

# Trophoblast-Specific Reduction of VEGFA Alters Placental Gene Expression and Maternal Cardiovascular Function in Mice<sup>1</sup>

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## ABSTRACT

Given the angiogenic function of vascular endothelial growth factor A (VEGFA), the function of its expression by trophoblast in the avascular placental junctional zone is unknown. In mice, cells from the trophoblast-specific protein alpha (*Tpbpa*) lineage populate this zone and, in late gestation, some of these cells invade the decidual layer. To diminish *Vegfa* expression in *Tpbpa* cells, we crossed *Vegfa*<sup>fllox/fllox</sup> females with males carrying *Tpbpa*-Cre. For single deletion (sd) of *Vegfa* in *Tpbpa* cells in 100% of conceptuses (SD<sub>100</sub> pregnancies, sd conceptuses) we crossed homozygous lines. For double deletion (dd) of both *Vegfa* alleles in 50% of the conceptuses (DD<sub>50</sub> pregnancies, 50% dd conceptuses and 50% no deletion [nd]), we crossed homozygous *Vegfa*<sup>fllox/fllox</sup> females with males heterozygous for *Tpbpa*-Cre and homozygous for *Vegfa*<sup>fllox/fllox</sup>. Controls were *Vegfa*<sup>fllox/fllox</sup> females bred to wild-type males (V-CTRL pregnancies). In SD<sub>100</sub> pregnancies, maternal plasma immunoreactive VEGFA significantly increased and arterial blood pressure decreased, whereas fetal body weight and placental *Flt1*, *sFlt1*, and *PrL3b1* mRNA were unchanged. In DD<sub>50</sub>, maternal immunoreactive VEGFA and arterial pressures were unaltered, but both dd and nd conceptuses exhibited significantly increased embryonic lethality, altered expression of *Flt1*, *sFlt1*, and *PrL3b1* mRNA in the decidual layer, and decreased fetal body weight relative to V-CTRL. Maternal cardiac output significantly increased in proportion to dd conceptuses in the pregnancy. In DD<sub>50</sub>, results are consistent with altered maternal function beginning in early gestation and adversely impacting both conceptus genotypes. We conclude that maternal function is influenced by *Vegfa* expression in trophoblast cells at the maternal-fetal interface, likely via an endocrine mechanism.

blood pressure, cardiac output, Cre-loxP, decidua, embryo loss, Flt1, hypotension, junctional zone, Kdr, labyrinth, placenta,

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placental circulation, placental hormones, placental lactogen, pregnancy, PrL3b1, sFlt1, spongiotrophoblast, transgenic mice, VEGF-A, vascular endothelial growth factor, vascular endothelial growth factor receptor-1, vascular endothelial growth factor receptor-2

## INTRODUCTION

During pregnancy, vascular endothelial growth factor (VEGF) A plays an irreplaceable role in mouse embryogenesis. Indeed, even inactivation of a single copy of *Vegfa* from the embryo and placenta results in early embryonic lethality [1, 2]. VEGFA appears to be important in maternal pregnancy adaptations as well; VEGFA protein increases in the maternal circulation during pregnancy in humans [3] and mice [4], and diminished VEGFA activity due to excess sFLT1 is associated with maternal hypertension and diminished cardiac output in pre-eclampsia, a life threatening complication of human pregnancies [5, 6].

Pre-eclampsia is believed to result from abnormal function of fetal-derived trophoblast cells in the placenta [7]. In pre-eclampsia, trophoblast cells invade less deeply into the maternal decidual layer, which ultimately is thought to result in reduced uteroplacental perfusion, and excessive trophoblast production of sFLT1 [6, 7]. High sFLT1 is thought to upset the VEGFA/sFLT1 balance in the maternal circulation, thereby leading to the clinical signs of gestational hypertension and proteinuria in pre-eclamptic pregnancies [6]. Whether reducing VEGFA production by trophoblast cells has a similar detrimental effect on the VEGFA/sFLT1 balance in the maternal circulation, and on adverse maternal outcomes, is unknown.

In the human placenta, VEGFA is expressed by trophoblast cells that invade the decidual layer [8, 9]. VEGFA expression is also high in the distal regions of the relatively avascular trophoblast cell columns, from which these invasive cells differentiate [9]. VEGFA expression is strikingly reduced in this area in pre-eclampsia [9, 10]. The mouse counterpart of the human trophoblast cell column is the junctional zone [11], which also expresses VEGFA [12] and is similarly quite avascular [13]. The junctional zone of the mouse placenta is believed to primarily play an endocrine role, whereas fetal-maternal exchange occurs in the highly vascular labyrinth, which is analogous to the villous region of the human placenta. Since trophoblast cells in the junctional zone are largely derived from the placental-specific [14] and rodent-specific [15] trophoblast *Tpbpa* lineage, the availability of *Tpbpa*-Cre transgenic mice and *Vegfa*-floxed mice provides an opportunity to use the Cre-loxP system to explore the function of VEGFA expression by trophoblast cells in the junctional zone in vivo.

In the current study, we crossed homozygous *Vegfa*<sup>fllox/fllox</sup> females with homozygous *Tpbpa*-Cre males to singly delete (sd)

a *Vegfa* allele in *Tpbpa* cells in 100% of conceptuses (SD<sub>100</sub> pregnancies) (Table 1). We found that conceptuses survived deletion of one copy of *Tpbpa-Vegfa*. We were therefore able to doubly delete (dd) both *Tpbpa-Vegfa* alleles in 50% of the conceptuses (with no deletion in 50% [nd]) by crossing homozygous *Vegfa*<sup>flox/flox</sup> females with males heterozygous for *Tpbpa-Cre* and homozygous for *Vegfa*<sup>flox/flox</sup> (DD<sub>50</sub> pregnancies) (Table 1). Phenotypes of three possible control lines (Table 1) were evaluated, and *Vegfa*<sup>flox/flox</sup> females bred to wild-type males were selected as the most relevant control (V-CTRL pregnancies). Note that, herein, upper case abbreviations are used for pregnancy group names, and lower case abbreviations for conceptus group names.

We hypothesized that VEGFA produced by *Tpbpa*-trophoblast cells in the junctional zone and adjacent decidual layer would primarily affect maternal function, given evidence supporting an important endocrine role for the junctional zone [16]. Furthermore, the maternal circulation receives maternal venous blood that has percolated through numerous tiny trophoblast-lined channels within the junctional zone [13]. Substances derived from the junctional zone may therefore enter maternal venous blood and then be carried into the systemic circulation to affect maternal function. Given the importance of VEGFA in promoting vascularity and cell migration [2], we also evaluated the effects of reducing *Vegfa* in trophoblast of the *Tpbpa* lineage on placental morphology, and on the uteroplacental and fetoplacental circulations.

## MATERIAL AND METHODS

### Animal Experiments

Experiments were approved by the Animal Care Committee of the Toronto Centre for Phenogenomics and Mount Sinai Hospital (Toronto, ON, Canada) and were conducted in accord with guidelines established by the Canadian Council on Animal Care. Mouse lines were maintained on an ICR outbred background (or the equivalent CD1 strain [17]) for >10 generations. Females (~8 wk) were studied in their first pregnancies. The presence of a sperm plug was defined as Gestational Day (GD) 0.5.

*Tpbpa-Cre* mice (Mouse Genome Informatics (MGI) transgene: Tg [*Tpbpa-Cre*, -EGFP] 5Jcc) [18] (a gift from Dr. J. Cross, University of Calgary, Calgary, AB, Canada) and *Vegfa-loxP* mice (MGI: *Vegfa*<sup>tm2Gne</sup>) [19] (courtesy of Dr. N. Ferrera) were bred to create two levels of tissue-specific VEGFA deficiency (Table 1). Genotyping was performed using PCR primers to detect *Tpbpa-Cre* and *Vegfa* (Table 2). In SD<sub>100</sub> pregnancies, Cre expression in *Tpbpa*-expressing cells would be anticipated to delete a single copy of *Vegfa* in all conceptuses (the sd conceptus group). In DD<sub>50</sub> pregnancies, Cre expression would be anticipated to delete both copies of *Vegfa* (i.e., cause double deletion [dd]) in 50% of the conceptuses (the dd group), whereas no deletion would be anticipated in the remainder of the litter (the nd group). Efficacy and tissue specificity of Cre deletion was assessed at GD8.5, 14.5, and 17.5 by breeding *Tpbpa-Cre* mice with Z/Red reporter mice (MGI: Tg [CAG-Bgeo,-DsRed\*MST] 1Nagy) [20]. All other experimental variables were evaluated at GD17.5 of pregnancy (2 days before normal term delivery). In control experiments, ICR outbred mice (Charles River, Saint-Constant, PQ, Canada) were used to test for possible phenotypes caused by inheritance of the paternal *Tpbpa-Cre* transgene (C-CTRL) or caused by the maternal homozygous *Vegfa*-

loxP transgene (V-CTRL). Phenotypes were compared to background ICR × ICR controls (B-CTRL) (Table 1).

### Cardiovascular Measurements at GD17.5

After recording maternal body weight, we measured maternal uterine arterial blood velocity [21], maternal cardiac output, blood velocity in the maternal arterial canals of the placenta, and fetal umbilical artery and vein blood velocities and diameters using micro-ultrasound (30- or 40-MHz transducer, Vevo770 or Vevo2100; VisualSonics, Toronto, ON, Canada) in isoflurane-anesthetized mice inspiring 50% oxygen. Duration of anesthesia was limited to ≤1 h. Maternal rectal temperature (THM100; Indus Instruments, Houston, TX) was maintained between 36° and 38°C using a heat lamp and heat pad. Ventricular area was used to calculate cardiac output (Vevo770 EKV function), pulsed-waved Doppler to quantify angle-corrected blood velocity (insonation angle <30°) and to calculate the Resistance Index (i.e., [peak systolic – end diastolic velocity]/peak systolic velocity), and calipers in B-mode images were used to measure vessel diameters. In DD<sub>50</sub> pregnancies, conceptus genotype was mixed, so mothers were killed while under anesthesia and imaged conceptuses were immediately identified. Three of ~10 conceptuses were imaged per pregnancy. Fetal tissue was collected from all conceptuses for genotyping.

Maternal ascending aortic blood pressure was measured using a catheter-tip transducer (SPR-671; Millar Instruments, Houston, TX) inserted via the carotid artery of isoflurane-anesthetized mice inspiring 50% oxygen, which were maintained at 36.5°C–37.5°C rectal temperature using a heating pad and lamp. Once stabilized (~2–3 min), arterial pressure was averaged over a 3-min recording period.

### Collection of Maternal Plasma and Placental Tissues at GD17.5

While mice were anesthetized with isoflurane, a heparin-coated needle was inserted into the maternal left ventricular cavity under micro-ultrasound guidance as previously described [22]. Maternal blood (1–1.5 ml) was collected, and plasma stored at –20°C until analysis. After death, the pregnant uterine horns were removed and placed in ice cold PBS. Maternal organ weights were measured. The number of conceptuses and reabsorption sites were recorded. One by one, the uterus was cut to isolate a conceptus. The myometrium was removed and fetal and placental weights recorded.

In a separate series, no ultrasound or blood pressure measurements were obtained, and samples for mRNA analysis were collected. Fetal weight was recorded. The uterus was collected as above. Each placenta was microdissected to obtain samples enriched for the decidual layer, junctional zone, labyrinth, and chorionic plate, as previously described [23]. Enriched samples for mRNA analysis were flash frozen in liquid nitrogen and stored at –80°C. Enrichment of decidual layer samples was shown by high mRNA expression of *Des* (desmin is a specific marker of decidualized stromal cells [24]) and low expression of *Ctsq* (cathepsin Q is a specific marker of sinusoidal trophoblast giant cells [TGCs] [18]) (Supplemental Fig. S1, A and C; all Supplemental Data are available online at [www.biolreprod.org](http://www.biolreprod.org)). Low contamination of junctional zone samples by decidual stromal cells was shown by low *Des* mRNA expression (Supplemental Fig. S1A). Low contamination of labyrinth by junctional zone and decidual stromal cells was shown by low expressions of *Prl8a8* (a prolactin-specific marker of spongiotrophoblast [16]) and *Des* (Supplemental Fig. S1, A and C), respectively. Some expression of the labyrinth-specific gene *Ctsq* was observed in junctional zone samples as anticipated (Supplemental Fig. S1C) due to interdigitation of the junctional zone-labyrinth interface.

TABLE 1. Breeding schemes, and parental and conceptus genotypes for control and experimental groups.\*

Pregnancy group	Mother	Father	Litter	Conceptus group	<i>Tpbpa-Vegfa</i>
Control					
B-CTRL	ICR	× ICR	→ 100% ICR	b-ctrl	No deletion
C-CTRL	ICR	× Cre/Cre	→ 100% Cre/+	c-ctrl	No deletion
V-CTRL	<i>Vegfa</i> <sup>flox/flox</sup>	× ICR	→ 100% <i>Vegfa</i> <sup>flox/+</sup>	v-ctrl	No deletion
Experimental					
SD <sub>100</sub>	<i>Vegfa</i> <sup>flox/flox</sup>	× Cre/Cre	→ 100% (Cre/+, <i>Vegfa</i> <sup>flox/+</sup> )	sd	Single deletion
DD <sub>50</sub>	<i>Vegfa</i> <sup>flox/flox</sup>	× (Cre/+, <i>Vegfa</i> <sup>flox/flox</sup> )	→ 50% (Cre/+, <i>Vegfa</i> <sup>flox/flox</sup> ) → 50% <i>Vegfa</i> <sup>flox/flox</sup>	dd nd	Double deletion No deletion

\* +, wild-type allele; Cre, *Tpbpa-Cre* transgene; *Vegfa*<sup>flox</sup>, *Vegfa*-floxed allele; *Vegfa*<sup>+</sup>, wild-type *Vegfa* allele.

TABLE 2. Primer sequences for genotyping and quantitative RT-PCR.

Target	NCBI RefSeq	Forward 5'→3'	Reverse 5'→3'
Allele			
<i>Tpbpa</i>		TTCAAGGACGACGGCAACTACAAGA	GGGGGTGTCTGCTGGTAGTGGTC
<i>Vegfa</i>		CCTGGCCCTCAAGTACACCTT	TCCGTACGACGCATTTCTAG
Gene			
<i>Vegf</i>	NM_001025250	GAG CAG AAG TCC CAT GAA GTG	TGT CCA CCA GGG TCT CAA TC
<i>Tpbpa</i>	NM_009411	CCA GCA CAG CTT TGG ACA TCA	AGC ATC CAA CTG CGC TTC A
<i>Kdr</i>	NM_010612	ATA GAA GGT GCC CAG GAA AAG	TCT TCA GTT CCC CTT CAT TGG
<i>sFlt1</i>	D88690*	AGA AGA CTC GGG CAC CTA TG	GCA GTG CTC ACC TCT AAC GA
<i>Flt1</i>	NM_010228	TCG TTA GAG ATT CGG AAG CG	GGT CGT AGA GCC ACT GAT GG
<i>Prl3b1</i>	NM_012535	GTC AAC CAT GCT CCT TCT CT	CAC CAC AAT CAC ACG
<i>Prl8a8</i>	NM_023741	GCA ATT CAG ACA ACC TCA CTT C	CTG TCA AAT GTT TCC ATC CGT G
<i>Ctsq</i>	NM_029636.3	TCA TCC CAG AAA GGT TGA G	CCA AAC TGG GAT CAA ATG CTG
<i>Des</i>	NM_010043.1	CTA AAG GAT GAG ATG GCC CG	GAA GGT CTG GAT AGG AAG GTT G
$\beta$ -actin	NM_007393	TCG TGC GTG ACA TCA AAG AGA	GAA CCGG CTC GTT GCC AAT A
TBP	NM_013684	CGG ACA ACT GCG TTG ATT TTC	AGC CCA ACT TCT GCA CAA CTC
<i>Capdh</i>	NM_008084	AGG AGT AAG AAA CC TGG AC	AGG CCC CTC CTG TTA TTA TGG

\* GenBank accession number.

### Histology and Immunohistochemistry

*Tpbpa*-Cre mice crossed to Z/Red reporter mice were killed at GD9.5, 14.5, and 17.5, and whole uterine horns were immersion fixed in 4% paraformaldehyde overnight at 4°C on a shaker. At GD17.5, uteri from the experimental and V-CTRL pregnancies (Table 1) were collected using the same procedure. Segments of uterus, containing individual placentas with the uterus still attached, were isolated. Fetal tissue samples were collected for genotyping. Placentas were further fixed for 12 h followed by dehydration, paraffin embedding, and routine histological methods (CMHD Pathology Services, Toronto Centre for Phenogenomics, Toronto, ON, Canada). Hematoxylin and eosin (H&E) stain was used to examine placental morphology and Martius Scarlet Blue stain was used to examine blood clots in GD9.5 implantation sites of homozygous *Tpbpa*-Cre females. Immunohistochemistry was used to detect red fluorescent protein in pregnancies containing Z/red conceptuses (ab34771, rabbit anti-RFP; Abcam, Toronto, ON, Canada), to detect cytokeratin as a marker of trophoblast cells (Z0622, rabbit anti-cytokeratin; Dako, Burlington, ON, Canada), and to detect CD<sub>34</sub> as a marker for fetal endothelial cells in the labyrinth (ab8158, rabbit anti-rat IgG; Abcam).

### PCR Analysis

Confirmation of Cre-mediated deletion of the loxP-flanked exon 3 of *Vegfa* was performed using DNA extracted from the decidual layer, junctional zone, and fetal tail samples. Primers (forward, 5'-CCTGGCCCTCAAGTACACCTT-3'; reverse, 5'-ACATCTGCTGTGCTGTAGGAAG-3') were designed to flank both loxP sites spanning a fragment of >1.8 kb. Upon cre activity, the fragment between the two loxP sites was excised, resulting in a 450-bp fragment (Fig. 1).

### Gene Expression

RNA was extracted from microdissected tissue collected from the decidual layer, junctional zone, and labyrinth using the Trizol method. DNA and RNA contaminants were removed using kits (RNase-Free DNase kit, no. 79254; Qiagen, Toronto, ON, Canada; RNeasyMinElute, no. 74204; Qiagen). Purified RNA samples were then reverse transcribed to cDNA (Taqman Reverse Transcription Reagents, no. N8080234; Life Technologies, Burlington, ON, Canada). Gene expression was measured in triplicate by quantitative RT-PCR using SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada). Results are expressed as  $\Delta\Delta CT$  relative to three housekeeping genes ( $\beta$ -actin, GAPDH, and TBP). Primer sequences are listed in Table 2.

### Progesterone, VEGFA, and sFLT1 Protein Quantification

Maternal plasma progesterone was measured by enzyme immunoassay (no. 582601; Cayman Chemical Company, Burlington, ON, Canada) and immunoreactive sFLT1 (ir-sFLT1) by ELISA (no. MVR100; R&D Systems, Burlington, ON, Canada). Immunoreactive VEGFA protein (ir-VEGFA) was measured in 2-fold diluted maternal plasma (no. MMV00; R&D Systems). Frozen placental tissue was crushed, then homogenized in RIPA buffer (Thermo Scientific; Rockford, IL) on ice. After centrifugation at 4°C, total protein concentration in the supernatant was measured using the Bradford

Assay (no. 500-0006; Bio-Rad). Tissue ir-VEGFA protein was measured by ELISA (no. MMV00), and was expressed per unit total protein.

### Histomorphometry

Placental histomorphometry at GD17.5 was performed on H&E-stained midline placental sections. Point counting at intersections of a superimposed grid (newCAST; Visiopharm, Denmark) was used to quantify total placental area, and the areas of the decidual layer, junctional zone, labyrinth, and chorionic plate, as well as the percent area occupied by glycogen cells in the decidual layer. Point counting was also used on CD34-stained sections to quantify the area of fetal blood spaces (brown CD34 staining) and the area of maternal blood spaces in the labyrinth.

### Statistical Analysis

Statistical significance ( $P < 0.05$ ) was determined using one- or two-way ANOVA as appropriate, followed by post hoc tests when ANOVA revealed significant differences (GraphPad Prism 5.0; GraphPad Software, Inc., San Diego, CA). Results are reported as the mean  $\pm$  SEM. N for maternal variables is the number of pregnancies. For conceptus variables, n is the number of conceptuses or conceptus pools. In some cases, pooling was necessary to obtain sufficient tissue volume for analysis.

## RESULTS

### *Tpbpa* and *Vegfa* mRNA Expression in the Mature Mouse Placenta

As anticipated, *Tpbpa* mRNA was highly expressed in tissue enriched for the junctional zone and decidual layer (Fig. 2A), whereas levels in the labyrinth were low. This result is consistent with prior work that used in situ hybridization to show strong and specific *Tpbpa* expression by spongiotrophoblast, glycogen trophoblast, and spiral artery TGCs (SpA-TGC) [18, 25, 26]. Thus, *Tpbpa*-Cre would be anticipated to be highly expressed in the decidual layer and junctional zone at this stage of gestation.

*Vegfa* mRNA was most highly expressed in the decidual layer, whereas it was similarly expressed at a lower level in the junctional zone and labyrinth layer (Fig. 2B). mRNA expression of the VEGFA receptor, *Kdr*, showed a similar pattern of expression to *Vegfa* (Fig. 2C). In contrast, *Flt1* mRNA expression was high in both the decidual layer and junctional zone, and low in the labyrinth layer (Fig. 2D), consistent with the distribution of LacZ staining at GD14.5 in prior work [27]. *sFlt1* mRNA showed a significant gradient: high levels in the decidual layer, moderate levels in the junctional zone, and low levels in the labyrinth (Fig. 2E). Thus,

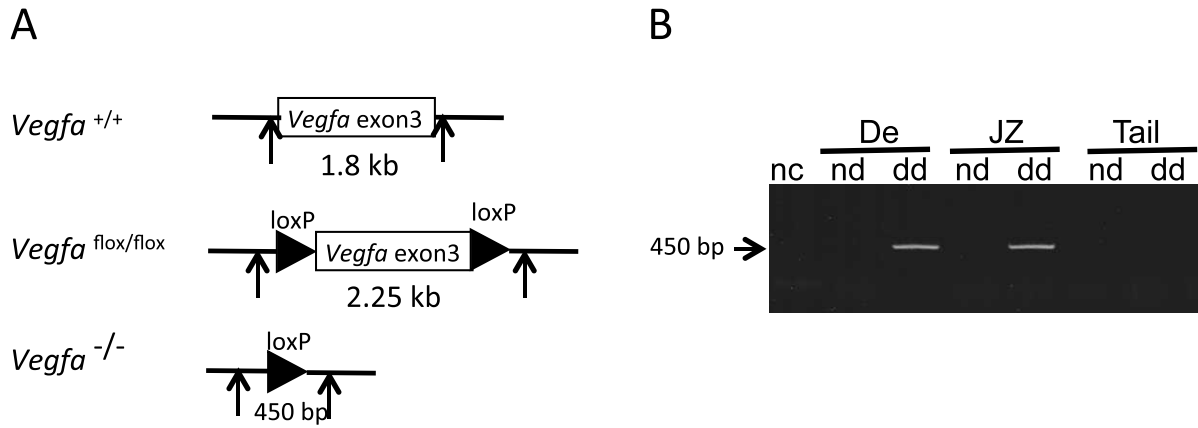


FIG. 1. Detection of *Tpbpa*-Cre mediated excision of exon3 of *Vegfa* by PCR. **A**) Primers spanning the floxed region of exon 3 of *Vegfa* (arrows) yield a 1.8-kb product in wild-type conceptuses (*Vegfa*<sup>+/+</sup>), a 2.25-kb region in floxed conceptuses (*Vegfa*<sup>flx/flx</sup>), and a 450-bp fragment, including one loxP site, after deletion of exon3 by Cre-recombinase. **B**) The 450-bp fragment was detected only in decidua (De) and junctional zone (JZ)-enriched placental tissue of dd conceptuses, but not in placental tissue from nd conceptuses. Deletion was also not detected in fetal tissue from the tail. nc, negative control; nd, no deletion; dd, double deletion.

in the decidual layer, *Tpbpa*-Cre-mediated *Vegfa* gene deletion would be anticipated to reduce *Vegfa* mRNA and its actions on *Kdr* and *Flt1* receptors, all of which are highly expressed in this layer. In the junctional zone, *Kdr* mRNA was low and *Flt1* mRNA high relative to expression levels in the decidual layer, suggesting that the *Flt1* receptor in the junctional zone may play a more prominent role, mediating the effects of *Tpbpa*-Cre-mediated *Vegfa* gene deletion at that site.

#### *Tpbpa*-Cre-Recombinase Expression Pattern and Efficacy of Excision

Consistent with previous work [26, 28, 29], we observed both *Tpbpa*-Cre expression and activity exclusively within the ectoplacental cone region in early gestation (Fig. 3, A–C), and within the junctional zone and adjacent maternal decidual layer in later gestation (Fig. 3, D–F). Histological analysis showed Cre activity in spongiotrophoblast cells in the junctional zone

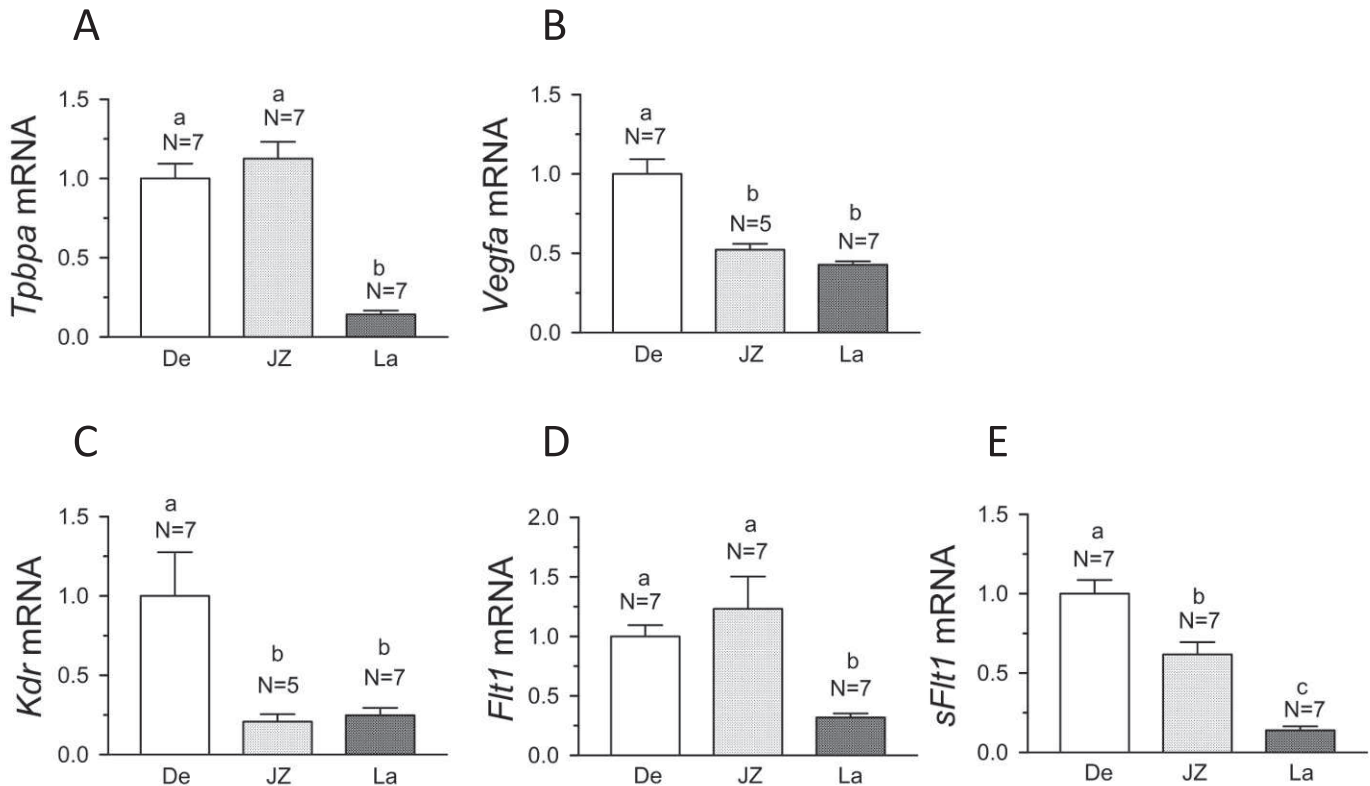


FIG. 2. Messenger RNA gene expression in layer-enriched samples of control *Vegfa*<sup>flx/+</sup> mouse placentas at GD17.5. Messenger RNA measured by quantitative RT-PCR is shown for samples enriched for decidua (De), junctional zone (JZ), or labyrinth (La) tissue relative to expression in the decidua (= 1). Above bars, N indicates number of pregnancies. If letters above bars differ, then results differ based on a one-way ANOVA, which, if significant, was followed by Tukey post hoc multiple comparison test ( $P < 0.05$ ). Mean  $\pm$  SEM.

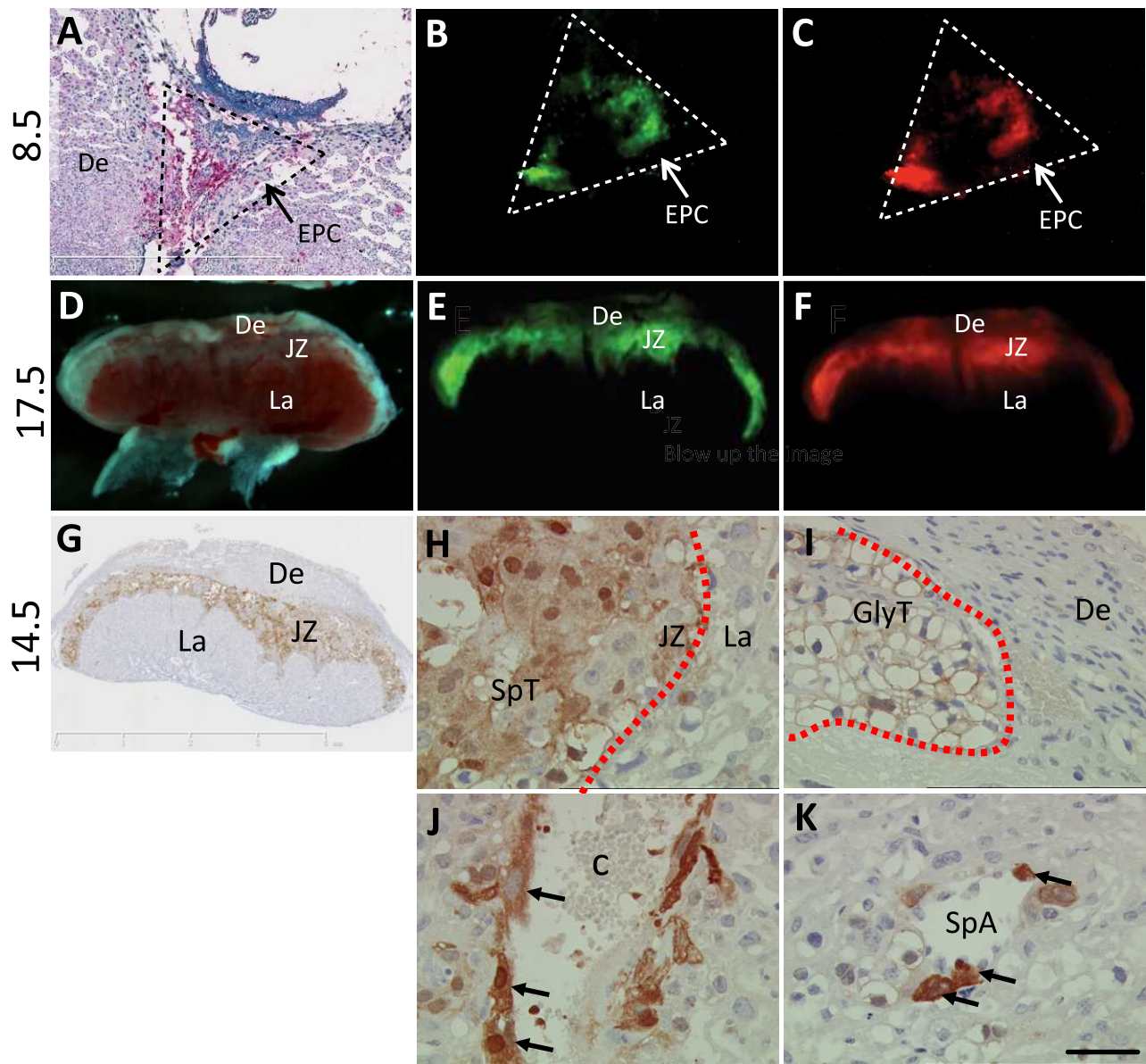


FIG. 3. Expression and activity of *Tpbpa*-Cre transgene in GD8.5 (A–C), 17.5 (D–F), and 14.5 (G–K) placentas. Placentas were obtained by crossing *Tpbpa*-Cre/+ males with Z/Red reporter females. **A**) Immunohistochemistry (IHC) for pancytokeratin (red stain) shows localization of trophoblast cells in the ectoplacental cone at GD8.5. Gross dissection of a conceptus at GD8.5 showing green fluorescence protein (GFP) fluorescence (green; **B**) indicating Cre expression, and RFP fluorescence (red; **C**) indicating Cre activity in the ectoplacental cone (EPC). **D**) Gross dissection of a placenta at GD17.5 showing GFP fluorescence (green; **E**) indicating Cre expression, and RFP fluorescence (red; **F**) indicating Cre activity in the junctional zone (JZ) and in invasive trophoblast cells in the decidua (De). **G–K**) IHC for RFP protein (brown stain) in midline placental sections at GD14.5. **G**) Brown stain for RFP shows high Cre activity in the junctional zone and adjacent decidua in a midline section from a whole GD14.5 placenta (uterine muscle intact). Under higher magnification, brown staining indicating Cre activity was observed in spongiotrophoblast cells (**H**), glycogen trophoblast cells (**I**), some canal-associated trophoblast cells (**J**; arrows), and some SpA-associated trophoblast cells (**K**; arrows). RFP, red fluorescence protein; La, Labyrinth; SpT, spongiotrophoblast cells; GlyT, glycogen trophoblast cells; C, canal lumen; SpA, spiral artery lumen. Bar in **K** = 60  $\mu$ m; **H–J** are at the same magnification as **K**.

(Fig. 3H), glycogen trophoblast cells in the junctional zone and proximal decidua (Fig. 3I), in some canal TGCs (C-TGCs) (Fig. 3J), and in SpA-TGCs (Fig. 3K). These results confirmed that the *Tpbpa*-Cre promoter construct is specifically expressed, and that the gene product, Cre-recombinase, is functional. No ectopic cre expression (green) was detected in any maternal organs examined by gross dissection, including uterus, vagina, liver, spleen, kidneys, ovaries, small and large intestine, lung, heart, brain, and skin.

#### Pregnancy Outcomes in Transgenic Lines

Homozygous *Tpbpa*-Cre mice were required to obtain SD<sub>100</sub> pregnancies (Table 1). Unexpectedly, homozygous *Tpbpa*-Cre females, when bred with ICR males, developed vaginal bleeding at ~GD9.5. They were often weak and pale, and most became moribund or aborted. Severe hemorrhage within all implantation sites was observed postmortem (Fig. 4). However, ICR females bred to homozygous *Tpbpa*-Cre males, and thus also carrying 100% heterozygous *Tpbpa*-Cre conceptuses, gained weight normally and had normal litter sizes of viable embryos near term (Supplemental Table S1).

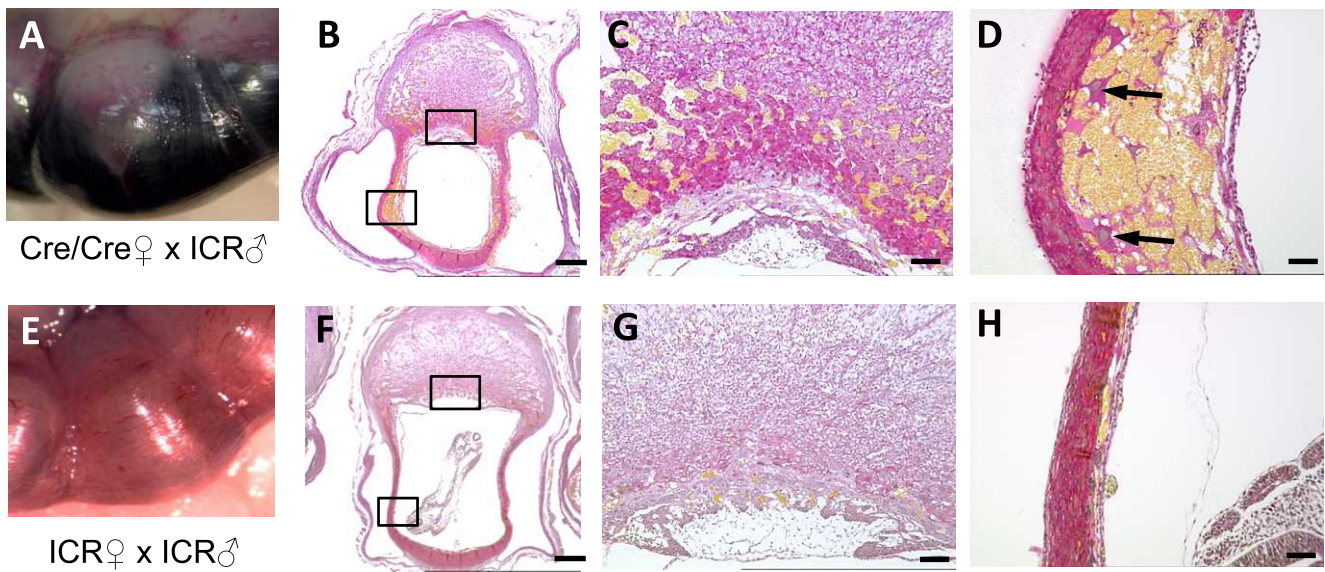


FIG. 4. Intrauterine bleeding occurred in pregnant homozygous *Tpbpa*-Cre females (cre/cre) mated with wild-type ICR males (A–D). A wild-type pregnant uterus is shown as a control (E–H). A) Uteri from pregnant homozygous *Tpbpa*-Cre females contained prominent black regions of hemorrhage around conceptuses by GD9.5. B) A histological cross-section of a conceptus at GD9.5 stained with Martius scarlet blue (MSB) showing hemorrhage (yellow) in the ectoplacental cone (C) and near the primary giant cells (D). Boxed regions in B are shown at higher magnification in C and D. Arrows in D show primary TGCs. E) Intrauterine bleeding was not observed in wild-type pregnant uteri at GD9.5 or in histological sections (F–H). Boxed regions in F are shown at higher magnification in G and H. Bar = 125  $\mu$ m in B and F; 70  $\mu$ m in C and G; and 35  $\mu$ m in D and H.

Therefore, paternal homozygous *Tpbpa*-Cre was used in SD<sub>100</sub> pregnancies (Table 1).

Pregnancy phenotypes of three possible control lines (Table 1) were evaluated. We tested paternal *Tpbpa*-Cre control (C-CTRL) or maternal *Vegfa*<sup>flox/flox</sup> control (V-CTRL) pregnancies versus background control (B-CTRL) pregnancies (Table 1). The only difference detected among the groups was the slightly smaller litter size of V-CTRL pregnancies (10 vs. 13 per litter; Supplemental Table S1). V-CTRL pregnancies were selected as the most relevant control, because both SD<sub>100</sub> and DD<sub>50</sub> experimental groups had *Vegfa*<sup>flox/flox</sup> mothers (Table 1). V-CTRL, C-CTRL, and B-CTRL groups did not significantly differ in maternal body weights, maternal organ weights, maternal hematology parameters, maternal cardiac outputs, maternal uterine arterial blood velocities, or their Resistance Index, litter sizes, percent reabsorption sites, fetal and placental weights, or in the maternal canal, umbilical artery, and umbilical vein blood velocities and diameters (Supplemental Tables S1 and S2).

#### Pregnancy Outcomes with Cre-Mediated *Vegfa* Deletion

As anticipated in DD<sub>50</sub> pregnancies, deletion of *Vegfa* exon 3 was detected in the junctional zone and decidual layer of dd placentas, whereas it remained intact in not deleted (nd) litter mates and in the tails of dd conceptuses (Fig. 1).

In dd placentas, Cre-mediated deletion significantly decreased expression of ir-VEGFA protein in the junctional zone and decidual layer relative to nd littermates (Fig. 5, A and D). In sd placentas of SD<sub>100</sub> pregnancies, expression of ir-VEGFA protein was significantly reduced in the decidual layer and junctional zone (Fig. 5, D and E) in comparison to V-CTRL placentas. Labyrinth ir-VEGFA protein was not significantly affected (Fig. 5F) in sd and dd placentas relative to their appropriate controls. Thus, we were successful in creating an incremental reduction of ir-VEGFA protein specifically in the decidual layer and junctional zone of sd and dd placentas using Cre-mediated *Vegfa* deletion.

**Maternal consequences.** We hypothesized that diminishing VEGFA expression in *Tpbpa* trophoblast cells would affect maternal function by diminishing VEGFA release into the maternal circulation. However, in SD<sub>100</sub> pregnancies, there was a paradoxical 25% increase in ir-VEGFA in maternal plasma relative to V-CTRL (Fig. 6A) despite reduced ir-VEGFA protein in both the decidual layer and junctional zone (Fig. 5, D and E). Immunoreactive sFLT1 levels in maternal plasma were not significantly changed in SD<sub>100</sub> pregnancies (Supplemental Table S2). Nevertheless, maternal function was significantly affected. Maternal arterial blood pressure was significantly reduced (Fig. 6B), whereas no change was detected in cardiac output expressed per unit maternal body weight in SD<sub>100</sub> pregnancies (Fig. 6C).

In DD<sub>50</sub> pregnancies, plasma ir-VEGFA levels and arterial pressure were not significantly altered relative to V-CTRL (Fig. 6, A and B). Possibly this was because decidual layer and junctional zone ir-VEGFA protein was only reduced in dd conceptuses, which comprised ~50% of each litter, while ir-VEGFA in nd placentas was not significantly different from v-ctrl (Fig. 5, D and E). Immunoreactive sFLT1 levels in maternal plasma were also not significantly altered in DD<sub>50</sub> pregnancies (Supplemental Table S2). However, cardiac output per unit maternal body weight was significantly increased by 20% compared to V-CTRL pregnancies (Fig. 6C). Interestingly, the maternal cardiac output per unit body weight in DD<sub>50</sub> pregnancies was positively correlated with the percentage of dd conceptuses ( $P < 0.05$ ; Fig. 6D), as was maternal heart weight per unit body weight ( $P < 0.008$ ; Fig. 6E). These relationships suggest that the presence of dd conceptuses led to a significant augmentation in maternal cardiac output.

SD<sub>100</sub> and DD<sub>50</sub> pregnancies did not significantly differ from V-CTRL<sub>50</sub> in other measured parameters, including maternal heart rate, maternal body weight, maternal kidney, spleen, heart, or liver weights, maternal hematology parameters, maternal uterine arterial blood velocities, and resistance index, litter size, and maternal progesterone (Supplemental Table S2). There were also no significant differences between

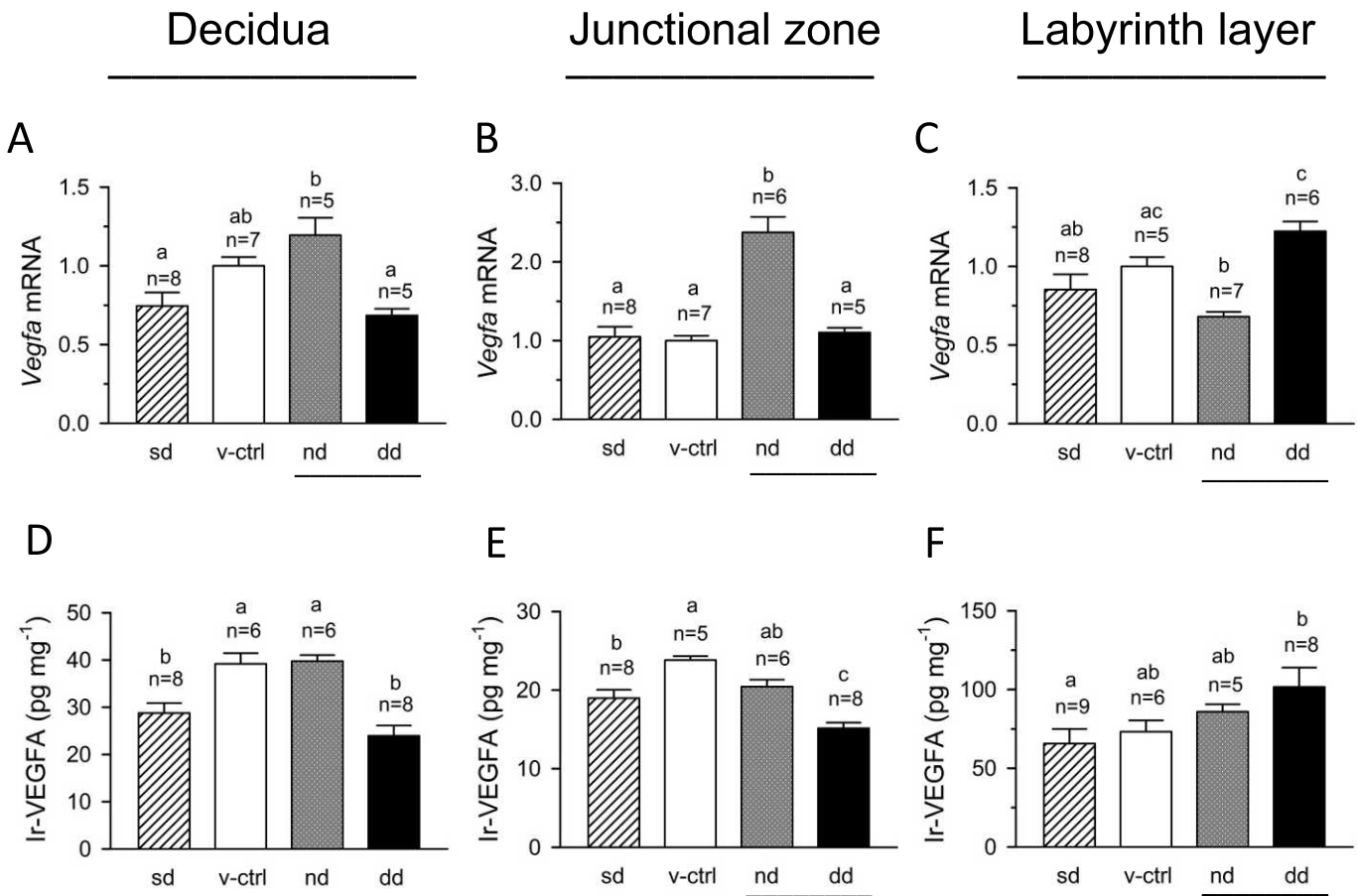


FIG. 5. Distribution of *Vegfa* expression in the placenta at GD17.5. *Vegfa* mRNA in decidua (A), junctional zone (B), and labyrinth (C) is shown relative to the v-ctrl mean (= 1). Immunoreactive VEGFA protein in decidua (D), junctional zone (E), and labyrinth (F) is expressed per milligram of total protein. dd and nd, double-deleted (dd) and no-deletion (nd) conceptuses from DD<sub>50</sub> pregnancies; sd, single-deletion conceptuses from SD<sub>100</sub> pregnancies; v-ctrl, control conceptuses from V-CTRL pregnancies. Above bars, n indicates number of conceptuses or number of conceptus pools assayed. If letters above bars differ, then results differ based on a one-way ANOVA, which, if significant, was followed by Tukey post hoc multiple comparison test ( $P < 0.05$ ). Mean  $\pm$  SEM.

groups in the velocity of maternal blood flowing in placental arterial canals (Supplemental Table S3).

**Fetal and placental consequences.** The proportion of implantation sites that were resorbed was significantly increased in DD<sub>50</sub> compared to V-CTRL pregnancies, implying that a disruption in early gestation had occurred (Table 3). The proportion of surviving dd and nd fetuses at GD17.5 (46:51) did not significantly differ from the expected 50:50 Mendelian ratio, implying that resorption had similarly affected both genotypes. In addition, DD<sub>50</sub> pregnancies had smaller fetuses ( $1.06 \pm 0.06$  g), regardless of dd or nd genotype, than did V-CTRL pregnancies ( $1.15 \pm 0.09$  g) (Table 3). There were no significant changes in resorption sites or fetal body weight in sd conceptuses from SD<sub>100</sub> pregnancies compared to V-CTRL (Table 3).

Interestingly, in DD<sub>50</sub> pregnancies, expression of *Flt1* and *sFlt1* mRNA in the decidua did not significantly differ between nd and dd placentas (Fig. 7, A and B), despite large genotype-dependent differences in *Vegfa* mRNA and protein expression (Fig. 5, A and D). Instead, *Flt1* and *sFlt1* mRNA levels in the decidua were significantly up-regulated by ~50% for both nd and dd genotypes when compared to v-ctrl (Fig. 7, A and B). A similar trend was observed in the junctional zone (Fig. 5, B and E; Fig. 7, D and E). *Kdr* mRNA expression did not differ between groups (not shown). For sd

placentas, no significant changes were detected in *Flt1*, *sFlt1*, or *Kdr* mRNA in the junctional zone or decidua layer.

*Prl3b1* (PL-II) is highly expressed in spongiotrophoblast [16] and plays an important role in maintaining pregnancy by regulating maternal corpus luteum function [30]. For placentas from DD<sub>50</sub> pregnancies, *Prl3b1* mRNA expression in the decidua layer and junctional zone was significantly down-regulated by 30–40% in both dd and nd compared to v-ctrl placentas (Fig. 7, C and F). In sd placentas, no significant changes in *Prl3b1* mRNA were detected (Fig. 7, C and F).

We were surprised to see that *Flt1*, *sFlt1*, and *Prl3b1* mRNA in nd and dd placentas were similarly abnormal relative to the v-ctrl group (Fig. 7). We wondered whether similar changes in gene expression in nd and dd conceptuses versus v-ctrl conceptuses could be explained by homozygous *Vegfa*<sup>flx/flx</sup> alleles in nd and dd, whereas v-ctrl conceptuses were heterozygous for this allele (Table 1). However, *Flt1*, *sFlt1*, and *Prl3b1* mRNA expression in the decidua layer of *Vegfa*<sup>flx/flx</sup> conceptuses did not significantly differ from v-ctrl conceptuses (n = 8/group; data not shown), indicating that this genotype difference cannot explain this finding.

In dd placentas, Cre-mediated deletion significantly decreased expression of *Vegfa* mRNA in the junctional zone and decidua layer relative to nd littermates (Fig. 5, A and D). However, unexpectedly, *Vegfa* mRNA in nd controls differed from the v-ctrl group; levels were significantly higher than v-

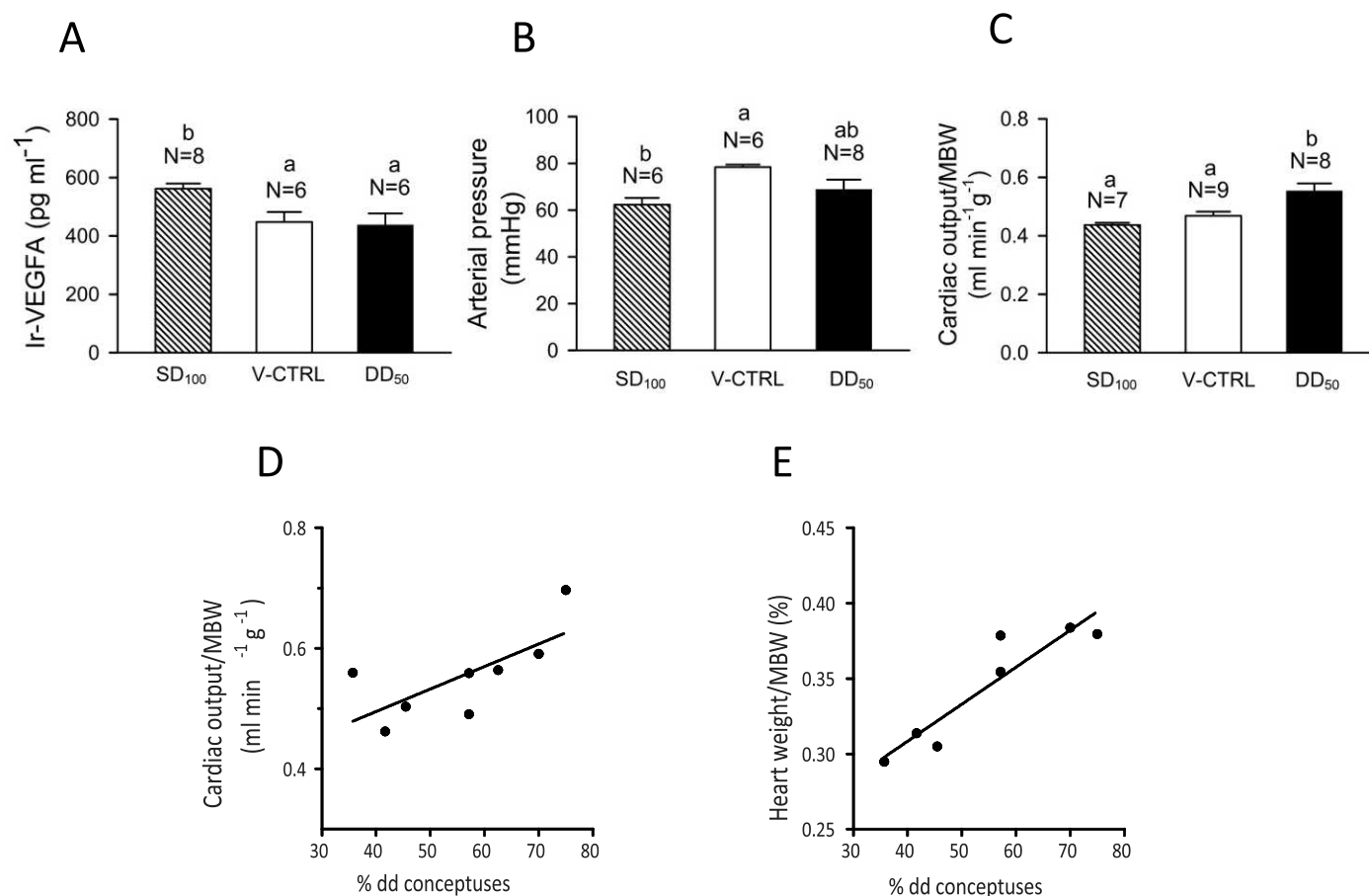


FIG. 6. Maternal circulating ir-VEGFA and maternal cardiovascular function at GD17.5. **A**) Maternal plasma ir-VEGFA ( $\text{pg ml}^{-1}$ ). **B**) Maternal mean arterial blood pressure (mm Hg). **C**) Maternal cardiac output expressed per unit maternal body weight (MBW) ( $\text{ml min}^{-1} \text{g}^{-1}$ ). **D** and **E**) In  $\text{DD}_{50}$  pregnancies, maternal cardiac output per unit MBW ( $\text{ml min}^{-1} \text{g}^{-1}$ ) in **D** and maternal heart weight to body weight ratio (%) in **E** were significantly correlated with the proportion of dd conceptuses in the litter (in **D**,  $R^2 = 0.52$ ,  $P < 0.05$ ; in **E**,  $R^2 = 0.79$ ,  $P = 0.008$ ). Above bars, N indicates number of pregnant mice. If letters above bars differ, then results differ based on a one-way ANOVA, which, if significant, was followed by Tukey post hoc multiple comparison test ( $P < 0.05$ ). Mean  $\pm$  SEM.  $\text{DD}_{50}$ , pregnancies with  $\sim 50\%$  conceptuses with double *Vegfa* deletion from *Tpbpa*-expressing cells;  $\text{SD}_{100}$ , pregnancies carrying 100% conceptuses with single *Vegfa* deletion from *Tpbpa*-expressing cells.

ctrl in the junctional zone and lower than v-ctrl in the labyrinth (Fig. 5, B and C). It is possible that altered *Vegfa* mRNA in nd littermates may be part of a compensatory response to the experimentally imposed limitation on *Vegfa* mRNA expression in *Tpbpa*-expressing trophoblast cells in this zone in dd conceptuses. Changes in *Vegfa* gene expression in nd conceptuses provide further evidence supporting effects on nd caused by dd conceptuses in the pregnancy, even though *Vegfa* mRNA changes (Fig. 5A) were not associated with parallel changes in ir-VEGFA protein (Fig. 5B).

Despite differences in VEGFA protein expression, and *Flt1*, *sFlt1*, and *Pr13b1* mRNA expression, no differences in placental morphology were detected between dd, nd, and v-ctrl placentas (Supplemental Fig. S2); there were no significant differences in the percent area of the placenta occupied by the decidual layer, junctional zone, labyrinth, or chorionic plate, in the percent area of the labyrinth occupied by fetal or maternal blood spaces, or in the proportion of glycogen cells in the decidual layer (Supplemental Table S4). Placental weight also did not differ between groups (not shown). There were also no

TABLE 3. Pregnancy outcome for control and experimental groups at GD17.5.

Parameter	V-CTRL	$\text{SD}_{100}$	$\text{DD}_{50}$	nd	dd	<i>P</i> value*
No. of pregnancies	14	15	11			ns
Litter size	10	11	10			ns
Embryo body weight ( $\text{g}$ ) <sup>†</sup>	$1.15 \pm 0.09^a$	$1.19 \pm 0.11^a$	$1.06 \pm 0.06^b$			$P < 0.05$
Resorption sites/total implantation sites	1/140 <sup>a</sup>	1/159 <sup>a</sup>	18/124 <sup>b</sup>			$P < 0.05$
No. of $\text{DD}_{50}$ live embryos by genotype				51	46	ns <sup>‡</sup>
Body weight of $\text{DD}_{50}$ live embryos ( $\text{g}$ ) <sup>†</sup>				$1.08 \pm 0.06$	$1.05 \pm 0.07$	ns

\* ns, not significant.

<sup>‡</sup> ns, not significantly different than anticipated 50:50 ratio.

<sup>†</sup> Values are presented as mean  $\pm$  SEM.

<sup>a,b</sup> Different superscript letters indicate statistically significant difference between groups.



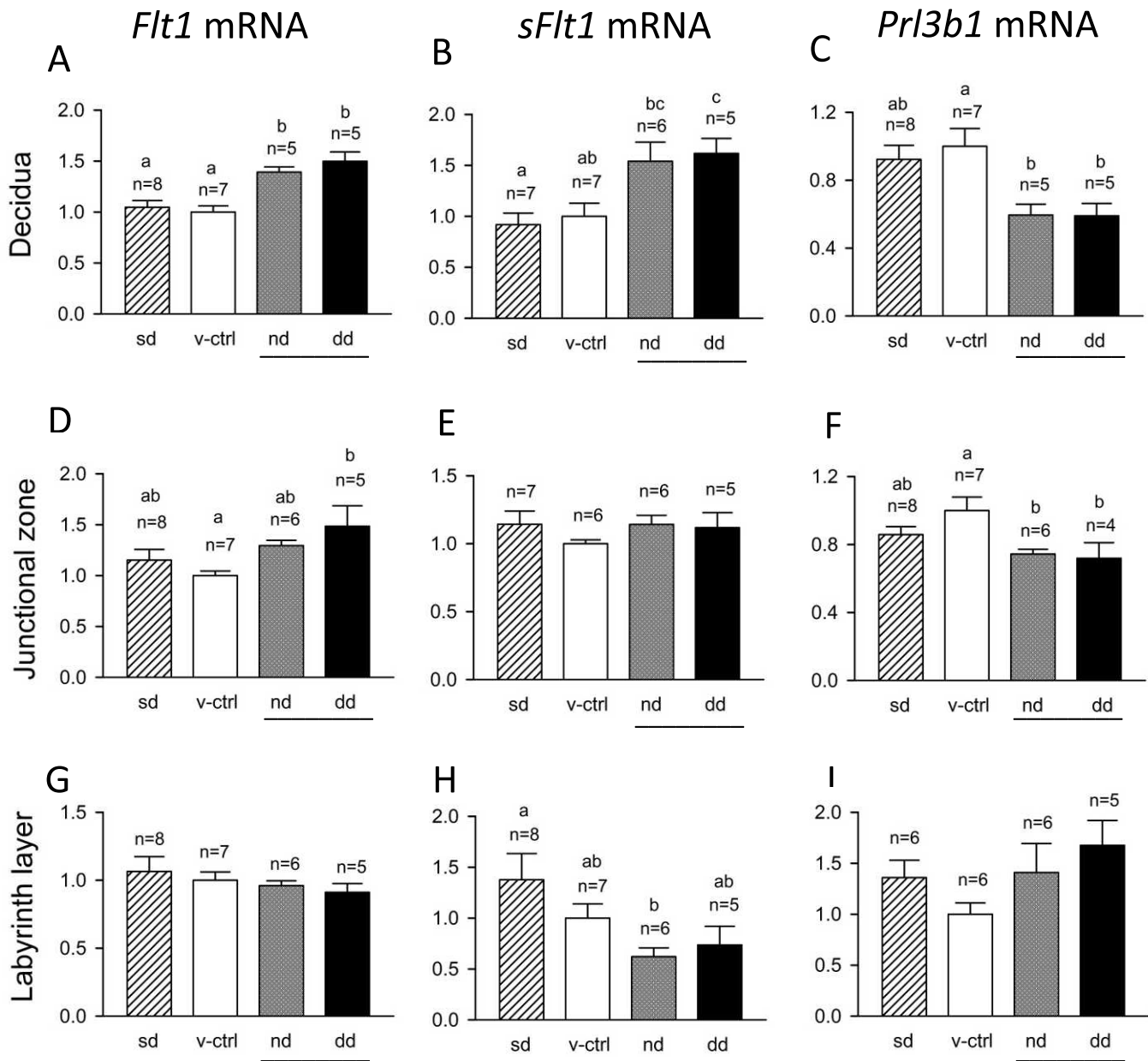


FIG. 7. mRNA gene expression in layer-enriched samples of placentas at GD17.5. *Flt1* (A, D, and G), *sFlt1* (B, E, and H), and *Prl3b1* (C, F, and I) mRNA expression measured by quantitative RT-PCR is shown for samples enriched for decidua (A–C), junctional zone (D–F), or labyrinth (G–I). Results are shown relative to expression in v-ctrl control conceptuses (= 1). dd and nd, dd conceptuses and nd conceptuses from DD<sub>50</sub> pregnancies; sd, sd conceptuses from SD<sub>100</sub> pregnancies; v-ctrl, control conceptuses from V-CTRL pregnancies. Above bars, n indicates number of conceptuses from which placental tissues were obtained. If letters above bars differ, then results differ based on a one-way ANOVA, which, if significant, was followed by Tukey post hoc multiple comparison test ( $P < 0.05$ ). Mean  $\pm$  SEM.

differences in blood velocity in the umbilical artery or vein, the umbilical artery Resistance Index, or the diameter of these vessels between the groups (Supplemental Table S3).

## DISCUSSION

The current study investigated the function of VEGFA expressed by trophoblast cells in the junctional zone, a relatively avascular region of the maternal-fetal interface of the placenta. Results showed that deleting a single copy of *Vegfa* in 100% of the litter in SD<sub>100</sub> pregnancies reduced ir-VEGFA protein expression in the decidua layer and junctional zone, while paradoxically increasing ir-VEGFA in the maternal circulation. Maternal arterial blood pressure was significantly

reduced, but other maternal, placental, and fetal parameters were unaffected. The more severe reduction in expression in dd conceptuses adversely impacted early gestation, resulting in increased litter-wide resorptions, layer-specific alterations in *sFlt1*, *Flt1*, and *Prl3b1* gene expression, and reduced fetal body weights. These changes affected the whole litter, regardless of genotype and independent of ir-VEGFA expression in the decidua layer and junctional zone. Maternal cardiovascular function was also altered by double deletion of *Vegfa* in 50% of the litter in DD<sub>50</sub> pregnancies, but, in this case, maternal cardiac output was significantly increased, whereas arterial pressure was unchanged. Maternal circulating ir-VEGFA was unaffected in DD<sub>50</sub> pregnancies. Findings from both models support our central hypothesis, that VEGFA expression in

*Tpbpa* trophoblast cells plays a role in maintaining normal maternal function during pregnancy. Results were inconsistent with a direct effect of reduced placental VEGFA protein expression on VEGFA protein levels in the maternal circulation. They were also inconsistent with a direct effect of reduced placental VEGFA on conceptus survival, growth, and *Flt1*, *sFlt1*, and *Pr13b1* mRNA expression in the decidual layer, because similar changes were seen in dd and nd conceptuses in DD<sub>50</sub> pregnancies. Instead, we infer that altering VEGFA in *Tpbpa* trophoblast most likely alters maternal function indirectly by influencing trophoblast endocrine function.

Cre expression in both *Vegfa* sd and dd placentas successfully reduced ir-VEGFA protein expression in the decidual layer and junctional zone relative to their controls (v-ctrl and nd, respectively). However, the observed reduction (20%–26% in sd, 30%–50% in dd) was less than the maximum possible percent reduction (50% in sd, 100% in dd). The less-than-maximal reduction in ir-VEGFA protein could be attributed to VEGFA produced by non-*Tpbpa*-expressing cells in these layers, VEGFA in matrix that may be derived from distant sources, and/or incomplete Cre-mediated deletion in *Tpbpa*-expressing cells. Nevertheless, ~50% reduction in *Vegfa* mRNA expression in the decidual layer and junctional zone was anticipated to be sufficient to exert a physiological effect. This was based on prior work using the same *Vegfa*-loxP mouse line for Cre-mediated deletion. In the lung, cre-mediated deletion in epithelial cells [31] or type II pneumocytes [32] caused ~50% reduction in lung *Vegfa* mRNA compared to no-deletion littermates, and resulted in pulmonary hypovascularity [31] or lung emphysema, and marked alterations in the lung's response to injury [32]. In the kidney, cre-mediated deletion in podocytes caused early postnatal renal disease [33]. Furthermore, a 50% reduction in *Vegfa* mRNA in heterozygous knockout mice was sufficient to cause embryonic lethality [1, 2].

Cre activity was detected by GD8.5, indicating that Cre-mediated *Vegfa* deletion would be occurring at the onset of the formation of the uteroplacental and fetoplacental circulations and the onset of embryonic cardiac function [34, 35]. Early deletion likely accounts for the increase in conceptus resorptions observed in DD<sub>50</sub> pregnancies. These resorptions were not dependent on conceptus genotype, suggesting that resorptions were caused indirectly by a maternal response to *Vegfa* deletion in a subset of her conceptuses. Interestingly, *Notch2* deletion from *Tpbpa* trophoblast also increases resorptions in a similarly conceptus-genotype-independent manner [36].

It is intriguing that cardiac output was higher in late gestation only in DD<sub>50</sub> pregnancies, the group in which early gestational defects were observed. In human pregnancy, cardiac output increases in early gestation, reaches maximal levels in midgestation, and thereafter remains stable or declines [37]. Presumably, the early increase is hormonally mediated, although the specific mechanism is unknown. In DD<sub>50</sub> pregnancies, it is likely that elevated cardiac output also occurred in early gestation, because heart weight, as well as cardiac output, was correlated with the percent of dd conceptuses, and cardiac hypertrophy would take time to occur. In contrast, in SD<sub>100</sub> pregnancies, the decrement in ectoplacental cone and/or junctional zone expression of VEGFA in sd conceptuses was insufficient to elicit early gestational lethality, and it also failed to alter cardiac output response in late gestation.

At GD17.5, significant reductions in ir-VEGFA protein were observed in the decidual layer, where SpA-TGCs are localized, and in the junctional zone, where canal TGCs (C-

TGC) are found. SpA-TGC are 100% derived from the *Tpbpa*-positive trophoblast lineage [18]. C-TGCs, which line the maternal arterial canals in the junctional zone and labyrinth, are also partially derived from the *Tpbpa* lineage [18]. Nevertheless, a reduction in VEGFA protein in SpA-TGCs and C-TGCs, which presumably occurred, caused no significant change in the histomorphometry of the decidual layer or in blood velocities observed upstream in the uterine artery or downstream in the maternal arterial canals of the placenta. This suggests that reduced VEGFA did not impair the ability of SpA-TGCs to tap into the maternal spiral arteries nor alter flow from the spiral arteries through the trophoblast-derived arterial canals. Normal uteroplacental arterial inflow also suggests that venous outflow is maintained and, hence, that the venous channels in the junctional zone [13] continued to function normally, despite the reduction in VEGFA induced in junctional zone trophoblast cells.

At GD17.5, there are abundant *Tpbpa*-expressing glycogen trophoblast cells in the decidual layer. However, they do not migrate there until after GD12.5 [38], which is after the uteroplacental circulation has been initiated [13]. Interestingly, histomorphometry showed no change in the proportion of the decidual layer occupied by glycogen trophoblast cells. This suggests that Cre-mediated *Vegfa* deletion did not impair their migration, despite a known role for VEGFA in promoting migration of human trophoblast cells [39] and other cell types, including endothelial cells [40] and monocytes [41, 42], in *in vitro* assays. Thus, there was no evidence that VEGFA derived from *Tpbpa*-expressing cells in the decidual layer played an important role in establishing the uteroplacental circulation, or in controlling the invasion of glycogen trophoblast cells into the decidual layer in later gestation.

In contrast to the placenta, which appeared to develop normally, maternal cardiovascular changes were observed. In human pregnancy, pre-eclampsia is associated with diminished VEGFA activity due to excess sFLT1, maternal hypertension, and diminished cardiac output [5, 6]. Thus, we wondered whether reducing VEGFA production by trophoblast cells would have a similar detrimental effect on the VEGFA/sFLT1 balance and would adversely affect maternal outcomes. Instead, we found that reducing ir-VEGFA in the decidual layer and junctional zone augmented maternal plasma ir-VEGFA levels in SD<sub>100</sub> pregnancies only, but, in both models, augmented a component of the normal maternal cardiovascular response to pregnancy (a decrease in arterial pressure in SD<sub>100</sub> or an increase in cardiac output in DD<sub>50</sub>). In SD<sub>100</sub> pregnancies, maternal arterial blood pressure was reduced by ~15 mm Hg relative to V-CTRL. Because of the hypotensive effects of VEGFA [2], the decrease in arterial pressure may have been mediated by the unexpected increase in ir-VEGFA in maternal plasma, which occurred despite reduced placental ir-VEGFA expression in 100% of the litter. In DD<sub>50</sub> pregnancies, maternal cardiac output was increased by ~20%, an increase that was correlated with the percentage of dd conceptuses. In this case, ir-VEGFA in maternal plasma and maternal arterial pressure were unchanged, so a potential mechanism was not apparent. In both cases, the magnitude of cardiovascular change was substantial relative to normal pregnancy given that, from early to midgestation in mice and humans, arterial pressure decreases transiently by ~10 mm Hg and cardiac output rises in a more sustained manner by ~50% [43–45].

In the current study, the cardiovascular change (i.e., arterial pressure versus cardiac output) differed between groups, which suggests that different placenta-derived mediators are responsible for decreasing arterial pressure and for increasing cardiac

output. In mice, this is consistent with their differing time courses: arterial pressure decreases transiently at GD11–13 [45], whereas cardiac output has already increased by GD9.5 and continues to rise to GD17.5 [44]. The hormonal mechanisms mediating these changes are unknown. With respect to human pregnancy, results of the current study do not support a role for reduced placental VEGFA in invasive cytotrophoblast cells in pre-eclampsia [9] as a mechanism causing maternal hypertension and reduced cardiac output [5].

During preliminary experiments, we discovered that homozygous *Tpbpa-Cre* females carrying heterozygous *Tpbpa-Cre* conceptuses had severe intrauterine bleeding and early embryo lethality. This was not caused by her heterozygous *Tpbpa-Cre* conceptuses, because it was not observed when the same genotype was generated in wild-type females using paternal cre. *Tpbpa-Cre* in maternal tissues is unlikely to be responsible based on the placental specificity of transcriptome tissue expression for *Tpbpa* (GEO dataset gds868), lack of detectable GFP expression in maternal organs (current study), and lack of *Tpbpa* expression by in situ hybridization in maternal ovaries [36]. In addition, if caused by Cre toxicity [46], then one would have expected an even greater bleeding phenotype in a prior study, in which *Tpbpa-Cre* was used to activate diphtheria toxin resulting in *Tpbpa*-cell ablation [26], but no bleeding was reported in that study. We speculate that the insertion site of the *Tpbpa-Cre* transgene may disrupt the function of a maternal gene that is required in maternal decidual tissues in early pregnancy. It would therefore be interesting to map the insertion site in future studies.

In the current study, both nd and dd conceptuses were present in DD<sub>50</sub> pregnancies. Having control conceptuses in the same pregnancy is often considered a strength in studies evaluating placenta and/or embryo phenotypes, because they serve as “internal controls” for the mutants. However, results of the current study show that these internal controls may themselves be affected by a maternal pregnancy phenotype elicited by the mutant conceptuses. It is likely that this caveat is especially relevant to mutations influencing the function of junctional zone cells or TGCs due to their endocrine roles [11, 16, 18], roles that are likely important in maternal pregnancy adaptations. In the current study, this caveat applies, because the presence of both genotypes in DD<sub>50</sub> pregnancies would enable nd conceptuses to compensate for the endocrine deficiencies of their dd littermates. Such compensation could result in pregnancy phenotypes that are relatively mild. In the current study, a mild maternal phenotype was advantageous in that there was no significant change in maternal litter size or body weight, and pregnancies went successfully to term; this facilitated comparisons of maternal phenotypes with control pregnancies. Mixed litters also allowed us to show that nd littermates were affected by changes in the maternal environment induced by the dd conceptuses, a novel finding that would not have been apparent otherwise. However, it is important to note that a more severe maternal pregnancy phenotype may occur in DD<sub>100</sub> pregnancies (in which all conceptuses are dd), such as a greater incidence of early embryonic lethality and/or greater abnormalities in maternal cardiovascular function. For this reason, it would be valuable to evaluate DD<sub>100</sub> pregnancies in future studies.

In summary, we used *Tpbpa-Cre*-mediated deletion of floxed *Vegfa* to evaluate the role of VEGFA in *Tpbpa*-expressing trophoblast cells in mouse pregnancy. Deleting a single copy of *Vegfa* in these cells in 100% of the litter significantly reduced maternal arterial blood pressure, but other maternal, placental, and fetal parameters were unaffected. A more severe reduction in *Vegfa* caused by double deletion in

50% of the litter adversely impacted early gestation, resulting in increased litter-wide resorptions, alterations in placental gene expression, reduced fetal body weight, and higher maternal cardiac output. These changes could not be explained on the basis of a direct effect of *Tpbpa-Vegfa* gene deletion within the conceptus itself. Rather, observed changes were more likely secondary effects caused by changes in maternal functions, resulting from altered endocrine function of *Tpbpa*-expressing cells. It was surprising, given the known roles for VEGFA in vascular development in other tissues, that reduced VEGFA expression in trophoblast had no detectable impact on the uteroplacental or fetoplacental circulation in this study. In conclusion, results from both models support our central hypothesis that the role of VEGFA expression in *Tpbpa* trophoblast cells is primarily to regulate maternal function during pregnancy.

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