SEE COMMENTARY

Pravastatin induces placental growth factor (PGF) and ameliorates preeclampsia in a mouse model

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Preeclampsia is a relatively common pregnancy-related disorder. Both maternal and fetal lives will be endangered if it proceeds unabated. Recently, the placenta-derived anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFLT1) and soluble endoglin (sENG), have attracted attention in the progression of preeclampsia. Here, we established a unique experimental model to test the role of sFLT1 in preeclampsia using a lentiviral vector-mediated placenta-specific expression system. The model mice showed hypertension and proteinuria during pregnancy, and the symptoms regressed after parturition. Intrauterine growth restriction was also observed. We further showed that pravastatin induced the VEGF-like angiogenic factor placental growth factor (PGF) and ameliorated the symptoms. We conclude that our experimental preeclamptic murine model phenocopies the human case, and the model identifies low-dose statins and PGF as candidates for preeclampsia treatment.

3-hydroxy-3-methyl-glutaryl-CoA | hemolytic anemia, elevated liver enzymes, and low platelet count | trophoblast

Preeclampsia originates from placental insufficiency and is observed in ≈5% of pregnant females (1). Although preeclampsia is a major cause of maternal and infantile morbidity and mortality worldwide, the fundamental therapy available is to terminate the pregnancy. Therefore understanding the pathogenesis of preeclampsia is highly important and the generation of an appropriate animal model would be helpful to develop therapeutic drugs. In preeclamptic women, Maynard et al. found that increased soluble fms-like tyrosine kinase-1 (sFLT1) associated with decreased circulating levels of free VEGF and placental growth factor (PGF) (2). Because sFLT1 is a truncated form of VEGF receptor 1 and antagonizes VEGF and PGF function, impaired angiogenic signaling most likely results in endothelial dysfunction and preeclampsia (3).

To establish an animal model of preeclampsia, placenta-specific gene manipulation is preferable because preeclampsia originates from a failure in placentation, and factors up-/ or down-regulated in the impaired placenta accentuate the symptoms. However, the lack of a facile and efficient method for placenta-specific gene manipulation has hampered the investigation of preeclampsia. The systemic administration of adenoviral vector (AdV-) expressing *sFLT1* into pregnant rats resulted in classic signs of preeclampsia such as hypertension, proteinuria, and glomerular endotheliosis. However, unlike in patients, the *sFLT1* expression in the rat model was transient and was mainly produced in the maternal liver, not in the placenta (2, 4). Previously, we and other groups have reported the placenta-specific transgenesis and expression by transducing blastocysts-stage embryos with HIV-I–based self-inactivating lentiviral vectors (5–7). The lentiviral vectors transduced the outermost layer of the blastocyst, the trophectoderm, that provides most of the main and functional components of the future placenta. By contrast, the vectors were not able to transduce the inner cell mass that constitutes the future fetus. Thus placenta-specific gene manipulation was successfully achieved.

Applying this technology, here we expressed human *sFLT1* (*hsFLT1*) specifically in the murine placenta to develop a unique preeclampsia model animal. Taking advantage of our method, we observed impaired placental vasculogenesis and intrauterine growth restriction (IUGR). We further demonstrated that a 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor, pravastatin, induces PGF and ameliorates the sFLT1-induced preeclampsia in the model mice. Our model establishes a system for investigating preeclampsia and developing treatments for the clinic.

Results and Discussion

We expressed human *hsFLT1* specifically in the murine placenta to develop a unique preeclampsia model (Fig. 1*A*). When we transduced blastocysts with a lentiviral vector expressing *hsFLT1*, LV-hsFLT1, and transplanted the blastocysts into pseudopregnant females, PCR and RT-PCR analysis demonstrated that the integration and expression of the LV-hsFLT1 transgene was specific to placenta (Fig. 1*B*). As we expected, the placenta-derived hsFLT1 circulated in the maternal blood (Fig. 1 *C* and *D*). The concentration of the circulating hsFLT1 at embryonic day 18.5 (E18.5) correlated with the lentiviral vector amount in transduced blastocysts (Fig. 1*C*). When we transduced blastocysts with LV-hsFLT1 at 100 ng of p24/mL, the circulating hsFLT1 concentration in the mother gradually increased along with placental growth during gestation, reaching its peak (average 5.84 ± 1.26 ng/mL) at E18.5 (Fig. 1*D*), which was comparable to the levels seen in human patients (≈4.38 ng/mL compared with 1.64 ng/mL in the control) (3).

After the elevation of hsFLT1, systolic as well as diastolic blood pressure significantly increased at E16.5 and continued during the rest of pregnancy ($P < 0.05$, Fig. 1*E*). It should be noted that the blood pressure promptly normalized postpartum, which mimics human recovery after delivery. The hypertensive effect was also observed in the group treated with LV-hsFLT1 at 20 ng of p24/mL. The pregnant female carrying the LV-hsFLT1–transduced placenta showed glomerular endotheliosis (Fig. S1) and proteinuria ($P < 0.05$ in albumin/creatinine ratio, Table 1). These data indicated that the placenta-specific overexpression of

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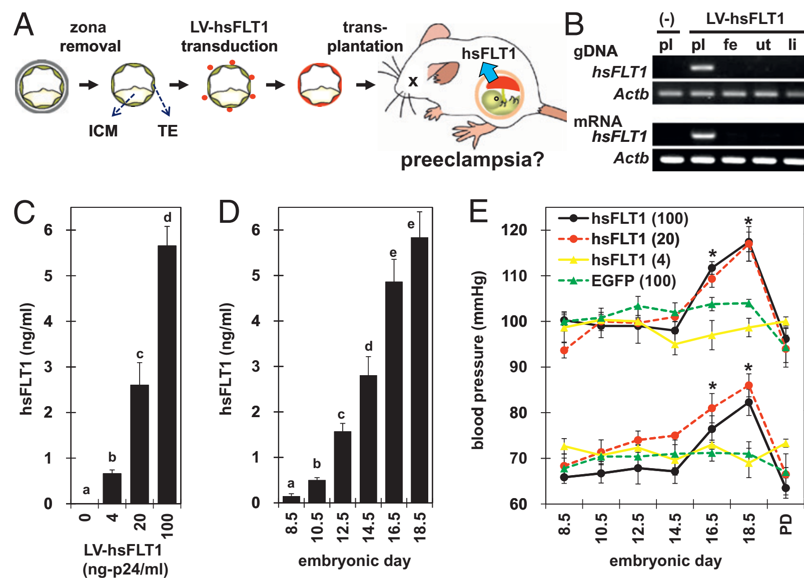

Fig. 1. Preeclampsia model generated by placenta-specific *sFLT1* expression. (*A*) Scheme for generating preeclampsia model mice. Zona pellucida–free blastocysts were transduced with a lentiviral vector expressing *hsFLT1* (LV-hsFLT1) and transplanted into pseudopregnant females. The transduced trophectoderm (TE) cell lineage provides the main components of the placenta and continuously expresses *hsFLT1*. ICM, inner cell mass. (*B*) Genomic DNA (gDNA) and mRNA collected from indicated tissues at E13.5 were subjected to PCR and RT-PCR, respectively. Actb was used as control. pl, placenta; fe, fetus; ut, uterus; li, liver. (*C*–*E*) Concentration of hsFLT1 (*C* and *D*) and blood pressure (*E*) were measured in the females carrying LV-hsFLT1–transduced placentas. (*C*) The circulating hsFLT1 in the mother at E18.5 depended on the lentiviral vector amount used for transduction (n = 5, 4, 5, and 7 in 0, 4, 20, and 100 ng/mL of LV-hsFLT1, respectively). (*D*) The circulating hsFLT1 in the mother increased during pregnancy (n = 5 in 100 ng of p24/mL of LV-hsFLT1). (*E*) Systolic and diastolic blood pressure were measured during pregnancy (n = 5, 3, 3, and 7 in 0, 4, 20, and 100 ng/mL of LV-hsFLT1, respectively). Both systolic and diastolic blood pressure at E16.5 and E18.5 in the group treated with 20 and 100 ng of p24/mL of LV-hsFLT1 were significantly higher than those in the control group treated with 100 ng of p24/mL of LV-EGFP (*$P < 0.05$). Elevated blood pressure promptly normalized after delivery of the placenta. PD, postdelivery. There are significant differences among the values labeled with different lowercase letters in *C* and *D* ($P < 0.05$).

hsFLT1 provided the basis for a unique and relevant animal model for preeclampsia.

Statins are drugs generally used for hypercholesterolemia, but it has been recently reported that statins have a protective effect on vascular endothelial cells (8, 9). Moreover, although it is not a preeclamptic model, the administration of pravastatin rescued placental dysfunction and prevented miscarriages in a spontaneous-abortion model mouse (10). To examine the therapeutic effect of pravastatin on our experimental preeclampsia model, we i.p. administered pravastatin at 5 μg/d, which is equivalent to a human therapeutic dose of 10 mg/d. It should be noted that pravastatin is not hypotensive in normal pregnant females. When we administered pravastatin every day from E7.5 ($P < 0.01$) or E10.5 ($P < 0.01$), a prophylactic/therapeutic effect on hypertension was observed at E16.5 and later (Fig. 2*A*). Administration of pravastatin from E13.5 also decreased the blood pressure, but it was not statistically significant. Glomerular endotheliosis and proteinuria were also improved when we treated with pravastatin starting at E7.5 (Fig. S1 and Table 1).

Table 1. Proteinuria observed in sFLT1-induced preeclamptic mice

Lentiviral vector	*n*	Albumin/creatinine	*P*
LV-EGFP	12	5.27 ± 0.74	—
LV-hsFLT1	14	7.47 ± 0.61	0.03
LV-hsFLT1 (PD)	5	4.50 ± 0.38	0.52
LV-hsFLT1 + pravastatin	8	4.06 ± 0.38	0.23
LV-hsFLT1 + LV-mPGF	15	6.41 ± 0.61	0.24

Blastocysts were transduced with lentiviral vectors at 100 ng of p24/mL. Pravastatin was administered at 5 μg per body every day starting at E7.5. Urine was collected at E18.5 or 1 wk postdelivery (PD) and analyzed. Average ± SEM. Student's *t* test was performed against LV-EGFP treatment.

In the next experiment, we investigated how pravastatin ameliorated sFLT1-induced hypertension. Because sFLT1 interacts with and antagonizes the angiogenic function of VEGF and PGF, we measured the maternal plasma levels of these factors at E18.5 (Fig. 2*B*). When pravastatin was administered every day starting at E7.5, the circulating hsFLT1 was significantly decreased from 5.84 ± 1.26 to 0.99 ± 0.65 ng/mL ($n = 5$, $P < 0.001$) in the pregnant females carrying LV-hsFLT1–transduced placentas. In contrast, mouse PGF (mPGF) was significantly increased by pravastatin treatment (from 60.3 ± 12.6 to 116.6 ± 13.2 pg/mL, $n = 7$, $P < 0.001$), whereas mouse VEGF (mVEGF) was not changed (from 44.7 ± 22.3 to 35.5 ± 13.6 pg/mL, $n = 6$, $P = 0.36$).

To determine whether PGF counteracts sFLT1 and shows therapeutic effects on preeclampsia, we simultaneously expressed *mPGF* with *hsFLT1* in the placenta by transducing the blastocyst with LV-hsFLT1 and LV-mPGF (100 ng of p24/mL for each vector). We used LV-EGFP instead of LV-mPGF in the control experiment. Excessive mPGF diminished the circulating hsFLT1 in the maternal blood ($n = 5$, $P < 0.001$, Fig. 2*B*) and ameliorated the hypertension ($n = 6$, $P < 0.01$, Fig. 2*A*). The ELISA kit used in the present study measures total hsFLT1, suggesting that the mPGF interaction might destabilize hsFLT1 in vivo. Although the mechanism is still unclear, it was reported that the reduction of sFLT1 after administration of recombinant VEGF in vivo (11). LV-mPGF transduction alone did not show any hypotensive effects in normal pregnant females. In addition to hypertension, glomerular endotheliosis and proteinuria were also improved by the mPGF (Fig. S1 and Table 1). These data support the idea that pravastatin-induced PGF counteracts the effect of sFLT1 and ameliorates the preeclamptic symptoms.

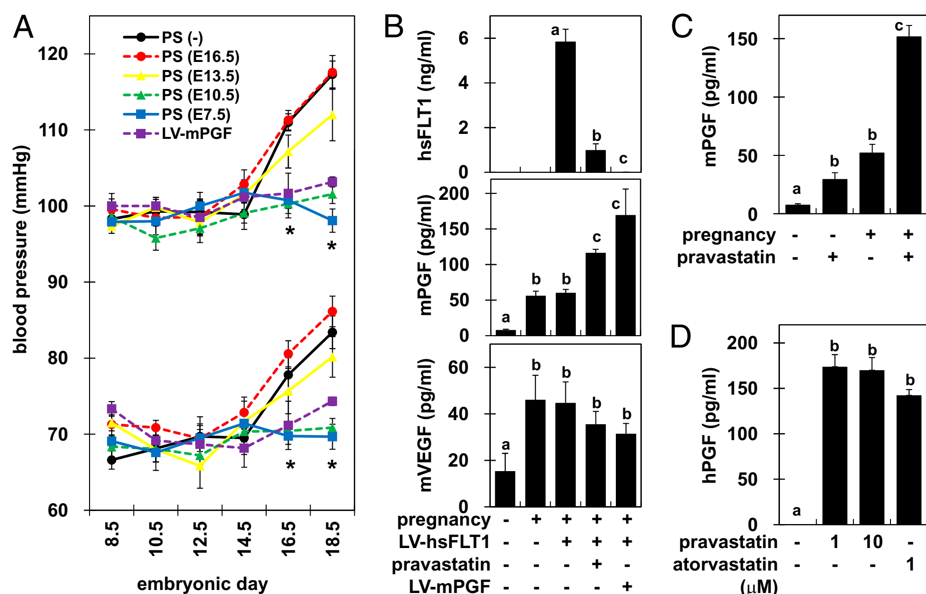

Fig. 2. Pravastatin induced mPGF and ameliorated the preeclampsia. (*A*) Hypertension induced by the placenta-specific *hsFLT1* expression was ameliorated by pravastatin (PS). Pravastatin (5 μg/d) was i.p. administered into the females every day starting at the indicated day (n = 12, 14, 6, 7, and 10 for E7.5, E10.5, E13.5, E16.5, and control, respectively). Both systolic and diastolic blood pressure at E16.5 and E18.5 in the group of pravastatin treatment starting at E10.5 or earlier and LV-mPGF were significantly lower than those in the control group treated with 100 ng of p24/mL of LV-hsFLT1 (*$P < 0.01$). (*B*) The concentrations of hsFLT1, mPGF, and mVEGF circulating in the mother were measured at E18.5. (n = 3–9) (*C*) Wild-type pregnant female mice were given pravastatin every day starting at E7.5. The concentration of mPGF was measured at E18.5. Age-matched nonpregnant females were treated with the same protocol (n = 3–7). (*D*) HUVECs were cultured with statins at the indicated concentration for 24 h. The human PGF concentration in the supernatant was measured by ELISA (n = 3). There are significant differences among the values labeled with different lowercase letters in *B–D* ($P < 0.05$).

To determine whether pravastatin induces mPGF independently of lentiviral vector transduction and/or *hsFLT1* expression, we administered pravastatin into wild-type females (Fig. 2*C*). The circulating mPGF was significantly increased in the pregnant females from 52.4 ± 20.2 pg/mL to 152.0 ± 19.7 pg/mL ($n = 4$, $P < 0.001$). It is interesting to note that detectable amounts of mPGF circulated in the nonpregnant females (7.8 ± 2.9 pg/mL) and this increased to 29.8 ± 13.2 pg/mL after pravastatin treatment ($n = 5$, $P < 0.01$). When we compared the *mPgf* mRNA amounts by quantitative RT-PCR, we confirmed that placenta is the main organ producing mPGF upon pravastatin treatment (Fig. S2). In addition, heart and aorta also showed the increased *mPgf* expression with pravastatin treatment. These data indicate that not only placenta but also other tissues are able to produce PGF upon pravastatin treatment. We next examined whether the vascular endothelial cells themselves are able to produce PGF upon statin treatment (Fig. 2*D*). Without statins, human umbilical vascular endothelial cells (HUVECs) did not produce PGF (<5 pg/mL, $n = 3$). Both pravastatin and atorvastatin induced PGF production (173.7 ± 13.5 and 142.3 ± 6.4 pg/mL at 1 μM, $n = 3$, $P < 0.001$ for both pravastatin and atorvastatin). It should be noted that pravastatin did not induce PGF production in HEK293 cells (<5 pg/mL, $n = 3$).

Taking advantage of our placenta-specific gene manipulation method, we evaluated the local effects of excess hsFLT1 on placentation and fetal development. The ratio of implantation to live birth was comparable to that of LV-EGFP–treated control mice (Table S1). The pups were delivered on the expected date in both groups. Although the LV-hsFLT1–transduced placentas looked normal and were able to support fetal development, maternal and fetal blood spaces were reduced in the labyrinthine layer (Fig. S3). When we immunostained for CD31 (platelet/endothelial cell adhesion molecule 1) as a marker for angiogenesis, the suppression of vascular bed development in the labyrinthine layer was observed at E13.5 (Fig. 3*A*). Both placenta and fetus from LV-hsFLT1–treated females (88.6 ± 1.9 mg and 1109 ± 1.9 mg, respectively) were significantly smaller than control placenta and fetus (109.8 ± 1.3 mg vs. 1293 ± 6.5 mg, respectively; $P < 0.001$ for both placenta and fetus) at E18.5 (Fig. 3 *B–D*). Other than the IUGR, we did not notice any abnormality in the pups ($n > 100$ in each group), and they developed healthily to term and were fertile. The impaired placentation and IUGR were also restored by the pravastatin treatment and the placenta-specific *mPGF* expression (Fig. 3), supporting the idea that statin-induced PGF counteracts sFLT1 and rescues impaired placentation and IUGR in preeclampsia.

In our model mice, we observed preeclamptic symptoms after placenta-specific overexpression of *hsFLT1*. We further demonstrated that excess placental sFLT1 causes impaired placentation and subsequent IUGR. Because IUGR is commonly associated with preeclampsia and increases the risk for adverse pregnancy outcomes (12), our model will also be beneficial for understanding these symptoms in the clinic. However, we did not observe the development of severe symptoms such as hemolysis, elevated liver enzymes, and low platelets (called HELLP syndrome). As for liver enzymes, aspartate transaminase and alanine transaminase remained comparable to controls in the LV-hsFLT1–treated animals (Table S2). One simple explanation could be the shorter gestation period in mice, but contributions of additional preeclamptic factors such as soluble endoglin (sENG) (13, 14), hypoxia-inducible factor 1, α subunit (HIF1a) (15), coagulation factor 3 (F3) (10), catechol-*O*-methyltransferase (COMT) (16), and heme oxygenase 1 (HMOX1) (17) might need to be taken into account. In fact, sENG in combination with sFLT1 synergistically aggravate endothelial-cell dysfunction and induce more severe symptoms such as HELLP syndrome symptoms and cerebral edema (14). Because we expressed *hsFLT1* and *mPGF* simultaneously, our mouse model can be used in future to examine the multiple factors involved in HELLP syndrome by transducing lentiviral vectors expressing different factors.

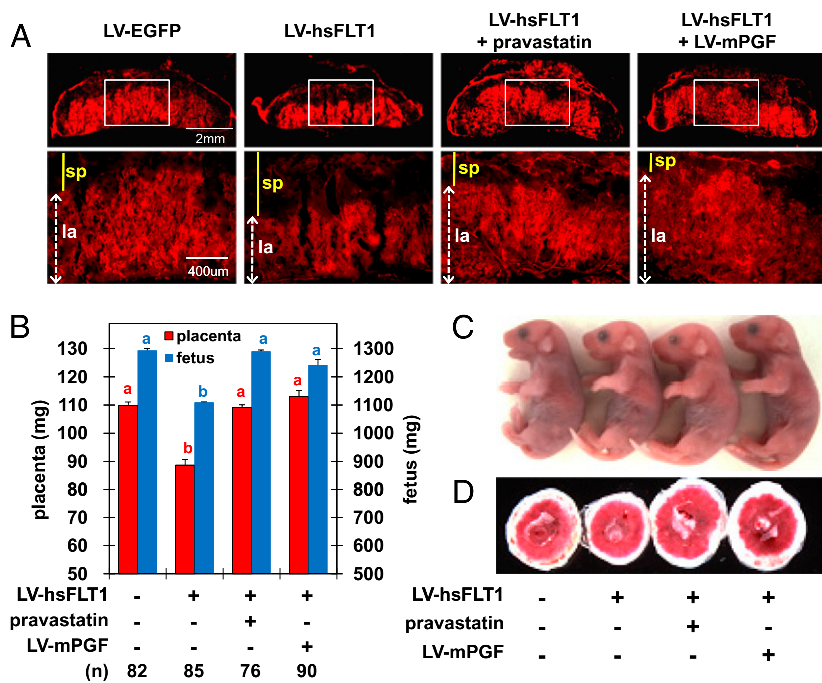

Fig. 3. Pravastatin and mPGF restored impaired placentation and IUGR in the preeclampsia. (*A*–*D*) Placenta-specific *hsFLT1* expression impaired placental bed development and caused IUGR. (*A*) Impaired vasculogenesis in the placenta was restored by pravastatin treatment and *mPgf* expression. Placentas were collected at E13.5, and vascular endothelial cells were stained with anti-CD31 antibody so that the region with more blood vessels appears in red. The labyrinthine area indicated with white box in *Upper* is magnified in *Lower*. la, labyrinthine layer; sp, spongiotrophoblast layer. (*B*–*D*) Fetuses and placentas collected by Caesarian section at E18.5. (*B*) Average fetal and placental weight. There are significant differences among the values labeled with different lowercase letters in the same color ($P < 0.05$). (*C*) Live and healthy pups were obtained in all groups. Malformation in gross appearance was not observed after pravastatin treatment. Because the difference in weight is ≈20% and is ≈6% in each dimension, it is difficult to see the weight differences in the photographs. (*D*) Placentas. Photos were taken from the fetal side.

Although the excess sFLT1 in the placenta caused some of the preeclamptic symptoms, it is still not clear whether human preeclampsia is initiated by elevated sFLT1 or is simply part of the end pathology. In addition to the sFLT1-induced models, preeclampsia model animals have been developed by other approaches, including the *Comt*$^{-/-}$ pregnant female resulting from an absence of 2-methoxyoestradiol (16), the angiotensinogen-expressing transgenic female mice mated with transgenic males expressing renin (18), and hypertensive pregnant rat model produced through the long-term reduction in uterine perfusion pressure (19). Comparative study of these models might shed light on the pathogenesis of human preeclampsia.

In humans, preeclampsia may represent at least two distinct entities: early-onset disease, being more severe and more often associated with HELLP syndrome, and late-onset disease, which is often milder. Our model might represent the late-onset case because blood pressure increased at the later stage of gestation and did not show severe symptoms such as cranial hemorrhage and HELLP syndrome. However, careful comparison will be required, because human and mouse placentas differ not only in the gestation length but also in some aspects of morphogenesis and endocrine function (20, 21).

As for IUGR, the transgenic *hsFlt1* is expected to be expressed by all trophoblast cells in our system (5), whereas endogenous *sFlt1* is expressed in the spongiotrophoblast cells and the maternal and fetal endothelial cells in the placenta (22). The different expression pattern might have different impacts on fetal development. However, when we implanted the control LV-EGFP–transduced embryos along with the LV-hsFLT1–transduced embryos into the same recipient, comparable amounts of hsFLT1 were detected in both fetuses at E18.5 (170.8 ± 76.1 and 164.4 ± 46.2 pg/mL, respectively, $n = 5$). This finding implies that the hsFLT1 circulating in the mother can pass through the placenta into the fetus and contribute in the IUGR.

In the present study, we demonstrated that preeclamptic symptoms, induced by hsFLT1, could be ameliorated by pravastatin treatment. Although both VEGF and PGF are known to ease endothelial dysfunction (23, 24), we propose that, with pravastatin treatment, endothelial dysfunction was ameliorated not by VEGF but by PGF. This report shows that the administration of therapeutic doses of pravastatin induces both placental and nonplacental *Pgf* expression in live animals. It is of great interest to know whether the nonplacental PGF also functions during the statin-mediated recovery from vascular problems such as coronary disease (25–27) and ischemia (28).

When we consider applying statin treatments to human preeclampsia, the teratogenicity of statins could become an obstacle because the Food and Drug Administration classifies statins as category X and discourages the use of statins during the first trimester. However, many recent reports indicate that statin usage in the first trimester is safe (29–32). We also did not notice any deformity in the pups derived from pravastatin-treated females during our experiments. Thus, the careful use of statins in the treatment of preeclampsia might be possible. Our experimental model can be used to develop further therapeutics for preeclampsia to save numerous pregnant women and infants from morbidity and mortality worldwide.

Methods

Primers and PCR. The *hsFLT1* cDNA was amplified by RT-PCR from HUVECs (Kurabo) with primers 5′-aaggatccgccgccatggtcagctactgggac-3′ and 5′-ttctcgagttaatgttttacattactttgtgtg-3′. The *mPgf* cDNA was amplified by RT-PCR from E13.5 murine placenta with primers 5′-aagaattcgccaccatgctggtcatgaagctgttc-3′ and 5′-ttctcgagtcacgggtggggttcctcag-3′. Primers used in Fig. 1*B* are 5′-aagtgtgacgttgacatccg-3′ and 5′- gatccacatctgctggaagg-3′ for *mActb* an d 5′-ggctgagcataactaaatctgcc-3′ and 5′-ggaatgacgagctcccttccttca-3′ for *hsFLT1*.

Lentiviral Vectors. The HIV-I based, self-inactivating, lentiviral vector plasmid pLV-EGFP was described previously (5). Other lentiviral vector plasmids, pLV-hsFLT1 and pLV-mPGF, were prepared by replacing the EGFP cDNA with the hsFLT1 and mPGF cDNA, respectively. Vesicular stomatitis virus glycoprotein–pseudotyped lentiviral vectors were generated, and p24 gag antigen concentration was measured as described previously (5, 33).

Mice and Lentiviral Transduction. Wild-type B6D2F1 females were superovulated by i.p. injection of pregnant mare's serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with wild-type B6D2 F1 males. Two to four cell-stage embryos were collected from the females at 1.5 d after copulation and then incubated in kSOM medium (34) for 2 d to obtain blastocysts. Zona pellucida was removed in acidic Tyrode's solution (Sigma) (35), and thus-prepared zona

pellucida–free blastocysts were incubated individually for 4 h in 5 μL of medium containing lentiviral vectors. The blastocysts were transduced with lentiviral vectors at 100 ng of p24/mL throughout the experiments unless otherwise indicated. When the LV-hsFLT1 amount was varied, LV-EGFP was used to keep the overall amount of LV at 100 ng of p24/mL. The transduced blastocysts were washed three times and then implanted into pseudopregnant ICR females. We transplanted 10 blastocysts into each horn of the uterus. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University.

Statins. Pravastatin sodium salt (Cayman Chemical) was dissolved in ethanol (1 mg/mL), and atorvastatin (Toronto Research Chemicals Inc.) was dissolved in methanol (25 mg/mL). For i.p. injection, the pravastatin stock solution was diluted by sterile PBS (25 μg/mL) at use and injected every day from E7.5 to E18.5 (10). For cell culture, the stock solutions were diluted in culture medium at use.

Blood and Urinary Samples. Blood samples were allowed to clot and were centrifuged to prepare serum samples. Concentrations of total hsFLT1, free mVEGF, total mPGF, and total human PGF were measured with ELISA kits, according to the manufacturer's instructions (R&D Systems). Aspartate transaminase and alanine transaminase were measured by Fuji DRI-CHEM 3500V and DRI-CHEM slides (Fujifilm). Urine albumin and creatinine concentrations were measured by using the Fuji DRI-CHEM 3500V and DRI-CHEM slides (Fujifilm).

Measurement of Blood Pressure. Blood pressure was measured by the tail-cuff method with BP98A (Softron). The mice were gently fixed in a small cage without anesthesia, and their blood pressures were measured after their behavior, heart rates, and blood pressures were stabilized. After stabilization, both systolic and diastolic blood pressure was recorded at least five times, and up to 10 times, until the stabilization was broken. The mean of both the systolic and diastolic blood pressures measured as above was used for further statistical analysis.

Histopathology of Placenta. Placentas were fixed in 4% paraformaldehyde/PBS for 12 h, rinsed with PBS for 4 h, and soaked sequentially in 40%, 70%, and 100% methanol at 4 °C. Sections prepared at a thickness of 5 μm were stained with anti-mouse CD31 antibody (BD Biosciences) and visualized with AlexaFluor 488–conjugated goat anti-rat IgG (Molecular Probes). The morphological alterations of the tissues were analyzed with Ehrlich H&E staining. Samples were visualized by using conventional microscopy (DM5500 B; Leica), and images were processed using Adobe Photoshop CS3 software (Adobe Systems) (36).

Histopathology of Kidney. Harvested kidneys were fixed, paraffin-embedded, sectioned, and stained by using standard H&E techniques.

Cell Culture. HUVEC and HEK293T cells were plated at 2×10^4 and 1×10^5 cells per well in 6-well plates, respectively, and incubated under 5% CO_2 at 37 °C. After 24 h, the medium was replaced with or without statins. The medium collected after another 24 h was centrifuged at $1,000 \times g$, and the supernatants were subjected to ELISA.

Quantitative Real-Time RT-PCR. Total RNA was prepared from placenta with TRIzol (Invitrogen), then 20 ng of each RNA sample was subjected to quantitative RT-PCR analysis with a Thermal Cycler Dice Real Time System using One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa). Each reaction was performed in triplicate, and the *Pgf* mRNA amounts were normalized to the amount of total RNA. Primer sets used were 5′-tgctgggaacaactcaacag-3′ and 5′-cctcatcaggtattcatcca-3′.

Statistical Analyses. All values are expressed as the mean ± SEM. We compared between groups by using the two-tailed unpaired Student's *t* test. We considered *P* values <0.05 as significant.

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