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# Adenoviral Delivery of VEGF<sub>121</sub> Early in Pregnancy Prevents Spontaneous Development of Preeclampsia in BPH/5 Mice

Ashley K. Woods, Darren S. Hoffmann, Christine J. Weydert, Scott D. Butler, Yi Zhou, Ram V. Sharma, Robin L. Davisson

**Abstract**—An imbalance in circulating proangiogenic and antiangiogenic factors is postulated to play a causal role in preeclampsia (PE). We have described an inbred mouse strain, BPH/5, which spontaneously develops a PE-like syndrome including late-gestational hypertension, proteinuria, and poor fetoplacental outcomes. Here we tested the hypothesis that an angiogenic imbalance during pregnancy in BPH/5 mice leads to the development of PE-like phenotypes in this model. Similar to clinical findings, plasma from pregnant BPH/5 showed reduced levels of free vascular endothelial growth factor (VEGF) and placental growth factor (PGF) compared to C57BL/6 controls. This was paralleled by a marked decrease in VEGF protein and *Pgf* mRNA in BPH/5 placentae. Surprisingly, antagonism by the soluble form of the FLT1 receptor (sFLT1) did not appear to be the cause of this reduction, as sFLT1 levels were unchanged or even reduced in BPH/5 compared to controls. Adenoviral-mediated delivery of VEGF<sub>121</sub> (Ad-VEGF) via tail vein at embryonic day 7.5 normalized both the plasma-free VEGF levels in BPH/5 and restored the *in vitro* angiogenic capacity of serum from these mice. Ad-VEGF also reduced the incidence of fetal resorptions and prevented the late-gestational spike in blood pressure and proteinuria observed in BPH/5. These data underscore the importance of dysregulation of angiogenic factors in the pathogenesis of PE and suggest the potential utility of early proangiogenic therapies in treating this disease. (*Hypertension*. 2011;57:94-102.) • **Online Data Supplement**

**Key Words:** hypertension ■ proteinuria ■ PGF ■ sFLT1 ■ angiogenesis ■ placenta

Preeclampsia (PE) is a pregnancy-specific syndrome defined by sudden onset of hypertension and proteinuria after 20 weeks gestation. A multisystem disorder that impacts both mother and fetus, PE is a major public health problem. Worldwide, PE affects 5% to 8% of pregnancies and is the leading cause of maternal and fetal deaths.<sup>1,2</sup> In developing countries, the incidence of PE is even higher.<sup>3</sup> Despite its common occurrence and serious consequences, treatment of PE has not changed over the last century. Even today, the only known effective means to avoid progression to eclampsia is delivery of the fetus and placenta. As such, PE accounts for up to 20% of preterm births worldwide.<sup>3</sup>

The etiology of PE remains unclear; however, there is growing evidence that an imbalance in several members of the vascular endothelial growth factor (VEGF) family and its receptors is linked to the clinical syndrome.<sup>1,4</sup> VEGF-A (VEGF), critical to angiogenesis and vasculogenesis required for placentation, is present in numerous isoforms.<sup>5</sup> VEGF<sub>121</sub>, the predominant isoform, lacks a membrane-bound motif, making it freely diffusible and therefore having the greatest

therapeutic potential.<sup>5</sup> The closely related placental growth factor (PGF) shares 42% sequence homology to VEGF and shares a common receptor, VEGFR-1 (ie, Fms-like tyrosine kinase 1 [FLT1]).<sup>6,7</sup> Many women who develop PE show decreases in circulating free VEGF and PGF starting in early gestation to midgestation.<sup>1,2,8</sup> In addition, high levels of the soluble form of the FLT1 receptor (sFLT1) and soluble endoglin (sENG), both antiangiogenic factors, are also reported in PE patients at various times during pregnancy.<sup>9–11</sup> On the other hand, increased serum sFLT1 levels are not always observed in women with PE,<sup>12</sup> and a recent study showed that not only were elevated sFLT1 levels at 11 to 14 weeks not predictive of PE in women but were, in fact, associated with reduced risk for delivery of a small-for-gestational-age baby.<sup>13</sup>

Despite discrepancies in clinical findings, significant progress in basic research has been made to support the hypothesis that an imbalance between proangiogenic and antiangiogenic factors is involved in the pathogenesis of PE. Maynard et al<sup>9</sup> found that adenoviral-mediated increases in

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circulating sFLT1 levels in normal pregnant rats induced a PE-like syndrome. A similar study in mice confirmed that exogenous delivery of sFLT1 during pregnancy can induce late-gestational hypertension.<sup>14</sup> To determine whether the effects of exogenous sFLT1 could be counterbalanced by increasing proangiogenic factors, Li et al<sup>15</sup> used repeated subcutaneous injections of VEGF<sub>121</sub> and found that this attenuated PE symptoms in pregnant rats previously made preeclamptic by an adenovirus encoding sFLT1. A similar strategy was adopted by Gilbert et al<sup>16</sup> in the context of a rat model in which the uteroplacental circulation is disrupted at midgestation, and increased circulating levels of sFLT1 late in pregnancy were observed. These studies provide important proof of concept that an angiogenic imbalance can induce some aspects of PE; however, the experimental design is somewhat predictive of the outcome. Testing whether there is endogenous angiogenic imbalance in an animal model that spontaneously develops PE is an important next step.

Several years ago, we described an inbred murine strain, BPH/5, which spontaneously develops a maternal and fetoplacental syndrome that bears striking resemblance to PE.<sup>17</sup> The model is characterized by late-gestational hypertension, proteinuria, renal glomerular lesions, and endothelial dysfunction.<sup>17,18</sup> In addition to the maternal systemic disorder, BPH/5 also show fetoplacental defects reminiscent of human PE, including defective trophoblast invasion of the maternal decidua and diminished remodeling of maternal spiral arteries.<sup>19</sup> This translates into dramatic decreases in end-diastolic blood flow in uterine arteries,<sup>19</sup> taken clinically to indicate placental vascular insufficiency.<sup>20</sup> BPH/5 also exhibit impaired endothelial cell branching and development of the fetal labyrinth, which is associated with fetal growth restriction, intrauterine fetal demise, and small litters of low-birth-weight pups.<sup>17–19</sup>

Based on our findings of fetoplacental defects indicative of poor angiogenesis/vasculogenesis in the BPH/5 model, together with clinical evidence of an angiogenic imbalance in women with PE, we hypothesized that BPH/5 would exhibit altered levels of endogenous proangiogenic and antiangiogenic factors during pregnancy, and this would translate into a maternal and fetoplacental syndrome that resembles human PE. Here we demonstrate that an angiogenic imbalance is present in BPH/5 mice during early gestation to midgestation, and this is due to decreased levels of proangiogenic VEGF and PGF but not increased levels of antiangiogenic sFLT1. Adenovirus-mediated delivery of VEGF<sub>121</sub> early in pregnancy (embryonic day 7.5 [e7.5]) in BPH/5 normalized VEGF levels, reduced fetal resorptions, and prevented spontaneous development of late-gestational hypertension and proteinuria in this model. These data underscore the importance of angiogenic imbalance in the pathogenesis of PE and further suggest the potential utility of early proangiogenic therapies in treating this disease.

## Methods

An expanded Methods section is available in the online Data Supplement available at <http://hyper.ahajournals.org>.

## Animals and Husbandry

Experiments were performed in 8- to 12-week-old BPH/5 and control C57BL/6 (C57) mice obtained from in-house colonies.<sup>17</sup> Mice underwent strain-matched mating, and presence of vaginal plug was defined as e0.5.<sup>17</sup> Gestational stages were defined as early (e9.5 to e12.5), middle (e13.5 to e15.5), and late (e18.5 to e19.5). All procedures were approved by the Institutional Animal Care and Use Committees at The University of Iowa and Cornell University.

## Adenoviruses

Adenoviral vectors encoding VEGF<sub>121</sub> (Ad-VEGF) or a control gene,  $\beta$ -galactosidase (Ad-LacZ), were prepared by The University of Iowa Gene Transfer Vector Core. Viruses were injected via tail vein on e7.5 (100  $\mu$ L, 10<sup>9</sup> PFU).

## Enzyme-Linked Immunosorbent Assays

Blood was collected from nonpregnant (NP), early, and midgestation BPH/5 and C57 females via cardiac puncture. ELISAs for VEGF, PGF, sFLT1, and sENG were performed according to the manufacturer's instructions.

## Quantitative Real-Time Polymerase Chain Reaction

Placental tissue was collected at various gestational time points, total RNA was harvested, and cDNA was generated. Templates were subjected in triplicate to real-time polymerase chain reaction (PCR) using Sybr Green and target-specific primers for Vegf, Pgf, and sFlt-1.

## Western Analysis

VEGF protein was measured by Western blot performed on placental lysates subjected to SDS-PAGE. Samples were incubated with goat polyclonal anti-VEGF (1:500; Sigma-Aldrich), followed by donkey anti-goat IgG peroxidase (1:10; Santa Cruz Biotechnology), and subjected to chemiluminescence.

## In Vitro Angiogenesis Assay

The in vitro angiogenesis assay was performed in human umbilical vein endothelial cells (HUVECs) utilizing a kit (Trevigen) according to the manufacturer's instructions. Total tube lengths per well were measured using ImageJ software. Data were normalized to the fetal bovine serum control.

## Radiotelemetric Measurement of Blood Pressure

NP females were implanted with radiotelemeters and allowed 1-week recovery as described.<sup>21</sup> Blood pressure (BP) was recorded for 3 days prior to strain-matched mating and then continuously following vaginal plug detection (e0.5) and through the postpartum period.<sup>17,18</sup> Titer-matched Ad-VEGF or Ad-LacZ was injected via tail vein on e7.5.

## Urine Protein Analysis

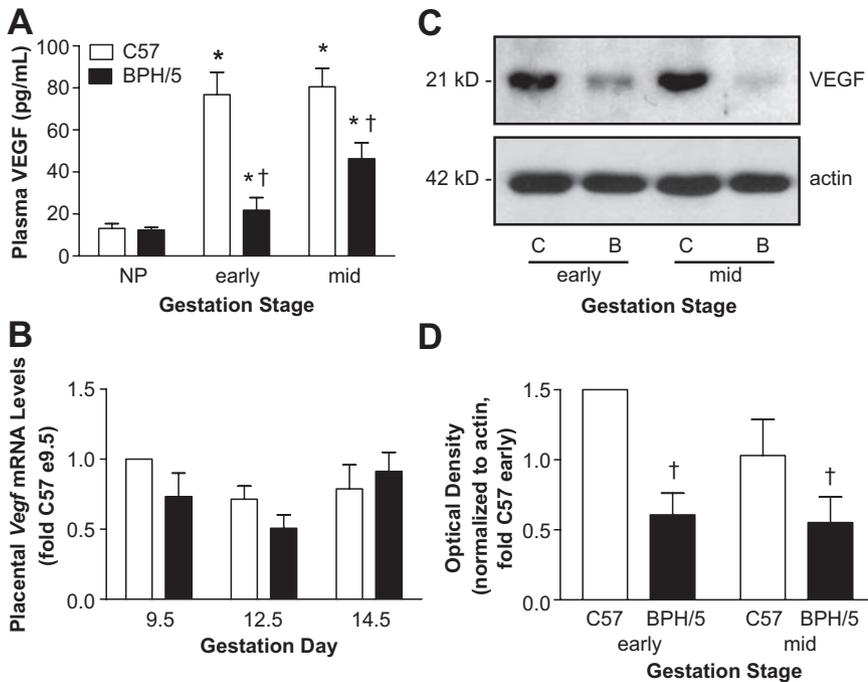
Urine samples from mice treated with viruses on e7.5 were collected at various gestational time points and frozen at  $-20^{\circ}\text{C}$  until Bradford analysis, as described.<sup>17,18</sup>

## Analysis of Pregnancy Outcomes

Mice treated with viruses on e7.5 were euthanized at midgestation, uterine horns were exposed, and fetuses were counted. Fetal resorptions were identified by necrotic/hemorrhagic appearance.<sup>17,18</sup> In 3 additional cohorts of virus-treated females, placental weights, fetal weights, and numbers of live pups born were recorded.

## Statistical Analysis

Data are expressed as mean  $\pm$  SEM. ANOVA followed by the Newman-Keuls test was performed for all data sets.  $P < 0.05$  was considered statistically significant.



**Figure 1.** Circulating and placental VEGF levels are decreased in BPH/5. A, Summary of circulating free VEGF as determined by ELISA in NP and at early gestation and midgestation C57 and BPH/5 (n=4 to 6 per group). B, Summary of real-time quantitative PCR analysis of placental *Vegf* mRNA levels at different days of gestation (n=5 per group). Data are expressed relative to C57 at e9.5. C, Representative Western blot of VEGF protein levels in placenta samples from C57 (C) and BPH/5 (B) at early gestation and midgestation.  $\beta$ -Actin served as the loading control. D, Summary of Western blot quantification (n=3 per group at each time point) normalized to actin and expressed relative to C57 early. \* $P < 0.05$  vs NP in matched strain; † $P < 0.05$  vs time-matched C57.

**Results**

**Angiogenic Imbalance in Pregnant BPH/5 is Due to Decreased Proangiogenic Factors**

Free VEGF levels were compared in BPH/5 and C57 plasma from early gestation and midgestation. We focused on these time points since clinical data point to early-gestational or midgestational decreases in VEGF in women with PE.<sup>1,8</sup> There was a marked increase in circulating free VEGF in C57 controls at both early gestation and midgestation compared to NP levels (Figure 1A). BPH/5 also showed pregnancy-induced increases in plasma VEGF, but this was significantly blunted at both time points compared to C57 (Figure 1A). Since the placenta is an important source of angiogenic factors during pregnancy,<sup>22</sup> we next compared *Vegf* mRNA levels in placental samples from C57 and BPH/5 at 3 time points. Summary data presented in Figure 1B show no differences in *Vegf* transcript levels between C57 and BPH/5 at any of the gestational time points examined. Since emerging evidence suggests that VEGF expression is regulated via microRNA (miRNA)-mediated repression of *Vegf* mRNA translation,<sup>23</sup> we next measured VEGF protein in placentae using Western analysis. As seen in Figure 1C and 1D, VEGF protein expression was significantly decreased in BPH/5 placentae at both early-gestational and midgestational time points compared to C57 controls, consistent with the plasma VEGF data.

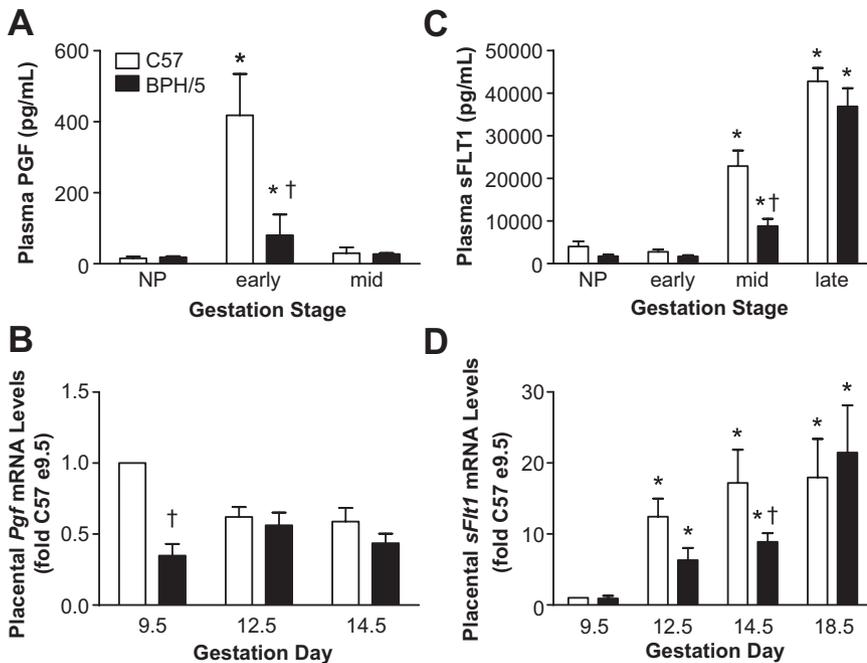
We next compared circulating free PGF in BPH/5 and C57 at early gestation and midgestation since decreased levels of this growth factor at these time points is associated with PE in women.<sup>8</sup> There was a marked early increase in plasma PGF levels in C57, and this returned to NP levels by midgestation (Figure 2A). Although BPH/5 showed a significant elevation in free PGF levels at early gestation compared to NP levels, this pregnancy-induced PGF increase was significantly reduced compared to C57 controls (Figure 2A). Since this

suggested that BPH/5 placentae produce only modest levels of PGF, we next examined *Pgf* transcript levels in this tissue using real-time PCR. As shown in Figure 2B, placental *Pgf* mRNA was reduced in BPH/5 compared to gestation-matched controls at e9.5 but not at other time points, reflecting the plasma PGF data.

Since a decrease in VEGF and PGF levels can be accompanied by an elevation in sFLT1 levels in women with PE,<sup>1,9</sup> next we compared the sFLT1 profile in BPH/5 and C57 at early, middle, and late gestation. Circulating sFLT1 levels were increased at midgestation and late gestation in both strains relative to NP levels; however, the increases in BPH/5 were either not different (late) or even decreased (mid) relative to C57 (Figure 2C). Since the placenta is the major source of sFLT1 during pregnancy,<sup>24</sup> we also analyzed placental *sFlt1* mRNA. Data in Figure 2D show that placental expression of *sFlt1* increased throughout pregnancy in C57 starting at e12.5, and although the temporal profile in BPH/5 mirrors that of C57, mRNA levels were either not altered or even decreased (e14.5) in BPH/5. Finally, since sENG has emerged clinically as a potentially important antiangiogenic factor in PE,<sup>11</sup> plasma sENG levels were compared in BPH/5 and C57 at early gestation and midgestation. As shown in Figure S1, sENG was modestly elevated in BPH/5 at e9.5 but not at the other time points examined.

**Adenoviral Delivery of VEGF<sub>121</sub> Restores Circulating Free VEGF Levels in BPH/5 and Rescues the Angiogenic Potential of Serum from Pregnant BPH/5**

Based on our findings of decreased proangiogenic factors in BPH/5, along with the promise of VEGF<sub>121</sub> as a therapeutic agent,<sup>5,15,16</sup> next we determined whether administration of an adenovirus encoding VEGF<sub>121</sub> (Ad-VEGF) early in pregnancy in BPH/5 would normalize circulating free VEGF



**Figure 2.** Proangiogenic PGF is down-regulated, whereas antiangiogenic sFLT1 is unchanged or even decreased in BPH/5. A, Summary of free plasma PGF levels as determined by ELISA in NP and at different gestational stages of C57 and BPH/5 (n=4 to 6 per group). B, Summary of *Pgf* mRNA expression from placental tissue at different gestational days (n=5 per group). Data are expressed relative to C57 at e9.5. C, Summary of circulating sFLT1 levels as determined by ELISA in NP and at different gestational stages of C57 and BPH/5 (n=4 to 6 per group). D, Summary of real-time quantitative PCR analysis of *sFlt1* from placental tissue of C57 and BPH/5 at different gestational days (n=5 per group). \**P*<0.05 vs NP in matched strain; †*P*<0.05 vs time-matched C57.

levels. We utilized an adenovirus rather than repeated daily injections of recombinant VEGF<sub>121</sub><sup>15,16</sup> to effect stable, long-term circulating levels of VEGF<sub>121</sub>.<sup>25</sup> As shown in Figure S2, tail vein injection of Ad-VEGF in NP C57 and BPH/5 females increased plasma VEGF levels more than 20-fold in both strains. Furthermore, Ad-VEGF injected at e7.5 increased circulating VEGF in BPH/5 to levels that were comparable to gestation-matched C57 controls at both early gestation and midgestation (Figure S2). Free VEGF levels of Ad-LacZ-treated mice of both strains were similar to those without viral treatments (Figure 1A), confirming that the viral vector itself had no effect on circulating free VEGF levels.

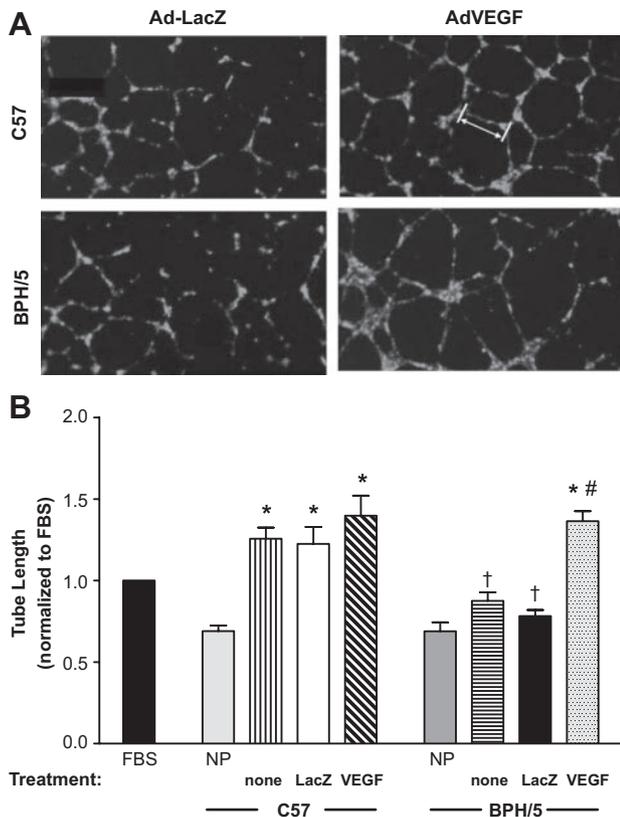
To confirm that the Ad-VEGF-induced elevation in circulating free VEGF leads to functionally active VEGF, we performed a well-established *in vitro* angiogenesis assay in HUVECs<sup>9</sup> using serum collected from NP or pregnant C57 and BPH/5 mice (e12.5) that had been treated with Ad-LacZ or Ad-VEGF (or no treatment) on e7.5. As seen in representative images and summary data in Figure 3, endothelial tube formation was significantly decreased with e12.5 serum from BPH/5 that had received no viral treatment or had been injected with the Ad-LacZ vector. Ad-VEGF treatment significantly enhanced tube formation elicited by BPH/5 serum compared to that of Ad-LacZ-treated controls, such that there were no differences in tube formation between serum of these mice and C57 controls (all treatments) (Figure 3). These results demonstrate that there was functional reconstitution of the angiogenic potential of serum from BPH/5 by systemic Ad-VEGF treatment.

**Ad-VEGF Therapy Early in Pregnancy Prevents the Hallmark Maternal PE Symptoms and Ameliorates Fetal Resorptions in BPH/5 Mice**

Next we tested the hypothesis that Ad-VEGF administered early in pregnancy in BPH/5 would ameliorate late-gestational hypertension and proteinuria in this model. NP

female BPH/5 and C57 mice were implanted with radiotelemeters for continuous measurement of BP before, during, and after pregnancy.<sup>17,18</sup> After a 1-week recovery, baseline BP was recorded for 3 days before timed strain-matched matings and the start of continuous BP measurements. On e7.5, BPH/5 and C57 mice underwent tail vein injections of Ad-VEGF or Ad-LacZ as above. As seen in Figure 4A and 4B, baseline mean arterial pressure (MAP) was significantly elevated in Ad-LacZ-treated BPH/5 compared to treatment-matched C57 (120±3 versus 95±4 mm Hg, *P*<0.05), consistent with our previous reports that BPH/5 have mildly elevated BP before pregnancy.<sup>17,18</sup> Importantly, neither viral vector altered baseline MAP in either strain (Figure 4A and 4B). However, pregnancy caused a late-gestational rise in MAP over baseline (days 16 to 20) in Ad-LacZ-treated BPH/5 mice, which is characteristic of this strain,<sup>17,18</sup> and this was prevented by Ad-VEGF therapy (Figure 4A). Notably, MAP returned to baseline following delivery in Ad-LacZ-treated BPH/5 mice, consistent with what we have shown previously<sup>17,18</sup> and with what occurs in women with PE.<sup>26</sup> MAP in C57 controls remained steady throughout pregnancy and was unaffected by either viral vector (Figure 4B).

In a separate cohort of C57 and BPH/5 mice that had undergone Ad-VEGF or Ad-LacZ injections at e7.5, 24-hour urine samples were collected at midgestation and late gestation and subjected to protein analysis. Proteinuria is detected only during late gestation in BPH/5 mice.<sup>17,18</sup> These data were confirmed here, wherein urinary protein levels were significantly elevated in Ad-LacZ-treated BPH/5 compared to treatment-matched C57 controls at late gestation but not midgestation (Figure 4C). Furthermore, similar to its effects on BP, Ad-VEGF administered early in pregnancy prevented development of proteinuria during late gestation in BPH/5 (Figure 4C). Ad-VEGF did not alter urinary protein levels in C57 mice at either gestational time (Figure 4C).



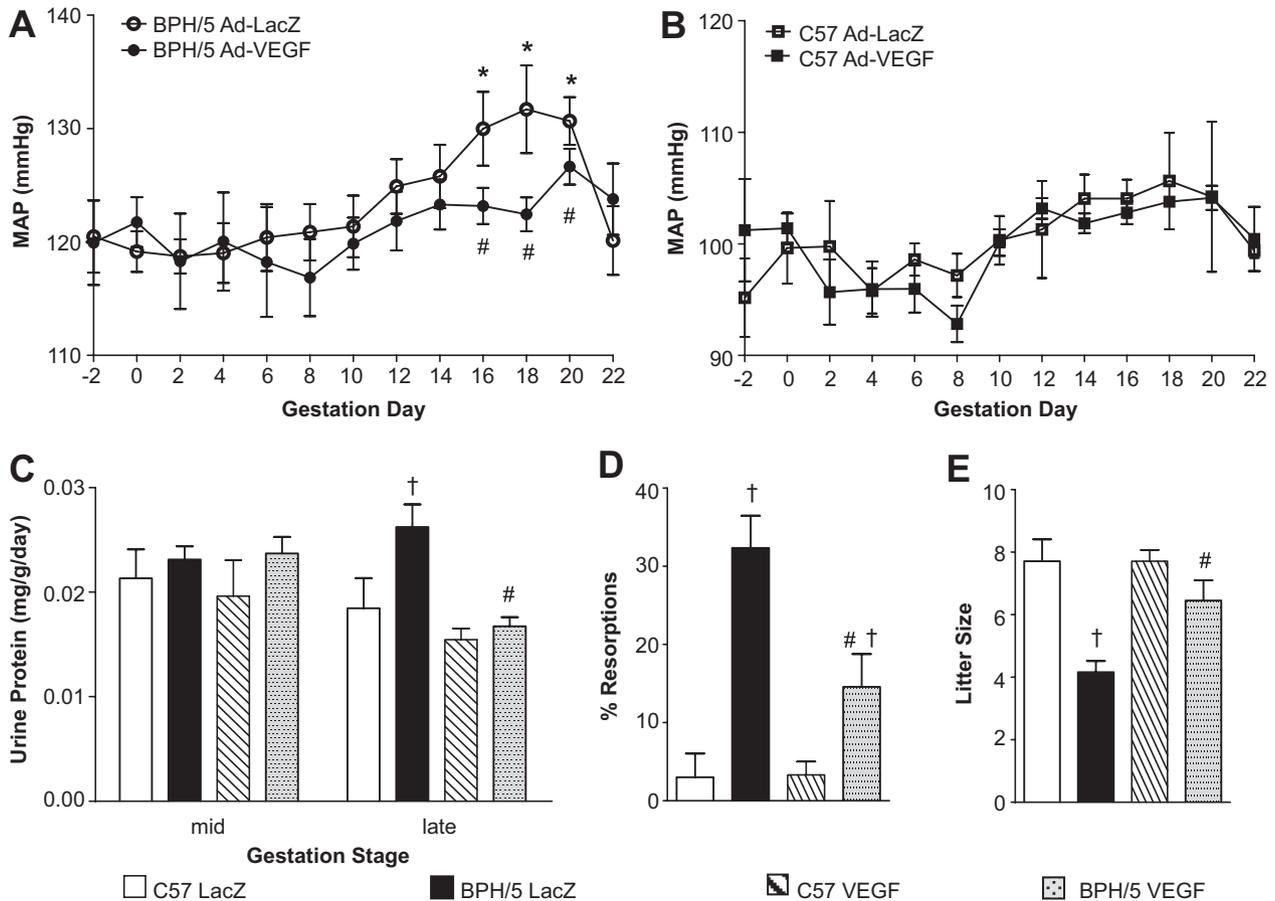
**Figure 3.** Ad-VEGF therapy rescues the angiogenic potential of BPH/5 serum as measured in an endothelial tube formation assay. A, Representative images of individual wells of HUVECs treated with serum collected from e12.5 C57 and BPH/5 that had undergone tail vein injections of Ad-LacZ or Ad-VEGF on e7.5. The white bar (top right) indicates a representative measurement of tube length. B, Summary of tube lengths normalized to 5% fetal bovine serum in cells treated with serum from NP mice or from e12.5 C57 and BPH/5 mice given no treatment (none) or administered Ad-LacZ or Ad-VEGF on e7.5. NP n=8 per strain; no treatment and Ad-LacZ n=5 to 8 per group and strain; Ad-VEGF n=5 to 12 per group and strain. \* $P < 0.05$  vs NP matched strain; † $P < 0.05$  vs C57 in matched treatment; # $P < 0.05$  vs BPH/5 none or Ad-LacZ.

To determine whether decreased VEGF production during early pregnancy is an important contributor to fetoplacental defects observed in BPH/5 mice,<sup>17,19</sup> we examined several fetoplacental outcomes in BPH/5 and C57 mice treated with Ad-VEGF or Ad-LacZ on e7.5 as above. Data presented in Figure 4D show that one-third of fetoplacental units in BPH/5 mice treated with the control vector were resorbed, whereas resorptions in Ad-LacZ-treated C57 mice were extremely rare. These results are consistent with our previous report of fetal resorption incidence in BPH/5<sup>17</sup> and confirm that systemic delivery of adenovirus itself does not elicit resorptions. Importantly, Ad-VEGF reduced this fetal demise observed in BPH/5 by  $\approx 50\%$  (Figure 4D), which resulted in a significant increase in term litter size compared to Ad-LacZ-treated mice (Figure 4E). On the other hand, VEGF therapy did not restore midgestational or late-gestational placental or fetal weights in BPH/5 (Table). It should be noted that in C57 mice, Ad-VEGF treatment had no significant effect on fetoplacental outcomes except a small reduction in fetal weight at e14.5 (Figure 4 and Table).

## Discussion

The angiogenic imbalance theory of PE, first posited by Karumanchi and colleagues, has gained significant traction over the years. Their landmark study in 2003 showed that the VEGF and PGF antagonist sFLT1 is upregulated in placentae of PE patients, and this is associated with increased circulating levels of sFLT1 and decreased free plasma VEGF and PGF.<sup>9</sup> A number of follow-up clinical studies supported these findings,<sup>1,10,27</sup> and an era began of determining whether these factors could be used as biomarkers and/or therapeutic targets in PE.<sup>27,28</sup> However, more recent clinical data<sup>13,29</sup> and retrospective reviews of clinical studies such as that by Widmer et al<sup>30</sup> reveal a much more complex picture. In parallel to the clinical studies, basic research in animal models has yielded important proof-of-concept support for this hypothesis.<sup>9,14,15</sup> However, the experiments have involved exogenous delivery or experimental induction of sFLT1 and subsequent injections of VEGF<sub>121</sub> to ameliorate sFLT1-mediated responses, which complicates interpretation about the endogenous role of these factors in PE. We used an animal model that spontaneously develops a PE-like syndrome to rigorously test the angiogenic imbalance hypothesis in the laboratory.

First, we found that the increase in plasma-free VEGF levels that occurs normally during early gestation and mid-gestation in murine (C57) pregnancies is significantly attenuated in BPH/5. This appears to be due, at least in part, to decreased translation of VEGF mRNA in the placenta. The decreased free VEGF levels observed in BPH/5 mice is consistent with human disease data<sup>9</sup>; however, our finding of diminished placental VEGF protein is in contrast with some reports in PE patients in which VEGF protein and total VEGF circulating levels may actually be higher than in normal pregnancies (but this is compromised by excess sFLT1).<sup>31</sup> Second, our results show that the normal pregnancy-induced increase in circulating free PGF in C57 is limited to early gestation, which mirrors the temporal expression pattern of this growth factor in murine placenta observed in this study and others.<sup>32</sup> Placental expression and plasma free PGF are also markedly blunted in BPH/5 pregnancies. Third, unlike the increased expression and circulating levels of sFLT1 that have been suggested as a causative factor in PE in women,<sup>1,9,10</sup> this antiangiogenic factor is either unchanged or even decreased in BPH/5 placentae and plasma relative to controls. This suggests that while there is marked angiogenic imbalance in the BPH/5 model, it may be due to decreased production of proangiogenic VEGF and PGF independent of sFLT1-mediated sequestration of these factors. However, it should be noted that sFLT1 is now known to have at least 4 isoforms, and the isoform that is most dominantly expressed in human PE placentae is not expressed in mice.<sup>33,34</sup> Fourth, sENG is modestly elevated transiently at early gestation in BPH/5, suggesting it is not a major contributor to the PE-like syndrome in BPH/5. Finally, we show that decreased expression of VEGF is functionally linked to the development of fetal demise and the maternal PE-like syndrome in BPH/5 mice since early (e7.5) systemic delivery of Ad-VEGF normalized circulating free VEGF, rescued the angiogenic capacity of serum from



**Figure 4.** Adenoviral-mediated delivery of VEGF<sub>121</sub> early in pregnancy prevents the hallmark maternal symptoms and rescues fetal demise in BPH/5. A, B, Summary of radiotelemetric measurements of MAP before, during, and after pregnancy in BPH/5 (A) and C57 (B) mice. Ad-VEGF or Ad-LacZ were injected by tail vein on e7.5; n=6 to 8 for each strain and treatment. C, Summary of urinary protein levels at midgestation and late gestation in BPH/5 and C57 mice that had undergone virus injections on e7.5; n=6 to 12 per group at each time point. D, Summary of incidence of fetal resorptions at e14.5 in C57 and BPH/5 mice that had undergone virus injections on e7.5; n=8 to 11 per group. E, Term litter sizes in each of the strains and virus treatment groups; n=11 to 18 litters per group. \* $P < 0.05$  vs baseline in matched strain; † $P < 0.05$  vs C57 in matched treatment; # $P < 0.05$  vs BPH/5 Ad-LacZ.

pregnant BPH/5, decreased fetal resorptions, and prevented the hallmark late-gestational hypertension and proteinuria observed in this model.

Although it is widely accepted that the placenta is the major source of angiogenic factors during pregnancy,<sup>19,22,32</sup> it has been difficult to directly link changes observed in plasma from PE patients to dysregulation of these genes in the placenta because of lack of availability of placental tissue from PE patients at early time points. However, a few studies have demonstrated that cytotrophoblasts from preeclamptic patients show lower staining of VEGF and its receptors, suggesting dysregulation of angiogenic genes in the placenta.<sup>31,35</sup> Our data highlight the possibility of posttranscriptional regulation of VEGF in the placenta because while there is a marked decrease in VEGF protein levels in BPH/5 placentae, mRNA levels of VEGF are not significantly changed at any time point compared to controls. Several mechanisms for posttranscriptional regulation of VEGF in other tissues have been postulated, including downregulation by miRNAs and translational regulation by cMyc.<sup>23</sup> In addition, hypoxia is known to stabilize *Vegf* mRNA,<sup>36</sup> and a stress-responsive “switch” in the 3' untranslated region of *Vegf* has been shown

to regulate its expression during hypoxia.<sup>37</sup> This is interesting since it has been postulated that PE is associated with premature breach of the trophoblast shell in early placental development and a subsequent burst of hyperoxia in the developing placental tissue.<sup>38</sup> Although decreased PGF in BPH/5 appears to be due to decreased placental transcription during early gestation, similar to VEGF, PGF regulation by miRNA-mediated repression of translation cannot be ruled out. Our unpublished data show that in early-gestational BPH/5 placentae, there is marked upregulation of several miRNAs that are known to bind and repress translation of both VEGF and PGF mRNA (Y. Zhou et al, unpublished data, 2010).

The mechanisms by which decreased circulating free VEGF or PGF during early gestation and midgestation result in the multisystem dysfunction observed in PE are not yet fully understood. Given their importance in angiogenesis and vascular remodeling during fetoplacental development, as well as in maintaining maternal cardiovascular and renal function, there are several possibilities. First, a decrease in placental synthesis and secretion of VEGF and PGF by villous cytotrophoblasts, fetal macrophages, and fetal fibro-

**Table. Placental and Fetal Weights**

Gestation Day and Sample	Treatment	Strain	Mean±SEM	Number
e14.5 Placental weight, mg	Ad-LacZ	C57	103.1±3.9	26
		BPH/5	81.7±5.1*	40
	Ad-VEGF	C57	109.4±4.2	19
		BPH/5	93.4±3.8*	13
e14.5 Fetal weight, mg	Ad-LacZ	C57	293.4±12.9	20
		BPH/5	196.4±6.8*	29
	Ad-VEGF	C57	236.9±5.4†	28
		BPH/5	174.1±8.6*	15
e18.5 Placental weight, mg	Ad-LacZ	C57	91.3±3.1	35
		BPH/5	105.3±4.1*	26
	Ad-VEGF	C57	90.8±3.1	42
		BPH/5	99.4±3.2	32
e18.5 Fetal weight, mg	Ad-LacZ	C57	1168±19.0	35
		BPH/5	1053±40.5*	23
	Ad-VEGF	C57	1193±18.5	44
		BPH/5	1022±22.3*	29

\* $P < 0.05$  vs C57 in matched treatment.

† $P < 0.05$  vs C57 Ad-LacZ.

Data are expressed as mean±SEM of the number given for each treatment and strain.

blasts may lead to decreases in vasculogenesis and angiogenesis, causing poor placentation.<sup>22,39</sup> Early defects in placentation attributed to decreased production of these proangiogenic factors could cause the so-called “stage 1” of the disease, characterized by reduced fetoplacental perfusion, placental oxidative stress, and inflammation.<sup>39,40</sup> Indeed, it is interesting to speculate that Ad-VEGF, by ameliorating poor placentation in the BPH/5 model, leads to reduced fetal resorptions as reported in Figure 4D and that this may be the critical upstream event in the beneficial effects of Ad-VEGF on maternal symptoms in this model. It should also be noted that another splice variant of the gene encoding a soluble form of the VEGF receptor-2 has recently been identified and has been shown to inhibit angiogenesis.<sup>41</sup> It will be intriguing to determine if this isoform is altered in BPH/5 placentae.

In addition to the placenta, angiogenic factors play an important role in various other organ systems. For example, VEGF expression is critical to maintaining the fenestrated and sinusoidal endothelium of the kidney and choroid plexus of the brain and liver, 3 distinct areas that are affected in PE patients.<sup>1,42</sup> Podocytes, renal epithelial cells that form the outermost glomerular filtration barrier, synthesize large amounts of VEGF, which is required for development and function of the glomerular filtration barrier.<sup>42,43</sup> Decreased circulating or local podocyte levels of VEGF could lead to damage of glomerular endothelium and increased protein secretion into the urine.<sup>1,42</sup> VEGF is also known to regulate vascular permeability, and dysregulation of this process can result in edema, another distinct feature of PE.<sup>1</sup> Furthermore, evidence from antiangiogenic cancer clinical trials using anti-VEGF antibodies shows that VEGF antagonism results

in hypertension, glomerular endotheliosis, and proteinuria,<sup>44</sup> again reinforcing the potential role of decreased VEGF in the development of gestational hypertension and proteinuria. Given the powerful effects of early Ad-VEGF treatment on BPH/5 fetal outcomes and maternal end points in this study, it will be important to determine the site(s) and mechanisms by which increased levels of this proangiogenic factor mediates its effects.

Emerging evidence suggests that decreased levels of circulating VEGF may also contribute to oxidative stress-induced endothelial dysfunction. Recent work from Granger and colleagues has shown that exogenous delivery of recombinant sFLT1 into pregnant rats results in reduced plasma-free VEGF levels, increased vascular oxidative stress, decreased NO-dependent vasorelaxation, and increased blood pressure.<sup>45</sup> Previous work from our laboratory has shown that increased reactive oxygen species scavenging by Tempol starting before and continuing throughout pregnancy ameliorates fetoplacental defects and development of PE symptoms in the BPH/5 model.<sup>18</sup> These results, along with our current findings of decreased placental and plasma-free VEGF in BPH/5 and our previous data showing that BPH/5 exhibit late-gestational endothelial dysfunction,<sup>17</sup> suggest that dysregulation of VEGF production in this model may lead to maternal oxidative stress and endothelial dysfunction. Certainly, many previous studies have implicated both increased oxidative stress and angiogenic imbalance in the development of PE; however, the pathogenic mechanisms have mostly been considered in parallel.<sup>39,40,46</sup> The studies discussed above suggest a possible connection between these 2 pathways.

### Perspectives

We have shown that an angiogenic imbalance in BPH/5 mice precedes the onset of maternal PE-like symptoms in this model, and this imbalance is likely caused by decreased placental synthesis and circulation of VEGF and PGF independent of increased antagonism by sFLT1. Furthermore, our studies suggest a causal link between decreased VEGF levels and the development of fetoplacental defects and maternal PE-like symptoms since early viral delivery of VEGF<sub>121</sub> was sufficient to reduce fetal resorptions and prevent late-gestational hypertension and proteinuria in this model. Since fetal loss occurs in advance of hypertension and proteinuria in this model, it is interesting to speculate that the beneficial effects of Ad-VEGF on fetal status contributed to amelioration of the maternal syndrome in this study. We believe the BPH/5 model will provide a unique opportunity to now examine the precise molecular underpinnings of changes in the angiogenic profile in the context of PE.

### Acknowledgments

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## Disclosures

None.

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## ONLINE SUPPLEMENT

### ADENOVIRAL DELIVERY OF VEGF<sub>121</sub> EARLY IN PREGNANCY PREVENTS SPONTANEOUS DEVELOPMENT OF PREECLAMPSIA IN BPH/5 MICE

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**Running Title:** VEGF therapy prevents pre-eclampsia in BPH/5 mice

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## METHODS:

**Animals and Husbandry.** Experiments were performed in 8-12 wk old BPH/5 and control C57Bl/6 (C57) mice obtained from in-house colonies. Animals were housed and maintained as previously described.<sup>1</sup> BPH/5 is an inbred strain derived from the spontaneously hypertensive BPH/2 strain, which was originally established through an eight-way cross that included C57Bl/6.<sup>1,2</sup> Mice underwent strain-matched mating and presence of a vaginal plug in the morning was defined as gestational day e0.5.<sup>1</sup> Gestational stages were defined as early: e9.5-12.5; middle: e13.5-15.5; and late: e18.5-19.5. All animal procedures were approved by the Institutional Animal Care and Use Committees at The University of Iowa and Cornell University, and are in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA regulations and the AUMA panel on Euthanasia.

**Adenovirus delivery.** Adenoviral vectors encoding VEGF<sub>121</sub> (Ad-VEGF) and a control gene  $\beta$ -galactosidase (Ad-LacZ) were prepared by the University of Iowa Gene Transfer Vector Core as described previously.<sup>3</sup> Briefly, cDNA for VEGFA<sub>121</sub> was cloned into the E1-deleted region of the replication-deficient E1/E3 deleted Ad5 adenovirus vector backbone.<sup>4</sup> Expression of the VEGF<sub>121</sub> gene in this viral construct was driven by the RSV promoter.<sup>3</sup> Virus was double-purified and the titer determined as described.<sup>3</sup> Ad-VEGF or Ad-LacZ were injected into C57 and BPH/5 via tail vein on e7.5 (100  $\mu$ l, 10<sup>9</sup> plaque forming units).

**Enzyme-Linked Immunosorbent Assay (ELISA).** To determine circulating levels of VEGF, PGF, sFLT1 and sENG, blood was collected from non-pregnant (NP), early, and mid-gestation BPH/5 and C57 females via cardiac puncture and fractionated using EDTA collection tubes. Plasma was isolated from blood samples spun within 30 minutes of collection at 3500 RPM for 15 minutes at 4°C and immediately frozen at -80°C. All ELISA kits were purchased from R&D systems (Minneapolis, MN) and assays were performed according to manufacturer's instructions. For measuring free VEGF, plasma was diluted 2-fold for NP mice and 5- to 10-fold for pregnant mice using assay diluent. The sensitivity for this assay was 3 pg/mL. To determine plasma free levels of PGF, ELISA analysis of undiluted plasma samples from NP, early, and mid-gestation were performed. The sensitivity for this assay was 2 pg/mL. Circulating levels of sFLT1 were determined by ELISA on plasma samples diluted 2-fold for NP mice and diluted 10-fold for pregnant mice. The sensitivity of this assay was 15 pg/mL. Finally, to measure sENG, ELISA was performed on plasma diluted 5-fold for NP, early- and mid-gestation samples. The sensitivity of this assay was 60pg/mL.

**Quantitative real-time PCR.** BPH/5 and C57 mouse placentae were collected at e9.5, e12.5, e14.5 and e18.5 (sFlt-1 only). Samples contained extraembryonic tissues including the decidua basalis and placenta, but excluded the umbilical cord and embryo

proper. Placentae from each litter were pooled to create one biological sample (n=1) and total RNA was isolated utilizing a RNeasy kit according to manufacturer's instructions (Qiagen). Quality of RNA samples was determined by denaturing agarose gel electrophoresis and total RNA content quantified by spectrophotometry.

Quantitation of *Vegf*, *Pgf* and *sFlt1* expression levels were performed by amplification of cDNA (equivalent to 10 ng input RNA) using SYBR Green and primers specific to each gene (ABI 7700, PE Biosystems, Foster City, CA). Primers were selected using a combination of Primer Express software (Applied Biosystems, Foster City, CA) and Primer Bank (Harvard University, Boston, MA <http://pga.mgh.harvard.edu/cgi-bin/primerbank>). All primers were purchased from integrated DNA technologies (IDT, Coralville, IA). The primers used were as follows: *Vegf* (NM\_001025250): forward 5'-CTT GTT CAG AGC GGA GAA AGC-3', reverse: 5'-ACA TCT GCA AGT ACG TTC GTT-3'; *Pgf* (NM\_008827): forward 5'-TCT GCT GGG AAC AAC TCA ACA-3', reverse 5'-GTG AGA CAC CTC ATC AGG GTA T-3'; *sFlt-1* (D88690): forward 5'-ACG TGT GTT TCC TGC TGT GT-3' and reverse 5'-TCA AAG CTT GGT GAA GGG CT-3'; and beta actin: forward 5'-CAT CCT CTT CCT CCC TGG AGA AGA 3' and reverse: 5'-ACA GGA TTC CAT ACC CAA GAA GGA AGG 3'. Samples were subjected to forty cycles of PCR (50°, 2 min; 95°, 10 min; 40X [95°, 0:15 min; 60°, 1 min]) followed by a dissociation protocol. Each sample was run in duplicate triplicate and gene expression was analyzed using the ddCt method. mRNA levels were normalized to  $\beta$ -actin (to generate dCt) and compared to C57 e9.5 (to generate ddCt). Sequence-specific amplification was confirmed by a single peak during the dissociation protocol following amplification.

**Western blot analysis.** Placentae were collected at early and mid-gestation from BPH/5 and C57 mice as described above. Samples were weighed, pooled from each litter (n=1) and homogenized in 50 mM potassium phosphate buffer (pH 7.8). Homogenates were sonicated for 30s at 30% amplitude (Sonics and Materials, Inc., Newtown, CT). Protein concentration was determined with the Bradford assay as previously described.<sup>5</sup> Equal amounts of protein (30  $\mu$ g protein) were separated on a 12.5% SDS-PAGE gel and were transferred to PVDF membrane. Membranes were blocked in 5% dry milk in TBST (0.01 mol/L Tris, 0.15mol/L NaCl buffer pH8.0 and 0.1% Tween 20) for 2 hours.<sup>5</sup> VEGF levels were measured using an antibody that recognized both VEGF<sub>121</sub> and VEGF<sub>165</sub> isoforms (anti-goat VEGF, 1:500, Sigma-Aldrich, St. Louis, MO). Blots were incubated for 1h at room temperature with primary antibody, washed three times in TBST, followed by incubation with secondary antibody for an additional hour (donkey anti-goat IgG peroxidase 1:10,000, Santa Cruz). Blots were stripped and re-probed with a monoclonal anti-actin antibody (Sigma Aldrich). Densitometry of VEGF and actin blots was performed utilizing the BioRad Quantity One (Hercules, CA). Band intensities were normalized to actin. Values were normalized to the early gestation C57 band for each blot. Experiments were run in triplicate.

***In Vitro Angiogenesis Assay.*** The *in vitro* angiogenesis assay was performed utilizing human umbilical vein endothelial cells (HUVEC) using an *In Vitro* Angiogenesis Kit (Trevigen) according to manufacturer's instructions. Briefly, black Optilux 96 well plates (Falcon) were coated with growth factor reduced matrigel (Trevigen) and were stored for 30mins at 37°C to allow polymerization. HUVEC cells obtained from B&D Biosciences were cultured in 75mm culture flasks according to manufacturer's instructions. Confluent cells in flasks were incubated with Calcein AM for 30mins and trypsinized. Approximately 25,000 cells were plated in matrigel-coated 96 well plates and cultured for 18-20hrs in medium supplemented with either 5% FBS or serum collected from different experimental animals. Plates were imaged using 1.25x objective on a Leica DMI 6000B inverted microscope equipped with green fluorescent protein imaging filters. Total tube lengths in the entire well were measured by experimenters blind to the treatments using Image J software. Data are normalized to the positive FBS control for total tube length.

***Radiotelemetric measurement of blood pressure throughout pregnancy.*** For longitudinal blood pressure measurements, NP female BPH/5 and C57 mice were surgically implanted with radiotelemeters as described previously.<sup>6</sup> Mice were allowed to recover fully for 7 days prior to baseline recording and strain-matched mating. Arterial blood pressure was recorded for 3 consecutive days prior to mating utilizing Dataquest ART data acquisition system (Data Sciences Int., Minneapolis, MN). Following strain-matched breeding and detection of a vaginal plug (e0.5), blood pressure was recorded as described previously.<sup>1,5</sup> Titer-matched Ad-VEGF or Ad-LacZ control vector (100 µL, 1x10<sup>9</sup> pfu) was injected via tail vein on e7.5 in both strains and arterial pressure recording was continued throughout the duration of pregnancy (~20 days) and for an additional week post-partum.

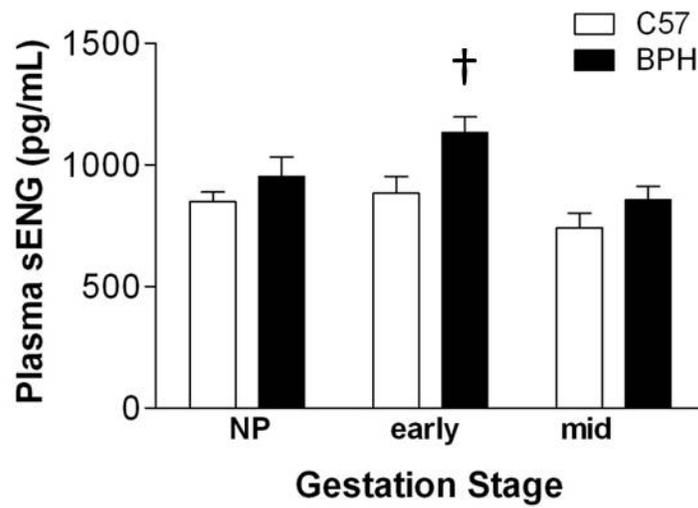
***Urine protein analysis.*** Urine samples were collected from mid- and late-gestation C57 and BPH/5 females injected with Ad-VEGF or Ad-LacZ on e7.5 and frozen at -20°C until analysis as described.<sup>1,5</sup> Protein analysis was performed using Bradford reagent as described.<sup>1,5</sup>

***Analysis of pregnancy outcomes.*** C57 and BPH/5 females treated with Ad-LacZ or Ad-VEGF on e7.5 were euthanized at mid-gestation. The uterine horns were exposed, and the fetuses were counted. Fetal resorptions were identified by necrotic/hemorrhagic appearance compared with normal viable fetuses.<sup>1,5</sup> In two additional cohorts of both strains treated with Ad-LacZ or Ad-VEGF at e7.5, placental and fetal weights at e14.5 and e18.5 were recorded for all pups from each litter. Finally, the numbers of live pups born were recorded in yet another cohort of BPH/5 and C57 females treated with Ad-LacZ or Ad-VEGF and allowed to deliver.

**Statistical analysis.** Data are expressed as mean  $\pm$  SEM. ANOVA followed by Newman-Keuls test for significance was performed for all data sets.  $p < 0.05$  was considered statistically significant.

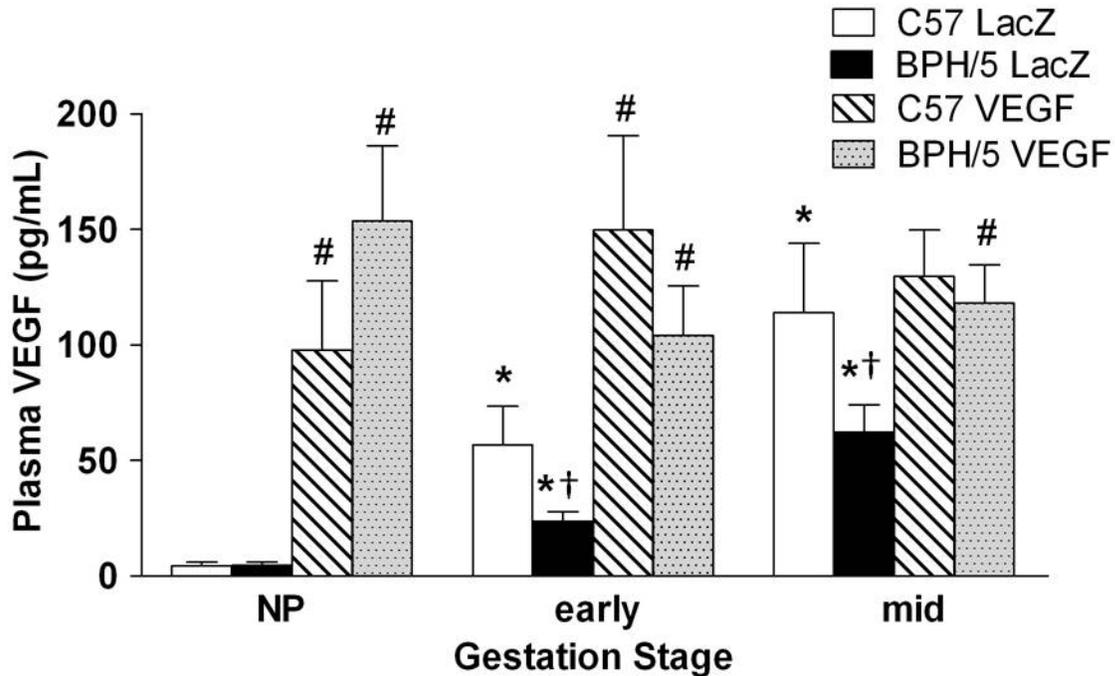
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**Figure S1**

**Figure S1:** Summary of circulating sENG levels as determined by ELISA in non-pregnant (NP) and at early- and mid- gestation C57 and BPH/5 (n=5-9 per group). † p<0.05 vs time-matched C57.



**Figure S2**

**Figure S2: Adenoviral-mediated delivery of VEGF<sub>121</sub> normalizes plasma VEGF levels in BPH/5.** Summary of circulating free VEGF levels as determined by ELISA in non-pregnant (NP, 3 days post-injection), early or mid-gestation C57 and BPH/5 mice that underwent tail vein injections of Ad-LacZ or Ad-VEGF. In pregnant mice, viral injections occurred on e7.5. NP: n=4-7 per group; early gestation: n=5-8 per group; mid gestation: n=5-10 per group. \*p<0.05 vs NP in matched strain and treatment; †p<0.05 vs time-matched C57 Ad-LacZ; #p<0.05 vs. Ad-LacZ in matched strain and time-point.