Causative Effects of Genetically Determined High Maternal/Fetal Endothelin-1 on Preeclampsia-Like Conditions in Mice

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Abstract —Endothelin-1 (ET-1) is implicated in the pathophysiology of preeclampsia. An association between an *EDN1* gene polymorphism with high ET-1 and preeclampsia was reported in humans, but their cause and effect relationships have not been defined. We examined the pregnancy effects in mice with a modified *Edn1* allele that increases mRNA stability and thus ET-1 production. Heterozygous $Edn1^{H/+}$ females showed no obvious abnormalities before pregnancy, but when mated with wild-type (WT) males developed a full spectrum of preeclampsia-like phenotypes, including increased systolic blood pressure, proteinuria, glomerular endotheliosis, and intrauterine fetal growth restriction. At 7.5 days post-coitus, the embryos from $Edn1^{H/+}$ dams, regardless of their *Edn1* genotype, lagged 12 hours in development compared with embryos from WT dams, had disoriented ectoplacental cones, and retained high E-cadherin expression. In contrast, WT females mated with $Edn1^{H/+}$ males, which also carried half of the fetuses with $Edn1^{H/+}$ genotype, showed a mild systolic blood pressure increase only. These WT dams had 2× higher plasma soluble fms-like tyrosine kinase-1 than WT dams mated with WT males. In human first trimester trophoblast cells, pharmacological doses of ET-1 increased the cellular *sFlt1* transcripts and protein secretion via both type A and B ET-1 receptors. Our data demonstrate that high maternal ET-1 production causes preeclampsia-like phenotypes during pregnancy, affecting both initial stage of trophoblast differentiation/invasion and maternal peripheral vasculature during late gestation. High fetal ET-1 production, however, could cause increased soluble fms-like tyrosine kinase-1 in the maternal circulation and contribute to blood pressure elevation. **(*Hypertension*. 2018;71:00-00. DOI: 10.1161/HYPERTENSIONAHA.117.10849.)** ● **Online Data Supplement**

Key Words: blood pressure ■ coitus ■ endothelin-1 ■ preeclampsia ■ vascular endothelial growth factor A

Preeclampsia is a pregnancy-related disease characterized by the onset of hypertension, proteinuria, and end-organ damage. It affects ≈5% to 8% of pregnancies and is one of the leading causes of maternal morbidity and mortality.[1] The pathogenesis of preeclampsia is largely unknown. Increased sFLT1 (soluble fms-like tyrosine kinase-1; soluble vascular endothelial growth factor [VEGF] receptor-1) in plasma is associated with preeclampsia, and evidence has been accumulating that sFLT1production in ischemic/hypoxic placentas seems to play a critical role in preeclampsia.[2,3] For example, in animal models, sFLT1 administered experimentally either by infusion or via adenoviral expression induces the preeclampsia-like phenotype, including hypertension, renal problems, and intrauterine growth restriction.[3–7]

Endothelin-1 (ET-1), a 21 amino acid proteolytic product of preproET-1,[8] is a potent vasoconstrictor involved in hypertension and kidney damage.[9–11] We and other investigators have shown that administering exogenous sFLT1 directly or by retroviral delivery increases mRNA levels of *Edn1* coding for preproET-1 and *Ednra* coding for endothelin type A receptor (EDNRA) in the kidneys.[5,6] In these models, EDNRA antagonist treatment reverses sFLT1-induced hypertension and proteinuria,[5,6] suggesting that upregulation of ET-1 signaling by sFLT1 plays a key role in preeclampsia. Compared with normotensive pregnant women, preeclamptic women have ≈2× to 3× higher circulating ET-1 levels.[12] Aggarwal et al[13] have reported an association between *EDN1* G5665T polymorphism and elevated plasma ET-1 with preeclampsia. Women with preeclampsia had an increased frequency of T-5665 allele, and both circulating ET-1 levels and maternal *EDN1* genotype were correlated with the severity of hypertension in preeclampsia.[13] Taken together, these observations in animal models and human polymorphisms have led investigators to propose that ET-1 is the key link between placental ischemia and hypertension in preeclampsia.[14] However, the cause and effect relationship between ET-1 and preeclampsia is still unclear.

Preeclampsia is a placenta-related disease associated with poor embryo implantation and low placental perfusion, and

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the maternal symptoms resolve with delivery.[15–17] The process of implantation and trophoblast invasion is a well-orchestrated epithelial–mesenchymal transition event.[18] E-cadherin mediates specific cell–cell adhesion in a Ca^{2+}-dependent manner[19,20] and plays a critical role in epithelial–mesenchymal transition.[21,22] In cancer cells, ET-1 inhibits E-cadherin via upregulation of E-cadherin transcriptional suppressors, such as Snail and Twist,[22,23] transcriptional factors that are also important ET-1 effectors in early embryo development. However, whether ET-1 affects embryonic implantation and plays a causative role in preeclampsia has not been investigated.

To further elucidate the relationships between ET-1 and preeclampsia, we studied mice with an *Edn1* allele modified to increase its mRNA stability leading to high ET-1 expression.[10] Because homozygosity for the high expressing allele is embryonically lethal,[10] we used $Edn1^{H/+}$ heterozygotes carrying 1 copy of the high expressing form of the *Edn1* gene and 1 copy of the wild-type (WT) gene. Our first objective was to measure the effect of maternal ET-1 overexpression on dam phenotype and trophoblast cell invasion in the early stage of pregnancy. Our second objective was to measure the effects of fetal ET-1 overexpression on dam phenotype and trophoblast invasion. Together, this study aimed to separate the effect of maternal ET-1 overexpression from that of embryonic/fetal ET-1 in a mouse model of preeclampsia.

Materials and Methods

The authors declare that all supporting data are available within the article (and its online-only Data Supplement files).

Mice

Mice (C57BL/6J) including both sexes and genotypes were housed in standard cages on a 12-hour light/dark cycle and were allowed free access to food and water. All experiments were performed in accordance with the National Institutes of Health guideline for use and care of experimental animals, as approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Genotypes were determined by polymerase chain reaction with primers: p1, 5′-AGACTAACTTAGCAGGAGGC-3′, p2, 5′-AGG TATGGAGCTCAGCTGCA-3′, and p3, 5′-AAAACACTGGGGG AGCTCTG-3′. A 520-bp fragment produced with p1 and p2 detects the targeted locus, and a 450-bp fragment produced with p1 and p3 detects the endogenous locus.

Matings

We compared the dams and embryos/fetuses from 3 different mating strategies. WT females mated with WT males (♀WT×♂WT) carrying all WT embryos, and $Edn1^{H/+}$ females mated with WT males (♀$Edn1^{H/+}$×♂WT) carrying both WT and $Edn1^{H/+}$ embryos, and WT females mated with $Edn1^{H/+}$ males (♀WT×♂ $Edn1^{H/+}$) also carrying both WT and $Edn1^{H/+}$ embryos. Females were checked for vaginal plugs each morning, and the day of plug detection was designated as 0.5 days post-coitus (dpc). To confirm pregnancy, mice were weighed 2× per week. Any females not gaining weight were not included in the study.

Determining Developmental Stage

For timed matings, males and females were housed together for 2 hour, and vaginal plugs were checked at the end of the 2 hours. We followed the Theiler staging criteria to determine the stage of development of the embryos for our comparisons.[24]

Blood Pressure Measurements by Telemetry During Pregnancy

Female virgin mice of reproductive age were anesthetized with inhaled isoflurane (2%–4% isofluorane/oxygen). The catheter (Data Sciences Inc, St. Paul, MN) was inserted into the left carotid artery and secured by ligation, and the transmitter was placed subcutaneously. Buprenorphine (analgesia) was first administered subcutaneously at anesthetic induction and then every 12 hours for the first 48 hours after surgery at a dose of 0.1 mg/kg body weight. One week after implantation, the blood pressure (BP) was recorded continuously for 3 to 5 days to establish the baseline.[6,7] After this, male mice were introduced into each cage for breeding, and females were checked for vaginal plugs each morning.

Plasma Analyses

Plasma ET-1, sFLT1, VEGF, and soluble endoglin were measured using ELISA kits (R&D Systems, Inc, Minneapolis, MN). The inter- and intravariability of these assays are <10%.

Urinary Albumin

Urine was collected by massaging the bladder at 1 time, and urinary albumin concentration and creatinine were determined using commercially available kits (Exocell Inc, Philadephia, PA) as described previously.[6,7,25]

Immunohistochemistry

Rabbit polyclonal antibodies against MMP9 (matrix metalloproteinase-9; ab19016, 1:200) and tissue inhibitor of metalloproteinase-3 (ab187297, 1:50) were from EMD Millipore (Billerica, MA) and Abcam (Cambridge, MA), respectively, and rabbit monoclonal E-cadherin (clone 24E10, no. 3195, 1:400) was from Cell Signaling Technology (Danvers, MA). Immunohistochemistry was performed in the Bond fully automated slide staining system (Leica Bicrosystems Inc, Vista, CA). Antigen retrieval was performed at 100°C in Bond-epitope retrieval solution 1 pH6.0 (AR9961) for 20 minutes for E-cadherin and MMP9 and in solution 2 (pH 9.0) for tissue inhibitor of metalloproteinase-3. Positive and negative controls (no primary antibody) were included for each antibody. Immunohistochemistry-stained sections were digitally imaged (×20 objective) in the Aperio ScanScope XT using line-scan camera technology (Leica Biosystems) and were stored within the Aperio eSlide Manager software.

Morphological Examination

Pregnant females and embryos were examined both visually and histologically at different time points: 7.5 (also 7.0 for WT because of developmental stage differences), 12.5, 14.5, and 18.5 dpc. Fixed tissues (4% paraformaldehyde) were sectioned (5 µm) and stained with hematoxylin and eosin or with Masson trichrome. For kidneys, periodic acid-Schiff staining was also used. Ultrastructural changes were examined by transmission electronic microscope (Jeol Jem 1230, Peabody, MA).[6,7]

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from tissues or cells was extracted using Trizol (Life Technologies, St. Paul, MN) following the manufacturer's instruction. NanoDrop spectrophotometer method and gel electrophoresis were used to check quantity and quality of RNA. mRNA was quantified with TaqMan real-time quantitative reverse transcription polymerase chain reaction (7500 real-time polymerase chain reaction system, Applied Biosystems, Foster City, CA) by using one-step RT-PCR Kit (Bio Rad, Hercules, CA) with *Hprt* as reference genes in each reaction for mouse tissue. For human cell experiments, *GAPDH* was used as a reference gene. The primer and probe sequences are in Table S3 online-only Data Supplement, and 100 ng of total RNA was used in each reaction.

Cell Culture

The HTR8/SVneo trophoblast cell line was kindly provided by Dr C.H. Graham, Queen's University, Kingston, Ontario, Canada,[26] and maintained in RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with 5% fetal bovine serum. Cells were starved for 24 hours with 0.5% fetal bovine serum and then treated with ET-1 at doses of 0.02, 0.1, or 0.5 µmol/L for 18 hours.[27] Different batches of cells were treated with ET-1 (0.5 µmol/L), ET-1 (0.5 µmol/L)+BQ123 (a selective EDNRA antagonist, 1 µmol/L), ET-1 (0.5 µmol/L)+BQ788 (a selective endothelin type B receptor [EDNRB] antagonist, 1 µmol/L),[28,29] and ET-1 (0.5 µmol/L)+bosentan (a nonselective EDNR antagonist, 1 µmol/L).[30] Another batch of cells was treated with ET-1 (0.5 µmol/L) or vehicle as control. After incubation, medium and cells were collected at 4, 8, 12, and 18 hours for analysis. ET-1, BQ123, and BQ788 were purchased from Sigma-Aldrich (St. Louis, MO). Bosentan was purchased from Cayman (Ann Arbor, MI).

Statistical Analysis

Data are presented as mean±SEM. Multifactorial ANOVA test was used with the program JMP 12.0 (SAS Institute Inc, Cary, NC). Post hoc analyses were done using the Tukey–Kramer honest significant difference test. Differences were considered to be statistically significant with $P<0.05$.

Results

Virgin Female *Edn1*$^{H/+}$ Mice With Higher ET-1 Are Normotensive

Consistent with our previously published data on male mice,[10] 2-month-old *Edn1*$^{H/+}$ females had 4× to 5× higher tissue *Edn1* expression and plasma ET-1 than WT females (1.30±0.26 pg/mL WT versus 5.4±1.2 pg/mL *Edn1*$^{H/+}$; $P<0.001$), but they had no obvious abnormalities (Table S1). The body weight, daily water and food intake, daily output of urine, plasma cholesterol, and glucose were not significantly different between WT and *Edn1*$^{H/+}$ mice. In addition, BP and urinary albumin excretion were not different between WT and *Edn1*$^{H/+}$ mice (Figure 1A; Figure S1). The glomerular structure of the virgin *Edn1*$^{H/+}$ female mice was indistinguishable from that of WT mice (Figure S2).

Edn1$^{H/+}$ Female Mice Develop Hypertension During Late Gestation

During the first week of pregnancy (up to 7.5 dpc) after mating with WT males, systolic blood pressure (SBP) of *Edn1*$^{H/+}$ pregnant dams was not significantly different from that of WT dams. However, at the beginning of the second week of pregnancy (8.5 dpc), SBP began to decrease in the WT dams (×♂WT), falling ≈10 mm Hg during the third week of pregnancy (starting at 14.5 dpc; red line in Figure 1A). In contrast, SBP began to rise in *Edn1*$^{H/+}$ dams during the second week until delivery, SBP increased ≈10 mm Hg, and returned to prepregnancy levels after delivery (black line in Figure 1A). Both diastolic blood pressure and mean arterial pressures of dams showed the same trend as their SBP (Figure S1A and S1B). The BP in the *Edn1*$^{H/+}$ dams and WT dams (×♂*Edn1*$^{H/+}$) clearly lacked the decrease seen in the WT dams.

In the reciprocal mating (♀WT×♂*Edn1*$^{H/+}$), where half of the pups are WT and half are *Edn1*$^{H/+}$ as in the *Edn1*$^{H/+}$ dams (×♂WT), BP of the WT dams did not significantly change during pregnancy (blue line in Figure 1A; Figure S1). However at the later stage, at ≈19.5 dpc, their SBP was 9.5 mm Hg higher than SBP before pregnancy.

Edn1$^{H/+}$ Dams, But Not WT Dams, Exhibit Proteinuria and Kidney Glomerular Endotheliosis

Urinary albumin excretion of the *Edn1*$^{H/+}$ dams was not significantly different from that of the WT dams at 7.5 and 14.5 dpc but was increased ≈3× at 18.5 dpc and returned to the prepregnancy levels when measured at postpartum day 7 (Figure 1B; Figure S1). In contrast, the urinary albumin excretion from WT dams (×♂*Edn1*$^{H/+}$) was indistinguishable from WT dams (×♂WT) at 18.5 dpc (Figure 1B).

Light microscopy of periodic acid-Schiff–stained kidneys at 18.5 dpc showed that the glomeruli of the pregnant *Edn1*$^{H/+}$ mice exhibited (Figure 1E) markedly reduced open capillary areas compared with those in WT dams (×♂WT) or WT dams (×♂*Edn1*$^{H/+}$; Figure 1C and 1D). Observations using transmission electron microscopy confirmed the loss of fenestrae in the glomerular capillary endothelial cells of *Edn1*$^{H/+}$ dams (Figure 1E). Podocyte foot processes were uniform, and no effacement was present. Ultrastructures of kidneys from the WT dams (×♂*Edn1*$^{H/+}$) were indistinguishable from those from WT dams (×♂WT; Figure 1D).

Fetal Growth Restriction and Intrauterine Fetal Demise Occur in the *Edn1*$^{H/+}$ Dams

WT dams (×♂*Edn1*$^{H/+}$) had similar litter size (Figure 2A and 2B) and fetal weights as WT dams (×♂WT; Figure 2C). In contrast, the average number of live pups born from the *Edn1*$^{H/+}$ dams (×♂WT) was about half of that from WT dams (Figure 2A), but the ratios of *Edn1*$^{H/+}$ pups and WT pups as well as sexes (M/F: 0.78/1; $P=0.5$; Fisher exact test) of the pups were not different from the expected 1:1 (Table S2). At 18.5 dpc, the number of live fetuses from *Edn1*$^{H/+}$ dams was already half of that from the WT dams (Figure 2B), and fetal weight (both WT and *Edn1*$^{H/+}$ fetuses) from *Edn1*$^{H/+}$ dams was significantly lower than fetal weights from WT dams (Figure 2C). The placental weight was not different among 3 groups of dams (0.101±0.01 g WT [×♂WT], 0.103±0.002 g WT [×♂ *Edn1*$^{H/+}$], 0.108±0.01g *Edn1*$^{H/+}$ [×♂WT]; $P=0.78$). At 14.5 dpc, the total number of fetuses (both alive and dead) was not significantly different between the 2 different maternal genotypes (Figure 2D), but the *Edn1*$^{H/+}$ dams had a higher percentage (41.6%) of resorption compared with WT dams (Figure 2D and arrows in Figure 2E). Furthermore, 2 of 7 (28.6%) *Edn1*$^{H/+}$ dams had poorly developed fetuses at 12.5 dpc, and these abnormal fetuses included both genotypes (Figure S3C). The placentas of these conceptuses show a strikingly reduced labyrinth zone, smaller junctional zone, and larger decidual basalis compared with placentas of WT dams (Figure S3E).

Implantation Sites From *Edn1*$^{H/+}$ Dams Have Abnormal Ectoplacental Cone (EPC) With Higher E-Cadherin Expression

At 7.5 dpc, when trophoblast cell invasion commences, the implantation sites and embryos from *Edn1*$^{H/+}$ dams were smaller than those from WT dams that were mated with either WT or *Edn1*$^{H/+}$ males. Careful determination of the stage of development of the 7.5 dpc embryos from the mutant dams revealed that development lagged behind by ≈12 hours,

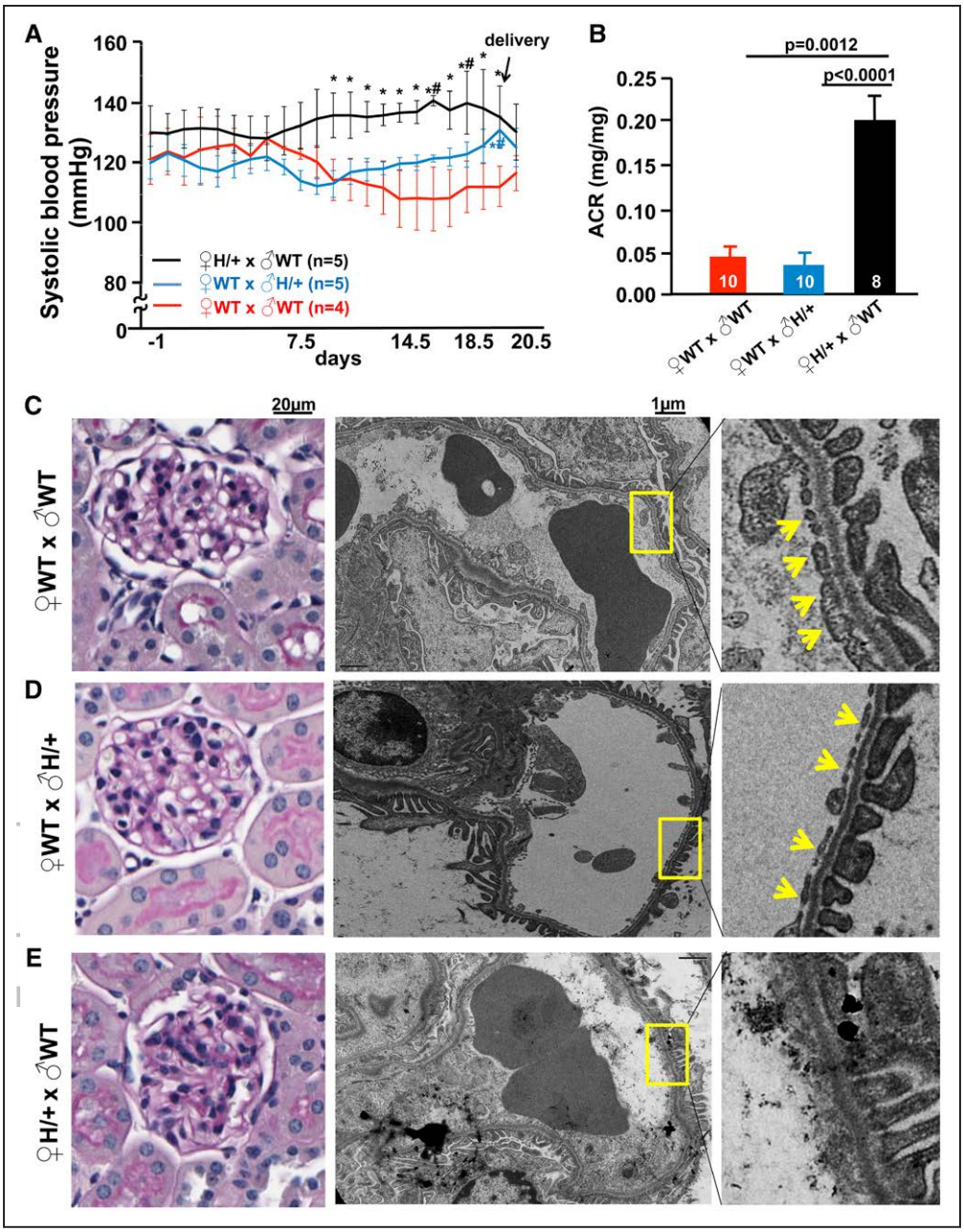

Figure 1. *Edn1*$^{H/+}$ dams (×♂ wild type [WT]) develop hypertension, albuminuria, and glomerular endotheliosis during late gestation. **A**, Systolic blood pressures of ♀*Edn1*$^{H/+}$ (×♂WT), ♀WT (×♂*Edn1*$^{H/+}$), and ♀WT (×♂WT) mice during the whole pregnancy. Day -1 corresponds to the day males and females were placed together for mating; the day the plug was detected as 0.5 days post-coitus (dpc). **P*<0.05 vs ♀WT (×♂WT) at the same gestational stage, *t* test; #*P*<0.05 vs before pregnancy, paired *t* test. **B**, Urinary albumin creatinine ratio (ACR) at 18.5 dpc. Numbers of mice are within the bars. Representative glomeruli from ♀WT (×♂WT) mice (**C**); ♀WT (×♂*Edn1*$^{H/+}$) mice (**D**); ♀*Edn1*$^{H/+}$ (×♂WT) mice (**E**) at 18.5 dpc. **Left**, Periodic acid–Schiff stain; **Right**, Electron micrographs of glomeruli. Yellow arrows: endothelial fenestrae. The endothelial fenestration is not present in *Edn1*$^{H/+}$ dams. Multiple comparisons used the Tukey–Kramer honestly significant difference test. Error bars are SEM.

making them comparable to 7.0 dpc embryos of WT dams. All the implantation sites of *Edn1*$^{H/+}$ dams at 7.5 dpc also showed a disoriented EPC (Figure 3A) regardless of the genotype of the embryo in comparison to those from WT dams at 7.0 and 7.5 dpc (Figure 3C; Figure S5). EPC regions of the embryos from WT dams (×♂*Edn1*$^{H/+}$) were not different from those of WT dams (×♂WT; Figure S5). As trophoblast cells differentiate and begin to migrate,[31] they normally downregulate E-cadherin expression as we observed at 7.0 to 7.5 dpc in the EPC region of WT dams regardless of whether they were mated with WT or *Edn1*$^{H/+}$ males (Figure 3D; Figure S5). In contrast, the expression of E-cadherin in the EPC region of *Edn1*$^{H/+}$ dams (×♂WT) at 7.5 dpc retained strong expression of E-cadherin (Figure 3B). However, immunohistochemistry showed that the expression of neither MMP9, a proteolytic enzyme from the embryo, nor tissue inhibitor of metalloproteinase-3, an inhibitor from the maternal endometrium, was different in the embryo implantation sites of *Edn1*$^{H/+}$ dams compared with WT dams (Figure S4). Negative isotype control with rabbit IgG staining is shown in Figure S6.

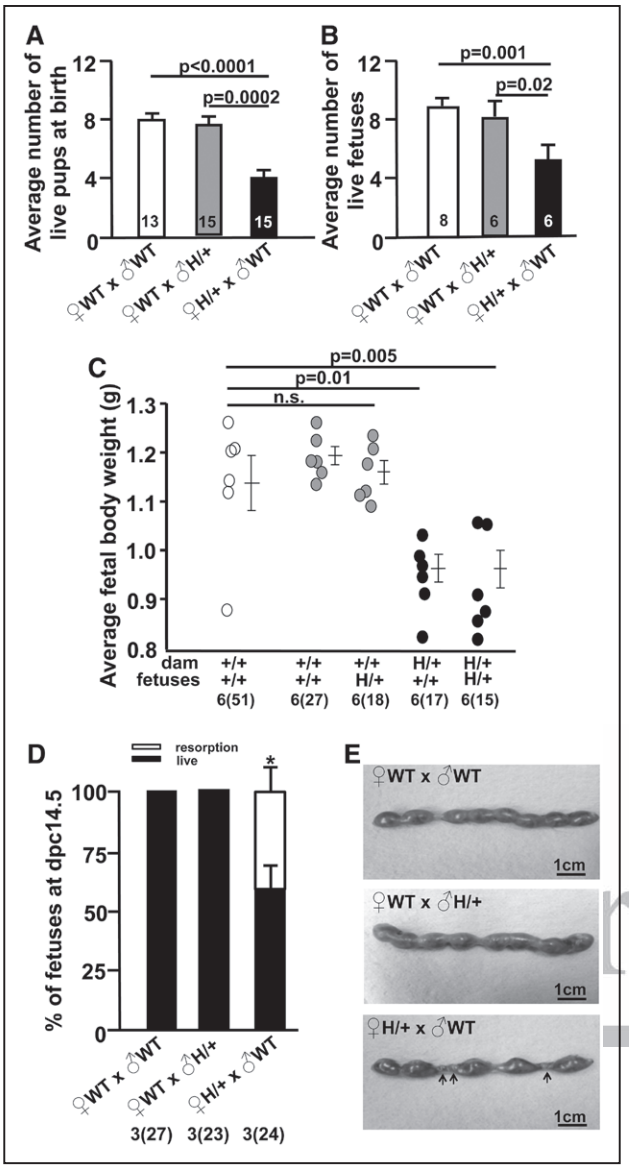

Figure 2. Fetal growth restriction and intrauterine fetal demise occur in pregnant *Edn1*$^{H/+}$ dams but not in wild-type (WT) dams. **A**, Average number of live neonates per pregnancy at the delivery day in ♀WT(×♂WT), ♀WT (×♂*Edn1*$^{H/+}$), and ♀*Edn1*$^{H/+}$ (×♂WT) mice, numbers of pregnant mice are within the bars. **B**, Average number of live fetuses per pregnancy at 18.5 days post-coitus (dpc) in the 3 groups of mice, numbers of pregnant mice are within the bars. **C**, Average fetal weight at 18.5 dpc from 3 groups of mice. Numbers of total fetuses shown in parentheses; numbers of pregnant mice are shown outside of the parentheses. **D**, Average percentage of fetuses at 14.5 dpc from 3 groups of mice. Number of total fetuses are shown in parentheses (including both resorption and live ones); number of pregnant mice are shown outside of the parentheses. **P*<0.01 vs ♀WT (×♂WT), ♀WT (×♂*Edn1*$^{H/+}$). **E**, Images of uteri harvested at 14.5 dpc. Arrows, resorptions of fetuses. Multiple comparisons used the Tukey–Kramer honestly significant difference test. Error bars are SEM. n.s. indicates not significant.

Plasma ET-1 Levels Markedly Increase in *Edn1*$^{H/+}$ Dams

The plasma ET-1 levels in WT dams (×♂WT) did not change during pregnancy (1.29±0.07 pg/mL at 18.5 dpc versus 1.30±0.26 pg/mL virgin; *P*=1.0). In contrast, a small but significant increase in the plasma ET-1 of WT dams (×♂*Edn1*$^{H/+}$) was observed during late pregnancy (2.76±0.78 pg/mL at 18.5 dpc; *P*=0.008; *t* test; Figure 4A). The embryos of these WT dams contributed to the increase because *Edn1* expression was 4-fold higher in the placentas from *Edn1*$^{H/+}$ fetuses than in those from WT fetuses (Figure 4B).

Plasma ET-1 levels in the *Edn1*$^{H/+}$ female mice before pregnancy (5.4±1.2 pg/mL) as well as at 12.5 dpc (4.1±1.0 pg/mL) were ≈4× higher than the levels in WT females and markedly increased in late pregnancy to (8.9±0.5 pg/mL) at 18.5 dpc (Figure 4A). *Edn1* mRNA in the placentas of the *Edn1*$^{H/+}$ females (×♂WT) differed according to the genotype of the embryos as it did in the WT females (×♂*Edn1*$^{H/+}$; Figure 4B). In the peripheral tissues, the *Edn1* mRNA levels in the kidneys of *Edn1*$^{H/+}$ females at 18.5 dpc were not different from pre-pregnancy although they were ≈5× higher than the levels in the WT females (Figure 4C). mRNA in the liver also showed a similar result (Figure S7).

Placentas of *Edn1*$^{H/+}$ Fetuses Contribute to Maternal Plasma sFLT1 Levels

Plasma sFLT1 in the *Edn1*$^{H/+}$ virgin females was higher than in WT virgin females (2.03±0.14 ng/mL *Edn1*$^{H/+}$ versus 1.35±0.06 ng/mL WT; *P*=0.003; *t* test). At 12.5 dpc, the plasma sFLT1 levels were higher in both WT dams (×♂*Edn1*$^{H/+}$; 8.7±0.9 ng/mL) and *Edn1*$^{H/+}$ dams (×♂WT; 8.2±1.1 ng/mL) than in WT dams (×♂ WT; 6.1±1.0 ng/mL) although the differences did not reach significance (Figure 4D). At 18.5 dpc, plasma sFLT1 levels in the C57BL/6J WT dams (×♂ WT) increased to 40.1±5.3 ng/mL, which were higher than in 129S6 WT dams we previously reported[7] but was comparable to the value reported by Woods et al[32] who also used female mice on a C57BL/6 background. In contrast, plasma sFLT1 levels in the WT dams (×♂*Edn1*$^{H/+}$; 77.1±5.8 ng/mL) were significantly higher than in the WT dams (×♂WT; *P*<0.01). Plasma sFLT1 levels in the *Edn1*$^{H/+}$ dams were not different from that in the WT dams (×♂ WT; 47.9±6.3 ng/mL; *P*=0.78) at 18.5 dpc. However, *Edn1*$^{H/+}$dams had on average half the number of live fetuses compared with the WT dams. After normalizing by the number of live fetuses, no difference was detected between WT dams (×♂*Edn1*$^{H/+}$) and *Edn1*$^{H/+}$ dams (×♂WT); both had twice higher plasma sFLT-1levels than WT dams (×♂WT; Figure 4E). Intriguingly, *sFlt1* mRNA in the placentas from both WT dams (×♂ *Edn1*$^{H/+}$) and *Edn1*$^{H/+}$ dams (×♂WT) was significantly lower than that in placentas from WT dams with all WT pups (Figure 4F): *sFlt1* mRNA levels in the placentas of WT fetuses were ≈45% while those in *Edn1*$^{H/+}$ fetuses were ≈65%.

Virgin *Edn1*$^{H/+}$ females had significantly higher plasma VEGF levels than virgin WT females (11±3 pg/mL WT versus 42±10 pg/mL *Edn1*$^{H/+}$; *P*=0.01; *t* test). The plasma VEGF levels increased by pregnancy, but at 18.5 dpc, they did not differ among 3 groups of dams (101±14 pg/mL WT [×♂WT], 93±12 pg/mL WT [×♂ *Edn1*$^{H/+}$], 98±20 pg/mL *Edn1*$^{H/+}$ [×♂WT]; *P*=0.9). Plasma soluble endoglin levels were not different among the 3 groups of dams (Table S1).

ET-1 Increases sFLT1 Expression in Human Trophoblast Cells via EDNRA and EDNRB Receptors

In human first trimester trophoblast cells (HTR8/SVneo), ET-1 treatment increased sFLT1 in the medium in a dose-dependent

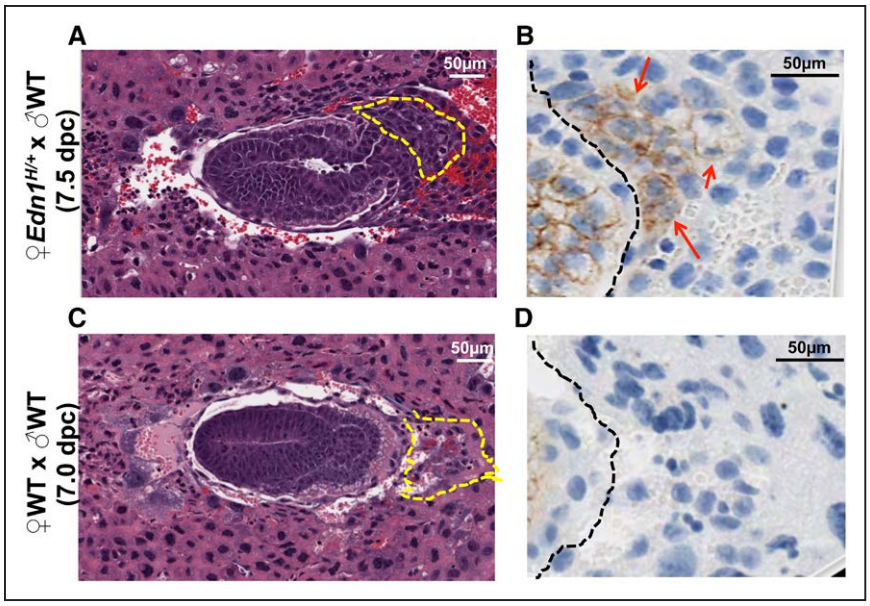

Figure 3. Implantation sites from pregnant *Edn1*$^{H/+}$ mice have abnormal ectoplacental cone (EPC) with higher E-cadherin expression. Midtransverse sections through implantation sites of ♀*Edn1*$^{H/+}$ (×♂ wild type [WT]; **A** and **B**) and ♀WT (×♂WT; **C** and **D**). **A** and **C**, Stained with hematoxylin and eosin. Section (**A**) shows the developmental stage of a 7.5 days post-coitus (dpc) embryos from ♀*Edn1*$^{H/+}$ (×♂WT) is comparable to a 7.0 dpc embryo from ♀ WT (×♂WT; **C**). EPC region outlined with the yellow dashed lines exhibits the disoriented alignment in the implantation site. **B** and **D**, Immunohistochemistry staining of E-cadherin in EPC region (right side of the black dashed lines) from (**A**) and (**C**), respectively. Red arrows, positive staining.

manner, and at 0.5 μmol/L differences reached the significant level (Figure 5A). When HTR8/SVneo cells were treated with ET-1 together with either BQ123 (a selective EDNRA antagonist) or with BQ788 (a selective EDNRB antagonist), sFLT1 concentration in the medium was decreased although the reductions did not reach significant levels. In contrast, bosentan (a nonselective EDNR antagonist) significantly abolished the induction of sFLT1 by ET-1 (Figure 5B). ET-1 at 0.5 μmol/L increased sFLT1 concentration in the medium in a time-dependent fashion (Figure 5C). The amount of the major sFlt1 transcript in human placenta, variant e15a,[33] increased in the cells by 4 hours after treatment and returned

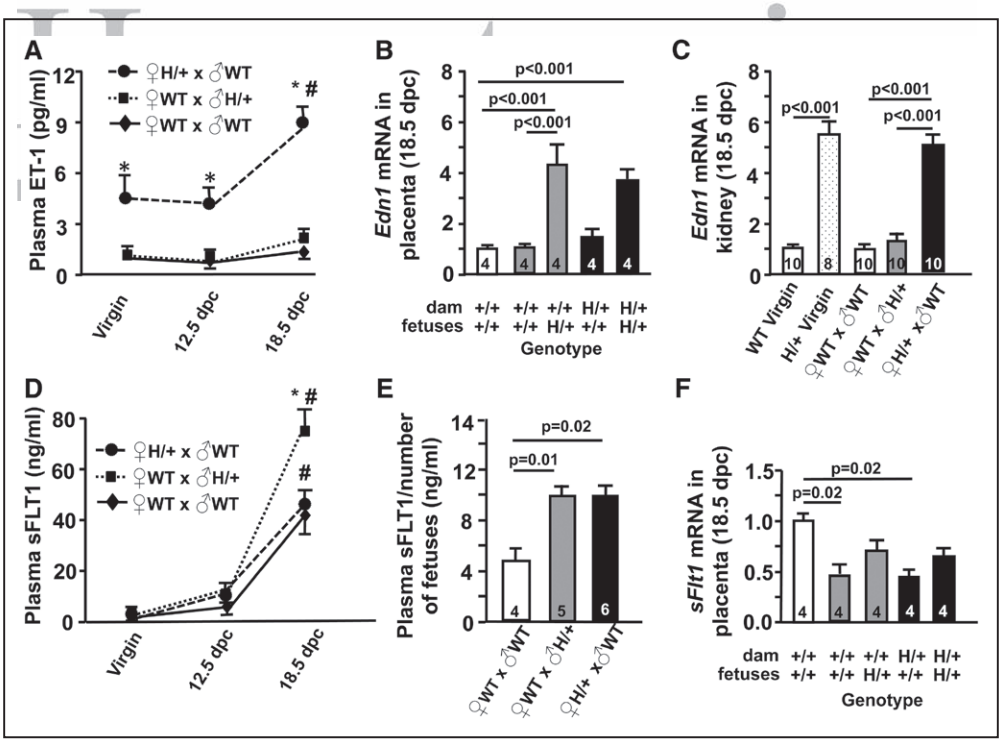

Figure 4. ♀*Edn*$^{H/+}$ (×♂ wild type [WT]) mice have elevated plasma endothelin-1 (ET-1) levels while ♀WT(×♂*Edn1*$^{H/+}$) mice have elevated plasma sFLT1 (soluble fms-like tyrosine kinase-1) levels in late pregnancy. **A**, Plasma ET-1 of virgin females and pregnant mice at 12.5 and 18.5 days post-coitus (dpc), **P*<0.001 vs WT at same gestational stage, #*P*<0.01 vs virgin *Edn1*$^{H/+}$ mice. n≥5. **B**, *Edn1* mRNA level in placenta from pregnant mice at 18.5 dpc. Numbers of dams are within the bars. **C**, *Edn1* mRNA level in kidneys from virgin females and pregnant mice at 18.5 dpc. Numbers of kidneys are within the bars. **D**, Plasma sFLT1 of virgin females and pregnant mice at 12.5 and 18.5 dpc, **P*<0.01 vs WT at same gestational stage, #*P*<0.01 vs virgin *Edn1*$^{H/+}$ mice. n≥5. **E**, Plasma sFLT1 concentration normalized to the number of live fetuses in each dam. Numbers of pregnant mice are within the bars. **F**, *sFlt1* mRNA level in placentas from pregnant mice at 18.5 dpc. Numbers of dams are within the bars. Multiple comparisons used the Tukey–Kramer honestly significant difference test. Error bars are SEM.

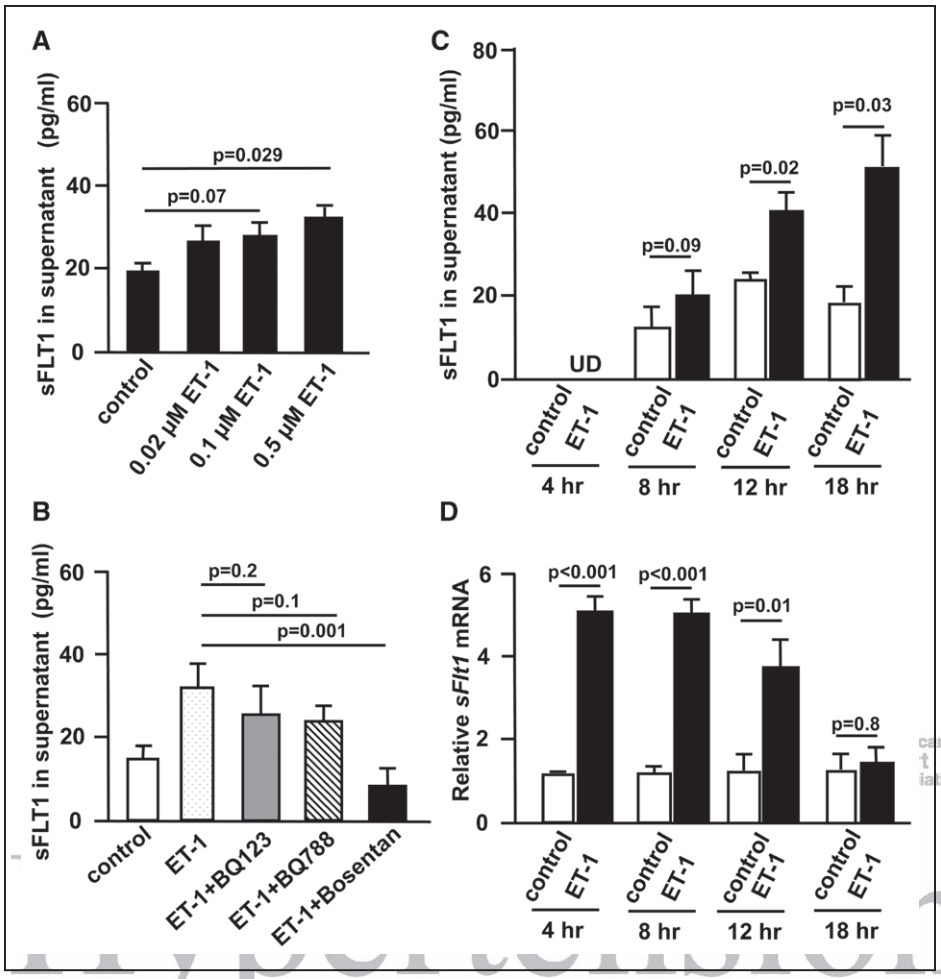

Figure 5. Endothelin-1 (ET-1) induces sFLT1 (soluble fms-like tyrosine kinase-1) expression in human trophoblast cells via endothelin type A receptor and endothelin type B receptor. After starving with 0.5% fetal bovine serum medium for 24 h, human first trimester trophoblast cells (HTR8/SVneo) were exposed to (**A**), ET-1 at doses of 0.02, 0.1, or 0.5 μmol/L for 18 h and sFLT1 concentration in culture medium was measured. **B**, ET-1 (0.5 μmol/L) alone, or with 1 μmol/L each of BQ123, BQ788, bosentan for 18 h and sFLT1 concentration in culture medium were determined. **C** and **D**, Cell culture medium and cells were harvested at 4, 8, 12, and 18 h after treatment with either vehicle or ET-1 (0.5 μmol/L). **C**, sFLT levels in culture medium. **D**, Cellular sFLT1 (variant e15a) mRNA levels (fold change relative to control cells). n=4. UD indicates undetectable.

to the levels of nonstimulated cells by 18 hours (Figure 5D). Similar results were obtained for the amount of another sFlt1 transcript, variant i13 (Figure S8).

Discussion

Our study demonstrates that ET-1 overproduction causes the preeclampsia-like phenotype in mice. Maternal but not fetal ET-1 overproduction seems to be the major contributor to delayed placental and fetal development. We further show that fetal ET-1 overproduction alone is not sufficient to induce a full spectrum of preeclampsia-like symptoms in dams. Taken together, these data suggest that although both maternal and fetal ET-1 affect the maternal phenotype, the maternal ET-1 plays a major role. In contrast, only maternal ET-1 affects fetal outcome.

Because we mated the *Edn1*$^{H/+}$ females with WT males, on average half of the fetuses were *Edn1*$^{H/+}$ genotype and produced higher levels of ET-1 compared with the WT fetuses. To separate contributions of maternal and fetal ET-1 to their preeclampsia phenotypes, we also studied WT pregnant mice mated with *Edn1*$^{H/+}$ males, which carry the same ratios of WT and *Edn1*$^{H/+}$ embryos/fetuses as the *Edn1*$^{H/+}$ females and observed mild hypertension during late gestation but no kidney damage or intrauterine growth restriction in these WT females. *Edn1* expression from placentas of *Edn1*$^{H/+}$ fetuses led to a 2-fold increase in the plasma ET-1 levels in these WT females. Thus, an increase of fetal ET-1 may directly affect maternal BP, but it is not sufficient to cause kidney damage consistent with preeclampsia.

Notably, although plasma ET-1 levels of *Edn1*$^{H/+}$ females are 4.5× higher than those in WT females, their BP is not elevated. This observation is consistent with our previous finding in male mice[10] in which SBP increased slightly in parallel with graded decrease of *Edn1* expression of 350%, 100%, 65%, and 25% normal. Despite ET-1 being a potent vasoconstrictor, various studies with genetically altered mice have shown that effects of ET-1 on BP are not simple. For example, Kurihara et al[34] reported that *Edn1*$^{+/-}$ heterozygotes expressing 50% normal level of ET-1 are hypertensive. However, both endothelial cell–specific ET-1–knockout mice as well as mice

overexpressing *EDN1* specifically in endothelial cells have near normal BP.[35] Presence of a complex feedback and developmental adjustment of the ET-1 system have been postulated and are supported by the finding that animals developed hypertension when endothelial cell–specific overproduction of ET-1 was induced in adults.[36] The homeostatic adjustment could involve the VEGF system because in the current study we also found that plasma VEGF was higher in virgin *Edn1*$^{H/+}$ female mice. Increased VEGF may counteract high ET-1 because VEGF is known to activate endothelial nitric oxidase synthase/nitric oxide.[37] Maternal physiological adjustments to pregnancy include induction of various vasodilators, such as VEGF. When *Edn1*$^{H/+}$ dams develop overt hypertension at the later stage of pregnancy, their plasma VEGF was not higher than that of WT dams, raising a possibility that there could be a ceiling to its upregulation.

We also note that plasma ET-1 in the *Edn1*$^{H/+}$ dams increased ≈7× of the WT levels at 18.5 dpc. This marked increase of ET-1 was not accounted for by the *Edn1* mRNA levels in the placenta or in peripheral tissues (kidneys and liver) although we did not examine the expression in the lung, which is the major producer of ET-1. The elevated plasma ET-1 in *Edn1*$^{H/+}$ dams could result from increased production of this 21 amino acid peptide through cleavage from its 38 amino acid precursor, big ET-1, by endothelin-converting enzyme. An elevated endothelin-converting enzyme and ET-1 have been reported in pregnant women with preeclampsia compared with women with a normal pregnancy.[38] A disturbance in ET-1 clearance mechanisms could also contribute to its marked increase in the *Edn1*$^{H/+}$ dams. Rapid but low capacity clearance of ET-1 takes place in the lung circulation via EDNRB receptor while the liver and kidneys clear ET-1 through nonreceptor-mediated mechanisms.[39,40] In the kidney, the transport of albumin across the glomerular basement membrane is predominantly by diffusion rather than by flow, whereas the transport of water and small molecules is entirely by flow.[6,41] Consequently, as the amount of fluid crossing a glomerulus decreases, the concentration of albumin in the glomerular effluent will increase without altering the concentration of small molecules. Thus, a reduced glomerular filtration rate and effective renal plasma flow because of high ET-1 and endotheliosis could lead to a reduction of the clearance of small molecules, such as ET-1, while increased concentration of albumin could saturate tubular protein reuptake systems, resulting in albuminuria. Future studies of kidney functions in preeclamptic animal models, including glomerular filtration rate in the pregnant *Edn1*$^{H/+}$ females, are necessary to shed light on the relationships between circulating ET-1 levels and albuminuria.

Since Maynard et al[3] published their seminal article in 2003, sFLT1 is perhaps the most studied molecule in preeclampsia. However, the precise mechanism(s) of sFLT1 regulation is still unclear. Previously, we and other investigators have shown that hypertension and renal injury induced by excess sFLT1 is through ET-1 because it was ameliorated by the treatment with EDNRA antagonists.[5,6] More recently, Amraoui et al[42] reported that sFLT1 augments vasoconstriction via ET-1, in part by inhibiting VEGF-mediated stimulation of nitric oxide production and in part by enhancing the cyclooxygenase-thromboxane signaling route downstream of ET-1. During pregnancy, the placenta is a major source of sFLT1, which increases throughout the pregnancy. In the current study, we found that the maternal circulation of sFLT1 at 18.5 dpc in WT dams (×♂*Edn1*$^{H/+}$) was twice that of WT dams (×♂WT). WT dams (×♂*Edn1*$^{H/+}$) had significantly higher plasma sFLT1 levels than *Edn1*$^{H/+}$ dams (×♂WT), at least in part because the number of functional placentas in the WT dams at this late stage of pregnancy was twice as many as those in *Edn1*$^{H/+}$ dams (×♂ WT). The modest increase in SBP of WT dams (×♂ *Edn1*$^{H/+}$) could have resulted from an increase in sFLT1 or other soluble factor(s) that are similarly induced by ET-1 in the placenta. Regardless, placentas of *Edn1*$^{H/+}$ fetuses produced more sFLT1 than those of WT fetuses within a single dam, suggesting that ET-1 affects sFLT1 expression and secretion in this model.

The in vitro data in cultured cells provide further evidence to support the theory that ET-1 increases the release of sFLT1. Human first trimester trophoblast cells, HTR8/SVneo, express sFLT1[43] as well as components of the ET-1 system.[44] A pharmacological dose of ET-1 (0.5 µmol/L) acutely increased *sFlt1* mRNA expression, followed by accumulation of its protein in the medium. However, 18 hours after treatment with ET-1, the mRNA level of s*Flt1* was no longer elevated, suggesting a potential feedback inhibition. Indeed, in vivo data also suggest this feedback inhibitory effect as indicated by decreased *sFlt1* mRNA levels in placentas from dams carrying mutant fetuses that could have higher sFLT1 protein levels. ET-1 executes its functions through 2 receptors, EDNRA and EDNRB in HRT8/SVneo cells.[44,45] Selective EDNRA or EDNRB receptor antagonists tended to decrease the elevated sFLT1 level mediated by ET-1, and the nonselective EDNR antagonist totally abolished the induction effect of ET-1, suggesting that ET-1 stimulates sFLT1 through both EDNRA and EDNRB receptors.

Many studies suggest that impaired trophoblast cell invasion is the root of preeclampsia.[16,17,46] At 7.5 dpc, before the placenta had formed, the embryos from *Edn1*$^{H/+}$ dams independent of their genotype showed a 12-hour delay in their developmental state, with disoriented EPC and abnormal E-cadherin expression. This suggests that high maternal ET-1 production impairs trophoblast invasion of embryos. We also found all embryos from dams with high ET-1 exhibit distortion of EPCs at 7.5 dpc. Because all embryos from *Edn1*$^{H/+}$ dams had the distorted EPCs regardless of their *Edn1* genotype, maternal high ET-1 is more likely to be the cause of this phenomenon. Studies show that both maternal and embryonic factors influence the EPCs, and similarly disoriented EPCs have been described in embryos from female mice carrying a dominant negative mutation in *Gjpa1* coding for connexin43 (maternal effects)[47] and in embryos lacking MMP9 completely (embryonic effects).[17] Transcriptional activation of MMP9 in the differentiated trophoblast giant cells is coincidental with their invasive characteristics while gap junction intercellular communication is involved in the development and differentiation process. In all 3 cases, intrauterine embryonic growth restriction occurs. Plaks et al[17] reported that MMP9$^{+/-}$ hetrozygous females develop hypertension, proteinuria, and renal injury during late pregnancy,

establishing the link between MMP9 and preeclampsia. It is important to note that the graded expression of *Edn1* is inversely correlated with the *Mmp9* gene expression in the heart.[10] ET-1, being a strong vasoconstrictor, could restrict blood supply in the maternal circulation, but because it is also expressed in most of the cells in the maternal-fetal interface, it may directly influence the terminal differentiation of the trophoblast giant cells.

In conclusion, our data suggest a chain of events that leads to preeclampsia in dams with genetically increased expression of ET-1. First, high ET-1 from maternal tissues negatively influences the differentiation and invasion of trophoblasts in early pregnancy. This is associated with increased sFLT1, which together with high expression of peripheral ET-1 production synergistically enhances vasoconstriction and vessel damage, resulting in the preeclampsia phenotype. This underscores the importance of screening maternal *EDN1* genotype to identify pregnant women who have higher ET-1 level and thus a higher risk of developing preeclampsia.

Perspectives

Preeclampsia is one of the leading causes of pregnancy-related maternal death, yet, the pathogenesis of preeclampsia is still largely unknown. Currently, the only definitive treatment is delivery. Our new mouse model of preeclampsia resulting from maternal over production of ET-1 will facilitate answering how maternal ET-1 influences the pathophysiology of preeclampsia: the mechanisms of trophoblast differentiation/invasion in embryo implantation stage and maternal vascular dysfunction in late stage of pregnancy. This also should stimulate development of novel blockers of the ET-1 system, which are not teratogenic, to treat preeclampsia.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This study of a novel mouse model of preeclampsia demonstrates that maternal endothelin-1 (ET-1) overproduction contributes to preeclampsia-like phenotypes, including hypertension, proteinuria, as well as glomerular endotheliosis and intrauterine growth restriction of fetuses.
- Maternal high ET-1 production impairs trophoblast cell differentiation/invasion in embryos and retention of high E-cadherin expression in ectoplacental cones.
- Fetal ET-1 stimulates soluble fms-like tyrosine kinase-1 expression in placenta, which contributes to the increase of maternal circulating soluble fms-like tyrosine kinase-1 and elevated blood pressure.

What Is Relevant?

- The causal link between ET-1 and preeclampsia underscores the importance of screening maternal *EDN1* genotype to identify pregnant women who have higher ET-1 production and thus a higher risk of developing preeclampsia. It also encourages the development of safe ET-1 system blockers for treatment of preeclampsia.

Summary

Preeclampsia is the leading cause of maternal morbidity and mortality, and there is no real treatment for this disease. The ET-1 system has been implicated for preeclampsia as the mediator of soluble fms-like tyrosine kinase-1 effects on preeclampsia, including hypertension and renal damage. However, whether ET-1 is a causative factor of preeclampsia is not clear. Here, we show that female mice with high production of ET-1 develop preeclampsia-like symptoms, which include elevated blood pressure, urinary albumin excretion, and fetal growth restriction. Maternal high ET-1 production also affects the trophoblast differentiation/invasion while fetal high ET-1 production leads to increased soluble fms-like tyrosine kinase-1 from placenta. These results encourage the development of ET-1 signaling blockers that are not harmful to babies to treat preeclampsia.

Hypertension
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Causative Effects of Genetically Determined High Maternal/Fetal Endothelin-1 on Preeclampsia-Like Conditions in Mice

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ONLINE SUPPLEMENT

CAUSATIVE EFFECTS OF GENETICALLY-DETERMINED HIGH MATERNAL/FETAL ENDOTHELIN-1 ON PREECLAMPSIA-LIKE CONDITIONS IN MICE

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Table S1. Characteristics of different groups of mice

Parameters	WT virgin	*Edn1*$^{H/+}$ virgin	♀WT x ♂WT	♀*Edn1*$^{H/+}$ x ♂WT	p (ANOVA)		
					G	P	GxP
Plasma ET-1 (pg/ml)	1.30±0.26	5.4±1.2*	1.29 ±0.07	8.9±0.5†	<0.01	<0.01	<0.01
Plasma sFLT1 (ng/ml)	1.35±0.06	2.03±0.14*	40.1±5.3	47.9±6.3	n.s.	<0.01	n.s.
Plasma Cho (mg/dL)	82±5	73±4	48±8	56±6	n.s.	<0.01	n.s.
Plasma Glu (mg/dL)	141±7	129±6	164±14	133±15	n.s.	n.s.	n.s.
Plasma VEGF (pg/dL)	11±3	42±10*	101±14	99±10	n.s.	<0.01	n.s.
Plasma sENG (pg/dL)	1.3±0.1	1.2±0.1	1.2±0.1	1.3±0.1	n.s.	n.s.	n.s.
Plasma AST (U/L)	35±3	98±13*	56±9	101±16†	<0.01	n.s.	n.s.
Body weight (g)	19.0±0.3	19.5±0.6	34.6±1.4	29.2±1.3	<0.01	<0.01	0.02
Food intake (g)	3.63±0.09	3.33±0.12	ND	ND			
Water intake (g)	2.77±0.39	3.26±0.42	ND	ND			
Urine volume (ml)	1.18±0.15	1.08±0.12	ND	ND			

Female virgin mice at the age of 8 wk were put into individual metabolic cages for 2 days. Every 24hr, body weight, food intake, water intake and urine volume were measured. Blood samples were collected at the end of the metabolic study. In order to avoid a miscarriage due to the stress induced by metabolic cages, pregnant mice were not subjected to this procedure. For pregnant dams, blood samples were collected at 18.5 dpc. Cho: cholesterol, Glu: glucose, AST: aspartate aminotransferase, Two way-ANOVA was used with genotype (G) and pregnancy (P) as two factors, GxP: interaction between genotype and pregnancy. *p <0.05 vs. WT virgin, †p <0.05 vs. ♀WT (x ♂WT). n≥4. ND: not determined. n.s. not significant.

Table S2. *Edn1* genotype of neonates from ♀*Edn1*$^{H/+}$ (X ♂ WT) and ♀WT (X ♂*Edn1*$^{H/+}$).

♀*Edn1*$^{H/+}$ X ♂ WT

genotype	Observed (#)	Expected (%)	outcome (%)
Edn1$^{H/+}$	20	50	44
Edn1$^{+/+}$	26	50	56

From 8 pregnancies (♀*Edn*$^{H/+}$ X ♂ WT). p=0.48 by Fisher's exact test

♀WT X ♂*Edn1*$^{H/+}$.

genotype	Observed (#)	Expected (%)	outcome (%)
Edn1$^{H/+}$	30	50	46
Edn1$^{+/+}$	35	50	54

From 8 pregnancies (♀WT X ♂*Edn1*$^{H/+}$). p=0.66 by Fisher's exact test

Table S3. Primers and probes for qRT-PCR

Gene	Type	Sequence (5'-3')
m-sFlt1	*Forward*	*TCG GAA GAC AGA AGT TCT CG*
	Reverse	TTG GAG ATC CGA GAG AAA AT
	Probe	FAM-AG AGG TGA GCA CTG CGG CAA AAA G- TAMRA
h-*sFLT1(e15a)*	Forward	ATG GTG GCT CAC GCC TGT
	Reverse	CTT GGA CTC CTG ACC TCA AAT
	Probe	FAM-TC CCA GCA CTT TGG GAG GCC AGG- TAMRA
h-*sFLT1(i13)*	Forward	GGGAAGAAATCCTCCAGAAGAAAGA
	Reverse	GAGATCCGAGAGAAAACAGCCTTT
	Probe	FAM-CA GTGCTCACCTCTGATTG- TAMRA [1]
m-Flt1	Forward	TCG GAA GAC AGA AGT TCT CG
	Reverse	ACC TCG TAG TCA CTG AGG TT
	Probe	FAM-AG ATT CGG AAG CGC CAC ACC TGC A- TAMRA
h-*FLT1*	Forward	CTT CAC CTG GAC TGA CAG CA
	Reverse	AGC CCC GAC TCC TTA CTT TTA
	Probe	FAM-AG GCC TCG CTC AAG ATT GAC ATC AG- TAMRA
m-*Edn1*	Forward	TGC CAC CTG GAC ATC ATC TG
	Reverse	ACG CTT GGA CCT GGA AGA AC
	Probe	FMA-TC CCG AGC GCG TCG TAC CGT ATG- TAMRA
m-*Cdh1*	Forward	GCT CTC ATC ATT GCC ACA GA
	Reverse	TTG TCG TTG ACG TCT GAC AG
	Probe	FAM-TC ACC CAT TGC CAC TGG CAC AGG- TAMRA
m-*Hprt*	Forward	GGA CTG ATT ATG GAC AGG AC
	Reverse	CAG AGG GCC ACA ATG TGA T
	Probe	FAM-CC TCC CAT CTC CTT CAT GAC ATC TCG- TAMRA
h-*GAPDH*	Forward	GAA GGT GAA GGT CGG AGT C
	Reverse	GAA GAT GGT GAT GGG ATT TC
	Probe	FAM-CA AGC TTC CCG TTC TCA GCC- TAMRA

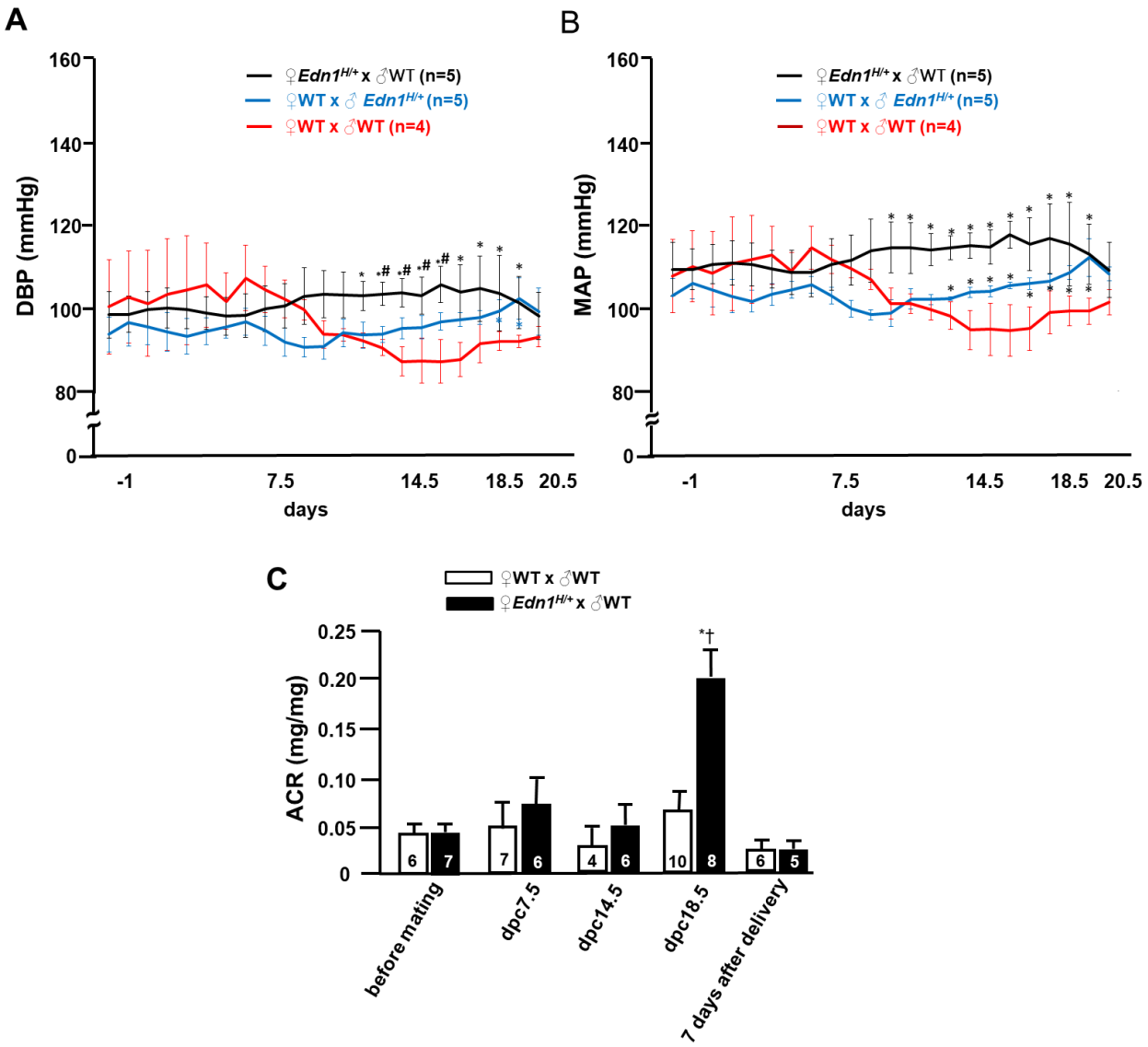

Figure S1. A, Diastolic blood pressure (DBP), B, Mean arterial pressure (MAP), and **C, Urinary albumin creatinine ratio (ACR) during different stages of pregnancy.** *p<0.05 vs. ♀WT (x ♂WT), t test; #p<0.05 vs. before pregnancy, paired t test; †p<0.05 vs. other time points of *Edn1*$^{H/+}$ mice**.** Numbers of mice are within the bars.

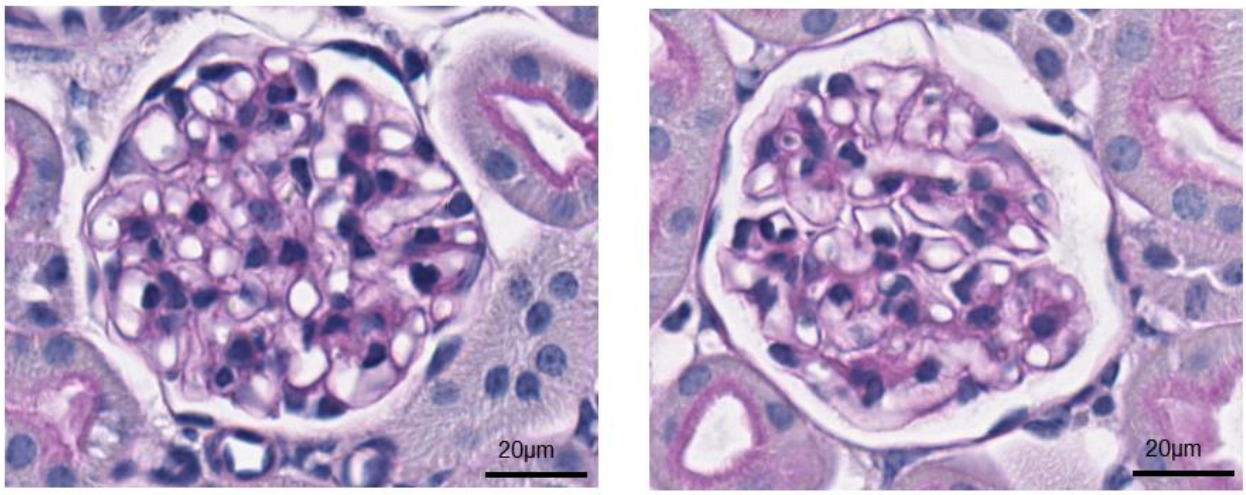

Figure S2. Representative glomerulus from WT and *Edn1*$^{H/+}$ virgin female mice. There is no obvious difference between the two genotypes.

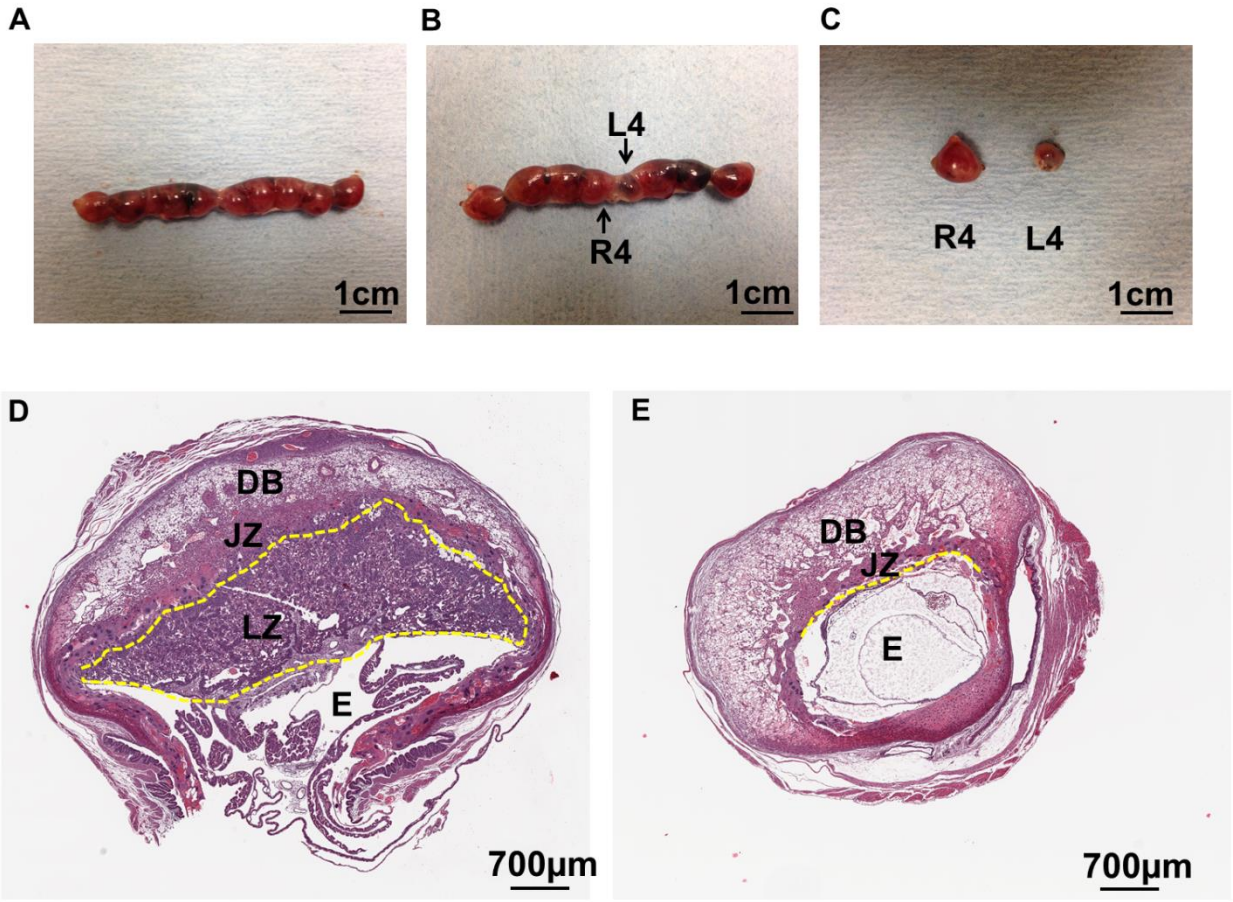

Figure S3. Abnormal conceptuses are present in ♀*Edn1*$^{H/+}$ (x ♂WT) mice at 12.5 dpc. At 12.5 dpc, pregnant mice were euthanized and uteri were isolated and total conceptuses were counted. Two out of seven pregnant *Edn1*$^{H/+}$ mice examined had runted conceptuses. **A**, Representative uterus from ♀WT (x ♂WT) mice. **B**, Representative uterus from ♀*Edn1*$^{H/+}$ (x ♂WT) mice with a runted conceptus, marked L4. **C,** The size of the runted conceptus L4 is approximately 1/3 of normal R4 from the same litter. The genotype of L4 is WT and R4 is *Edn1*$^{H/+}$. **D,** H&E staining of normal placenta from ♀WT (x ♂WT). **E**, The placenta of a runted WT fetus from ♀*Edn1*$^{H/+}$ (x ♂WT) mice. DB:decidual basalis; JZ: junctional zone; LZ: labyrinth zone within yellow dashed line; E: embryonic cavity. Note: the runted placenta did not have a LZ and had a smaller JZ.

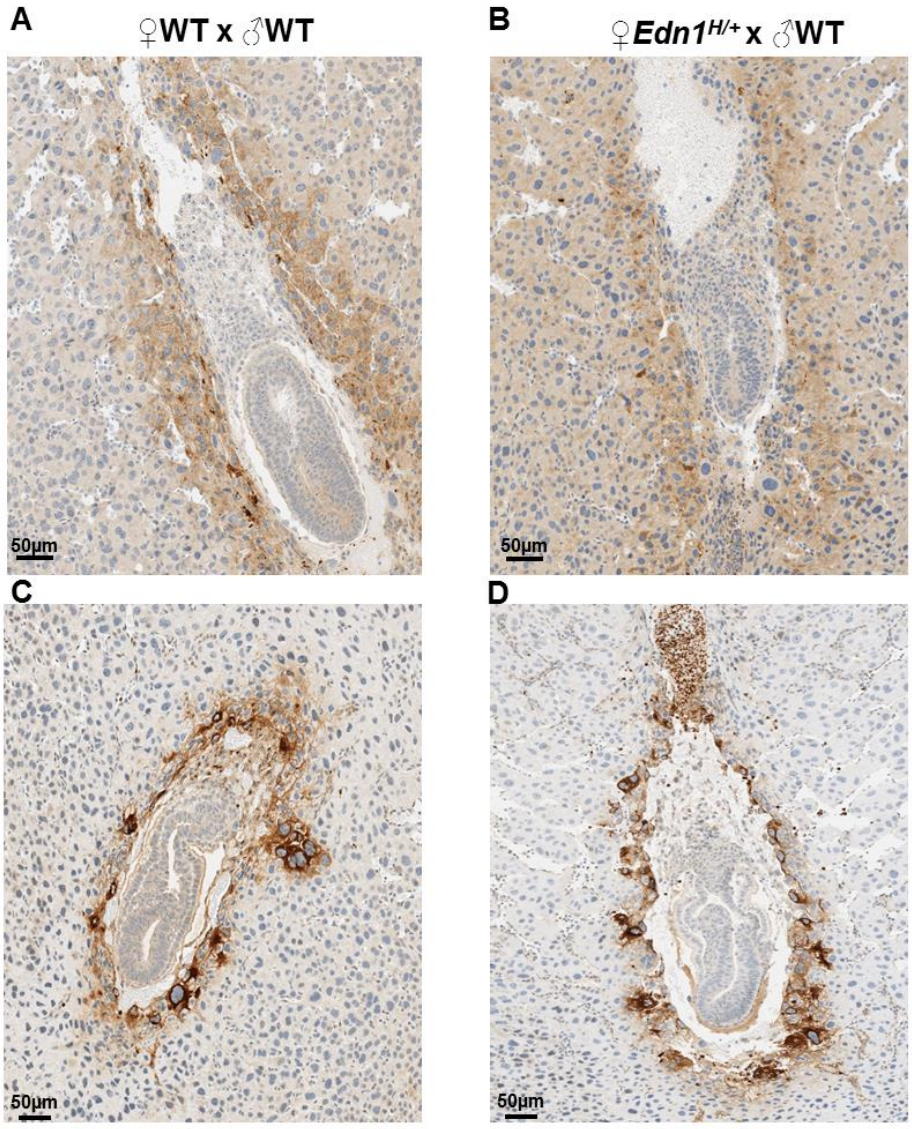

Figure S4. MMP9 and TIMP3 expression are not altered in the *Edn1$^{H/+}$* embryo implantation site. A&**B**, TIMP3 IHC staining. **C**&**D**, MMP9 IHC staining.

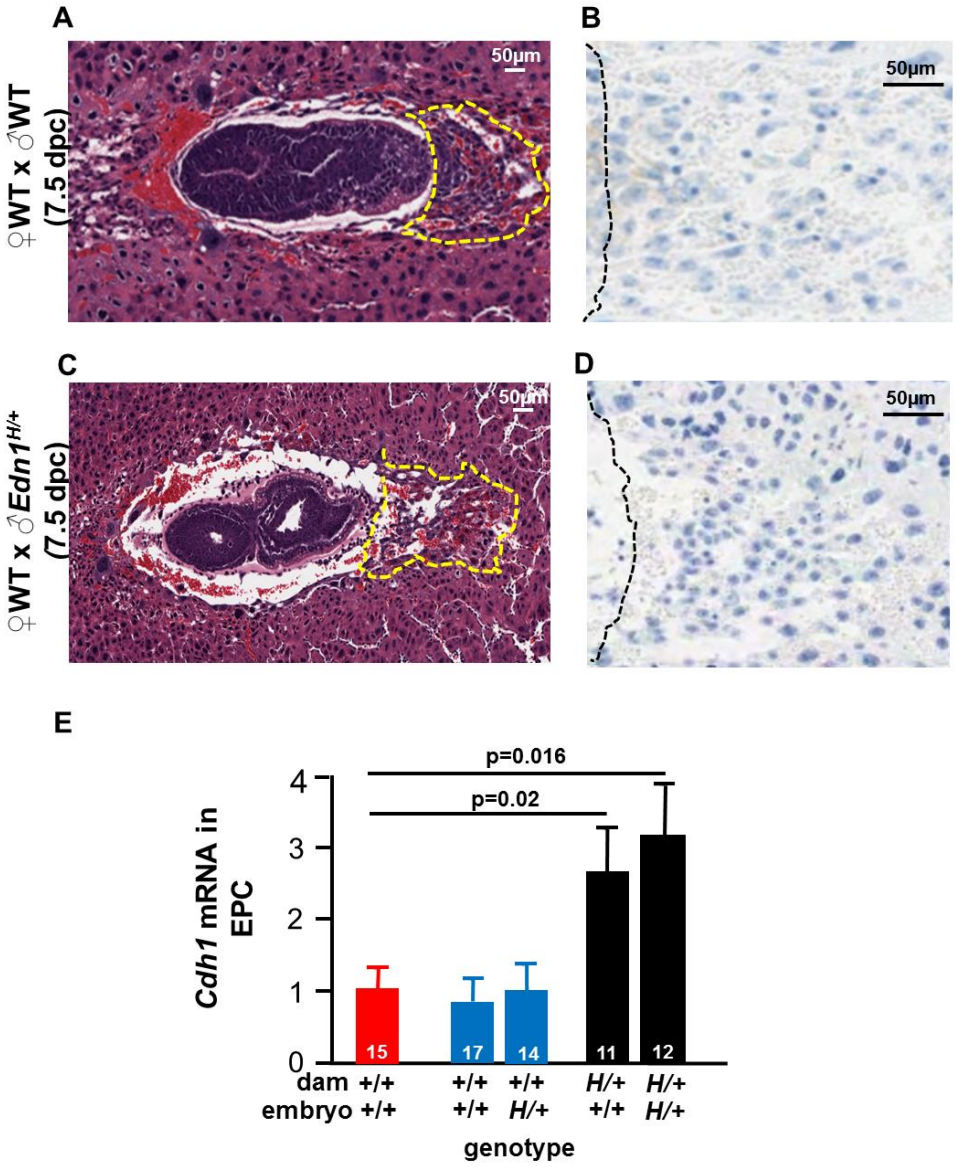

Figure S5. The expression of E-cadherin in EPC region is not different between ♀WT (x ♂WT) and ♀WT (x ♂*Edn1*$^{H/+}$) mice. **A**&**C**, Midtransverse sections of embryo implantation sites, H&E staining. **B**&**D**, immunohistochemistry staining of E-cadherin in EPC region (right side of the black dashed lines) from (**A**) and (**C**). **E**, mRNA expression of *Cdh1* (coding E-cadherin) in EPC region from 5 groups of embryos. Numbers of EPC examined are within the bar.

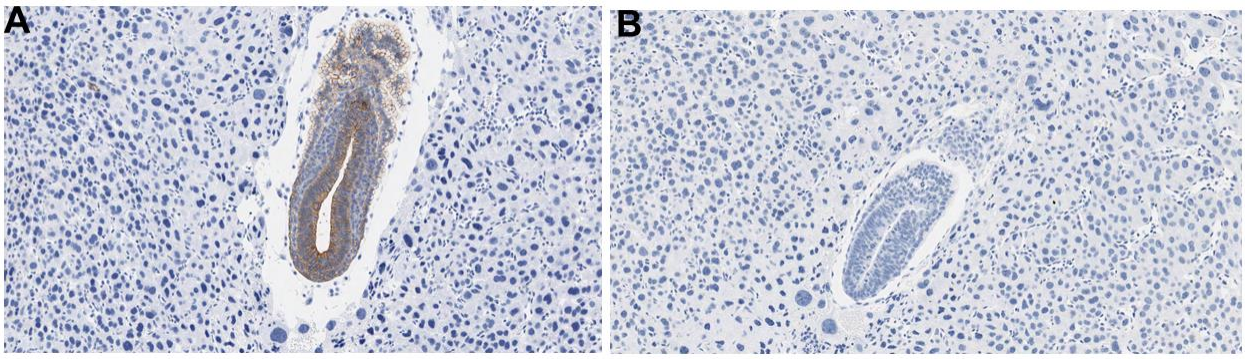

Figure S6. Immunohistochemistry staining. A, positive staining of E-cadherin. B, Isotype control with rabbit IgG [Abcam (ab171870)].

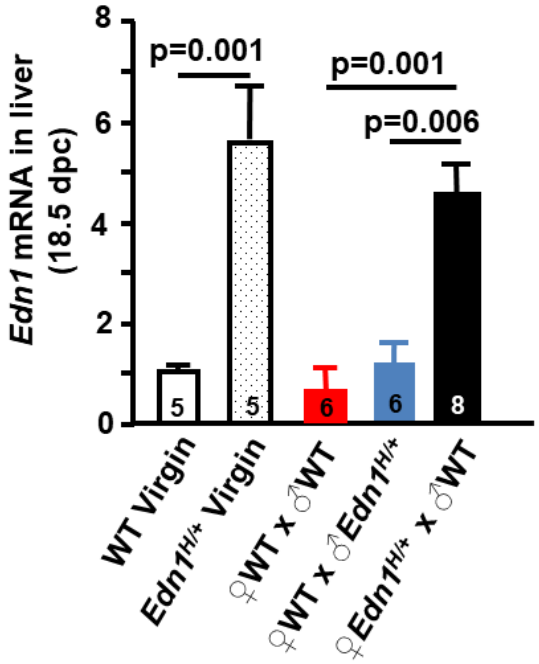

Figure S7. mRNA level in livers from virgin females and pregnant mice at 18.5 dpc. Numbers of livers analyzed are within the bars. Multiple comparisons used the Tukey–Kramer HSD test. Error bars are SEM.

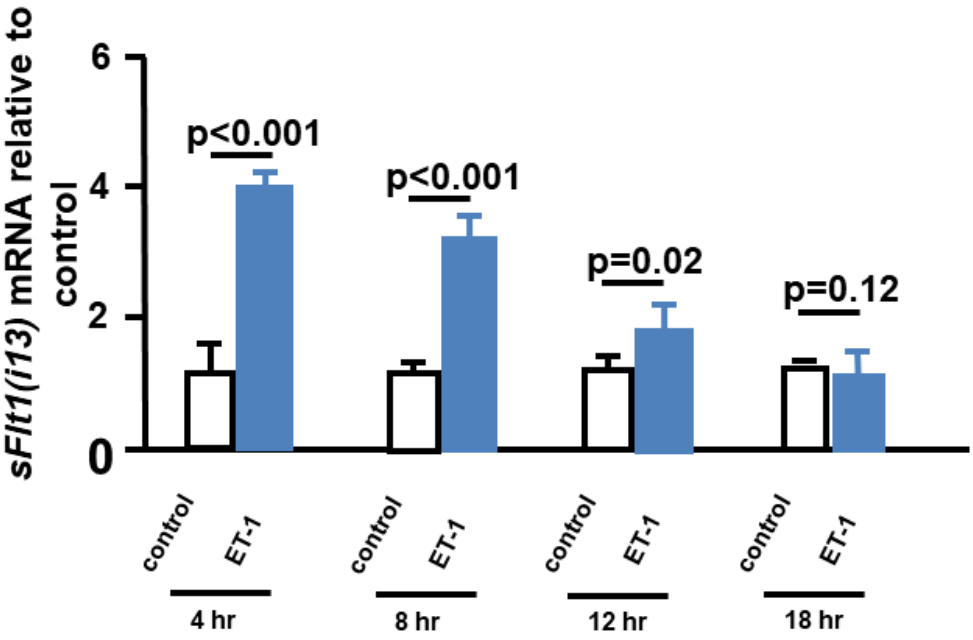

Figure S8. Cellular mRNA levels of *sFlt1* transcript for the variant i13.
HTR8/SVneo cells treated with 0.5 µM ET-1 (fold difference relative to control cells without ET-1). n=4