vivo biological effects of the full-length human sFlt-1-e15a and the truncated mouse sFlt-1(1-3) could be directly compared;
15. The full-length mouse transmembrane mFlt-1 was expressed most abundantly in the placenta, while the soluble mouse Flt-1-i13 was only expressed in the placenta;
16. Utilizing a fiber-mutant adenovirus and a placenta-specific promoter increased the placental expression of both the hsFlt-1-e15a and control transgenes;
17. Both hsFlt-1-e15a and msFlt-1(1-3) induced the symptoms of preeclampsia, including blood pressure elevation, glomerular damage, proteinuria and endothelial dysfunction;
18. One msFlt-1(1-3)-treated dam had constantly high blood pressure, severe proteinuria and extensive glomerular damage, suggesting increased sensitivity to sFlt-1 of this animal;
19. The overexpression of hsFlt-1-e15a increased litter size, while msFlt-1(1-3) did not have this effect, underlining the important role of the full-length sFlt-1 in early pregnancy;
20. These observations point to the difference in the biological effects of full-length and truncated sFlt-1 and the changes in the effect of full-length sFlt-1 during pregnancy, and thus, may have important implications in the management of preeclampsia.

6. LIST OF PUBLICATIONS

6.1. Publications related to the PhD Thesis

1. **Szalai G**, Romero R, Chaiworapongsa T, Xu Y, Wang B, Ahn H, Xu Z, Chiang PJ, Sundell B, Wang R, Jiang Y, Plazyo O, Olive M, Tarca AL, Dong Z, Qureshi F, Papp Z, Hassan SS, Hernandez-Andrade E, Than NG. Full-length human placental sFlt-1-e15a isoform induces distinct maternal phenotypes of preeclampsia in mice. *PLoS One*, 2015, 10(4):e0119547. **IF: 3.234**

2. **Szalai G**, Xu Y, Romero R, Chaiworapongsa T, Xu Z, Chiang PJ, Ahn H, Sundell B, Plazyo O, Jiang Y, Olive M, Wang B, Jacques SM, Qureshi F, Tarca AL, Erez O, Dong Z, Papp Z, Hassan SS, Hernandez-Andrade E, Than NG. In vivo experiments reveal the good, the bad and the ugly faces of sFlt-1 in pregnancy. *PLoS One*, 2014, 9(11):e110867. **IF: 3.234, Citations: 5**

6.2. Publications unrelated to the PhD Thesis

1. Than NG, Romero R, Xu Y, Erez O, Xu Z, Bhatti G, Leavitt R, Chung TH, El-Azzamy H, LaJeunesse C, Wang B, Balogh A, **Szalai G**, Land S, Dong Z, Hassan SS, Chaiworapongsa T, Krispin M, Kim CJ, Tarca AL, Papp Z, Bohn H. Evolutionary origins of the placental expression of chromosome 19 cluster galectins and their complex dysregulation in preeclampsia. *Placenta*, 2014, 35, 855-865. **IF: 2.710, Citations: 6**

2. Krishnamurthy U, **Szalai G**, Neelavalli J, Shen Y, Chaiworapongsa T, Hernandez-Andrade E, Than NG, Xu Z, Yeo L, Haacke M, Romero R. Quantitative T2 changes and susceptibility-weighted magnetic resonance imaging in murine pregnancy. *Gynecol. Obstet. Invest.* 2014, 78, 33-40. **IF: 1.696, Citations: 2**

3. Hernandez-Andrade E, Ahn H, **Szalai G**, Korzeniewski SJ, Wang B, King M, Chaiworapongsa T, Than NG, Romero R. Evaluation of utero-placental and fetal hemodynamic parameters throughout gestation in pregnant mice using high-frequency ultrasound. *Ultrasound Med. Biol.*, 2014, 40, 351-60. **IF: 2.214, Citations: 2**

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