CHAPTER 1: INTRODUCTION

1.1 The pathogenesis of preeclampsia

Preeclampsia, a leading cause of maternal and fetal mortality and morbidity, is a potentially devastating disorder with an unknown etiology. Although the underlying cause of preeclampsia is still not clear, the placenta ischemia/hypoxia is thought to be centrally involved in the pathogenesis of this syndrome.

There are two stages in the development of preeclampsia. The first stage concerns defective early trophoblast invasion and remodeling of the spiral arteries which lead to insufficient blood supply to the placenta. Two overarching factors contribute to the relative failure of early trophoblast invasion (Redman, 2005). One is the immunological factor, it has been suggested that preeclampsia may result from maternal immune rejection of the genetically foreign fetus due to maternal-fetal immune mal-adaptation (Renaud, 2008; Saito, 2007). Specifically, decreased expression of human leukocyte antigen-G (HLA-G) interacts abnormally with decidual natural killer (NK) cells which play a major role in trophoblast invasion through the production of immuno-regulatory cytokines such as TNF-α (Sargent, 2007), therefore leading to defective trophoblast invasion; the other factor is an abnormal angiogenesis caused by the imbalance between pro-angiogenic factors and anti-angiogenic factors such as vascular endothelium growth factor (VEGF), soluble fms-like tyrosine kinase (sFlt-1) and placental growth factor (PlGF) (Bdolah, 2004). These two components interact and contribute to a hypoxic placenta, stimulating the synthesis and the release of increased amount of vasoactive factors and cytokines, leading to endothelial activation/dysfunction, which could then trigger the second stage of the maternal syndrome characterized by hypertension,

proteinuria, liver dysfunction and other systemic manifestations of end organ damage. (Lyall, 2005; Redman, 2005; van den Brule, 2005; Gilbert 2008)

1.1.1 ANIMAL MODELS OF PREECLAMPSIA

There have been several attempts at creating an animal model of preeclampsia. These animal models exhibiting some aspects of preeclampsia have been described in a number of species including rats, rabbits, dogs, guinea pigs, sheep and non-human primates. The common theme of these animal models is the altered placental vascular function. The attempted methods include surgical, pharmacological and genetic manipulations. Specifically, the methods used to produce a preeclampsia-like syndrome fall into the following general categories: reduction of utero-placental perfusion by surgical means such as ligation of abdominal aorta, internal iliac artery and uterine artery (Ogden, 1940; Lunell, 1984; Woods, 1989); alteration of vascular function via impairment in the nitric oxide system by knocking out the endothelial NOS (nitric oxide synthase) gene (Zatz, 1998; Hefler, 2001), chronic blockade of the endothelial derived relaxing factor (Baylis, 1992) or using a NOS inhibitor such as N (G)-nitro-L- arginine methyl ester (L-NAME)(Greenberg, 1997), over-activity of the renin-angiotensin systems (Kanayama, 1996; Takimoto, 1996; Kananyama, 1997; Bohlender, 2000;Takiuti, 2002), or activation of systemic inflammatory responses (Faas, 1994; Hung, 2004). None of them however has produced an acceptable animal model. Most recently, the imbalance in angiogenic factors has been found to be involved in the pathogenesis of preeclampsia. Maynard et al induced a dose-dependent hypertension, proteinuria and glomerular endotheliosis at the day 16 to 17 of gestation in pregnant rats by administering adenovirus carrying sFlt-1 at day 8 of gestation (Maynard, 2003), which suggested a possible role for sFlt-1 in the pathogenesis of preeclampsia.

1.1.2. UTEROPLACENTA UNIT

Preeclampsia has long been suspected of being a placental disorder (Zuspan, 1988; Redman, 1991). The paucity of placental lobes, abnormal maturation of the terminal villi, reduced mass of syncytiotrophoblasts, relative excess of cytotrophoblasts and multiple placental infarcts all support a role for the placenta in the etiology of preeclampsia (Hustin, 1983; Khong, 1986; Shanklin & Sibai, 1989; Redman, 1993; Pijnenborg, 1996). Reduced placenta perfusion observed in preeclamptic women occurs due to the failure of vascular remodeling of the maternal uterine spiral arteries. The placental vascular remodeling depends on invasion of the wall of the spiral arterioles by the cytotrophoblasts; this process allows the establishment of an adequate vascular connection between the intervillous space and the maternal blood flow that is independent of humoral or neuronal influences, providing a progressive increase in blood supply to the growing fetus. The invasion in preeclampsia is confined to the decidual part of the spiral arteries and about one third of these arteries completely escape trophoblast invasion (Figure 1). Therefore, preeclampsia is associated with decreased trophoblast invasion and poor vascular remodeling leading to reduced placenta perfusion and oxidative stress.

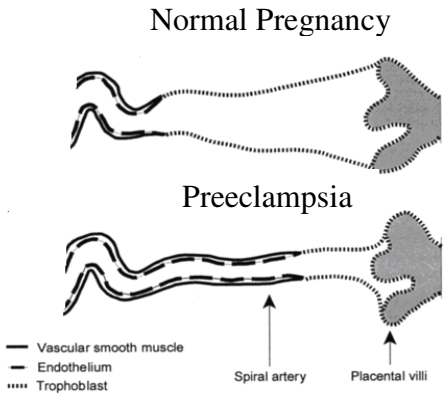


Figure 1 Trophoblast invasion into the spiral arteries in the placental bed in normal pregnancy and in preeclampsia (Figure modified from VanWijk et al, 2000)

1.1.2 GENES INVOLVED IN HYPOXIC PLACENTA

Hypoxic-inducible factor-1 (HIF-1) is known to activate the transcription of genes in response to hypoxia. It is composed of two subunits, the constitutively-expressed HIF-1β and the inducible HIF-1α. Under low oxygen conditions, HIF-1α dimerizes with HIF-1β to form the active HIF complex, which can bind the promoters of oxygen-responsive genes and activate their transcription. Therefore, it has been suggested that HIF-1α plays an important role in signaling hypoxia. HIF-1 is believed to be involved in the pathogenesis of several human diseases such as ischemic cardiovascular disorders, pulmonary hypertension and preeclampsia (Semenza, 2000). It has also been shown that HIF-1α regulates trophoblast differentiation by up-regulating the expression of transforming growth factor β3 (TGFβ3). TGFβ3 is involved in oxygen-dependent differentiation processes during placental development as well as in various pregnancy disorders (Hernandez-Valencia, 2001). The decrease in TGFβ3 expression occurs immediately prior to the peak of trophoblast invasion, suggesting a role for TGFβ3 as an inhibitor of early trophoblast differentiation (Caniggia, 2000a; Caniggia, 2000b; Caniggia, 1999). HIF-1α and TGFβ3 are over-expressed in placental tissue from preeclamptic women (Caniggia 2000a).

Therefore, the expression of TGFβ3 and HIF-1α seems to be a good indicator of the health of the placenta (Figure 2).

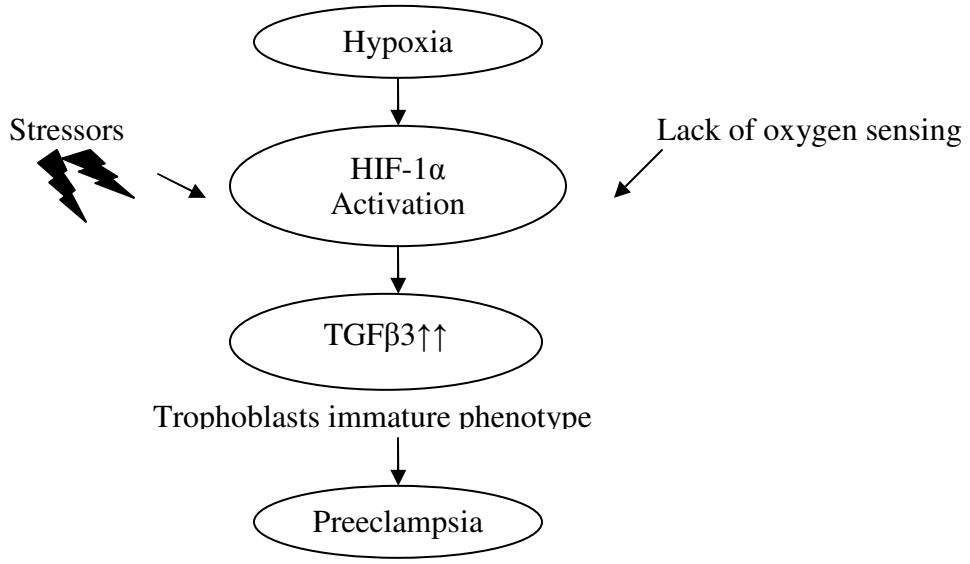


Figure 2 Putative model of HIF-1α activation in abnormal placental development. In pre-eclampsia the abnormal activation of HIF-1 α may be due to (1) a failure in increasing oxygen tension which normally occurs at 10–12 weeks' gestation, (2) a lack of oxygen sensing by trophoblast cells, and (3–4) a release of inflammatory cytokines and/or ROS following oxidative stress. (Modified from Caniggia, 2002)

Glial Cells Missing (GCM) gene is the primary regulator of glial cell determination in the nervous system of *Drosophilia* (Chen, 2004). There are two isoforms of GCM genes, GCM1 and GCM2. GCM1 is selectively expressed in the placentas of human and mouse, and plays an important role in placental angiogenesis and vascular branching. The placenta from GCM1-deficient mice shows a complete block of vessel branching in the chorioallantoic interface and chorionic trophoblast cells which do not fuse to form the syncytiotrophoblast (Anson-Cartwright, 2000). A decrease in placental GCM1 gene expression has been found in the placentas from preeclampsia patients; recently, GCM1 has been shown to contribute to constitutively high trophoblast placental growth factor expression, supporting its role for trophoblast differentiation and placental vascular function (Chen, 2004; Chang, 2008).

1.2. Role of Angiogenic Factors in the Pathogenesis of Preeclampsia

During normal pregnancy, the placenta undergoes high levels of vasculogenesis and angiogenesis. Angiogenesis is the process of neovascularization from preexisting blood vessels, whereas vasculogenesis is the process of blood vessel generation from de novo vessel formation. Placental perfusion is dependent on angiogenesis, a major mechanism responsible for the increase in placental blood flow throughout gestation. Numerous factors have been implicated in this process. Based on in vivo and in vitro studies, it has been shown that the VEGFs family is the main regulator of the angiogenesis process. The same factors are involved in normal and pathological processes including tumor growth and wound healing (Ferrara, 1997, Fraser, 2000). VEGF/VEGFR mRNA and their protein are abundantly present in the placenta and are essential for normal placental vascular development (Maglione, 1991, Cooper, 1995, Charnock-Jones, 1994; Bdolah, 2004). Moreover, gene knockout studies provide convincing evidence for a key role of VEGF in fetal and placental angiogenesis. Mice embryonic stem cells lacking the VEGFR1 (Flt-1) show an increased endothelial cell mitotic index, indicating that aberrant endothelial cell division occurs in vivo due to lack of VEGFR1 (Flt-1) (Kearney, 2002). Homozygous knockout mice lacking the VEGFR2 gene have altered fetal and placental angiogenesis, characterized by the failure of blood-island formation, organization and patterning (Shalaby, 1995). Similarly, heterozygous gene knockouts for VEGF have an abnormal development of the embryo and placental vasculature (Carmeliet, 1996). The placenta also expresses anti-angiogenic factors such as soluble VEGFR1 (sFlt-1) and neurokinin B (Page, 2000). Soluble Flt-1, the circulating form of the receptor, binds to VEGF and PlGF, preventing them from binding to their

endothelial surface receptor, and leading to the inhibition of their angiogenic action, and an imbalance between pro-angiogenic and anti-angiogenic factors in the placenta.

1.2.1 SOLUBLE FLT-1 AND ITS ROLE IN PREECLAMPSIA

The soluble Flt-1 is generated by differential mRNA splicing of VEGF receptor 1 (VEGFR-1). However, sFlt-1 lacks the cytosolic domain compared with VEGFR-1. sFlt1 acts as a potent antagonist of VEGF and PlGF in two ways: firstly, by binding directly to VEGF and PlGF thus decreasing their available free levels for their angiogenic action; secondly, sFlt-1 hetero-dimerizes with the extra-cellular ligand binding region of the membrane spanning VEGF receptors, thereby blocking the phosphorylation and activation of the downstream signal transduction pathways for endothelial cell proliferation (Kendall, 1993; Kendall, 1996).

Studies show that sFlt-1 is an anti-angiogenic molecule, and thus seems to be centrally involved in the pathogenesis of preeclampsia. High levels of circulating sFlt-1 detected in early pregnancy are associated with later onset of preeclampsia (Maynard, 2003; Thadhani, 2004; McKeeman, 2004). Moreover, a fall in sFlt-1 levels following delivery of the placenta correlates with improvement in clinical syndromes (Maynard, 2003). However, the etiology of increased sFlt-1 level in preeclamptic women is still not known. The source of sFlt-1 is considered to mainly come from placenta as well as other sources such as endothelial cells and activated mononuclear cells (Rajakumar, 2005). In vitro studies demonstrated that sFlt-1 inhibits tube formation of endothelial cells from human umbilical vein and trophoblast migration and differentiation (Maynard, 2003; Ahmad, 2004; Zhou, 2002). In primary cytotrophoblast cell culture, sFlt-1 production and mRNA expression is inversely related to oxygen saturation (Nagamatsu, 2004). A two-fold elevation in the level of sFlt-1 was also observed when normal villous explants were exposed to a hypoxic state (1% O_2) compared to the physiological 5% O_2 exposure

(Ahmad, 2004). Hence, sFlt-1 is up-regulated in response to hypoxia in vitro. An in vivo study (Makris, 2007) had shown that increased circulating level of sFlt-1 correlates with the onset of clinical syndromes and the severity of disease in an artificial model of preeclampsia induced by decreasing the blood flow to the utero-placental units, this has also been confirmed by other investigators in clinical studies (Wathen, 2006; McKeeman, 2004; Hertig, 2004). Thus, sFlt-1 may act as a mediator in inducing maternal endothelial dysfunction, hypertension and proteinuria in preeclampsia, and therefore operate as a key part of the mechanism linking the placenta with maternal endothelial dysfunction. Although the above studies indicate that excess sFlt-1 production noted in preeclampsia may be secondary to placental hypoxia, it is still unclear whether sFlt-1 production or placental hypoxia is the trigger event in the pathogenesis of preeclampsia. Genetic factors as well as inflammatory factors could lead to increased sFlt-1 level and decreased free VEGF and PlGF levels which contribute to abnormal placentation, resulting in placental hypoxia (Figure 3). Recently, an increase in other angiogenic molecules have also been observed in preeclampsia; endostatin, an inhibitor of angiogenesis (Hirtenlehner, 2003), and soluble endoglin (sEng), a truncated form of endoglin which is a cell surface receptor for TGF-β (Venkatesha, 2006), are elevated in preeclampsia in a pattern similar to that of sFlt-1. The precise roles of these two molecules and their relationships with sFlt-1 have not been discovered yet.

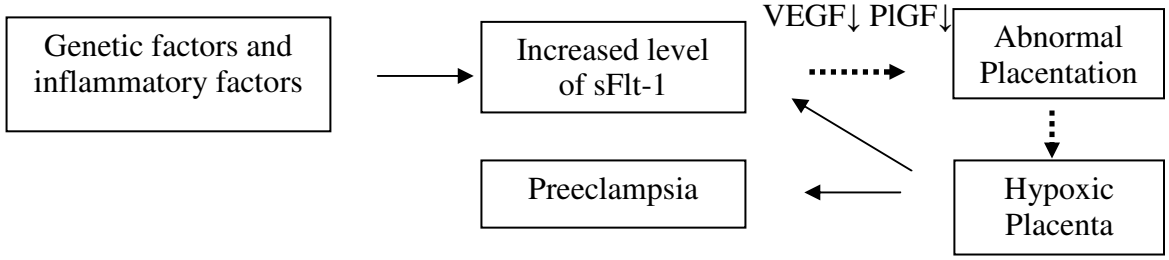


Figure 3 Hypothesis of the role of sFlt-1 and placental hypoxia in the pathogenesis of preeclampsia (modified from Karumanchi, 2004). ⋯→ Hypothesis ⟶ Known

1.3. Regulation of Vascular Function and Blood Pressure

1.3.1 THE ROLE OF ENDOTHELIUM

The endothelium, a single cell line covering the inner wall of all blood vessels in the body, has long been known to act as a "plasma-tissue barrier", preventing the egress of large plasma proteins while transferring of nutrients and wastes between blood and tissues. It was also recognized to be an important regulator of vascular function until late 20^{th} century. In 1980, Furchgott and Zawadski first described endothelium-derived relaxing factor produced by endothelium which was later identified as nitric oxide (Furchgott, 1980). Since then, large amount of molecules synthesized by endothelium have been reported and found to play a major role in the control of blood clotting, pro-thrombotic and anti-thrombotic as well as fibrinolytic activity, and in the cardiovascular disease states and inflammatory condition by binding to platelets, monocytes and leukocytes (Hurairah H, 2004). Moreover, endothelium expresses a number of vasodilators and vasoconstrictors to regulate vascular tone. An increase in free cytoplasmic Ca^{2+} levels, which is achieved by various mechanisms including influx of extracellular Ca^{2+}, Na^{+}-Ca^{2+} exchange and liberation of Ca^{2+} from intracellular stores (e.g. sarcoplasmic reticulum) (Singer and Peach, 1982; Winquist et al., 1985, Luckhoff and Busse, 1986), triggers the release of endothelial relaxing factors in the endothelial cells (Furchgott, 1983). A number of factors such as nitric oxide (NO) (Palmer et al., 1987), prostacyclin (Moncada and Vane, 1979), and endothelium derived hyperpolarizing factor (EDHF) (Rubanyi and Vanhoutte, 1987) have been involved in the endothelium-dependent relaxation of the vasculature; while vasoconstrictors, namely endothelin-1, thromboxane A_2 and angiotensin II, have been identifies as being involving in the endothelium-dependent vasoconstriction (Hurairah H, 2004).

The regulation of vascular tone by endothelium during pregnancy is extremely important. The endothelium appears to be up-regulated and produces more vasodilatation by either an increased release of vasodilators or a decreased vasoconstrictor output (Poston, 1995). There is a critical balance between endothelium-derived vasoconstrictors and vasodilators. In preeclampsia, this balance is disrupted and the vasculature is predisposed to vasoconstriction (VanWijk, 2000).

1.3.2 NITRIC OXIDE (NO)

Endothelium-derived relaxing factor first described by Furchgott and Zawadski (1980) (Furchgott, 1980) and later identified as NO (Palmer, 1987; Ignarro, 1987), is a very small molecule with only a few seconds of half life. It can diffuse rapidly across cell membranes and has a high affinity for the heme iron or S-nitrosothiols to form the bioactive pool, which could be the precursors or effectors in the nitric oxide pathway (Stamler 1992).

NO synthase (NOS) is the enzyme responsible for the production of NO from its substrate L-arginine (Bredt & Snyder, 1990). Three isoforms of NOS: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) have been described (Forstermann, 1995; Wu, 1995). nNOS is the constitutive isoform mainly expressed in neural tissues, while iNOS becomes active in the presence of inflammation. In the vasculature, eNOS is the main isoform responsible for the production of NO (Wu, 1995).

After synthesis, nitric oxide diffuses from its original production site to the target cells where it binds and interacts with specific molecules containing iron in either a heme or iron-sulfur complex (Sato and Murota 1995). NO acts via activation of the soluble guanylate cyclase in the vascular smooth muscle (VSM), increasing cyclic-GMP levels, which is thought to be the main mediator of the cellular effects produced by nitric oxide

(Bina, 1995; Kannan and Johnson, 1995; Moro, 1996; Peng, 1996; Quignard, 1997). Cyclic GMP then binds to one of three possible groups of receptor proteins: cGMP-regulated ion channels, cGMP-binding phosphodiesterases or cGMP-dependent protein kinases; and triggers the relaxation of the VSM via activating an intracellular molecular cascade which results in a reduction of intracellular Ca^{2+} concentration and an reduction of the sensitivity of the contractile system to the Ca^{2+} (Holzmann, 1982; Carvajal, 2000). Many agents produce the effect of vascular smooth muscle relaxation via NO-cGMP pathway such as acetylcholine (Pussard, 1995; Iranami, 1996), estrogens (White, 1995; Rosenfeld, 1996) and insulin (Trovati 1995 and 1996). NO is also an inhibitor of smooth muscle proliferation and plays an important role in vascular remodeling in response to injury (Rudic, 1998; Ledingham, 2001). The state of vasodilatation during pregnancy is partly mediated by nitric oxide which is important for the maintenance of adequate utero-placental perfusion (Langille, 1986; Yallampalli, 1993). Findings in pregnant guinea pigs confirm that NO is responsible for the physiological dilation of the uteroplacental arteries (Nanaev, 1995). A decrease of nitric oxide could contribute to the pathogenesis of hypertension in preeclampsia, atherosclerosis and intrauterine growth restriction (Diket, 1994; Molnar, 1994; Sankaralingam, 2006). There are several mechanisms of reducing NO bioavailability, increased oxygen free radicals can scavenge nitric oxide or increased plasma levels of S-nitrosoalbumin in preeclampsia can result in a decrease in the release of NO. The decreased release of nitric oxide could also be partly due to decreased vitamin C levels (Tyurin, 2001; Gandley, 2005).

1.3.3 PROSTACYCLIN AND OTHER EICOSANOIDS IN PREGNANCY

Prostacyclin (PGI_2) and prostaglandin (PG) E_2 are potent vasodilators which may contribute to the reduced peripheral vascular resistance in pregnancy. Prostaglandins are

generated from arachidonic acid by cyclooxygenases. Their biosynthesis is stimulated by numerous agonists that make free arachidonic acid available as a substrate for cyclooxygenase by increasing the activity of phospholipase enzymes. PGI_2 is mainly biosynthesized in the endothelium of large blood vessels (Moncada and Vane, 1979) and in human glomeruli (Sraer, 1982), and may be increased locally in the uterine and renal circulations or in placenta during pregnancy possibly in response to increased circulating levels of angiotensin II (Magness, 1995; Glance, 1985). PGI_2 exerts its vasodilatory effects via the stimulation a G-protein-coupled, cell-surface receptor termed IP, which in turn activates the intracellular enzyme adenylyl cyclase, leading to an increase in intracellular cyclic AMP levels, cAMP then activates protein kinase A which decreases myosin light-chain kinase (MLCK) activity, hence inhibiting contraction and causing vasodilatation (Nakagawa, 1994; Hebert, 1998). In addition, a nuclear receptor, the peroxisomal proliferator-activated receptor α (PPARα) is also activated by PGI_2, and together with IP receptor activation may mediate the anti-platelet and vasodilatation effects of PGI_2 in the vasculatures (Marx, 2003; Vu-Dac, 1995).

Thromboxane A_2 (TxA_2), a potent vasoconstrictor and platelet aggregator, is produced by platelets and has a half-life of approximately 30 sec under physiological conditions. It increases three to five folds during pregnancy and remains elevated throughout (Fitzgerald, 1987a), which is consistent with evidence of the enhanced platelet activation associate with pregnancy (McKay, 1981). It diffuses into vascular smooth muscles where TxA_2 can stimulate endoperoxide/thromboxane receptors to induce an increase in cytosolic Ca^{++} and hence vasoconstriction (Mayeux, 1989; Morinelli, 1989; Smith, 1994).

One probable cause of preeclampsia is thought to be caused by underproduction of PGI_2 from the endothelium coupled with overproduction of TxA_2. Reduced PGI_2

production in preeclampsia is possibly due to chronic damage to the maternal vascular endothelium (Roberts, 1989; Roberts and Redman, 1993), which is reflected indirectly by increased concentrations of serum fibronectin and factor VIII antigen/coagulant ratio. Reduced PGI_2 itself could contribute to over-production of TxA_2 because of insufficient PGI_2 anti-aggregatory activity (Fitzgerald, 1987b; Walsh, 1990). Increased TxA_2 production is reflected in increased excretion of its major urinary metabolites; also, it has been shown that placenta from preeclampsia biosynthesize more TxA_2 compared with healthy placenta (Fitzgerald, 1990; Walsh, 1985).

1.3.4 CIRCULATING VASOACTIVE SUBSTANCES

Vascular tone is regulated by several circulating vasodilatory factors which includes kinins and atrial natriuretic peptide (ANP). Kinins consisting of bradykinin and kalidin trigger the release of the endothelium-derived relaxing factors and hence cause vasorelaxation. Interestingly, bradykinin receptors are up-regulated in preeclampsia, which may heterodimerize with angiotensin II type I receptors and increase responsiveness to angiotensin II in vitro (AbdAlla, 2001). ANP is a vasodilatory hormone produced mainly in the atrial myocytes in response to wall stretch and in the placenta. Atrial natriuretic peptide acts on the particulate guanylate cyclase, increasing cGMP and hence causing relaxation of the smooth muscle (Stjernquist et al., 1995). Plasma ANP has been found to be elevated in preeclampsia (Fievet, 1988)). ANP induces vasodilatation in the uteroplacental vasculature of women with preeclampsia and small reduction in blood pressure (Grunewald, 1994), opposing the vasoconstrictive action of endothelin (Kublickiene, 1995) and angiotensin (McQueen, 1990). In addition, there are several circulating vasoconstrictors such as vasopressin and angiotensin II. Vasopressin is released by the posterior pituitary and causes Ca^{2+} entry through voltage-gated Ca^{2+}-

channels, hence increasing intracellular $Ca2^+$ levels as well as IP_3 production (Noguera et al., 1997). Active angiotensin II is generated by angiotensin-converting enzyme (ACE) from angiotensin I, The effects of angiotensin II, including vasoconstriction, are mainly mediated by AT1 receptors, which has a more wide-spread distribution than more recently discovered AT2 receptor (Hurairah, 2004; Wang, 1999).

1.3.5 NEURAL REGULATION OF VASCULAR TONE

The sympathetic nervous system is present in all vascular beds and can alters vascular tone by releasing catecholamines which can induce vascular smooth muscle contraction. In preeclampsia, it has been found that sympathetic nerve activity of fibers innervating blood vessels in skeletal muscle was increased (Schobel, 1996); however, the underlying mechanisms of this increased sympathetic activity are still unclear.

Some vascular beds, including the uterine bed, have cholinergic nerves (parasympathetic vasodilator innervation). In addition, peptidergic nerves which can release vasodilator (calcitonin gene-related peptide, vasoactive inhibitory peptide, substance P and NO) and vasoconstrictors peptides (neuropeptide Y) (Mentlein and Roos, 1996; Lundgaard et al., 1997; Ignacio et al., 1997) have also been found.

1.4. Intrauterine fetal vascular programming

The origins of several adult diseases such as hypertension, cardiovascular disease and diabetes can be tracked back to the fetal development. Fetal programming is defined as a process in which the fetus adapts in order to survive stimuli or insults occurring during a critical period of fetal development. Although genetic factors, including the maternal and paternal genes as well as the phenomenon of genomic imprinting that influence the fetal phenotype and contribute to the occurrence of fetal origin of adult disease via the intrauterine environment, these stimuli or insults are usually maternal

factors which affect the fetus via the altered the intrauterine environment. Therefore our project specifically focuses on the situation of abnormality of placenta such as in preeclampsia. In support of this hypothesis, low birth weight has been found to be associated with an increased risk of developing coronary heart disease, hypertension, stroke and diabetes in adulthood (Barker, 1992; Fall, 1995; Vijayakumar, 1995; Rich-Edwards, 1997). Maternal hypertension, either chronic or acute, leads to inadequate vascular adaptations during pregnancy, alteration in the circulation at the utero-placental interface and consequently poor perfusion of the placenta-fetal unit (Rotmensch, 1994; Henriksen, 2002). In addition, the placenta, as a low resistance organ, impacts fetal cardiovascular development due to its role in determining fetal cardiac load which in turn has a major effect on cardiac and vascular development (Thornburg, 2000 & 2001). These mal-adaptations lead to fetal hypoxemia and reduction in fetal perfusion, which can convey long lasting physiological and structural alterations that predispose the fetus to diseases in later life (Henriksen, 2002).

1.4.1 FETAL PROGRAMMING AND ANIMAL MODELS

In the past few years, significant research efforts have been directed towards unraveling the underlying mechanism of the fetal origin of adult disease. Since it is somewhat challenging to conduct this type of experimental research in humans, laboratories have turned to animal models. Species studied include rat, mouse, guinea pig, sheep and non-human primates (Bertram, 2002). Experimental methods employed to produce an unfavorable uterine environment and induce fetal growth restriction have included dietary, pharmacological, and surgical manipulations (Benediktsson, 1993; Block, 1989 and 1990; Murotsuki, 1997; Langley-Evans, 1999). Protein restriction in rats during pregnancy, such as low protein diet supplemented with methionine, has been found to result in hypertension in the offspring later in life (Petrie, 2002). Reduction of

the uterine perfusion by placing a silver clip around the aorta below the renal arteries during mid-late gestation in pregnant rats caused low-birth weight offspring predisposed to development of hypertension (Alexander, 2003). Another example is the transgenic animal model used by Longo et al (Longo, 2005) in which female eNOS knockout mice ($NOS3^{-/-KO}$) were bred with wild-type mice ($NOS3^{+/+WT}$) to produce maternally-derived heterozygous ($NOS3^{+/-mat}$) and paternally-derived heterozygous ($NOS3^{+/-pat}$) litters that are genomically-similar. Impaired vascular function has been seen in the $NOS3^{+/-mat}$ offspring that developed in a NOS3 deficient maternal/uterine environment compared with the normal vascular function seen in $NOS3^{+/-pat}$ offspring born to wild-type mothers. Embryo transfer experiments confirmed the predominance of the uterine environment over the genetic background in determining fetal programming (Longo, 2004). This study provides direct evidence in support of the role of uterine environment in determining an increase risk of onset of cardiovascular in later life.

1.5 Research project objectives

Based on the published evidence, we speculate that the soluble fms-like tyrosine kinase1 (sFlt-1) induces an imbalance between the active circulating pro-angiogenic factors (VEGF and PlGF) and anti-angiogenic factors (sFlt-1), consequently leading to an abnormal placentation, placental insufficiency, and ultimately preeclampsia. The abnormal maternal vascular adaptation to pregnancy, and the utero-placental insufficiency, may also alter fetal vascular programming resulting in long lasting effects on the vascular function in adult life.

Our overall hypothesis is that abnormal utero-placental adaptation caused by altered angiogenesis leads to preeclampsia and altered fetal vascular programming. As a step toward the overall goal, we propose to test the hypothesis that

sFlt-1, an inhibitor of angiogenic factors, induces a condition similar to preeclampsia in pregnant mice by inhibition of VEGF and PlGF action, and leads to altered fetal vascular programming. This general hypothesis was tested in a mouse model by examining the following specific hypotheses:

Specific Hypothesis 1: Over-expression of sFlt-1 in pregnant mice results in hypertension and other manifestations that mimic human preeclampsia.

Specific Hypothesis 2: High level of sFlt-1 during pregnancy leads to altered central and peripheral vascular reactivity.

Specific Hypothesis 3: sFlt-1 leads to changes in hypoxia-induced genes in the placenta and kidney by affecting the circulating angiogenic factors levels of VEGF and PlGF.

Specific Hypothesis 4: Elevated levels of sFlt-1 results in an adverse intrauterine environment which leads to an altered fetal vascular programming manifesting as hypertension in the offspring later in life.

The following aims are proposed to test the corresponding hypotheses:

Specific Aim 1: To determine the effect of over-expression of sFlt-1 in pregnant mice on blood pressure and other systems affected in preeclampsia using a mouse model transfected with adenovirus carrying sFlt-1.

Specific Aim 2: To evaluate vascular function of the carotid and uterine arteries in pregnant mice transfected with adenovirus carrying sFlt-1 by in vitro vascular reactivity.

Specific Aim 3: To compare mRNA expression level of the hypoxic induced factors in pregnant mice over-expressing sFlt-1 versus controls.

Specific Aim 4: To evaluate fetal vascular programming by determining blood pressure in adult offspring born to pregnant mice over-expressing sFlt-1.

1.6 Significance

This investigation provides a new insight into the role of sFlt-1 in the pathogenesis of preeclampsia, and a novel approach to studying fetal vascular programming, particularly as it applies to preeclampsia. This animal model helps to elucidate the mechanism induced by sFlt-1 leading to preeclampsia and its effect on the uterine environment and fetal vascular development. Given that preeclampsia complicates 10% of pregnancies its potential impact on health and disease in adult offspring can be tremendous. The experiments shed light on fetal and placental growth and development. The findings from this study can improve our understanding and prevention of adverse pregnancy outcomes, and their consequences on fetal growth and development.

CHAPTER 2: MATERIALS AND METHODS

Animal Care

Pregnant CD-1 mice at day 6 of gestation and non-pregnant CD-1 mice were purchased from Charles River (Wilmington, MA). The mice were maintained in the animal care facility at the University of Texas Medical Branch. All procedures were approved by the Animal Care and Use Committee (ACUC) of the University of Texas Medical Branch. The mice were housed separately in temperature and humidity controlled quarters with constant light: dark cycles of 12h: 12h. They were provided with food and water ad libitum. The same chow was used for all animals, including the offspring. Regular maintenance and care were provided by certified personnel and veterinary staff according to the guidelines of the ACUC. Surgical procedures were performed according to the ACUC guidelines under anesthesia with ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (Gemini, Rugby, Rockville Center, NY). The animals were sacrificed by CO_2 inhalation per the ACUC and the American Veterinary Medical Association guidelines.

Amplification of virus vectors

The adenovirus vectors' stock, Ad-Flt-1 (1-3) (first generation, E1 and E3 deleted) as the active vector, and Ad-mFc as the adenovirus control were prepared and titered by the Research Vector Core, Harvard Medical School. We used the 293 cell line to grow and transfect the virus. The cells were cultured in 150 mm plates and seeded for 3 days, when the cells reached 70%-80% confluence they were considered ready for transfection. The transfection medium containing the virus (2×10^7 PFU; transfection medium: 1% P/S and 2% FBS) was added to each plate. About 20 hrs later, around 50%

of the cells have started to detach from the plates and showed the cytopathic effect (CPE). At this point, the cells were collected and spun at 2,000 rpm for 15 minutes at 4^{o}C, the supernatant was removed and the cells were re-suspended in 10 ml cold PBS again and centrifuged at 2,000rpm/4^{o}C/15min. The supernatant was removed and the cells were stored at -80^{o}C for purification. For the purification process, 2mL of sterile 10mM Tris (pH=8) was added to the cell pellet which was then placed in a water bath to thaw, then vortexed briefly, and placed on the dry slurry to be frozen. The cycle was repeated 6 times. The cells were lysed after spinning them at 2,000rpm/15min/4^{o}C and the supernatant was collected. The supernatant containing virus was added into a CsCl step gradient (d=1.43 and d=1.34). This preparation was centrifuged at 30,000 rpm for 3 hours at 4^{o}C in a SW40 rotor. The adenovirus band was apparent at this point and easily collected using an 18 gauge needle and 5mL syringe, and added to a tube containing 1.34g/ml CsCl. The sample was again centrifuged at 35,000 rpm for 16 hours at 4^{o}C in a VTi90 rotor. The adenovirus band, which was now more evident, was collected in a clean dialysis tube containing the dialysis buffer to isolate the virus. The concentration of adenovirus particles was determined by spectrophotometric analysis of an appropriate dilution of the test sample (typically 1/10) in a solution of 10mM Tris/1mM EDTA/0.1% SDS.

In vivo blood pressure measurement

We utilized a telemetric method for an in vivo blood pressure recording that requires the insertion of a 0.4 mm catheter into the carotid artery and tunneled into aortic arch. In this system (PA-C20, Data Sciences International, St. Paul, MN), the catheter was connected to a combination pressure transducer, transmitter and battery, all encased in a miniature capsule. The capsule transmits to a pad under the cage and can be turned

on and off magnetically. This system allows monitoring of arterial blood pressure (BP) in unrestrained conscious mice.

The protocol for blood pressure measurement was the following: The mice were anesthetized with a mixture of ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (Gemini, Rugby, Rockville Center, NY). A vertical midline skin incision was made along the neck and the left common carotid artery carefully isolated. The catheter was inserted into the carotid artery through a small incision in the vessel wall and tunneled to the aortic arch (Figure 4), and the body of the transducer (PA-C20 model, Data Sciences, St. Paul, MN) was secured in a subcutaneous pouch along the animal's right flank through the same ventral neck incision. The incision was closed using 6–0 silk. Mice were kept warm on a heating pad and monitored closely until full recovery from anesthesia.

BP data was transmitted to RLA 1020 telemetry receivers (Data Sciences), multiplexed by BCM consolidation matrix (Data Sciences), and sent to an adapter, where the signal was de-multiplexed, sampled at 1024 Hz, and converted to analog output (UA-10 universal adapter, Data Sciences). This output was subsequently band-pass filtered and amplified and the information fed to a recording system (MacLab 16/s, AD Instruments, Castle Hill, Australia).

Data Analysis

Dataquest A.R.T.3.1 software was used for data acquisition. Collected systolic and diastolic BP, mean arterial pressure (MAP) data was plotted as mean values ± SEM over each 12 hours. Data was analyzed by One-way and/or Two-way ANOVA, as well as Student-Newman-Keuls post-hoc test as appropriate. A p value less than 0.05 was considered significant.

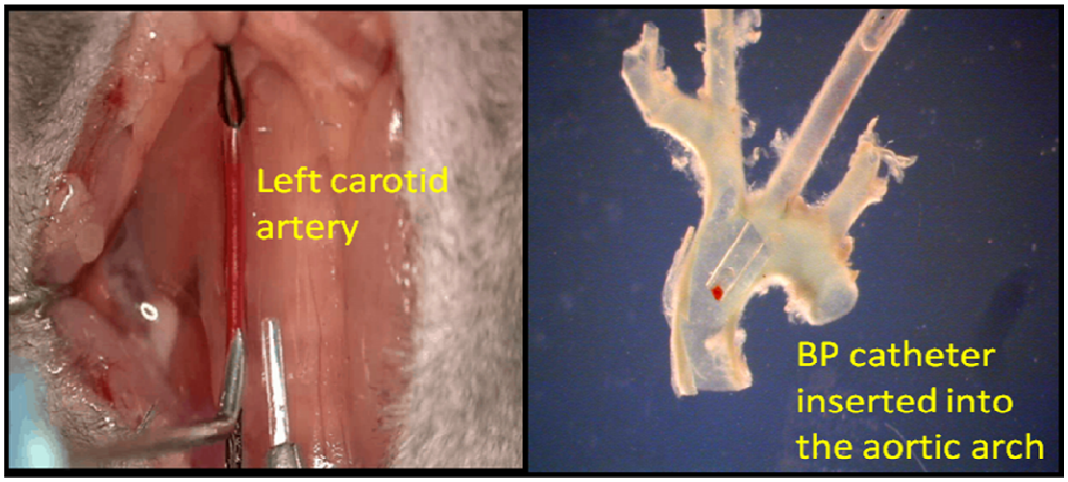


Figure 4 *Left:* in-vivo photograph demonstrating the exposure of the left common carotid artery which has been ligated proximally and distally to the incision site. *Right:* ex-vivo aortic arch and main arteries showing the catheter insertion through the left carotid artery and placement of the catheter tip within the lumen of the aortic arch. (Pictures courtesy of Esther H. Tamayo, University of Texas Medical Branch, Galveston, Texas)

ELISA assay for plasma sFlt-1 and VEGF level measurement

This assay (R&D systems, Minneapolis, MN) employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse VEGF or mouse VEGFR has been pre-coated onto a microplate. Standards, control and samples are pipetted onto wells and any mouse VEGF or soluble VEGFR1 (sFlt-1) present is bound by the immobilized antibody. After washing away any unbound substances, the enzyme-linked polyclonal antibody specific for mouse VEGF or mouse VEGFR is added to wells. Following a wash to remove any unbounded antibody-enzyme reagent, a substrate solution is added to the wells and the enzyme reaction yields a blue product that turns yellow when stop solution is added. The intensity of the color measured is in proportion to the amount of mouse VEGF or sFlt-1 bound in the initial step. In detail, the plasma was obtained from the CD-1 mice every other gestational day. The samples were diluted

6-fold by Calibrator Diluent and incubated in a 96-well plate pre-coated with a capture antibody directed against sFlt-1 or VEGF for 2 hours at room temperature. The wells were washed and incubated with a secondary antibody against sFlt-1 or VEGF conjugated to horseradish peroxidase for an additional 2 hours. The plates were then washed and the substrate solution was added to each well and incubated for 30 minutes at room temperature. The plates were read at the optical density of 450nm. All assays were run in duplicate.

Data Analysis

Data were calculated using a standard curve derived from known concentrations of the recombinant protein and showed as Mean ± SEM. Data were analyzed by Student *t*-test or One-way ANOVA, as well as Student-Newman-Keuls post-hoc test as appropriate. A p value less than 0.05 was considered significant.

In Vitro Vascular Studies

Pregnant CD1 mice treated with adenovirus carrying sFlt-1 or mFc were used for these studies. Each experimental group consisted of ≥6 mice. The carotid and uterine arteries were separated from the connective tissue, taking care not to damage the endothelium or the intima. The carotid arteries were taken from the segment just proximal to their bifurcation into internal and external carotid artery. The carotid arteries and uterine arteries were dissected immediately and immersed in physiological salt solution (see below). Two-millimeter segments of the vessels were mounted between the two jaws of a wire myograph (Model 410A, J.P. Trading I/S, Aarhus, Denmark) using 25 μm tungsten wires. One jaw in these myographs is attached to a micromanipulator for adjustment of tension in the vessel while the other jaw is fixed to an isometric force transducer (Figure 5). The preparations were bathed in physiological salt solution

maintained at 37°C, pH ~7.4. A mixture of 95% O_2 and 5% CO_2 was bubbled continuously through the solution. The force was continuously recorded by an isometric force transducer and analyzed using PowerLab system and Chart 5 data acquisition and playback software (AD Instruments, Castle Hill, Australia).

Drugs and Solutions

The drugs used in the in-vitro experiments were acetylcholine hydrochloride (ACh), phenylephrine hydrochloride (PE), and sodium nitroprusside (SNP) and N (G)-nitro-L- arginine methyl ester (L-NAME) from Sigma (St. Louis, MO) and thromboxane A_2 (TxA_2)(U-46619, Cayman chemical, Ann Arbor, MI). Stock solutions of the drugs (10^{-2} mol/L) were prepared in deionized water and stored at -20°C. The composition of physiological salt solution was as follows: NaCl 115 mmol/L, KCl 5 mmol/L, NaH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, $MgCl_2$ 1.2 mmol/L, $CaCl_2$ 2.5 mmol/L, ethylene diamine tetra acetic acid (EDTA) 0.026 mmol/L, glucose 11 mmol/L.

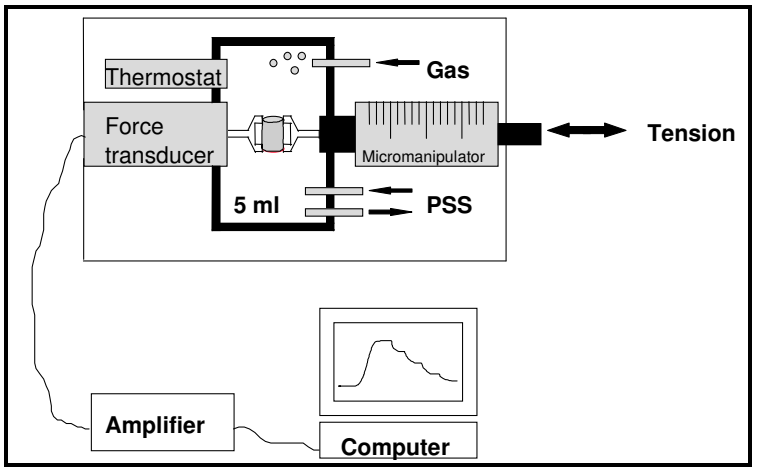


Figure 5 Methods: *in vitro* contractility of carotid/uterine artery. Vascular segments of the arteries were mounted in a small vessel myograph in 5 ml chambers filled with PSS and bubbled with gas mixture (95% O_2 and 5% CO_2). The chambers were maintained at 37°C by a thermostat. The preparations were mounted between two jaws of the myograph with 25 µm tungsten wires. One jaw was fixed with a micromanipulator which allowed change in the distance between the wires and hence the tension in the vessel while the other was

connected to the force transducer. The signal was amplified and recorded with a data acquisition system.

Vascular Reactivity Protocol

The vessels were allowed to stabilize for one hour. After stabilization of the tone, and in order to enhance reproducibility of responses, the vessels were given two successive stimulations of 10 min duration with 60 mM KCl separated by 30 min equilibration. The second response to KCl was used as the reference contraction in the final calculations. The presence or absence of endothelium in the preparations was confirmed by contracting with α_1-adrenergic agonist phenylephrine PE (10^{-6} M) and testing for relaxation with endothelium-dependent vasodilator ACh (10^{-6} M).

After equilibration in physiological salt solution, contractile responses to PE (10^{-10}-10^{-5} mol/L) and TxA_2 were assessed. In addition, vessels were pre-contracted with PE (10^{-7}-10^{-6} M) to produce matching contractions in the different study groups and relaxant responses to cumulative concentrations of acetylcholine (ACh) and sodium nitroprusside (SNP) were assessed.

Data Analysis

Data were expressed as the mean ± SEM. Response to KCl was used as a reference to calculate the percent of contraction achieved by PE. Responses to ACh and SNP were expressed as percentage relaxation of the PE-induced precontraction. The area under the concentration response curves (AUC), logarithm of the concentration producing 50% of the maximal effect (log IC_{50}, a measure of sensitivity to the agent), and the maximal effect (Emax) were calculated. One-way ANOVA, as well as Student-Newman-Keuls post-hoc test was used as statistical analysis. A p value less than 0.05 was considered significant.

Table 1 Compounds used to investigate selected pathways involved in the regulation of vascular function

Agents used in dose-response curves			
Compound	*Type*	*Action*	*Dose*
PE	α_1-adrenergic agonist	smooth muscle contraction	10^{-10}-10^{-5} M
ACh	Muscarinic-receptor agonist	endothelium-dependent-vasorelaxation	10^{-10}-10^{-5} M
TxA_2	anti-platelet aggregation	smooth muscle contraction	10^{-10}-10^{-5} M
SNP	smooth muscle relaxant	Endothelium-independent vasorelaxation	10^{-10}-10^{-5} M
L-NAME	NO antagonist	Nonselective NOS inhibitor	10^{-4} M

Histological Examination

At the time the mice used for the in vitro vascular studies were sacrificed, the kidney and liver were collected, and fixed in 10% formalin. After the embedding process in paraffin, 8- to 10-µm sections were cut and stained with hematoxylin and eosin (H & E). H & E stains the nuclei dark blue and stains everything else various shades of pink. Inflammatory loci and glomeruli with sclerosis per high power field (X40) were determined in the liver and kidney.

Gene Expression Studies

SAMPLE PREPARATION AND RNA ISOLATION

Gene expression in the placenta and kidney from the various groups was analyzed using real time RT-PCR analysis. The placentas and kidneys were rapidly dissected and flash frozen in liquid nitrogen. The samples were labeled and stored at –80°C until ready

for extraction of mRNA. Subsequently, frozen tissues were ground with liquid nitrogen in a pre-chilled mortar and pestle. Using a pre-chilled metal spatula, the powdered tissue were scraped into the lysis/binding solution (Ambion Co., Austin, TX) with a ratio of 200 to 700 µl solution to 100-10^7 cells and total RNA was isolated using RNAqueous- small scale phenol-free total RNA isolation kit (Ambion Co.) and 10µg of RNA for target genes and endogenous control were used. RNA concentrations in the samples were measured by ultraviolet absorbance at 260 nm, using a spectrophotometer (DU-64, Beckman Instruments, Palo Alto, CA). The samples were considered non-degraded when the ratio falls in the range of 1.8-2.0 at 260nm and 280nm.

TWO-STEP REAL-TIME RT-PCR METHODOLOGY

This technique involves the use of specifically-designed primers (forward and reverse) and a probe that binds the gene of interest. During the reverse transcription (RT) step, the reverse primer synthesizes the cDNA. Then in the PCR cycles, the forward primer and the probe anneal to the cDNA. During primer extension, the Taq DNA polymerase, that has a fork-like structure-dependent 5' nuclease activity, cuts the TaqMan probe. The probe contains a reporter dye (FAM for the gene-specific probes, and VIC for the 18s rRNA probe) at its 5'-end and a quencher dye (MGB) at its 3'-end. When the two dyes are close to each other, the quencher dye quenches the signal of the reporter dye. However, the DNA polymerase action and the resulting strand cutting separate the reporter dye from the quencher dye resulting in the release of fluorescence from the reporter. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

In the reaction tube, there is another dye (ROX, passive reference dye) giving a signal. This signal provides an internal reference to which the reporter dye signal can be normalized since the passive reference dye does not participate in the reaction.

During the TaqMan Two-Step RT PCR, the signal detected from the reporter dye (FAM and VIC) is normalized to the signal from ROX, the calculated ratio is defined as Rn (i.e. the normalized reporter) (Figure 6). Rn^+ is the Rn value of a reaction containing all components including the template. Rn^- is the Rn value of an un-reacted sample. This value can alternately be obtained from the early cycles prior to a detectable increase in fluorescence. ΔR is the difference between the Rn^+ value and the Rn^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions. The threshold cycle or C_t value is the fractional cycle number at which the fluorescence passes the fixed threshold.

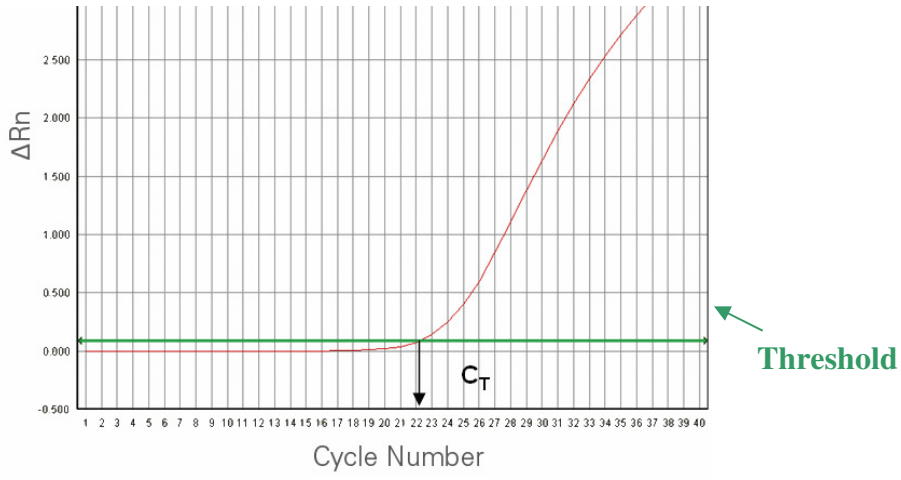


Figure 6 Representation of a Typical Amplification Curve. The X-axis shows the cycle number, while the Y-axis is ΔRn (Rn minus the baseline).

PROBES AND PRIMERS

Specific primers and probes for the genes of interest (HIF-1α, TGFβ3 and GCM1) and for the 18s rRNA (internal control) were obtained from Applied Biosystems (Foster

City, CA) (Table II). The Assays-by-Design 20× assay mix contained the specific primers as well as the TaqMan MGB probes. The probes 5'-end FAM™ dye-labeled for the target genes and 5'-end VIC™ dye-labeled for the for18S rRNA and 3'-end MGB™ dye-labeled. These assays were designed to span exon-exon junctions so as not to detect genomic DNA.

Table 2 Probe used for the Assays-on-Demand™

HIF1α	NM_010431.1	Mm00468869_m1
TGFβ3	NM_009368.2	Mm00436960_m1
GCM1	NM_008103.3	Mm00492310_m1

TWO-STEP REAL-TIME RT-PCR REACTIONS

The reactions were conducted with 1μg RNA for both target genes and endogenous control (18s rRNA). The cycling parameters for reverse transcription (Perkin-Elmer, Applied Biosystems, CA, 94404), 25 ^{o}C for 10 min, extension at 37 ^{o}C for 120 min and inhibition at 85 ^{o}C for 5 sec, keep at 4 ^{o}C after finish. The cycling parameters for real-time PCR were: stage 1 included one step for initial denaturation at 95 ^{o}C for 20 sec; stage 2 included two steps, step one for denaturation at 95 ^{o}C for 3 sec and step two annealing/extension 60 ^{o}C for 30 sec (repeated 40 times) on 7500 Fast Real Time PCR (Applied Biosystems)

Duplicate C_t values were used and analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems).

DATA ANALYSIS

The cyclic threshold (C_t) was determined for each sample; The results were expressed as mean $\pm$ SEM of HIF1α, TGFβ3 and GCM1 mRNA expression relative to a reference sample (one wild-type control sample) and normalized to 18s rRNA (internal control). The comparative C_t ($\Delta\Delta C_T$) was used for data analysis. One-way ANOVA followed by Newman-Keuls post-hoc test was used for statistical analysis (significance: $p<0.05$).

CHAPTER 3: RESULTS

Specific Hypothesis 1:

Over-expression of sFlt-1 in pregnant mice results in hypertension and other manifestations that mimic human preeclampsia.

SPECIFIC AIM 1:

To determine the effect of over-expression of sFlt-1 in pregnant mice on blood pressure and other systems affected in preeclampsia using a mouse model transfected with adenovirus carrying sFlt-1.

INTRODUCTION

Based on the recently described association between the occurrence of preeclampsia and an imbalance in angiogenic factors, we thought to focus on the inhibition of angiogenesis as a method to alter placental vascular function (Levine, 2004). Administration of adenovirus carrying sFlt-1 to pregnant rats at day 8 of gestation has been shown by others to induce a dose-dependent hypertension, proteinuria and glomerular endotheliosis on day 16 to 17 of gestation (Maynard, 2003). This study, however, was limited by small numbers, evaluation of a single time point in gestation, and measurements in anesthetized animals. We therefore decided to test the hypothesis that the elevation in sFlt-1 levels in pregnant mice leads to a condition that mimics preeclampsia, characterized by sustained hypertension in late gestation in the unrestrained conscious animal, fetal growth restriction, and histo-pathological evidence of maternal end-organ damage. We believe that this approach more closely resembles the

clinical situation of hypertension in pregnancy and minimizes the confounding effects of manipulation of the animals.

SPECIFIC AIM 1: STUDY DESIGN

Pregnant CD-1 mice at day 8 of gestation were randomly divided into three groups and injected through the tail vein with either adenovirus carrying sFlt-1 (10^9 PFU in 100µl; sFlt-1 group), adenovirus carrying the murine IgG2α Fc fragment (10^9 PFU in 100µl; mFc group used as a control for the virus), or saline (100µl; saline group). Two days later, blood pressure (BP) catheters were inserted through the left carotid artery into the aortic arch and tunneled to a telemetric transmitter. Blood pressure was recorded continuously in the conscious and unrestrained mice from day 10 of gestation until day 18. Mean blood pressure was averaged over 12-hour intervals. In a second set of similarly treated pregnant CD-1 mice, blood (100µl) was drawn serially from the time of injection until day 18 of gestation and circulating levels of sFlt-1 and free VEGF were measured by ELISA assay. In a third set of CD-1 mice, the mice were sacrificed at day 18 of gestation, and the placenta and pups were counted and weighed. Liver and kidney were collected for histological examination Blood was also collected from these animals for maternal complete blood count (CBC), liver and kidney function.

A group of non-pregnant CD-1 mice were injected with the same amount of sFlt-1 or saline as used in the pregnant mice. Two days later, BP catheters were inserted into the carotid artery to reach the aortic arch for BP recording. The BP was monitored continuously in the non-pregnant mice from day 3 to 6 after injection.

SPECIFIC AIM 1: RESULTS

Plasma sFlt-1 and free VEGF levels

Injection of the adenovirus carrying sFlt-1 at day 8 of gestation resulted in significant elevation of plasma sFlt-1 levels as early as 10 days of gestation. Plasma sFlt-1 levels increased slightly toward the end of gestation in the animals injected with the adenovirus carrying mFc or saline, but these levels remained significantly lower than those in the animals injected with the adenovirus carrying sFlt-1(Figure 7). In both animals injected with adenovirus-carrying sFlt-1 and mFc, free VEGF levels increased until day 10 and decreased abruptly in mid-term and then reduced slightly toward the end of gestation. Free VEGF levels in mFc group was significantly increased in mid-term and remained higher throughout the gestation compared with that in sFlt-1 group (Figure 8).

Figure 7 Plasma sFlt-1 Levels

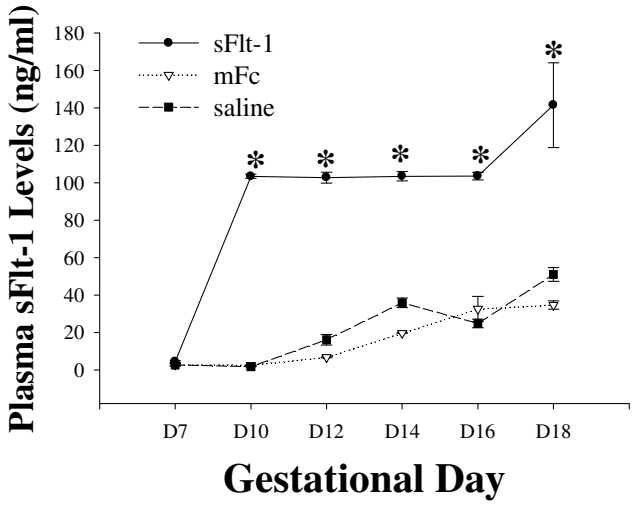


Figure 7 Plasma sFlt-1 levels in pregnant mice at different days of gestation before and after injection (on day 8) of adenovirus carrying sFlt-1 (sFlt-1), adenovirus carrying mFc

(mFc), or saline (Saline). Data shown as mean + SE. Asterisk denoted $p<0.05$ compared with mFc and saline.

Figure 8 Plasma Free VEGF Levels

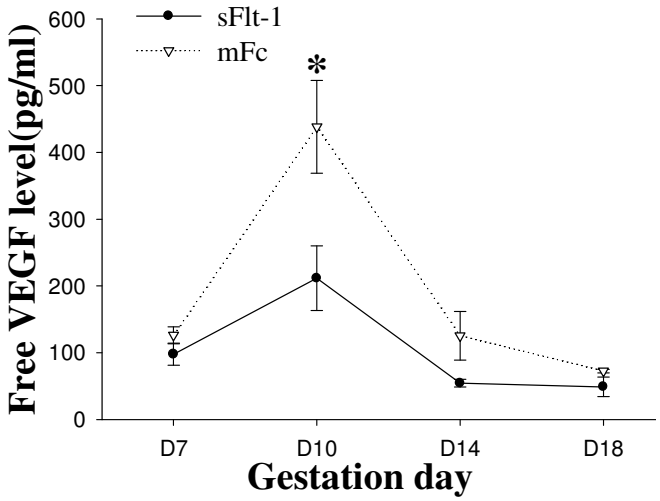


Figure 8 Plasma free VEGF levels in pregnant mice at different days of gestation before and after injection (on day 8) of adenovirus carrying sFlt-1 (sFlt-1) and adenovirus carrying mFc (mFc). Data shown as mean $\pm$ SE. Asterisk denoted $p<0.05$ compared with mFc.

BP curves in pregnant mice and non-pregnant mice

In pregnant mice, blood pressure in the mFc and saline groups were not significantly different, and demonstrated normal gestational changes as pregnancy progressed. Blood pressure in the sFlt-1 group was higher compared with the mFc and saline groups, and increased significantly toward the end of gestation (Figure 9). When injected into non-pregnant mice, the adenovirus carrying sFlt-1 did not result in hypertension compared to injection with saline (Figure 10).

Figure 9 Mean Blood Pressure in Pregnant Mice

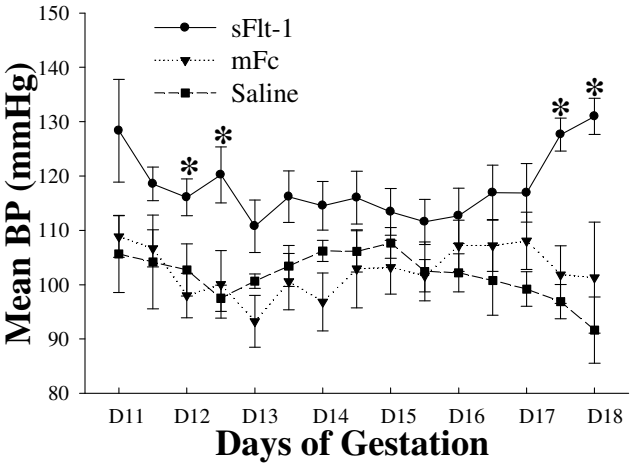


Figure 9 Mean blood pressure, averaged over 12-hour periods, in pregnant mice at different days of gestation after injection (on day 8) of adenovirus carrying sFlt-1 (sFlt-1), adenovirus carrying mFc (mFc), or saline (Saline). Data shown as mean $\pm$ SE. Asterisk denoted $p<0.05$ compared with mFc and saline.

Figure 10 Mean Blood Pressure in Non-Pregnant Mice

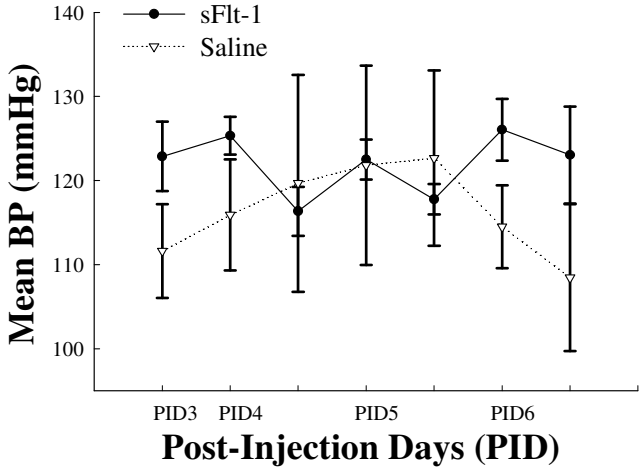
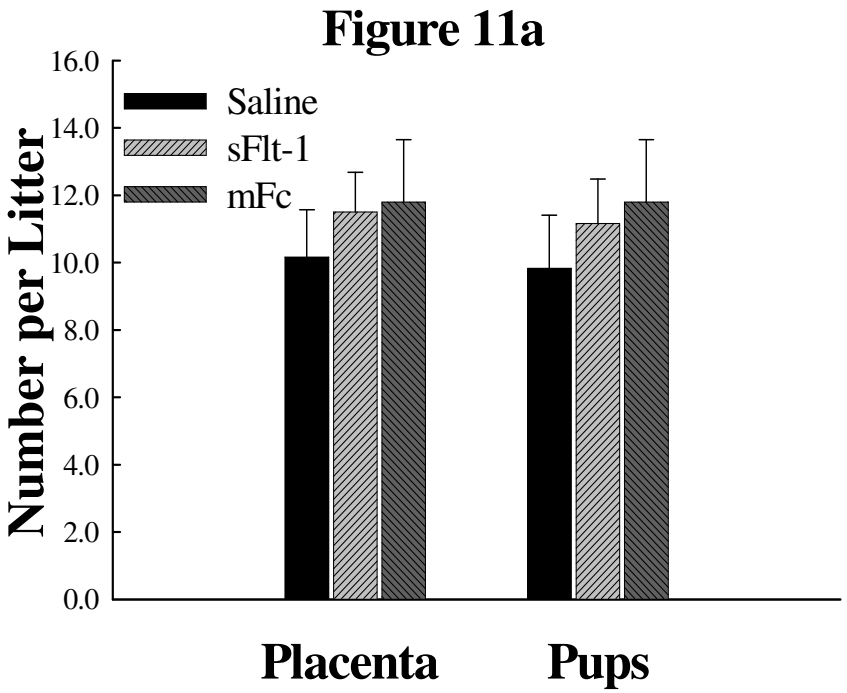


Figure 10 Mean blood pressure in non-pregnant mice from day 3 to 6 following injection with adenovirus carrying sFlt-1 (sFlt-1) or saline (Saline). Data shown as mean $\pm$ SE.

Litter characteristics The number of pups and placentas per litter was not significantly different between the groups (Figure 11a). However, the average pup and placenta weights (in gram) per litter were significantly lower in the sFlt-1 group, but not mFc group, compared with the saline group (Pup weights: sFlt-1 group 1.16 ± 0.06, mFc 1.37 ± 0.11, and saline group 1.49 ± 0.09; placenta weights: sFlt-1 0.11 ± 0.01, mFc 0.25± 0.06, saline mice 0.24± 0.05) (Figures 11b and 11c, respectively).

Figure 11 Litter Characteristics



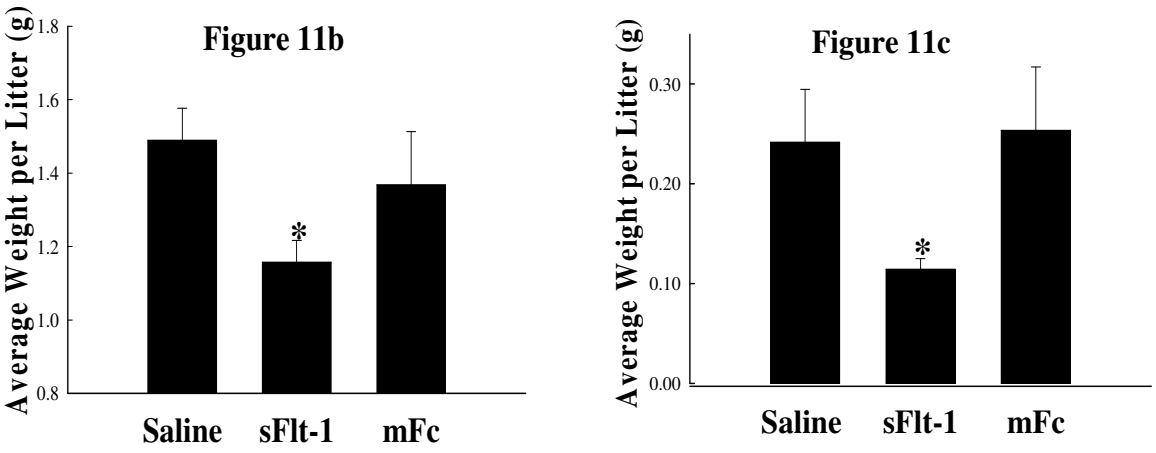


Figure 11 Number of pups and placentas (11a), average pup weight (11b) and average placenta weight (11c) per litter at day 18 of gestation in pregnant mice injected on day 8 with adenovirus carrying sFlt-1 (sFlt-1), adenovirus carrying mFc (mFc), or saline (Saline). Data shown as mean $\pm$ SE. Asterisk denoted $p<0.05$ compared with mFc and saline.

Complete Blood Count (CBC)

Maternal platelet counts were significantly lower and white blood cell count significantly higher in the sFlt-1 group compared with the mFc and saline groups (Figures 12a and 12b, respectively). Maternal hemoglobin concentrations were higher in the sFlt-1 group compared to the other 2 groups, but the difference did not reach statistical significance (Figure 12c).

Figure 12 Complete Blood Count

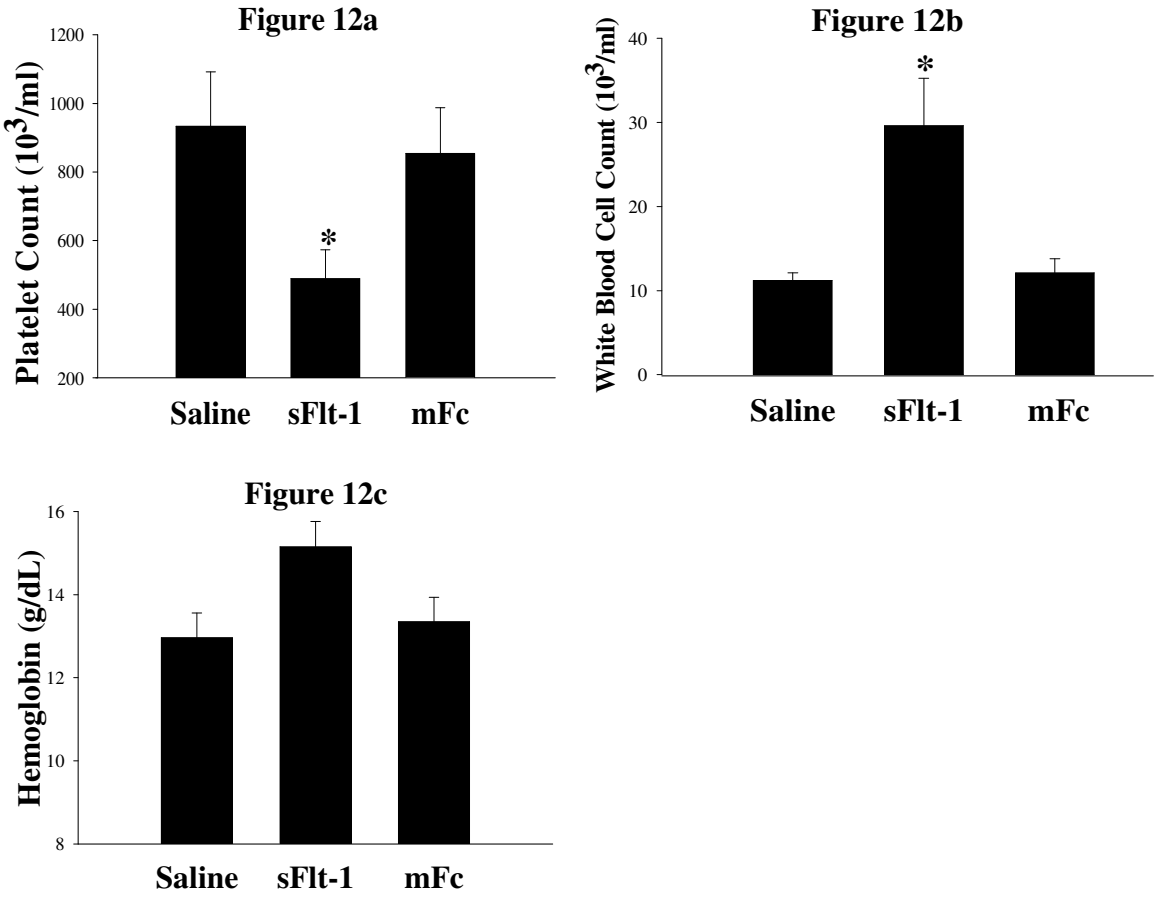


Figure 12 Maternal platelet count (12a), white blood cell count (12b) and hemoglobin concentration (12c) at day 18 of gestation in pregnant mice injected on day 8 with adenovirus carrying sFlt-1 (sFlt-1), adenovirus carrying mFc (mFc), or saline (Saline). Data shown as mean $\pm$ SE. Asterisk denoted $p<0.05$ compared with mFc and saline.

Renal and liver histopathological studies (40×)

All of the animals (n=5) treated with sFlt-1 and mFc showed inflammatory infiltrates mostly localized in the periportal area (Figure 13a), the enlargement of hepatocytes, the presence of polymorphs in the sinusoids and numerous acidophilic bodies with periportal and mid-zonal distribution (Figure 13b); while these inflammatory

changes were not seen in the saline group (n=5). The kidneys of half the animals treated with sFlt-1 showed glomerular sclerosis (Figure 13c); proteinaceous deposits in the tubules (Figure 13d) and mild focal tubular necrosis, but none of the controls have these alterations.

Figure 13 Liver and Renal Histopathological Pictures

Figure 13a

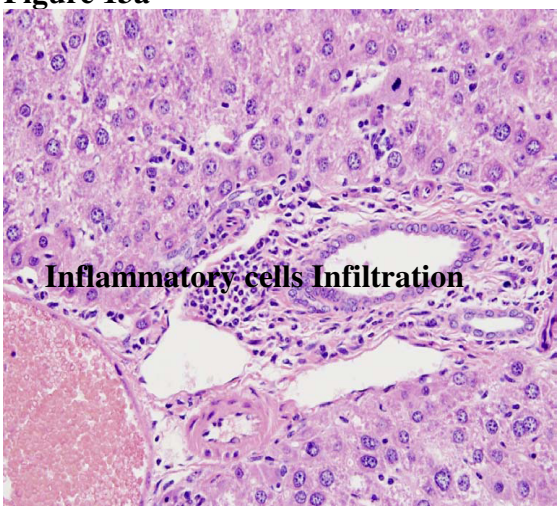


Figure 13b

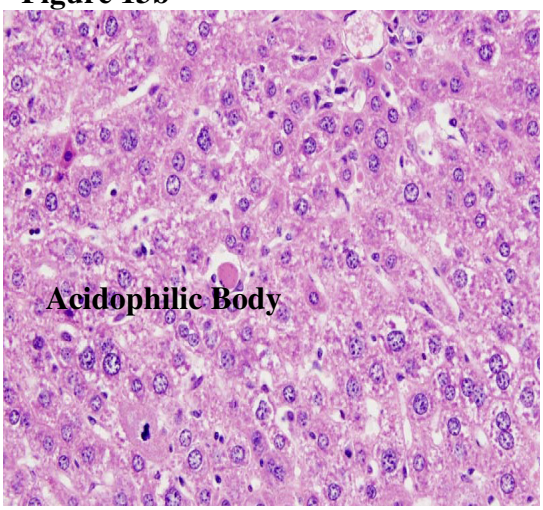


Figure 13c

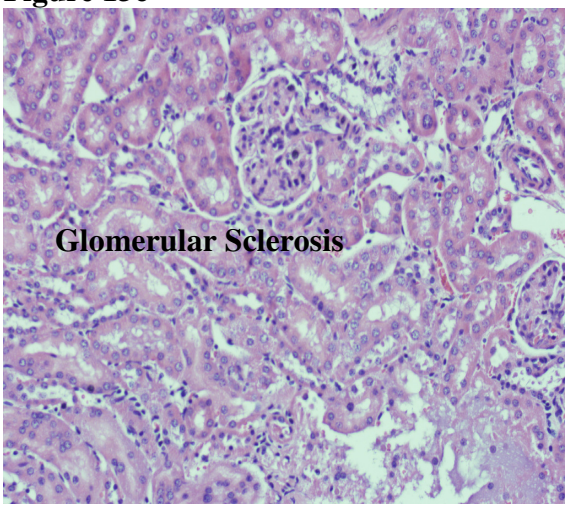
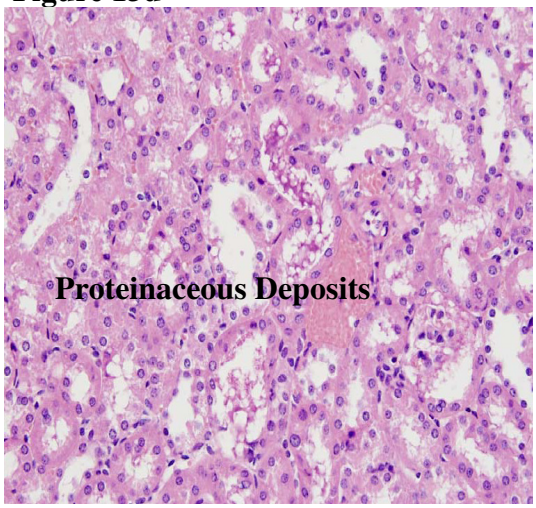


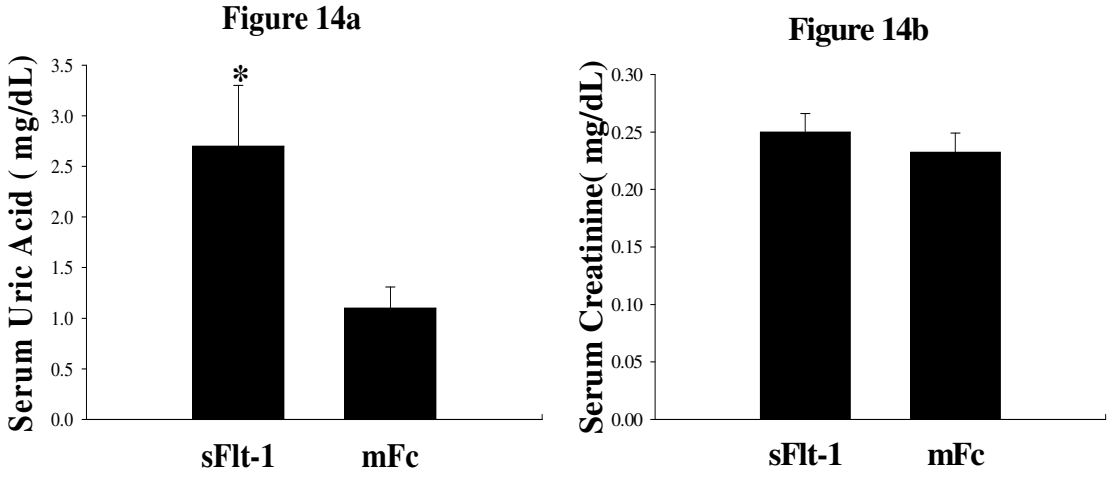
Figure 13d

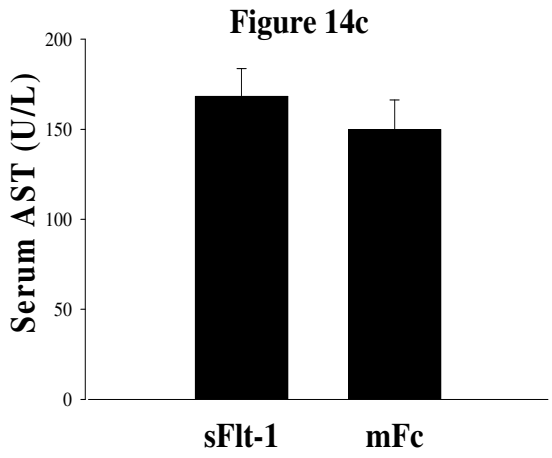


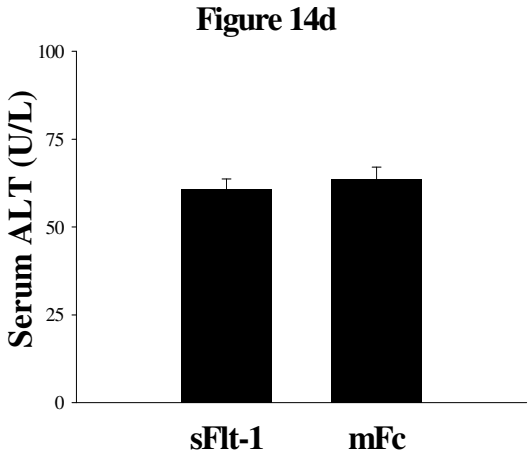
Renal function and liver function at day 18 of gestation

We investigated the renal function of pregnant mice at day 18 of gestation by measuring the concentration of serum uric acid and creatinine. Serum uric acid level was significantly increased in pregnant mice treated with sFlt-1 when compared with the control group (Figure 14a). There was no difference in serum creatinine levels between these two groups (Figure 14b). We also examined liver function at the day 18 of gestation by determining the serum alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH). Serum AST level, ALT, and LDH in sFlt-1 treated group was similar to those in mFc-treated group (Figure 14c, d, e, respectively).

Figure 14 Renal Function and Liver Function







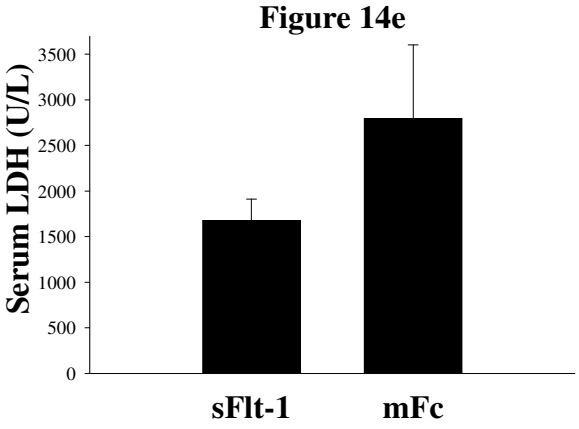


Figure 14 Renal function shown as serum uric acid(Figure 14a) and serum creatinine (Figure 14b), liver function test shown as serum AST(Figure 14c), serum ALT(Figure 14d) and serum LDH(Figure 14e) at day 18 of gestation in pregnant mice injected on day 8 with adenovirus carrying sFlt-1 (sFlt-1) and adenovirus carrying mFc (mFc). Data shown as mean $\pm$ SE. Asterisk denoted $p<0.05$ compared with mFc.

SPECIFIC AIM 1 CONCLUSION

The results have shown that the transfection of pregnant mice with adenovirus carrying sFlt-1 leads to an increase in circulating levels of sFlt-1 followed by

hypertension and a decrease in free VEGF levels. The animals transfected with the adenovirus carrying sFlt-1 also demonstrate a decrease in fetal and placental weights, as well as hepatic, renal, and hematologic findings consistent with preeclampsia. Because of the pitfalls in measuring urinary protein excretion in animals, particularly mice, we opted to confirm the other characteristics of preeclampsia first. However, protein deposition within the collecting tubules was detected on histological examination, these changes are induced by sFlt-1, specifically in pregnancy, is supported by the lack of effect when the adenovirus carrying the murine IgG2α Fc fragment as a control and when the adenovirus carrying sFlt-1 was injected into non-pregnant animals.

These results confirm the described association between the occurrence of preeclampsia and an imbalance in angiogenic factors, and provide the evidence that the increased level of sFlt-1 could induce maternal hypertension as well as a preeclampsia-like picture.

Specific Hypothesis 2:

High level of sFlt-1 during pregnancy leads to altered central and peripheral vascular reactivity.

SPECIFIC AIM 2:

To evaluate vascular function of the carotid and uterine arteries in pregnant mice transfected with adenovirus carrying sFlt-1 by in vitro vascular reactivity.

INTRODUCTION

The mice model created in specific aim 1 confirmed the association between the occurrence of preeclampsia and an imbalance in angiogenic factors induced by sFlt-1.

However, the underlying mechanisms of the preeclampsia-like condition in this animal model are still lacking. Preeclampsia is mainly characterized by vascular dysfunction including generalized endothelium dysfunction which is shown by mis-adaptation of multiple organ systems. Decreased perfusion is a characteristic of hemodynamic alterations commonly seen in preeclampsia. Our hypothesis is **high level of sFlt-1 during pregnancy leads to altered central and peripheral vascular reactivity.**

During pregnancy, vessels are remodeled to form a low-resistance arteriolar system which serves to promote delivery of a greatly increased blood and oxygen supply to organs including developing fetus. The incomplete remodeling contributes to an inadequate response to the increasing demand of blood supply to various organs (Anderson, 2005). The cervical part of common carotid arteries is mostly involved in cerebral vascular changes; while determination of functional responses in uterine artery reflects the utero-placental perfusion.

SPECIFIC AIM 2: STUDY DESIGN

CD1 mice (n=6-14/group) at day 8 of gestation were randomly injected with adenovirus carrying sFlt-1 or mFc. In vitro carotid and uterine vascular studies were carried out in these CD1 mice at both the day 14 (mid-term) and 18 of gestation (at term).

SPECIFIC AIM 2: RESULTS

In vitro carotid vascular activity

Responses to KCl were not significantly different between the groups (Figure 15). The PE contraction (AUC: 221.35±31.1) as well as its maximal effect (Emax: 131.85±16.59) in the sFlt-1-treated group at term was significantly higher compared with mFc-treated group at term (Figure 16a; Table 3&4). The sFlt-1 and mFc-treated groups at

mid-term (AUC: 129.31±8.0 and 108.74±18.49 respectively) and the mFc-treated group at term (AUC: 139.15±16.23), had similar contractile responses at all concentrations of phenylephrine with similar maximal effects (Figure 16b; Table 3&4). In the presence of L-NAME, responses to phenylephrine in the mFc-treated pregnant mice both at term and at mid-term, as well as in sFlt-1-treated mothers at mid-term, increased and became similar to responses in the sFlt-1-treated mothers at term (Figure 17a & b & Table 3). In addition, there was a decrease in the sensitivity to PE in the mFc group (-log IC_{50} 7.14±0.2) at term compared with the other 3 groups (Table 4). No difference was noted in TxA_2 contractile response across all groups (Figure 18). Similarly, relaxant responses to ACh and SNP did not differ between the groups (Figure 19 and Figure 20).

Figure 15 KCl contraction in the carotid arteries

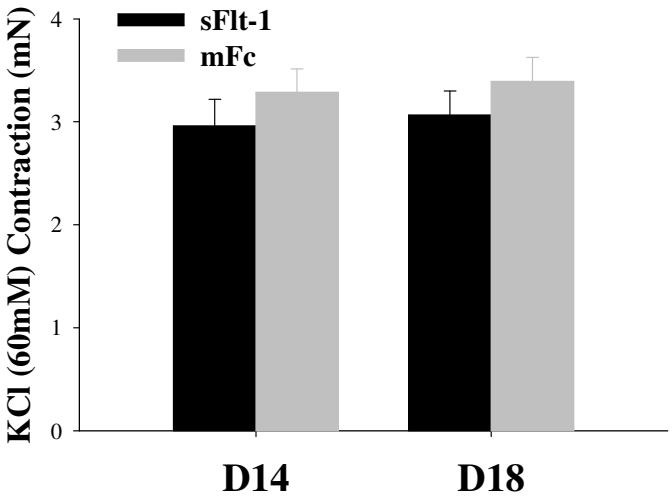


Figure 15 KCl contraction in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term, sFlt-1- treated (n=8) and mFc-treated pregnant mice (n=8) at mid-term.

Figure 16 Phenylephrine (PE) concentration-response curves in the carotid arteries

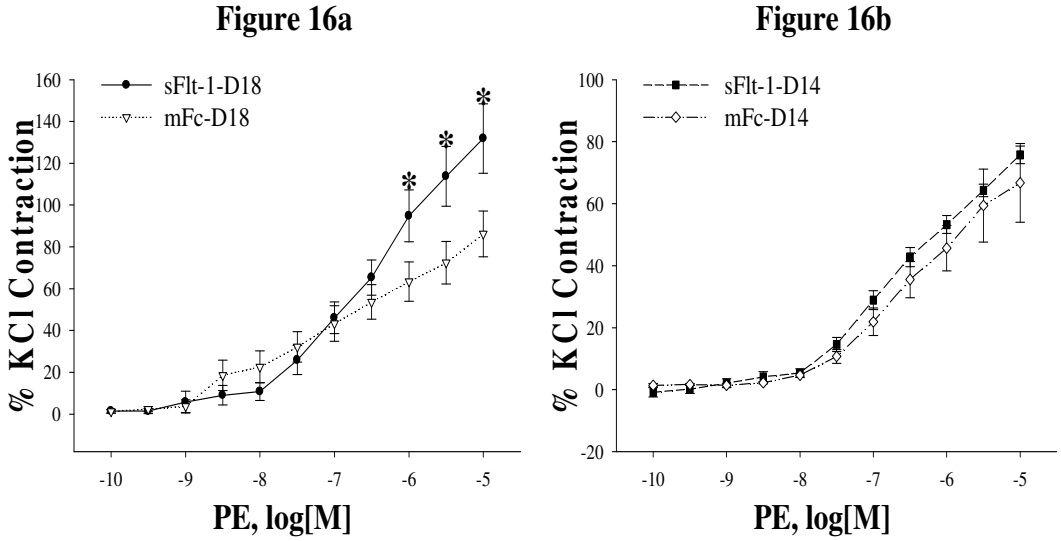


Figure 16 Phenylephrine (PE) concentration-response curves in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term (a), sFlt-1- treated (n=8) and mFc-treated pregnant mice (n=8) at mid-term (b). The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs all other groups.

Figure 17 Phenylephrine (PE) concentration-response curves in the carotid arteries in the presence of L-NAME

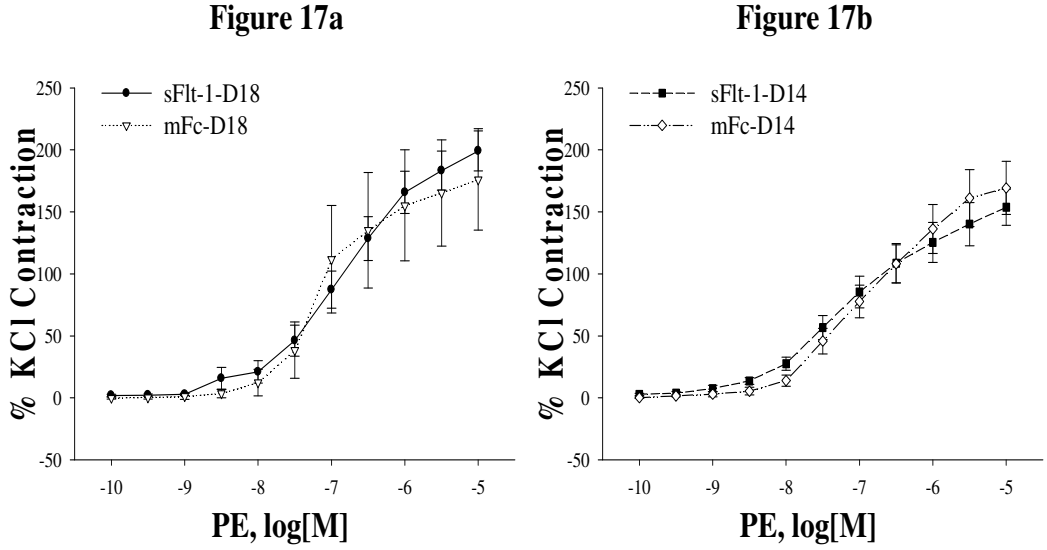


Figure 17 Phenylephrine (PE) concentration-response curves in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term (a), sFlt-1- treated (n=8) and mFc-treated pregnant mice (n=8) at mid-term (b) in the presence of the nonspecific NOS inhibitor L-NAME.

Table 3 Area under the phenylephrine concentration curve in the absence or presence of L-NAME, The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs all other groups.

Groups	Area Under Curve (PE)	Area Under Curve (PE+LN)
sFlt-1-D14	129.31±8.0	323.57±44.06
mFc-D14	108.74±18.49	319.68±42.99
sFlt-1-D18	221.35±31.1*	376.80±48.46
mFc-D18	139.15±16.23	357.33±44.88

Table 4: Maximal Effect and Area under the phenylephrine concentration curve (AUC; arbitrary units), logarithm of molar concentration that produces log IC_{50} and maximal effect (expressed as the percentage of the reference contraction to 60 mM KCl) in the carotid arteries of sFlt-1 or mFc treated pregnant mothers at mid-term or term (n=6-14). The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs mFc-treated mice at term.

Groups	Area Under Curve	Maximal Effect (Emax)	IC50
sFlt-1-D14	129.31±8.0	75.73±2.88	6.69±0.12
mFc-D14	108.74±18.49	66.71±12.66	6.58±0.11
sFlt-1-D18	221.35±31.1*	131.85±16.59*	6.59±0.1*
mFc-D18	139.15±16.23	88.17±10.91	7.14±0.2

Figure 18 Thromboxane A_2 (TxA_2) concentration-response curves in the carotid arteries

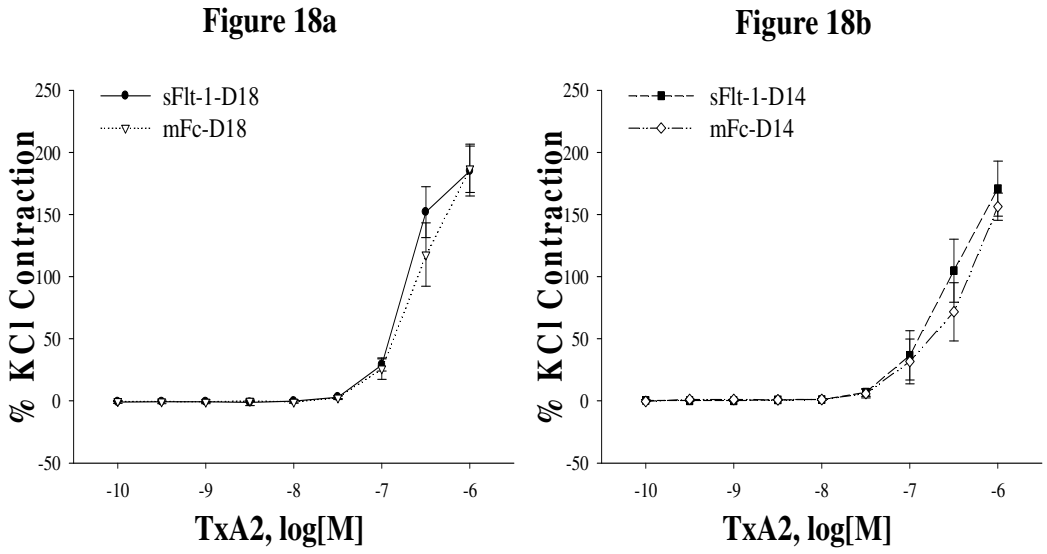


Figure 18 Thromboxane A_2 (TxA_2) concentration-response curves in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term (a), sFlt-1- treated (n=6) and mFc-treated pregnant mice (n=6) at mid-term (b).

Figure 19 Acetylcholine (ACh) concentration-response curves in the carotid arteries

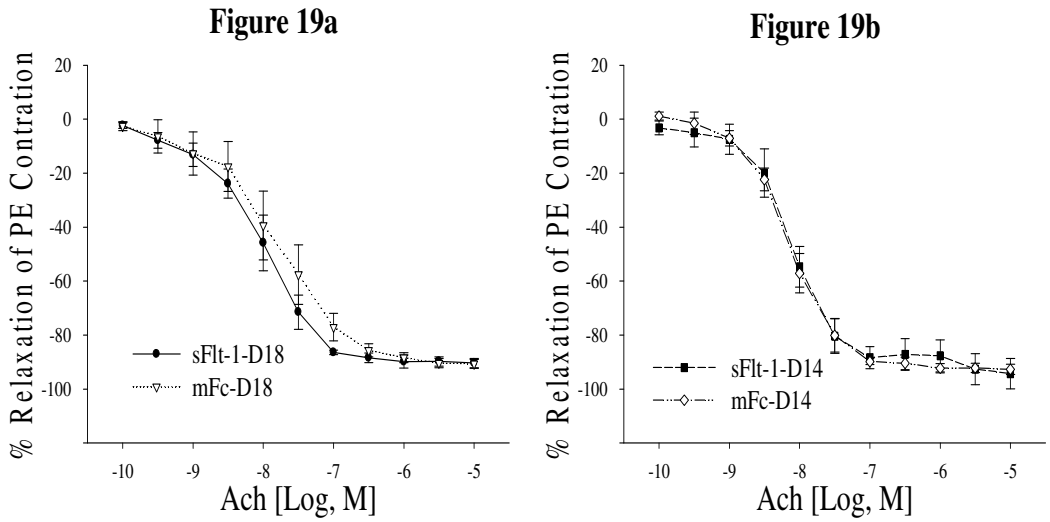


Figure 19 Acetylcholine (ACh) concentration-response curves in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term (a), sFlt-1- treated (n=8) and mFc-treated pregnant mice (n=6) at mid-term (b). Responses are presented as percent relaxation of the PE contraction.

Figure 20 Sodium Nitroprusside (SNP) concentration-response curves in the carotid arteries

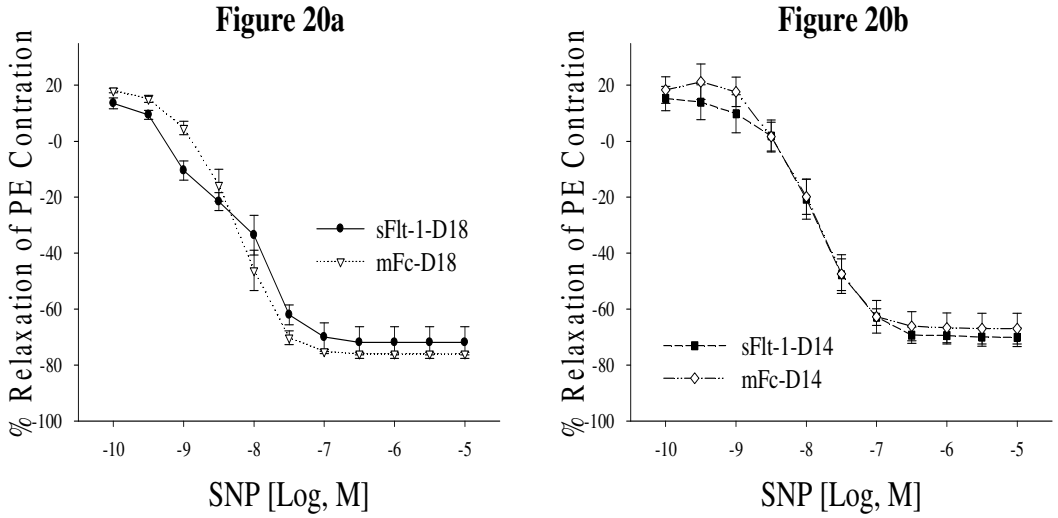


Figure 20 Sodium Nitroprusside (SNP) concentration-response curves in the carotid arteries of sFlt-1- treated (n=14) and mFc-treated pregnant mice (n=9) at term (a), sFlt-1-treated (n=8) and mFc-treated pregnant mice (n=6) at mid-term (b). Responses are presented as percent relaxation of the PE contraction.

In Vitro Uterine Artery Vascular Activity

Responses to KCl were not significantly different between the groups (Figure 21). PE contraction was significantly higher in the sFlt-1-treated groups at term compared with control group respectively (Figure 22a & table 5). In the presence of L-NAME, responses to PE in the mFc-treated pregnant mice at term and at mid-term, as well as in sFlt-1-treated mothers at mid-term, increased and became similar to responses in the sFlt-1-treated mothers at term (Figure 23).

The relaxation to ACh as well as the maximal effect and the area under curve was significantly decreased in the sFlt-1 group at term compared with sFlt-1-treated group at mid-term and mFc-treated groups both time points (Figure 24 and table 6).

No differences were noted in the SNP and TxA_2 responses across all groups (Figure 25, 26).

Figure 21 KCl contraction in the uterine arteries

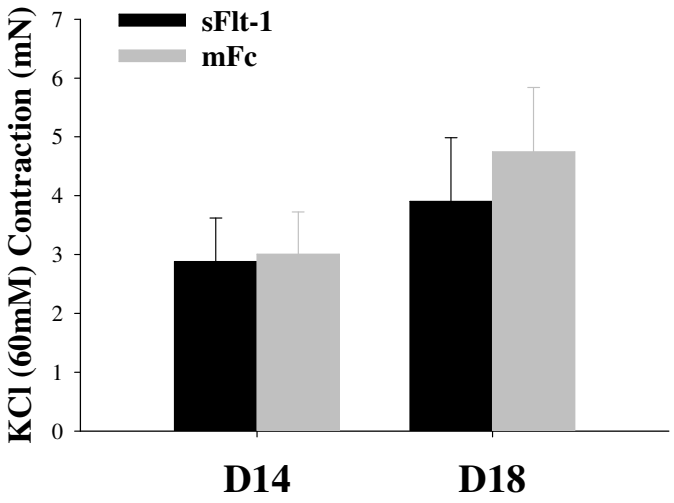


Figure 21 KCl contraction in the uterine arteries of sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at term, sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term.

Figure 22 Phenylephrine (PE) concentration-response curves in the uterine arteries

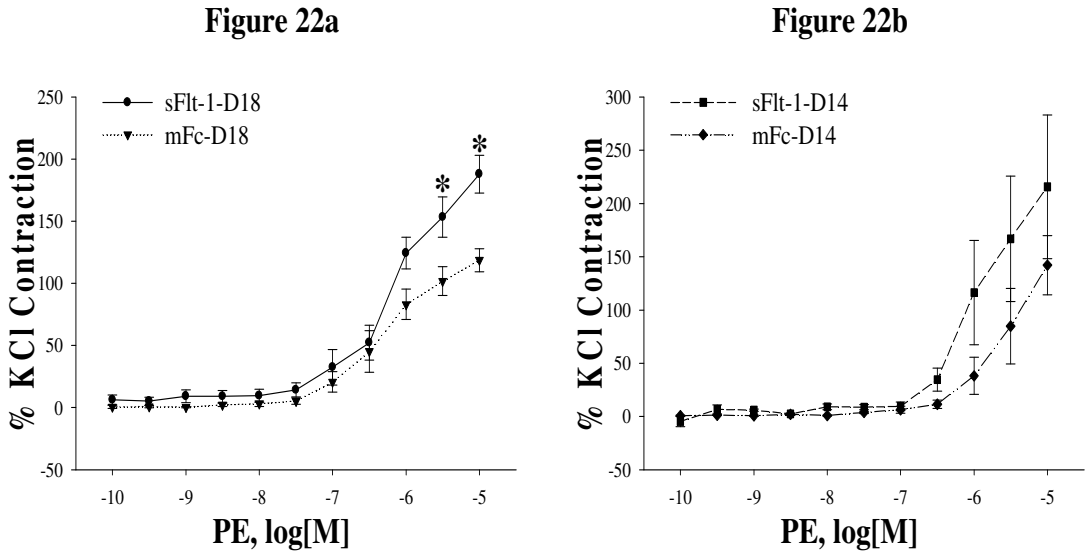


Figure 22 Phenylephrine (PE) concentration-response curves in the uterine arteries of sFlt-1- treated (n=8) and mFc-treated pregnant mice (n=4) at term (a), sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term (b).

Table 5: Maximal Effect and Area under the phenylephrine concentration curve (AUC; arbitrary units), logarithm of molar concentration that produces log IC_{50} and maximal effect (expressed as the percentage of the reference contraction to 60 mM KCl) in the uterine arteries of sFlt-1 or mFc treated pregnant mothers at mid-term or term (n=6-14). The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs mFc-treated at term.

Groups	Area Under Curve	Maximal Effect (Emax)	IC50
sFlt-1-D14	235.7±76.1	215.6±68	5.7±0.1
mFc-D14	110.3±35	142.1±27.8	4.5±1.2
sFlt-1-D18	253.24±22.5*	188±15.1*	6.1±0.3
mFc-D18	161.6±20.4	118.7±9.3	6.28±0.2

Figure 23 Phenylephrine (PE) concentration-response curves in the uterine arteries in the presence of L-NAME

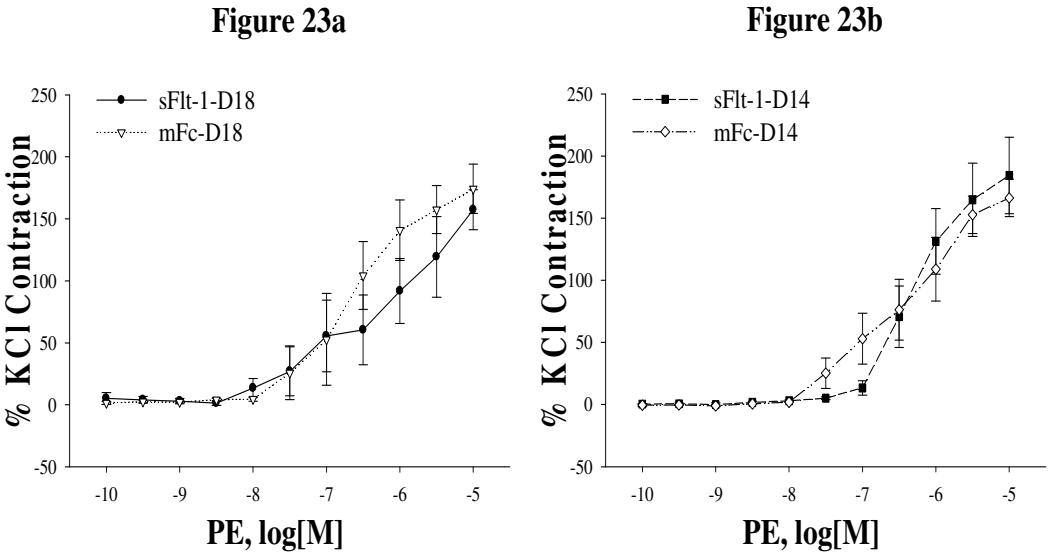


Figure 23 Phenylephrine (PE) concentration-response curves in the uterine arteries of sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at term (a), sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term (b) in the presence of the nonspecific NOS inhibitor L-NAME.

Figure 24 Acetylcholine (ACh) concentration-response curves in the uterine arteries

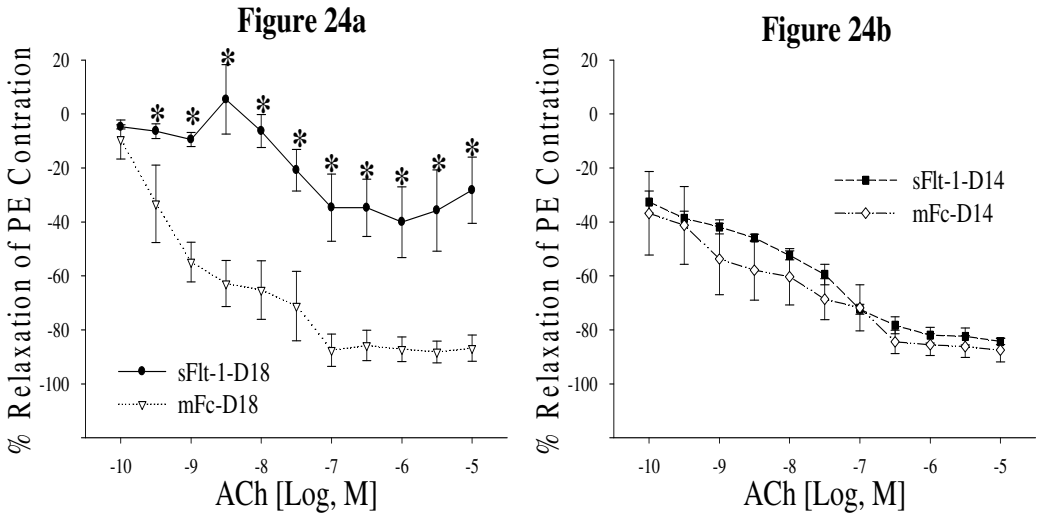


Figure 24 Acetylcholine (ACh) concentration-response curves in the uterine arteries of sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at term (a), sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term (b). Responses are presented as percent relaxation of the PE contraction. The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs all other groups.

Table 6 Maximal Effect and Area under the acetylcholine concentration curve (AUC; arbitrary units), logarithm of molar concentration that produces log IC_{50} and maximal effect (expressed as the percentage of the reference contraction to 60 mM KCl) in the uterine arteries of sFlt-1 or mFc treated pregnant mothers at mid-term or term (n=4-5). The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs all other groups.

Groups	Area Under Curve	Maximal Effect (Emax)	IC50
sFlt-1-D14	305.96±12.67	-84.31±1.17	7.78±0.05
mFc-D14	336.08±22.73	-87.52±4.39	7.66±0.33
sFlt-1-D18	122.29±22.67*	-26.27±12.3*	9.64±2.03
mFc-D18	343.45±23.96	-86.79±4.86	9.36±0.51

Figure 25 Sodium Nitroprusside (SNP) concentration-response curves in the uterine arteries

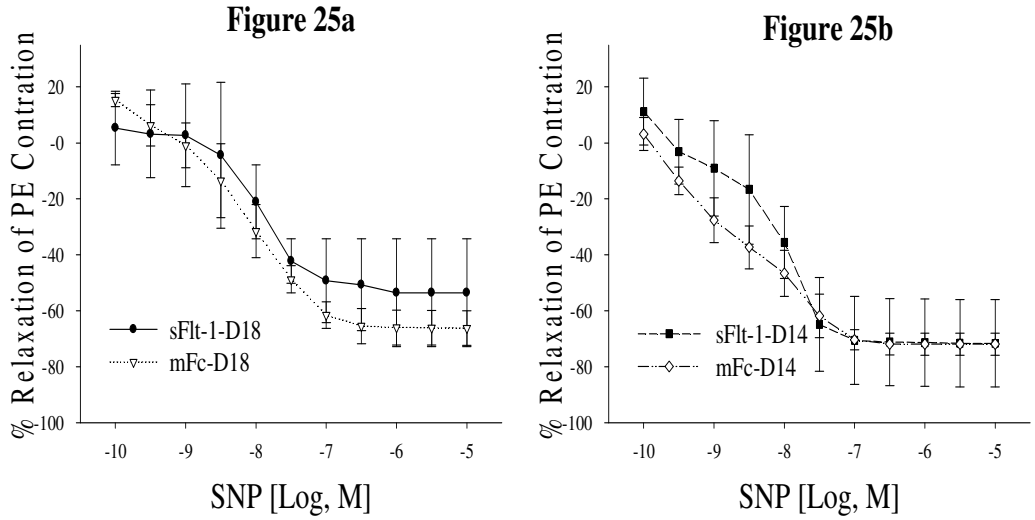


Figure 25 Sodium Nitroprusside (SNP) concentration-response curves in the uterine arteries of sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at term (a), sFlt-1-treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term (b). Responses are presented as percent relaxation of the PE contraction.

Figure 26 Thromboxane A_2 (TxA_2) concentration-response curves in the uterine arteries

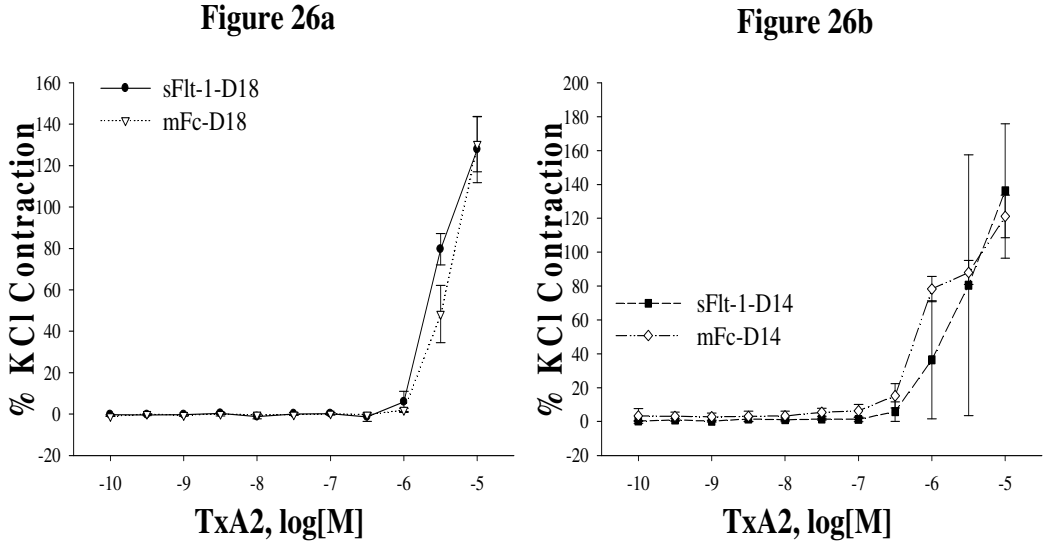


Figure 26 Thromboxane A_2 (TxA_2) concentration-response curves in the uterine arteries of sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at term (a), sFlt-1- treated (n=5) and mFc-treated pregnant mice (n=4) at mid-term (b).

SPECIFIC AIM 2: CONCLUSIONS

The results confirm that our murine model has a similar vascular reactivity profile to what has been described in humans. No significantly difference was seen in smooth muscle contractile responses to membrane depolarization by high-KCl solution, which indicates no structural changes occurred in both carotid and uterine arteries. Responses to the endothelium-dependent vasorelaxant ACh in the uterine arteries were significantly decreased in sFlt-1-treated pregnant mice at term compared with the other three groups, while no difference in Ach relaxation response was noted in carotid arteries across all groups. The impaired uterine vascular reactivity was likely due to endothelial dysfunction

in this preeclampsia-like model, which could lead to impaired placenta perfusion and preeclampsia.

In carotid arteries, we have found a significantly increased contractile response to incremental doses of phenylephrine in sFlt-1-treated pregnant mice toward the end of pregnancy compared with that in mFc-treated control mice at term; which suggest that altered carotid vascular function probably involves receptor-dependent mechanism. These changes in the carotid artery may have significance in relation to brain blood flow in preeclampsia. The signifant increase in PE contraction in sFlt-1-treated mice had also been seen in the uterine arteries; we did not see any difference in TxA_2 and SNP in both carotid and uterine arteries across all groups.

Specific Hypothesis 3:

sFlt-1 leads to changes in hypoxia-induced genes in the placenta and kidney by affecting the circulating angiogenic factors levels of VEGF and PlGF .

SPECIFIC AIM 3:

To compare mRNA expression level of the hypoxia-induced factors in the placentas and kidneys from mice over-expressing sFlt-1 versus controls.

SPECIFIC AIM 3: INTRODUCTION

The results in the specific aim 2 have demonstrated the impaired uterine vascular functions in sFlt-1 treated mice at term, which reflected indirectly that decreased utero-placental blood flow was present in this animal model, it is consistent with clinical data shown by other researchers (Lunell, 1984). A number of pro-angiogenic proteins in

endothelial and tumor cells including endothelin (Kourembanas et al., 1991), VEGF (Shweiki, 1992) and Flt-1 (Gerber, 1997) were known to be stimulated by hypoxic conditions. VEGF and Flt-1 gene transcription are also regulated by a transcription factor hypoxia-inducible factor 1 (HIF-1α)(Forsythe, 1996; Gerber, 1997), which was found to be elevated in the preeclamptic placenta (Caniggia 2000; Rajakumar, 2003; Rajakumar, 2001). Transforming growth factor beta 3 (TGF β3) is a mediator to the signal pathway of HIF-1α leading to the altered trophoblast differentiation. The altered trophoblast differentiation could also be affected by another gene—Glial cells missing (GCM1), which was shown to be down-regulated in the preeclamptic placentas. Moreover, recent evidence highly suggests that the glomerular injury in preeclampsia is mediated by sFlt-1, causing subsequent new-onset of hypertension and proteinuria (Stillman 2007). We tested our hypothesis that **sFlt-1 leads to a hypoxia induced change in placenta and kidney, and consequently to a maternal hypertension by determining the expression of hypoxia regulated genes in this animal model.**

SPECIFIC AIM 3: STUDY DESIGN

CD1 mice (n=6-14/group) at day 8 of gestation were randomly injected with adenovirus carrying sFlt-1 or mFc. Placentas and kidneys were collected in these CD1 mice at both the day 14 (mid-term) and 18 (term) of gestation for the detection of the expression levels of hypoxic genes.

SPECIFIC AIM 3: RESULTS

Hypoxic gene expression in placenta and kidney

The expression levels of HIF-1α in both placenta and kidney were significantly increased in sFlt-1-treated groups at term compared with the other groups (Figure 27a and b). The significantly higher expression of TGFβ3 has only been detected of sFlt-1-

treated group in placenta at term (Figure 28a) with no differences in TGFβ3 expression in kidney either at mid-term or at term (Figure 28b). GCM1 mRNA expression level was significantly decreased in sFlt-1-treated group in placenta at term (Figure 29a); while there were no differences in GCM1 expression in kidney from both sFlt-1-treated and mFc-treated groups either at mid-term or at term (Figure 29b).

Figure 27 HIF-1α mRNA Expression

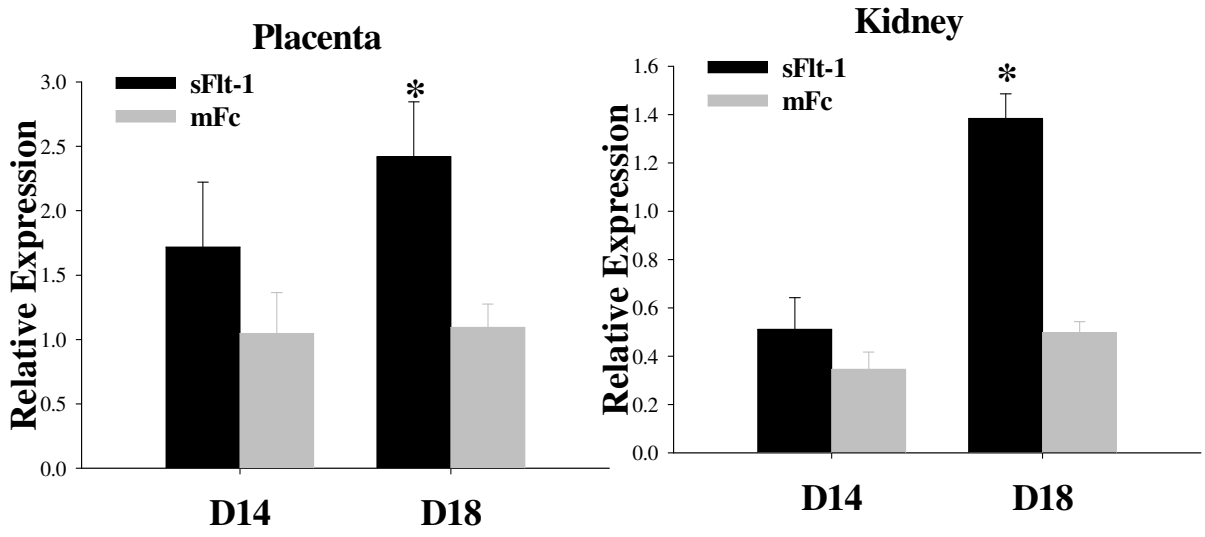


Figure 27 Fold expressions of HIF1α mRNA were shown by $\Delta\Delta C_t$ (RQ) values in both placenta (Figure 27a) and kidney (Figure 27b) at day 14 and day 18 from sFlt-1 and mFc-treated groups. The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs mFc-treated groups at term.

Figure 28 TGFβ3 mRNA Expression

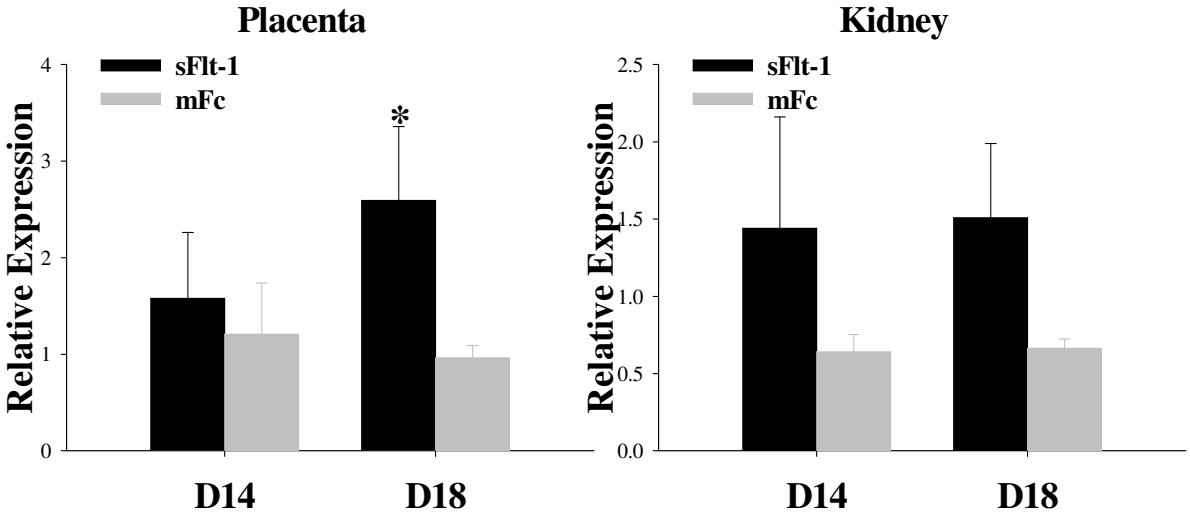


Figure 28 Fold expressions of TGFβ3 mRNA were shown by $\Delta\Delta C_t$ (RQ) values in both placenta (Figure 28a) and kidney (Figure 28b) at day 14 and day 18 from sFlt-1 and mFc-treated groups. The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs mFc-treated groups at term.

Figure 29 GCM1 mRNA Expression

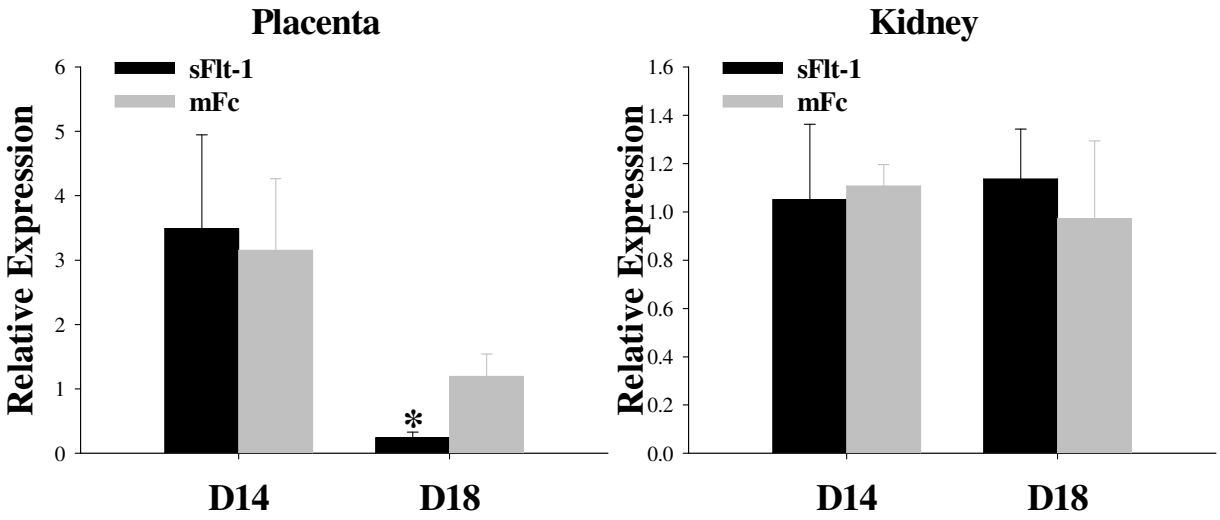


Figure 29 Fold expressions of GCM1 mRNA were shown by $\Delta\Delta C_t$ (RQ) values in both placenta (Figure 29a) and kidney (Figure 29b) at day 14 and day 18 from sFlt-1 and mFc-treated groups. The asterisk denotes a probability value of <0.05 for sFlt-1-treated pregnant mice at term vs mFc-treated groups at term.

SPECIFIC AIM 3: CONCLUSIONS

Pregnant mice over-expressing sFlt-1 display a progression toward smaller placentas which was demonstrated in specific aim 1, increased expression of HIF1α, TGF β3 and decreased expression of GCM1 in the placenta, with a more pronounced difference at term. This study confirms the presence of hypoxic placenta in this mouse model of preeclampsia and elucidates the relationship between sFlt-1 and the hypoxic placenta. Meanwhile, the increased expression of HIF1α in the kidney indicates the hypoxia condition is present in kidney, possibly mediated by sFlt-1 that deprives glomerular endothelial cells of VEGF they require. We didn't detect the changes in the mRNA expression levels of TGFβ3 and GCM1 in the kidney from sFlt-1-treated and mFc-treated groups. We speculated that the hypoxic condition induced by sFlt-1 in the

kidney could possibly involve the other transcription factor namely TGFβ1, which play an important role in the formation of fenestrated glomerular endothelium (Liu, 1999; Ballermann, 2007)

Specific Hypothesis 4

Elevated level of sFlt-1 results in an adverse intrauterine environment which leads to an altered fetal vascular programming manifesting as hypertension in the offspring later in life.

SPECIFIC AIM 4

To evaluate fetal vascular programming by determining blood pressure in adult offspring born to pregnant mice over-expressing sFlt-1.

SPECIFIC AIM 4: INTRODUCTION

The "Barker's Hypothesis" states that the intrauterine environment is involved in the well being of fetal development, that insults occurring during critical periods of fetal development lead to fetal programming, a series of adaptations that allow the fetus to survive in altered uterine conditions. The resulting fetal adaptations may have permanent specific short and long-term effects on the development of various organ systems, including the cardiovascular and metabolic systems (Barker, 1986; 1989; 1994; 1998). In support of this hypothesis, Barker and Osmond found that systolic blood pressure in adults is inversely related to birth weight, and this inverse relationship was independent of gestational age at birth, suggesting that the intrauterine environment can influence blood pressure in adult life (Barker, 1989). Since then, several studies have confirmed the

association between low birth weight and cardiovascular diseases later in life, including coronary artery disease, stroke and hypertension (Hoy, 1999).

The milieu in which the fetus develops is created by the interactions between the fetal genome and the intrauterine environment, and this interaction may be operational in pregnancy complicated by diabetes, hypertension and preeclampsia. However, the observed association between an unfavorable/hostile fetal environment and diseases in later life may be confounded by the presence of a genetic predisposition for the particular disease in both mother and offspring. This is particularly applicable to cardiovascular and endocrinologic/metabolic disorders since genetic predispositions for these disorders may affect the fetal environment, as well as the risk for adult diseases when present in both mother and offspring. In these cases, epidemiological studies may not differentiate or apportion the risk of disease in later life between the unfavorable uterine environment versus the hereditary predisposition that is common to both mother and fetus (Dunger, 2006). Animal models may be of significant help in addressing such an issue.

Preeclampsia figures prominently among the various causes of altered uterine and fetal environment. Preeclampsia is thought to result from an imbalance between placental perfusion and placental metabolic needs leading to placental hypoxia (Zuspan,1988; Redman, 1991), providing a hostile intrauterine environment in which the fetus is developing (Zusterzeel, 2001; Naicker, 2003). Thus the utero-placental insufficiency present in preeclampsia can potentially lead to altered fetal vascular programming and risk of cardiovascular disease later in life.

The animal model we created in specific aim 1 has been shown that pregnant mice over-expressing the soluble fms-like tyrosine kinase1 (sFlt-1) are hypertensive, deliver growth restricted fetuses, have lower platelet counts compared with controls, as well as

other manifestations of a preeclampsia-like syndrome (Lu, 2007). The association between over-expression of sFlt-1 and a preeclampsia-like condition was also shown by others in pregnant rats (Maynard, 2003). Therefore, we decided to test the hypothesis that the elevated levels of sFlt-1 results in an adverse intrauterine environment which leads to an altered fetal vascular programming manifesting as hypertension in the offspring later in life.

SPECIFIC AIM 4: STUDY DESIGN

Pregnant CD1 mice at day 8 of pregnancy were randomly divided into two groups and injected through the tail vein with either adenovirus carrying sFlt-1 (10^9 PFU in 100µl; sFlt-1 group) or adenovirus carrying the murine IgG2α Fc fragment (10^9 PFU in 100µl; mFc group used as a control for the virus). We allowed the pregnant mice to deliver. Offspring was weighted weekly after weaning. At the 6 month of age, we measured blood pressure of female and male offspring using telemetry system.

SPECIFIC AIM 4: RESULTS

Post weaning weight

The male and female offspring from sFlt-1-treated and mFc-treated pregnant mice were followed until adulthood. The weight from weaning until adulthood of male offspring born to sFlt-1 treated pregnant mice was significantly lower than male offspring born to mFc-treated pregnant mice (Figure 30a). There was no significant difference in the post weaning weight in female offspring between the 2 groups (Figure 30b).

Figure 30 Post-Weaning Weight

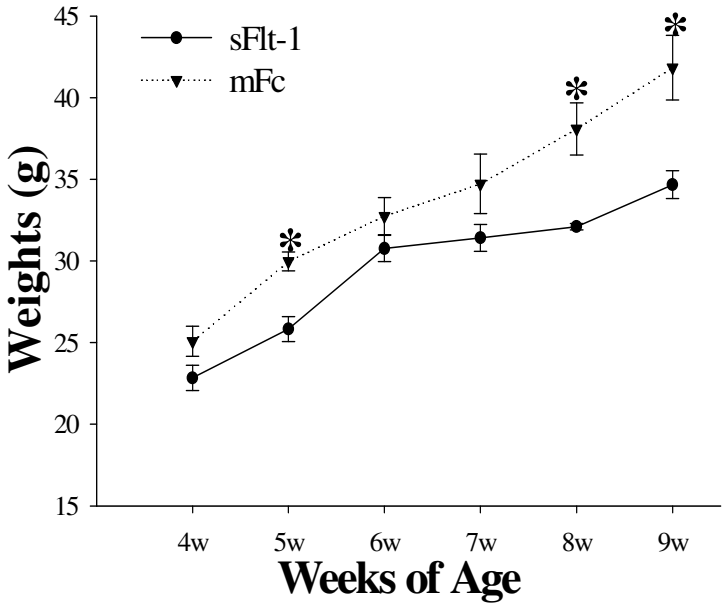


Figure 30a Post weaning weight of male offspring, at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1 (sFlt-1)(n=19) or adenovirus carrying mFc (mFc)(n=8). Data are shown as mean ± SEM. The asterisk denotes a probability value of <0.05 for male offspring born to sFlt-1-treated pregnant mice vs male offspring born to mFc-treated pregnant mice.

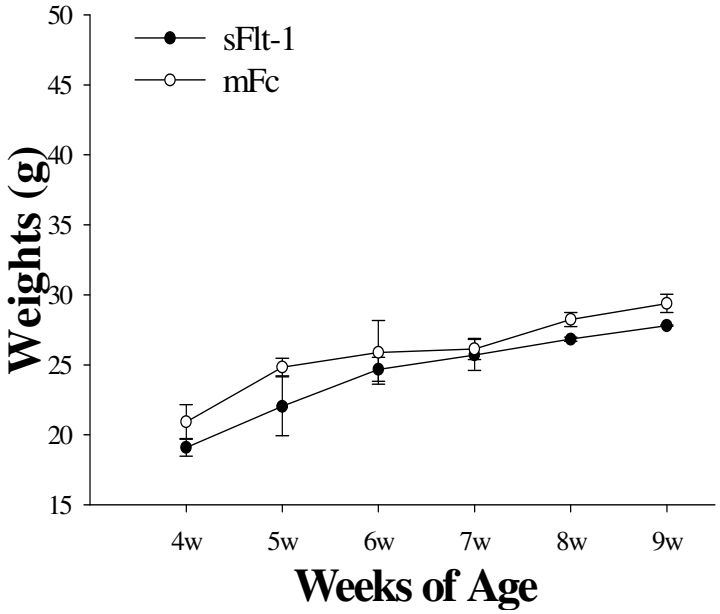


Figure 30b Post weaning weight of female offspring, at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1 (sFlt-1)(n=19) or adenovirus carrying mFc (mFc)(n=12). Data are shown as mean ± SEM.

Blood Pressure in male offspring

Mean blood pressure (BP) was significantly higher during the entire measurement period in male offspring born to sFlt-1-treated mothers (D1: 146.33±4.98 and D6: 136.54±2.17mmHg) compared with offspring born to mFc-treated mothers (D1: 120.76±2.88 and D6: 113.54±2.17mmHg) (Figure 31a). In addition, the average systolic BP and diastolic BP for the entire measurement period were significantly higher in the male offspring born to sFlt-1-treated mothers compared with control (Figure 31b and 31c). This difference between the 2 groups of offspring was maintained during daytime and nighttime measurements. BP was significantly higher in the male offspring born to sFlt-1 treated mothers compared with offspring born to mFc treated mothers both during

the daytime as well as nighttime periods. However, BP did not differ between day and night within each group (Figure 31d).

Figure 31 Blood Pressure in Male/Female Offspring

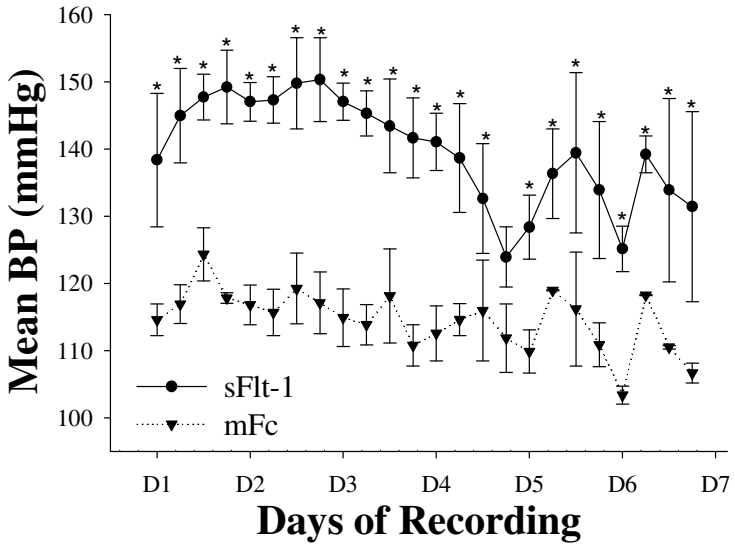


Figure 31a Mean blood pressure, averaged over 6-hour periods, in male offspring (n=4/each group) at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1 (sFlt-1)or adenovirus carrying mFc (mFc). Data are shown as mean ± SEM. The asterisk denotes a probability value of <0.05 for male offspring born to sFlt-1-treated pregnant mice vs male offspring born to mFc-treated pregnant mice.

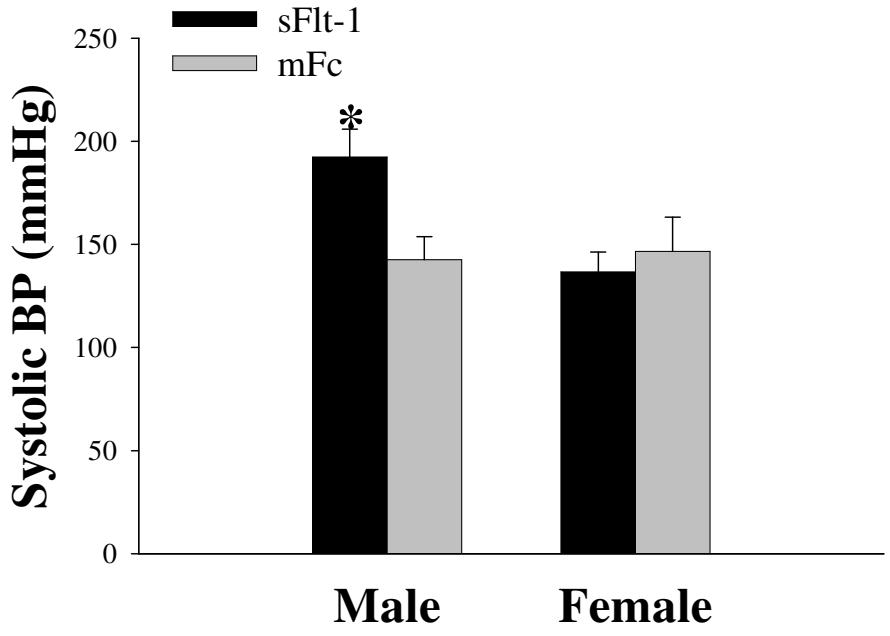


Figure 31b Systolic blood pressure, averaged over the entire measurement period, in male and female offspring, at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1(sFlt-1) or adenovirus carrying mFc (mFc). Data are shown as mean ± SEM. The asterisk denotes a probability value of <0.05 for male offspring born to sFlt-1-treated pregnant mice vs male offspring born to mFc-treated pregnant mice, female offspring born to sFlt-1 and mFc-treated mice.

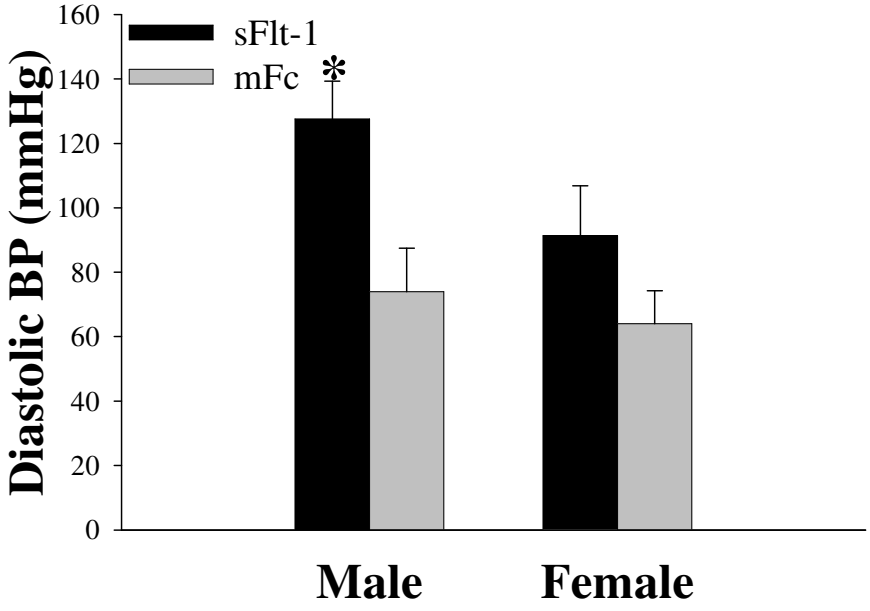


Figure 31c Diastolic blood pressure, averaged over the entire measurement period, in male offspring and female offspring, at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1(sFlt-1) or adenovirus carrying mFc (mFc). Data are shown as mean ± SEM. The asterisk denotes a probability value of <0.05 for male offspring born to sFlt-1-treated pregnant mice vs male offspring born to mFc-treated pregnant mice, female offspring born to sFlt-1 and mFc-treated mice.

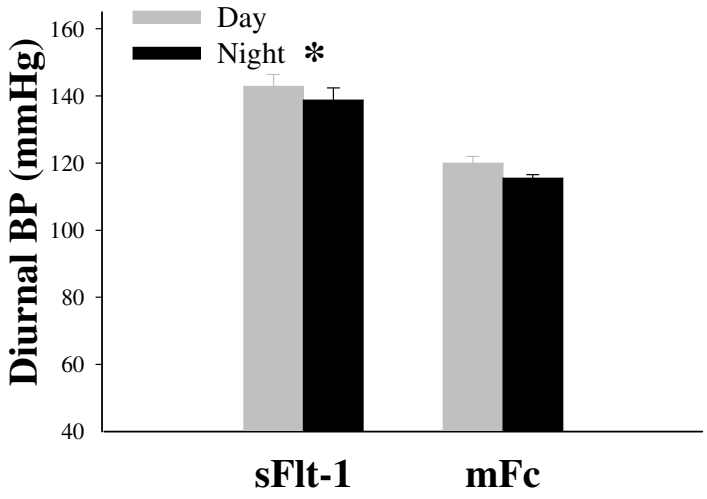


Figure 31d Mean blood pressures, averaged over the entire measurement period, in male offspring during daytime (shown in gray) and nighttime (shown in black) in both groups. Data are shown as mean ± SEM. The asterisk denotes a probability value of <0.05 for male offspring born to sFlt-1-treated pregnant mice vs male offspring born to mFc-treated pregnant mice.

Blood Pressure in female offspring

There were no significant differences in mean blood pressure, systolic or diastolic blood pressure in female offspring born to sFlt-1-treated mothers and mFc-treated mothers. This lack of difference was also evident during daytime and nighttime measurement (Figures 32a, 31b, 31c, 32b).

Figure 32 Blood Pressure in Female Offspring

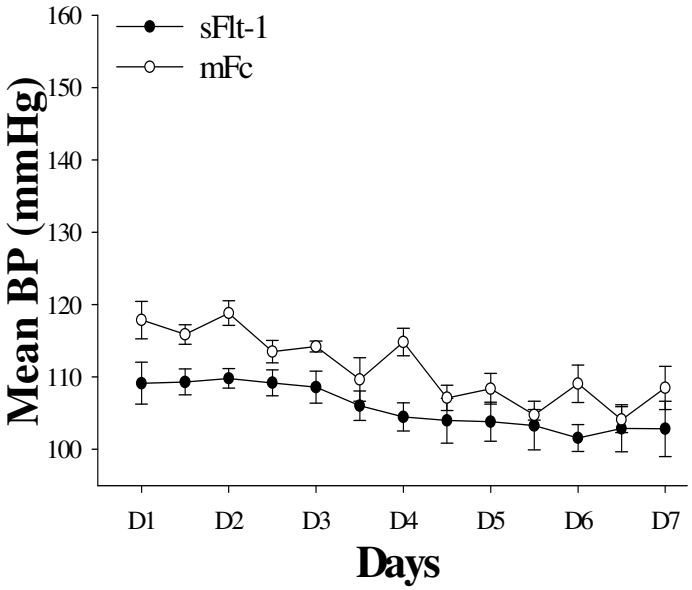


Figure 32a Mean blood pressure, averaged over 12-hour periods, in female offspring (n=5/each group), at 6 months of age, born to pregnant mothers treated with adenovirus carrying sFlt-1(sFlt-1) or adenovirus carrying mFc (mFc). Data are shown as mean ± SEM.

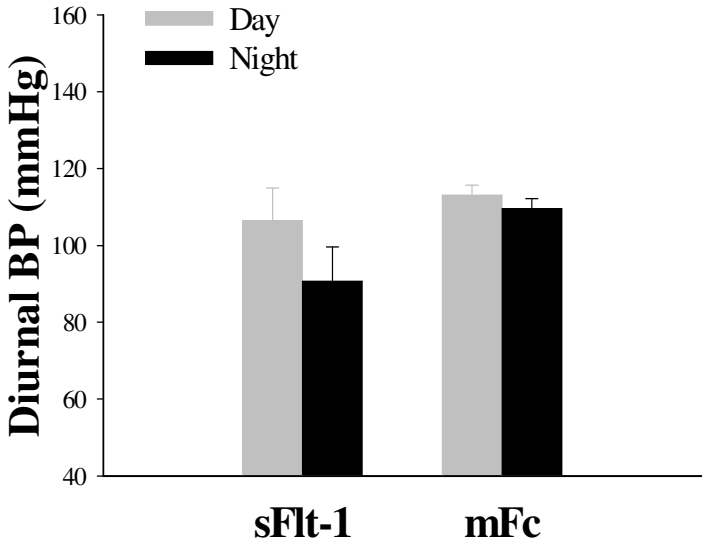


Figure 32b Mean blood pressure, averaged over the measurement period, in female offspring during daytime (shown in gray) and nighttime (shown in black) in both groups. Data are shown as mean ± SEM.

SPECIFIC AIM 4: CONCLUSION

We found that systolic, diastolic, and mean blood pressures of male offspring at 6 months of age born to pregnant mother injected with adenovirus carrying sFlt-1 were significantly higher than the similarly aged male offspring from mFc-treated pregnant mothers (adenovirus carrying the murine IgG2α Fc fragment as a control), and these differences were not present in female offspring at the same age born to sFlt-1-treated and mFc-treated mothers. There were no differences in plasma sFlt-1 levels in these offspring either born to sFlt-1-treated or mFc-treated mothers at three growth courses (at birth, 3 months of age and 6 months of age) (data not shown). In specific aim 1, we have shown that this animal model is characterized by over-expression of sFlt-1 and

hypertension in the pregnant animal, as well as growth restriction of the pups. The hypertension in the male offspring in association with altered post-weaning growth, and its absence in the female offspring where post-weaning growth is normal, support the developmental origin of the hypertension. Our results also confirm a gender-sensitive developmental programming of blood pressure in this particular animal model of adverse uterine environment.

CHAPTER 4: DISCUSSION

We had hypothesized that abnormal intrauterine environment caused by altered utero-placenta perfusion leads to preeclampsia and altered fetal vascular programming. This hypothesis was tested in a mouse model of preeclampsia induced by sFlt-1, a potential antagonist VEGF and PlGF. The purpose of this project was to elucidate the mechanism induced by sFlt-1 leading to preeclampsia and its effect on the uterine environment and fetal vascular development.

SFlt-1 and animal model of preeclampsia

We have shown that transfection of pregnant mice with adenovirus carrying sFlt-1 leads to an increase in circulating levels of sFlt-1 and a decrease in free VEGF level, causing hypertension and limited fetal and placental growth, as well as hepatic, renal, and hematologic changes at late gestation. We also detected proteinaceous deposition in collecting tubules of kidney from sFlt-1-treated pregnant mice at term by histological examination. The reports in humans that perturbation in VEGF and PlGF levels weeks before the clinical onset of preeclampsia point to abnormal angiogenesis as a possible mechanism, but cannot differentiate between a cause and effect (Taylor, 2003; Levine, 2004). However, our results, coupled with the prior report in the rat by Maynard and colleagues, strongly support an etiological role for sFlt-1 in preeclampsia, either primary or intermediary (Maynard, 2003).

The search for a suitable animal model of preeclampsia has been ongoing for decades. Since preeclampsia is a condition observed only in human pregnancy with an exception of patas monkey (Palmer, 1979), the efforts to create a model of preeclampsia have been hampered by a number of limitations, particularly the confounding effects of

the interventions and manipulations required in these animal models. This includes a number of factors such as the handling of the animals, the route of administration of any agent, and the methods used to measure the different variables, mainly blood pressure. Administration of any agents by the oral route is affected by the amount of food or water intake and by gastrointestinal motility or absorption, all of which may vary tremendously during pregnancy or in response to the agent used. The decreased intake of drinking water containing L-arginine analogues is one such example (Greenberg, 1997). Long-term intravenous catheters for infusion of drugs, blood drawing or intravascular pressure measurements may require restraining or tethering the animal, a potential source of artifacts. Measuring blood pressure is another challenge. The effect of stress in the animals caused by all the required manipulation cannot be over-emphasized and has been clearly shown to result in changes which could mimic preeclampsia (Kanayama, 1997). Using telemetric blood pressure monitoring similar to what was used in the current study, hypertension was not observed in a previously reported animal model of preeclampsia induced by inhibition of nitric oxide synthase (Buhimschi, 1999). In fact, the blood pressure in the pregnant rats treated with the nitric oxide synthase inhibitor L-NAME increased initially after treatment and then returned to normal levels, as well as demonstrated the normal decrease in blood pressure closer to term. Prior studies had used restrained or anesthetized animals, and/or measured blood pressure sporadically. These findings underscored the need for continuous monitoring of blood pressure in the unrestrained and conscious animal.

Recently, more and more work is being done in the field of pathogenesis of preeclampsia, with emphasis on the role of angiogenic factor as potential mediators of the clinical syndromes of preeclampsia. High level of circulating sFlt-1 was the first

abnormality detected in the later onset preeclamptic patients (Maynard, 2003). There are the overwhelming evidences to support that disrupting VEGF/PlGF signaling induced by excess sFlt-1 could mediate endothelial dysfunction and lead to altered placental vascular development. Therefore, the use of over-expression of sFlt-1 to induce a preeclampsia-like condition has biological plausibility. The finding of a disturbance in circulating angiogenic factors preceded the clinical onset of preeclampsia, and is more convincing than prior evidence relating to other mechanisms where the data were obtained at the time of disease. We would also like to focus on the inhibition of angiogenesis as a method to alter placental vascular function. Maynard et al established a preeclampsia model in pregnant rats by administrating adenovirus carrying sFlt-1 at day 8 of gestation (Maynard, 2003), this study, however, was limited by small numbers, evaluation of a single time point in gestation, and measurements in anesthetized and restrained animals. In our project, we measured blood pressure continuously for 7 days in the unrestrained conscious pregnant mice after administration of single dose of adenovirus carrying sFlt-1. This new model more closely resembles the clinical situation of hypertension in pregnancy and minimizes manipulation and should be a valuable addition to the tools available to the quest to uncover the mystery of preeclampsia.

SFlt-1 and altered vascular function

Normal pregnancy is associated with extensive anatomical and functional changes of maternal cardiovascular system, all geared toward accommodating the demands of pregnancy and the growing fetus. Preeclampsia, in contrast, is characterized by abnormal vascular remodeling which results in endothelial dysfunction, abnormal placental perfusion and disturbed cardiovascular adaptations.

Our previous findings in hypothesis 1 have shown a drop in blood pressure in the control (mFc) group in late pregnancy; while an increase in BP was seen in the in sFlt-1-overexpressing animals group (Lu, 2007). Since growing evidences support that sFlt-1 may be a mediator inducing maternal endothelial dysfunction and hypertension in preeclampsia, we investigated the contractile and relaxant properties of the carotid arteries which represent conduit vessels and control blood flow to the brain. We also studied uterine arteries which represent resistant vessels and control the utero-placenta perfusion.

Normal pregnancy is characterized by the attenuated responses to vasoconstrictor agents namely, phenylephrine and Angiotensin II in many species including human, ewe, sheep and ovine (Gant NF, 1973; Magness, 1986; Magness, 1988; Birds, 1997). However, these contractile responses are exaggerated in pregnancies complicated by preeclampsia (Lindheimer, 1992; Ariza, 2007; Verlohren, 2008). Our findings in sFlt-1-induced animal model of preeclampsia also demonstrated significantly increased contractile responses to phenylephrine in carotid arteries in sFlt-1-treated mice at term when compared with mFc control at term; An significant increase in the phenylephrine contractile response toward the end of pregnancy was also found in uterine arteries of sFlt-1-treated mice compared with controls; The vascular reactivities to phenylephrine in carotid and uterine arteries from control pregnant mice were significantly increased and became similar to sFlt-1-treated group in the presence of L-NAME. Therefore, we have concluded that the increased vascular reactivity to vasoconstrictors in preeclampsia probably results from the decreased endothelium-dependent vascular relaxation and /or enhanced vascular smooth muscle contraction (Khalil, 2002). The lack of a difference in KCl response among the study groups supports the notion that there is no gross structural

change in both the carotid arteries and uterine arteries in these sFlt-1 and mFc-treated pregnant animals.

Recently, growing evidence implicate the renin-angiotensin system (RAS) in the pathogenesis of preeclampsia (Ariza, 2007; Shah, 2007). It has been suggested that vascular mal-adaptation in preeclampsia to some extent results from an increased level renin and Ang II and increased sensitivity to Ang II (Chesley, 1965). However, only transient and small contractile response to Ang II was determined in our model (data not shown). We speculated that this unexpected finding was probably mouse CD1 species-specific.

Responses to the endothelium-dependent vasorelaxant ACh were not significantly different in sFlt-1-treated pregnant mice at mid-term and at term in carotid arteries. However, as expected, the impaired endothelium-dependent vasorelaxation mediated by ACh in uterine arteries was demonstrated in this model. Although we did not examine the uterine blood flow by Doppler, we speculate that there is a decrease in utero-placental perfusion in sFlt-1-treated mice, which in turn is probably responsible for creating the deleterious intrauterine environment in this animal model. These inconsistent vaso-reactivity results have also been identified in other animal models of preeclampsia (Podjarny, 1999; Bobadilla, 2001; Martinez-Orgado, 2004). The preeclampsia-like condition induced in rats by reduction of utero-placental perfusion pressure (RUPP model) was associated with a normal vasorelaxation to ACh in the mesenteric arteries (Anderson, 2006). We speculate that the endothelium-dependent relaxation is model- or vascular bed-specific. The observation that relaxation in carotid and uterine arteries in response to the exogenous NO donor SNP is not significantly different in sFlt-1 versus

mFc animals, which suggests that endothelium-independent relaxation in response to NO is not impaired in sFlt-1-treated mice.

Besides producing the relaxation factor, the endothelium may produce contracting factors, such as endothelin I and thromboxaneA_2 (TxA_2). The latter is a potent vasoconstrictor with a strong platelet aggregation action. Increased biosynthesis of TxA_2 appears to correlate with severity of preeclampsia (Fitzgerald, 1990). We did not find a difference in the sensitivity to TxA_2 between sFlt-1-treated mice and control mice in either carotid arteries or uterine arteries.

The findings of an exaggerated response to phenylephrine and an impaired endothelium-dependent relaxation in sFlt-1 over-expressing pregnant mice are likely to be important contributors to the hypertension and other clinical parameters we previously reported in this animal model. The sFlt-1 model of preeclampsia is a valuable tool to study the etiology of this disease.

SFlt-1 and placental hypoxia

It is believed that the placental ischemia/hypoxia is centrally involved in the pathogenesis of preeclampsia. This stimulates excessive production of sFlt-1, leading to a binding of circulating VEGF and inhibition of its function. Maternal endothelium becomes dysfunctional when is deprived of VEGF, leading to the development of the clinical signs and symptoms of preeclampsia. Hence, the sFlt-1 could act as mediator linking hypoxic placenta and clinical syndrome of preeclampsia. Moreover, based on in vivo and in vitro models of human hypoxic placenta, elevated expression of sFlt-1 under hypoxic conditions is induced by a cellular pathway which is mediated by HIF-1α (Nevo, 2006).

Recently, a differing view comes up to question that placenta hypoxia is the main stimulus to release sFlt-1 and emphasizes the placental oxidative stress in the pathogenesis of preeclampsia (Redman, 2009). There are two main hypotheses to explain how pathology seen in preeclamptic placenta arises. The first is that a reduced utero-placental perfusion resulting from inadequate remodeling of the spiral arteries (Kaufmann, 2003) leads to a hypoxic placenta. The second is that intermittent placental perfusion due to the incompletely remodeled spiral arteries leads to the fluctuations in the oxygen tension within the placenta and provides basis for a hypoxia-reoxygenation type insult, creating the oxidative stress condition and reactive oxygen species (Hung, 2006). The latter view also points out that inflammatory mechanisms may contribute or even predominate the release of sFlt-1 from placenta without hypoxia, for example, sFlt-1 release can be stimulated by TNFα from cultured placental explants in a dose-response manner (Ahmad, 2004). Other pro-inflammatory products of syncytiotrophoblast, namely, inhibin A, activin A, and leptin, together with sFlt-1 and soluble endoglin (sEng) may also contribute to the maternal inflammatory stress, leading to the clinical signs of preeclampsia.

Although the excess sFlt-1 production is a consequence of the placental hypoxia, supported by in vitro and in vivo studies (Nagamatsu, 2004; Makris, 2007), the relationship between placental hypoxia and sFlt-1 production is still unclear - is it a cause or a consequence of preeclampsia? Our study provided the evidence that an increase in sFlt-1 levels during pregnancy was a primary event leading to the abnormal placentation and hypoxic placenta. The etiology of increased sFlt-1 level in preeclampsia remains unknown, and inflammatory factors may contribute to the increased level of sFlt-1 as mentioned above.

SFlt-1 and renal histopathology

In preeclampsia, the characteristic lesion in kidney is glomerular endotheliosis, which is demonstrated by the loss of glomerular endothelial cell fenestrae and glomerular endothelial swelling and proteinuria (Ballermann, 2007; Stillman, 2007). It has been known that VEGF plays an important role in the glomerular endothelial cell fenestrae formation and the reduced VEGF production by 50% in a mouse glomerulus could lead to glomerular endotheliosis and the loss of glomerular endothelial fenestrae (Eremina, 2003). Therefore, inhibitions of VEGF by sFlt-1 or neutralizing VEGF antibodies could result in glomerular injury (Sugimoto, 2003; Karumanchi, 2006), which was confirmed by our study. Proteinuria is a characteristic clinical manifestation of preeclampsia which differentiates preeclampsia from gestational hypertension. However, the mechanism for proteinuria in preeclampsia is not well understood. It might be due to the loss of charge selectivity in the glomerular filtration barrier (Moran, 2003; Hladunewich, 2007) or due to the defect in the glomerular filtration barrier caused by high level of circulating angiogenic factors (Hladunewich, 2007).

A variety of methods have been used to examine the quantity of proteins in urine. The methods include ELISA, colorimetric and proteomics. Proteomic profiling of urine from preeclamptic pregnant women detected specific fragments of SERPINA1 (SERine Protease Inhibitor A1) and albumin (Buhimschi, 2008). The urine excretion of orosomucoid and albumin were increased in preeclamptic women as determined by sandwich ELISA (Kronborg, 2007). Because of difficulty in collecting the mouse urine, we were unable to complete investigations into establishing the urine proteins levels in sFlt-1 treated pregnant mice and control mice. However, the detection of proteinaceous

deposits in renal tubules in sFlt-1-treated mice by histological examination provides indirect evidence of glomeruli injury in the animal model of sFlt-1-induced preeclampsia.

SFlt-1 and fetal vascular programming

SFlt-1, as an inhibitor of VEGF and PlGF, leads to inadequate vascular adaptations during pregnancy, alteration in the circulation at the utero-placental interface and consequently poor perfusion of the placenta-fetal unit, these mal-adaptations lead to fetal hypoxemia and reduction in fetal perfusion, which can convey long lasting physiological and structural alterations that predispose the fetus to diseases in later life.

Our results strongly support the above hypothesis that the elevated levels of sFlt-1 leads to an altered fetal vascular programming manifesting as hypertension in the offspring in adult life. Moreover, the increased blood pressure levels only occurred in male offspring born to pregnant mother treated with adenovirus carrying sFlt-1, which suggested gender sensitivity to fetal programming. These findings are consistent with prior studies of altered uterine environment induced by manipulation of maternal diet in which fetal vascular programming and hypertension later in life were more pronounced in male versus female offspring (Kwong, 2000; Ozaki, 2001). Kwong et al. found increased systolic blood pressure and abnormal organ/body mass ratios in male rat offspring born to pregnant mothers on a low protein diet regimen during the pre-implantation period of development. Thus they speculated that male pre-implantation embryos have a greater susceptibility to respond to the maternal environment and lower possibility to adapt and may consequently exhibit increased sensitivity to specific fetal programming effects. Gender-related hypertension was also demonstrated in another rat model in which dietary intake was manipulated to induce maternal under-nutrition. In this model, increased systolic, diastolic and mean arterial blood pressures were more pronounced in male rat

offspring, and these abnormalities increased with age (Ozaki, 2001). In addition, prenatal glucocorticoids can program adulthood cardiovascular and metabolic physiology in a gender-specific pattern (O'Regan, 2004; McMullen, 2005; Roghair, 2009). Interestingly, hepatic LDL-receptor-mediated programming of altered lipid metabolism and aortic vascular dysfunction was found in the female offspring of mothers fed a high saturated fatty acid, while male offspring had only dyslipidemia (Chechi, 2009). Although there are growing evidences to support gender sensitivity to fetal programming, the mechanisms are not really well understood. We speculate that it may be related to the differences in the hormonal status between male and female which can have the effect in relation to blood pressure and metabolism.

Overall our findings highlight the role of the intrauterine environment in the developmental origin of adult vascular disease using a mice model over-expressing sFlt-1 in the mother during pregnancy. This mechanism leads to altered placental perfusion and consequently fetal vascular programming and onset of hypertension in the male offspring later in life. In addition, it demonstrates the role played by the gender in the fetal programming of adult hypertension later in life.

Conclusions

Recent work suggests that angiogenic imbalance plays an important role in the pathogenesis of preeclampsia. In order to study the underlying mechanisms of the development of clinical signs and symptoms of preeclampsia, animal models are needed. In this study we present an animal model that provides direct evidence in support of the hypothesis that altered angiogenesis induced by adenovirus carrying sFlt-1 leads to preeclampsia-like syndrome. Altered vascular functions in central vascular system represented by carotid arteries and peripheral vascular system represent by uterine

arteries, together with hypoxic placenta contribute to the development of maternal clinical syndrome of preeclampsia.

Because altered angiogenesis results in the abnormal utero-placental adaptation which created a harmful intrauterine environment for the growing fetus, our model can also be used as an animal model of fetal programming to explore the role of in utero programming in the development of adult disease. We also demonstrated the gender-specific fetal vascular programming in this animal model. Additional studies are needed to further elucidate the mechanisms of gender-specific fetal vascular programming in-utero and in later life. All these studies can improve our understanding of the pathogenesis of preeclampsia and the in utero fetal programming, and can guide the intervention strategies aimed at preventing chronic adult diseases, which originates during fetal development.

Appendix: List of Abbreviations

sFlt-1	Soluble fms-like tyrosine kinase
sEng	Soluble Endoglin
VEGF	Vascular endothelium growth factor
PlGF	Placental growth factor
HIF1α	Hypoxic-inducible factor 1alpha
GCM1	Glial cells missing1
TGFβ3	Transforming growth factor-beta 3
TNFα	Tumor necrosis factor alpha
PE	phenylephrine
ACh	acetylcholine
SNP	Sodium nitroprusside
TxA2	thromoboxane
L-NAME	Nω-nitro-L-arginine methyl ester
VSM	Vascular Smooth Muscle
IP3	inositol 1,4,5-triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
RT-PCR	reverse transcription polymerase chain reaction
NO	Nitric oxide
NOS	Nitric oxide synthase
MLCK	Myosin light chain kinase
EDHF	Endothelium-derived hyperpolarizing factor

PSS	physiological salt solution
Emax	Maximal Effect
AUC	Area Under Curve
AST	Alanine Transaminase
ALT	Aspartate Transaminase
LDH	Lactate Dehydrogenase
mFc	Murine IgG2α Fc fragment

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PULICATIONS:

1. **Lu F**, Bytautiene E, Tamayo E, Gamble P, Anderson GD, Hankins GD, Longo M, Saade GR Gender-specific effect of over-expression of sFlt-1 in pregnant mice on fetal programming of blood pressure in the offspring later in life. *Am J Obstet Gynecol.* 2007 Oct; 197(4):418.e1-5.

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ABSTRACTS:
Oral Presentations:

1. **Lu F**, Goharkhay N, Tamayo E, Hankins GD, Longo M, Saade GR In vitro vascular reactivity in a mouse model of preeclampsia induced by over-expression of sFlt-1 *Program of The Central Association of Obstetricians and Gynecologists 75th annual meeting 2008*
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