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Trophoblast VIP deficiency entails immune homeostasis loss and adverse pregnancy outcome in mice

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ABSTRACT: Immune homeostasis maintenance throughout pregnancy is critical for normal fetal development. Trophoblast cells differentiate into an invasive phenotype and contribute to the transformation of maternal arteries and the functional shaping of decidual leukocyte populations. Insufficient trophoblast invasion, inadequate vascular remodeling, and a loss of immunologic homeostasis are associated with pregnancy complications, such as preeclampsia and intrauterine growth restriction. Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide synthetized in trophoblasts at the maternal–placental interface. It regulates the function of trophoblast cells and their interaction with decidual leukocytes. By means of a murine model of pregnancy in normal maternal background with VIP-deficient trophoblast cells, here we demonstrate that trophoblast VIP is critical for trophoblast function: VIP gene haploinsufficiency results in lower matrix metalloproteinase 9 expression, and reduced migration and invasion capacities. A reduced number of regulatory T cells at the implantation sites along with a lower expression of proangiogenic and antiinflammatory markers were also observed. Findings detected in the implantation sites at early stages were followed by an abnormal placental structure and lower fetal weight. This effect was overcome by VIP treatment of the early pregnant mice. Our results support the relevance of trophoblast-synthesized VIP as a critical factor *in vivo* for trophoblast-cell function and immune homeostasis maintenance in mouse pregnancy.—Hauk, V., Vota, D., Gallino, L., Calo, G., Paparini, D., Merech, F., Ochoa, F., Zotta, E., Ramhorst, R., Waschek, J., Leirós, C. P. Trophoblast VIP deficiency entails immune homeostasis loss and adverse pregnancy outcome in mice. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: trophoblast function · immunoregulation · early pregnancy

The maintenance of immune homeostasis at the maternal–placental interface involves trophoblast–immune cell regulatory loops in a concerted action with hormones and locally synthesized mediators (1–4). Trophoblast cells invade the decidua and promote the selective recruitment and functional shaping of different maternal leukocyte populations from the beginning of pregnancy (3–6). Defects in these processes underlie deep placentation disorders with exacerbated inflammation like preeclampsia or intrauterine growth restriction (7–10). Invasive trophoblast cells secrete proteases like matrix metalloproteinase (MMP) 2 and especially MMP9, which help to degrade extracellular matrix components (11, 12). The inhibition of MMP9 activity in human trophoblast cells prevents their invasion *in vitro*, whereas cytotrophoblast cells produce less MMP9 in women with preeclampsia with or without intrauterine growth restriction (13).

In mouse pregnancy, the ectoplacental cone (EPC) develops from d 5.5 of pregnancy. Stem cells in the base of the cone migrate toward the tip, where they differentiate to trophoblast giant cells (TGC) with invasive capacity, and by d 7.5, these cells contact maternal vessels (14, 15). TGC present high MMP9 activity and intense phagocytic activity, with erosion and displacement of epithelium and the decidual stroma. Accordingly, the extracellular matrix around decidual cells in $MMP9^{-/-}$ mice is not properly removed, resulting in debris accumulation and reduced EPC area; as a consequence of a deficient differentiation and migration of trophoblast cells, by d 10.5 the layer of TGC appears wider compared to wild-type (WT) mice (16).

ABBREVIATIONS: EIA, enzyme immunoassay; EPC, ectoplacental cone; H&E, hematoxylin and eosin; KO, knockout; LCM, laser capture microdissection; MMP, matrix metalloproteinase; sENG, soluble endoglin; TGC, trophoblast giant cell; T_{reg}, regulatory T; VIP, vasoactive intestinal peptide; WT, wild type

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doi: 10.1096/fj.201800592RR

Multiple hormonal and peptidic regulatory factors are known to regulate trophoblast-cell invasiveness and their interaction with maternal immune cells (17–20). The vasoactive intestinal peptide (VIP) is a 28 aa neuropeptide with vasodilating, prosecretory, and CNS modulatory effects (21–23). VIP also has potent antiinflammatory and immunosuppressive effects (24–28). VIP is expressed in the human uterus (29), and VIP plasma levels are increased in pregnant *vs.* nonpregnant women (30). Human first- and third-trimester placentas and different human trophoblast cell lines express VIP (31–33). Moreover, VIP increases progesterone and human chorionic gonadotrophin secretion in human trophoblast cells (31), activates trophoblast migration and invasion through VIP receptor type 1 and 2, and regulates the interaction of trophoblast cells with maternal leukocytes through autocrine and paracrine loops (32, 34). Consistent with an immunomodulatory role during pregnancy, VIP promoted antiinflammatory and tolerogenic responses in cocultures of human leukocytes and trophoblast cell lines (32–37). In mouse pregnancy, VIP was shown to stimulate mouse embryo weight gain *in vitro* (38), and the injection of a VIP receptor antagonist at d E9 to E11 impaired neurodevelopment, inducing microcephaly through microcephalin signaling (23, 39). Mature VIP is expressed in the murine implantation sites until d 12, whereas the mRNA for VIP was not detected until E12 in the embryonic structures or the fetal mouse, suggesting a role for maternal rather than embryonic VIP on embryo development at early stages (23, 40, 41). Likewise, a single VIP injection at d 6.5 improved pregnancy outcome and increased antiinflammatory and tolerogenic markers in resorption-prone models (42, 43). Cytokeratin-positive TGCs, the phenotype that better resembles human invasive extravillous trophoblast cells (44, 45), are the major cell type expressing VIP in mouse implantation sites (42). On the other hand, VIP knockout (KO) mice exhibited a disrupted estrous cycle compared to controls, with longer periods between gestations, and produce about half the offspring of their WT sisters even when mated to the same males (46). However, there is little information on the mechanisms underlying these abnormalities. Moreover, neonates born to $VIP^{+/-}$ C57BL/6 mothers presented a reduced birth weight compared to those born to WT mothers, a deficit which was restored by postnatal d 5 (47).

Here we explored the relevance of VIP synthesized by trophoblast cells in immune homeostasis maintenance and pregnancy outcome. To probe further into the mechanisms of endogenous VIP as a pregnancy regulatory peptide, we developed a mouse pregnancy model with normal maternal background and VIP-deficient trophoblast cells by mating VIP WT females with either WT or VIP KO males. This strategy allowed us to compare trophoblast/maternal interactions of WT and $VIP^{+/-}$ embryos in WT mothers. We demonstrate that VIP deficiency in TGCs disrupts the formation of the maternal–placental interface, affects the functional phenotype of trophoblast cells with lower MMP9 expression, reduces trophoblast migration and invasion capacities, and reduces regulatory T (T_{reg}) cell migration to the implantation sites; it also reduces the expression of proangiogenic and antiinflammatory markers. Finally, deficiency of VIP in trophoblast cells affects placental structure and fetal growth.

MATERIALS AND METHODS

Mouse pregnancy model

Mice were bred and maintained on a 12/12-h light–dark schedule in the Central Animal Care facility at the School of Exact and Natural Sciences at the University of Buenos Aires. WT C57BL/6 or FOXP3-GFP knock-in (48) mice on a C57BL/6 background (3–6 mo old) were paired with WT male C57BL/6 or VIP KO males on a C57BL/6 background (49). Animals were checked daily for vaginal plugs and separated once mated. The day of the vaginal plug was considered as d 0.5 of pregnancy. Pregnant mice were humanly killed on d 8.5, 14.5 or 17.5 of gestation. For the VIP treatment studies, pregnant mice were treated with PBS intraperitoneally or with VIP (2 nmol/mouse) at d 6.5 of gestation. All studies were conducted according to standard protocols and were approved by the Animal Care and Use Committee of the School of Sciences at the University of Buenos Aires.

Implantation sites and EPC explant isolation

Pregnant uteri were carefully dissected at d 8.5 of pregnancy, and implantation sites were defined as previously described (42, 50) and isolated for cytokine and transcription factors measurement by RT-PCR, or preserved in paraformaldehyde 4% for histologic features or in optimal cutting temperature compound (Biopack, Buenos Aires, Argentina) for laser capture microdissection (LCM). For the preparation of EPC explants, the embryos at gestational d 8.5 were dissected from the implantation sites, and then the EPC was separated from each embryo using sterile fine forceps (51).

Fetal weight and placenta isolation

At gestational d 14.5 or 17.5, whole uteri were removed, and different end points of pregnancy outcome were analyzed macroscopically such as the number of viable implantation sites with resorption processes ongoing, and placental and fetal size and weight. Individual fetuses were dissected carefully from the uterine tissue and separated from the placenta. Each fetus and placenta was weighed individually.

RT-PCR and quantitative RT-PCR

The expression of different mediators was determined by RT-PCR (**Table 1**). Briefly, total RNA was isolated according to the manufacturer's instructions with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), cDNAs were generated from 1 μg or RNA using a Moloney murine leukemia virus reverse transcriptase, RNAse inhibitor, and oligodT kit (Promega, Madison, WI, USA), and samples were stored at −20°C for batch analysis. For RT-PCR, each cDNA was amplified using specific primers (Table 1). PCR products and DNA size markers were fractionated on 2% agarose gels, visualized with ethidium bromide staining, and band density expressed in arbitrary units normalized to *GAPDH*. Bands were semiquantified with ImageJ software (Image Processing and Analysis in Java; National Institutes of Health, Bethesda, MD, USA; *https://imagej.nih.gov/ij/*) and intensity expressed in arbitrary units relative to *GAPDH*. For real-time PCR, samples were incubated with SYBR Green PCR Master Mix and the forward and reverse primers. Quantitative RT-PCR was performed on a Bio-Rad iQ5 Real-time PCR system (Bio-Rad, Hercules, CA, USA). The relative gene expression levels were determined using the cycle threshold (C_t) method ($2^{-\Delta\Delta Ct}$) normalized to the endogenous *GAPDH* or *S29* gene control.

TABLE 1. *Primers used in RT-PCR assays*

Gene	Primer sequence, 5′–3′	
	Forward	Reverse
S29	GGAGTCACCCACGGAAGTTCGG	GGAAGCACTGGCGGCACATG
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT
PRL3D1	GTCTTGAGGTGCCGAGTTGTC	CTGGGTGGGCACTCAACATT
MMP9	CCATGCACTGGGCTTAGATCA	GGCCTTGGGTCAGGCTTAGA
ANGPT1	-GGGGGAGGTTGGACAGTAA	CATCAGCTCAATCCTCAGC
VEGFA	CACGACAGAAGGAGAGCAGAAG	CTCAATCGGACGGCAGTAGC
sENG	GCCCTGACCTGTCTGGTAAAG	GGGTGGAGGCTTGGGATAC
IL-10	GTTGCCAAGCCTTATCGGAAATG	CACTCTTCACCTGCTCCACTG
TNF-α	TACTGAACTTCGGGGTGATCGGTCC	CAGCCTTGTCCCTTGAAGAGAACC
TGF-β	GACTCTCCACCTGCAAGACCA	TTGGGGGACTGGCGAGCCTT
FOXP3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
IL-6	ACCGCTATGAAGTTCCTCTCTGC	AGTGGTATCCTCTCTGTGAAGTCTCC

Trophoblast invasion assays

To evaluate invasiveness, Transwell chambers with 6.5-mm diameter polycarbonate filters of 8 μm pore size were placed into wells of a 24-well plate. Each chamber was coated with 30 μl of reduced-growth factors Geltrex (Thermo Fisher Scientific). Isolated EPCs were set on the top chamber, and the bottom chamber was filled of culture medium. Treatments were performed 24 h later. After 48 h of treatment, the Transwell inserts were washed, and the lower surface was fixed and stained with DAPI. This 2-chamber system assesses both cell invasion and migration. The number of cells that had traversed the filter was quantified by fluorescent microscopy. Each experiment was carried out at least 3 separate times.

Laser capture microdissection

LCM (ArcturusXT; Thermo Fisher Scientific) of fresh frozen tissue cryosections previously stained with Histogene kit was directed to selected TGCs that were then captured on an RNase-free cap with a transfer film (CapSure LCM Cap; Arcturus; Molecular Devices, San Jose, CA, USA). Total RNA was extracted from TGCs captured by the LCM transfer film using a PicoPure RNA Isolation Kit (ArcturusXT). Total RNA was treated with DNAasa I according to the manufacturer's instructions (MilliporeSigma, Burlington, MA, USA) to avoid DNA contamination, and then samples were reverse transcribed using a Moloney murine leukemia virus reverse transcriptase, RNAse inhibitor, and oligodT kit (Promega) as previously described.

Histologic studies

Implantation sites and placentas at gestational d 8.5 and 14.5, respectively, from WT mothers crossed with WT or VIP KO males were fixed in 4% paraformaldehyde overnight at 4°C. The tissues were embedded in paraffin, and 4 μm sections were cut and placed on silanized glass slides. Hematoxylin and eosin (H&E) staining was performed as previously described (50).

Flow cytometry

To evaluate the percentage of $CD4^+FOXP3^+$ cells at the implantation sites, FOXP3-GFP knock-in mice were crossed with WT or VIP KO mice. At gestational d 8.5, decidual cells were obtained by disaggregating the tissue and filtering them through a sterile mesh. Then the cells were washed and incubated with an anti–CD4-PE antibody (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at room temperature in darkness. Events were acquired in a FACSAria cytometer, and results were analyzed by FlowJo software (Treestar, Ashland, OR, USA).

VIP determination

VIP secretion was quantified in supernatants obtained from EPC explant cultures after 48 h of culture using the VIP enzyme immunoassay (EIA) kit (Phoenix Pharmaceutical, Burlingame, CA, USA). In brief, 25 μl of antiserum and 50 μl of the standard or sample were incubated in 96-well immunoplates for 1 h at room temperature. Then25 μl of biotinylated tracer was added and incubated for 2 h. After 5 washings with EIA buffer, 100 μl of streptavidin–horseradish peroxidase was added and incubated at room temperature for 1 h. After washing, tetramethylbenzidine solution and 2 N HCl were sequentially added for color development. Absorbance was determined using an iMark Absorbance Microplate Reader (Bio-Rad) at 650 and 450 nm for the blue and yellow products, respectively. The specificity of the EIA is 100% for VIP (human, rat, mouse, pig, sheep, guinea pig). The specificity for VIP (10–28) and VIP from chicken is 34.6 and 28%, respectively, and for PACAP-27-NH2 (human, rat, sheep), it is $<0.02\%$. The kit has no cross-reactivity with substance P, endothelin 1, secretin, glucagon, galanin, somatostatin, or PACAP-38. The linear range of the EIA for VIP is 0.12 to 0.93 ng/ml.

Statistical analysis

Statistical significance of differences was determined by the Mann-Whitney *U* test for nonparametric samples. When multiple comparisons were necessary, Kruskal-Wallis with Dunn's multiple comparison posttest was performed. Statistical significance was defined as $P < 0.05$ using Prism6 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Histologic alterations in implantation sites with VIP-deficient trophoblast cells

To disclose the *in vivo* effect of VIP synthesized by trophoblast cells in pregnancy outcome, C57BL/6 WT female mice were bred with $VIP^{-/-}$ males so that resulting trophoblast cells were deficient in VIP in a WT maternal context. We first performed histologic studies to evaluate the structure of implantation sites at gestational d 8.5, a

time at which trophoblasts are believed to be the only embryonic source of VIP.

In control mice, a single monolayer of TGCs that migrate from the EPC is observed; however, in VIP-deficient implantation sites, an expansion of the TGC layer was evident (**Fig. 1*A***, left and right). Likewise, vascular defects were also detected in implantation sites from WTxKO pregnancies (Fig. 1*B*). The top panel of Fig. 1*B* shows well-defined control TGCs (asterisk) and blood vessels (arrow).

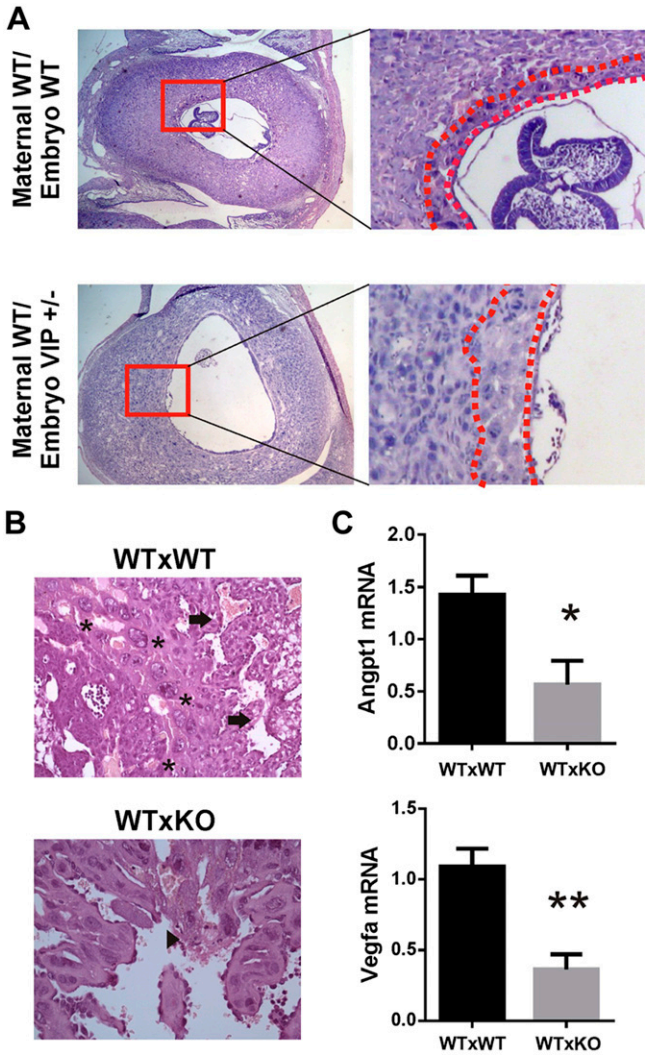

Figure 1. Broad TGC layer and vascular defects in implantation sites from WTxKO pregnancies. *A*) H&E staining of implantation sites at gestational d 8.5 from WT females crossed with WT or VIP KO males. Higher-magnification images of boxed regions are shown at right. TGC layer is indicated within dotted line. *B*) H&E staining of implantation sites at gestational d 8.5 from WT females crossed with WT (top) or VIP KO males (bottom). Asterisks show TGCs, arrows indicate blood vessels, and arrowheads point to loss of normal histology with cellular necrosis. Images *A* and *B* are representative of at least 6 implantation sites from 6 different pregnancies in each group. *C*) mRNA expression of *Angpt1* and *Vegfa* was evaluated by quantitative RT-PCR in implantation sites from WT mothers crossed with WT or VIP KO males at gestational d 8.5. Data are means ± SEM of at least 4 samples of different pregnancies in each group. $*P < 0.05$, $**P < 0.01$.

TGCs at the interface between decidua and the placenta are present within the capillaries. In contrast, a disruption of the endothelium with necrotic structures was found in WTxKO implantation sites (Fig. 1*B*, bottom). Consistent with these observations, the expression of 2 crucial proangiogenic factors that participate in vascular transformation, such as VEGF-α and angiopoietin 1 (*Vegfa* and *Angpt1*), is reduced (Fig. 1*C*).

VIP deficiency affects the functional phenotype of trophoblast cells during placentation

TGCs resemble human invasive trophoblast cells (44, 45), and differentiation of trophoblast cells into an invasive phenotype is a critical process for proper placentation. On the basis of the pathologic findings observed in VIP-deficient implantation sites, and considering that VIP autocrine pathways appear to be involved in human trophoblast-cell migration and invasion (34), we next evaluated the functional profile *ex vivo* of trophoblast cells from WT mothers crossed either with VIP KO males (VIP-deficient trophoblast cells) or to WT males (control trophoblast cells).

First, we studied the migration capacity of trophoblast cells by EPC outgrowth assessment. After 48 h of culture, we observed that VIP-deficient EPC displayed reduced outgrowth compared to control EPC (**Fig. 2*A***). To evaluate if MMP9 expression was affected in VIP-deficient trophoblast cells, we isolated TGC using LCM. We confirmed the TGC phenotype through assessing the expression of *Prl3d1*, a marker of TGC, which was enriched more than 100-fold (Fig. 2*B*, left). The expression of *MMP9* was significantly reduced in VIP-deficient TGC (Fig. 2*B*, right). We next assessed trophoblast invasiveness using Geltrex-coated Transwell inserts using control or VIP-deficient EPC in the presence or absence of 100 nM VIP or a VIP receptor antagonist (50 nM), respectively. VIP receptor antagonist prevented WT EPC invasiveness, whereas in VIP-deficient EPC the addition of VIP restored cell invasion (Fig. 2*C, D*). When we analyzed the levels of VIP in the EPC explants in culture. The VIP concentration in the supernatant of WT EPC was 152 ± 42 pg/ml ($n = 4$), whereas in the WTxKO EPC animals ($n = 4$), the levels of VIP appeared under the detection limit of the assay.

VIP deficiency in trophoblast cells promotes proinflammatory microenvironment with reduced migration of T_{reg} cells

We have previously shown that VIP acts as an immunomodulatory peptide in murine pregnancy (42, 43, 50). On this basis, we next analyzed if VIP deficiency in trophoblast cells from WTxKO mating could modulate the immune microenvironment at the implantation sites. **Figure 3*A*** shows a lower mRNA expression of *FOXP3*, a regulatory T cell marker, and *IL-10*, with no changes in *TGF-β, IL-6, and TNF-α* in VIP-deficient implantation sites. Considering that T_{reg} cells express both FOXP3 and IL-10, we next studied the frequency of $CD4^+FOXP3^+$ cells present at the implantation sites. For this purpose, we crossed

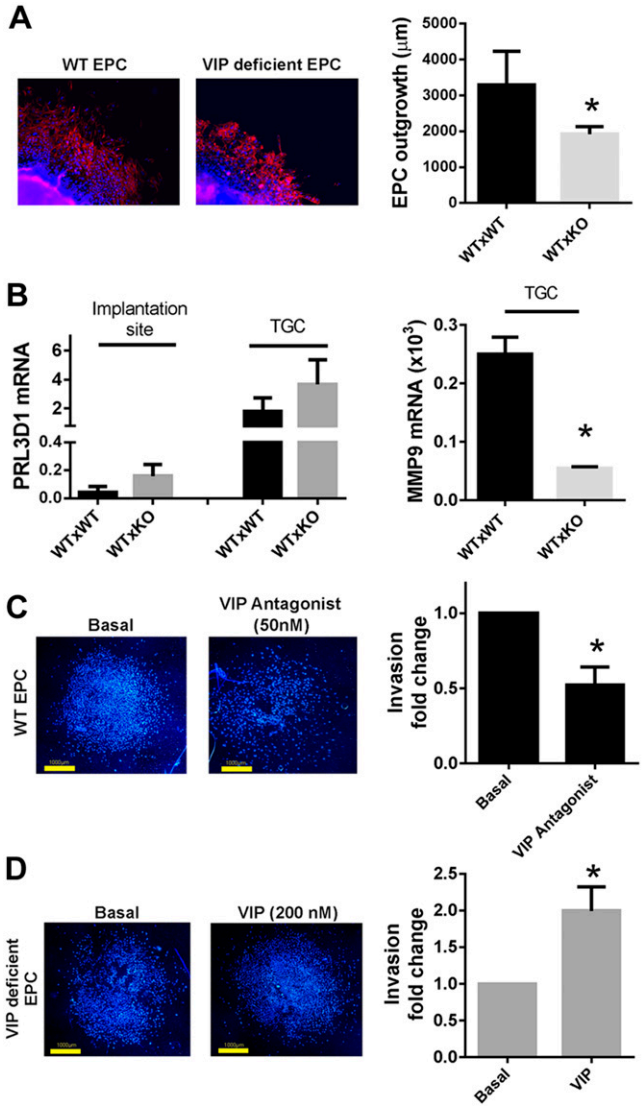

Figure 2. Functional phenotype of trophoblast cells affected by VIP deficiency. *A*) For study of outgrowth migration capacity, gestational d 8.5 mouse EPC explants were cultured for 48 h, fixed in fresh 4% paraformaldehyde, and stained with tetramethylrhodamine isothiocyanate–phalloidin and DAPI. Representative images of outgrowths from at least 3 cultures are shown. Outgrowth extension was measured as distance between farthest migrated cells and central EPC, defined as central, single mass within which individual cell nuclei could not be determined. Values are means ± SEM of at least 3 experiments. *$P < 0.05$. *B*) mRNA expression of *MMP9* and *Prl3d1* was quantified by quantitative RT-PCR in TGC isolated by LCM and implantation sites (Prl3d1) from WT mothers crossed with WT or VIP KO males. Values are means ± SEM of at least 4 determinations.*$P < 0.05$ *vs.* corresponding marker in WTxWT. *C*, *D*) For study of invasive capacity, EPC explants were cultured on Geltrex-coated Transwell inserts for up to 48 h. VIP receptor antagonist (50 nM) for WT EPC (*C*) or VIP (100 nM) for VIP-deficient EPC (*D*) was added for last 24 h of culture. Left: representative images of 4 experiments run similarly. Right: values are means ± SEM of at least 4 determinations. *$P < 0.05$.

FOXP3-GFP C57BL/6 females with VIP KO C57BL/6 males or control C57BL/6 males, and the number of $CD4^+$ $FOXP3\text{-}GFP^+$ cells at the implantation sites or in the maternal inguinal draining lymph nodes was evaluated by flow cytometry. A decreased frequency of T_{reg} cells in VIP-deficient implantation sites compared to control mating was found (Fig. 3*B*). This difference was not observed in draining inguinal lymph nodes (Fig. 3*C*).

Placental abnormalities and adverse pregnancy outcome in WTxKO pregnancies

We next assessed whether these early alterations had any impact later on during gestation, either in placental structure or fetal growth. **Figure 4** shows that VIP-deficient fetuses exhibited reduced size (Fig. 4*A*) and weight for their gestational age at d 14.5 (Fig. 4*B*), and this difference persisted later in gestation, at d 17.5 (Fig. 4*C*). The lower fetal weight observed in VIP WTxKO pregnancies was not related to differences in placental weight, which showed similar values for the WTxKO and control pregnancies (Fig. 4*D*). In addition, there was an increased resorption rate in VIP WTxKO pregnancies at d 14.5 but rarely in the WT counterparts (Fig. 4*E*). Soluble endoglin (sENG) is a coreceptor for TGF-β with antiangiogenic properties. Pregnancy with fetal growth retardation but without maternal symptoms are characterized by elevated sENG in circulation (52), and a significant correlation was found between high levels of sENG and the occurrence of growth retardation among severe preeclamptic patients (53). Following these observations, we evaluated the expression of sENG in placentas of both normal and VIP-deficient pregnancies. Figure 4*F* shows that placentas from VIP WTxKO pregnancies express higher levels of *sENG*. Finally, histologic analysis performed in placentas from mice with VIP-deficient embryos showed differences in the extent and characteristics of the placental functional layers, with an evident disruption of their structure and organization. Moreover, in control placentas, the labyrinth and junctional zone appeared integrated, whereas in the placentas from the VIP WTxKO mating, a severely reduced labyrinth layer was found, which could result in impaired communication between maternal and fetal circulation (Fig. 4*G*).

VIP treatment restores fetal weight loss in VIP WTxKO pregnancies

In previous studies using 2 resorption-prone mouse models, we showed that VIP treatment at gestational d 6.5 improved pregnancy outcome. To address whether the lower fetal weight in VIP WTxKO pregnancies was related to VIP deficiency, we treated pregnant mice with 2 nmol VIP at d 6.5 of pregnancy, considering that implantation has already occurred and therefore the peptide would only affect postimplantation processes. **Figure 5** shows that VIP treatment increased fetal weight in VIP WTxKO pregnancies, reaching values similar to control fetuses. The effect of VIP was also observed when we compared the average of fetal weights of each litter: WTxWT, 0.27 ± 0.03 g; WTxKO, 0.18 ± 0.01 g; and WTxKO treated with VIP, 0.22 ± 0.01 g. Results are means ± SEM of at least 4 pregnant mice in each group. *P* was <0.05 for WTxWT *vs.*

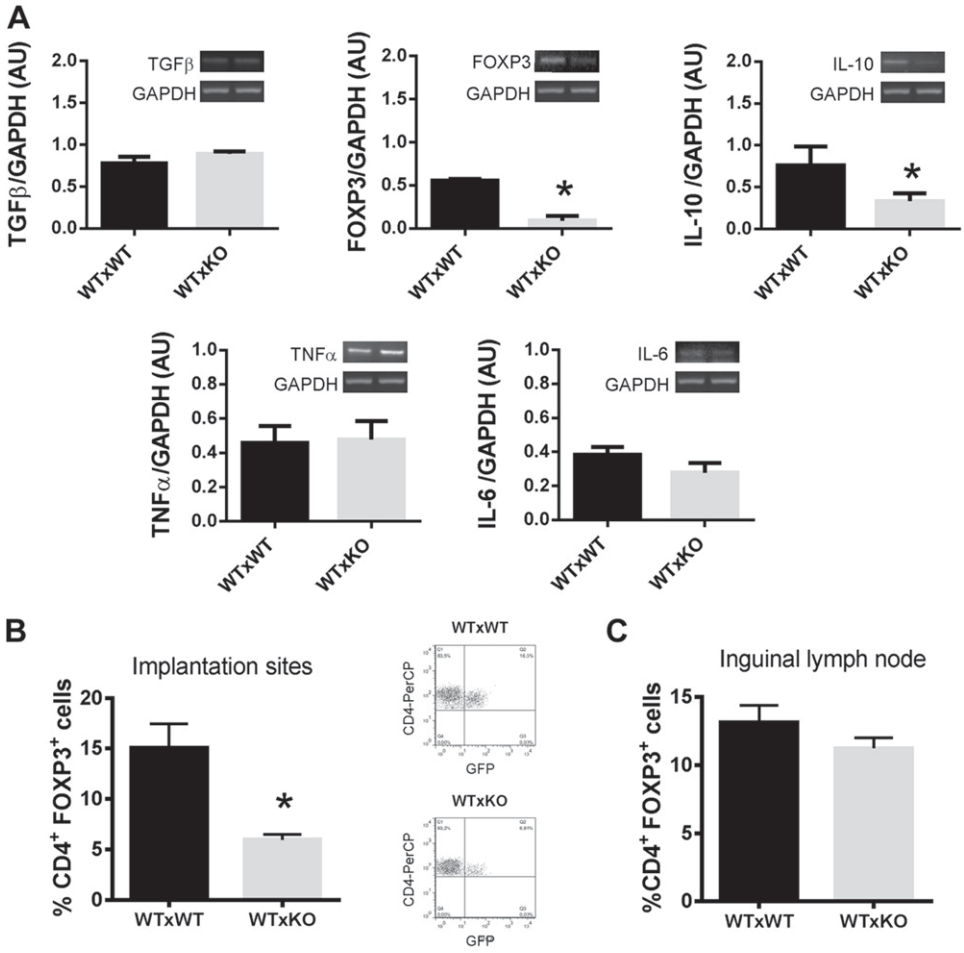

Figure 3. Lower expression of immunosuppressive markers and decreased frequency of T_{reg} cells in implantation sites from VIP WTxKO pregnancies. *A*) Implantation sites were obtained at gestational d 8.5 from WT females mated to WT or VIP KO males. *TGF-β*, *FOXP3*, *IL-10*, *TNF-α*, and *IL-6* mRNA levels assessed by RT-PCR as indicated in Materials and Methods. Representative gels from at least 3 experiments and band densitometry using ImageJ software representing means ± SEM of at least 4 determinations are shown. $^*P < 0.05$. *B*, *C*) C57BL/6 FOXP3-GFP females were crossed with C57BL/6 WT or VIP KO males. At gestational d 8.5, frequency of $CD4^+$ FOXP3-GFP^+ cells at implantation site (*B*) or at inguinal lymph nodes (*C*) was analyzed by flow cytometry. Representative dot plots from implantations sites and values of means ± SEM of at least 3 independent experiments are shown. $^*P < 0.05$.

VIP WTxKO and WTxKO *vs.* WTxKO treated with VIP. Finally, similar to the observations in NOD or CBA, CBA/J x DBA/2 resorption, JxDBA/2 resportion-prone models, VIP treatment at d 6.5 also reduced the rate of resorption in the VIP WTxKO mating (data not shown).

DISCUSSION

Here we tested the hypothesis that trophoblast-synthesized VIP is critical *in vivo* for trophoblast-cell function and immune homeostasis maintenance in a mouse pregnancy model. Results presented here point to the relevance of trophoblast cells as one of the major cellular sources of the peptide, and that its deficiency affects placentation, reduces the local expression of proangiogenic and antiinflammatory factors, impairs T_{reg} recruitment, and restricts fetal growth. These conclusions are based on the following observations. First, an abnormal expansion of the TGC layer, vascular defects, and disruption of the endothelium with necrotic structures were found in implantation sites from WTxKO pregnancies. Reduced expression of *VEGF*-α and *angiopoietin 1* was also found. Second, LCM studies revealed that TGC from VIP-deficient pregnancies showed a 3-fold decrease of MMP9 expression, which was paralleled by a lower EPC migration capacity at this gestational stage compared to normal pregnancies. VIP restored the impaired invasiveness of VIP-deficient trophoblast explants *ex vivo*, and a VIP receptor antagonist prevented cell invasion. Third, implantation site explants from VIP WT mothers crossed with $VIP^{-/-}$ males presented a reduced percentage of FOXP3-GFP^+ T_{reg} cells within $CD4^+$ cells and decreased *IL-10* and *FOXP3* expression compared to WTxWT mating at d 8.5 of pregnancy. Finally, the placental defects detected at early stages in VIP-deficient pregnancies were followed by a reduced size of the labyrinth layer of the placenta, an increased resorption rate, and a reduced fetal weight at gestational d 14.5. Fetal weight loss was restored by a single injection of VIP at d 6.5 of pregnancy.

Placentation is a tightly regulated process that involves trophoblast differentiation and migration and the transformation of maternal arteries to supply the demands of the growing fetus. Mouse TGCs are the functional analogs of the human extravillous trophoblast invasive phenotype (44, 45). The invasive behavior of trophoblasts cells in mice involves degradation and remodeling of uterine extracellular matrix, partly regulated by MMP9 activation (11, 12). Factors that affect the expression of MMPs at the maternal–fetal interface may have an effect on early placentation. In line with this, MMP9 deficiency is associated with impaired trophoblast differentiation and invasion shortly after implantation, along with intrauterine growth restriction or embryonic death (16). Our results provide

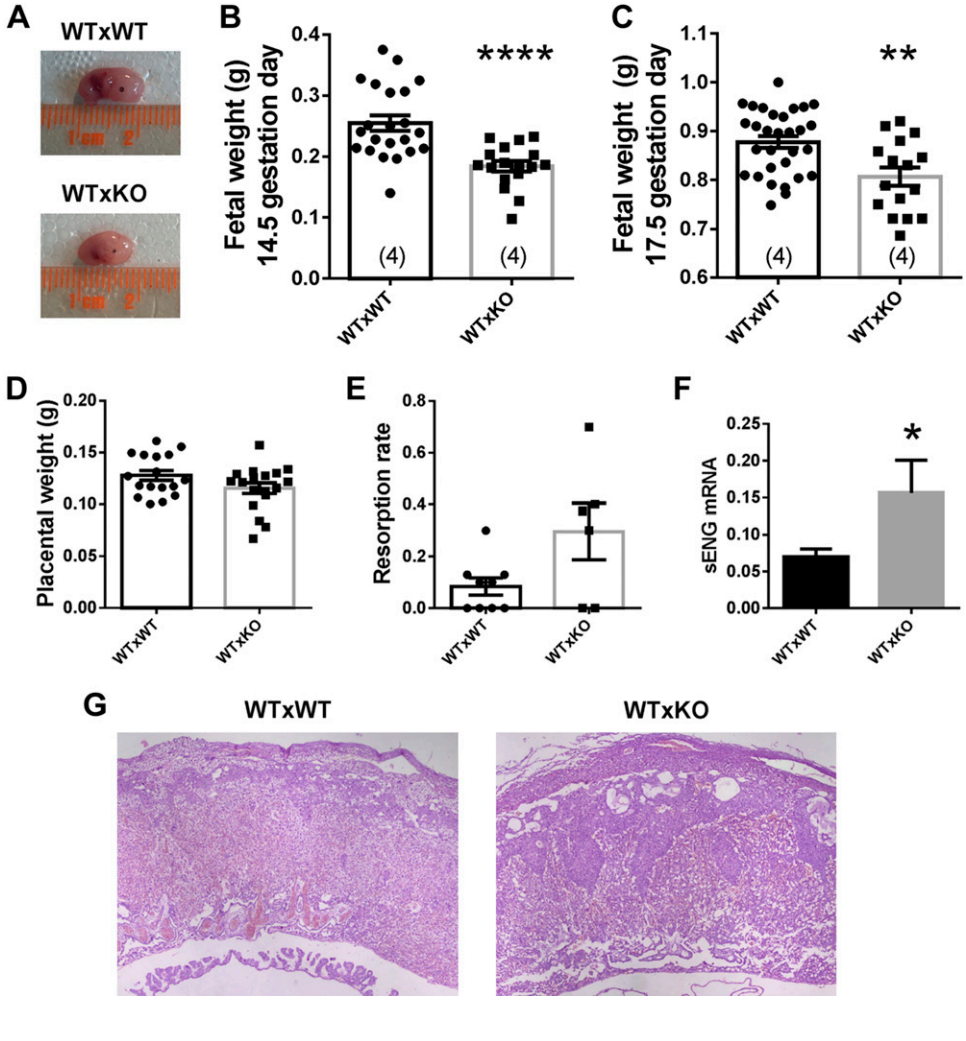

Figure 4. Adverse pregnancy outcome and reduced fetal weight in WTxKO pregnancies. *A*) Representative images of fetal size differences at gestational d 14.5 from WT females crossed with WT (WTxWT) or VIP KO (WTxKO) males. *B*, *C*) Fetal weight was evaluated at gestational d 14.5 and 17.5. Values are means ± SEM of number of individuals shown. $^*P < 0.05$. Numbers of litters analyzed are depicted in brackets inside each bar. *D*) Placental weight was evaluated at gestational d 14.5 in WTxWT or VIP WTxKO pregnancies. Values are means ± SEM of number of individual placenta shown. *E*) Resorption rate was calculated at gestational d 14.5 as number of resorbed fetus over total fetus counted in WT females crossed with WT (WTxWT) or VIP KO (WTxKO) males. Values are means ± SEM of number of pregnancies shown. ($^*P < 0.05$. *F*) Expression of *sENG* mRNA was evaluated by quantitative RT-PCR in placentas from WT mothers crossed with WT or VIP KO males at gestational d 14.5. Values are means ± SEM of at least 4 determinations corresponding to 4 different pregnant mice in each group. $^*P < 0.05$. *G*) H&E staining of placentas at gestational d 14.5 from WT females crossed with WT or VIP KO males. Images are representative of 4 placenta from 4 different pregnant mice in each group.

new evidence on VIP as a factor synthesized by trophoblast cells that modulates the migration and invasion of these cells, affecting the normal placentation *in vivo*. VIP would act in autocrine and paracrine loops to modulate trophoblast function because VIP-deficient TGC showed reduced expression of MMP9 on one side, and the inclusion of an antagonist of VIP receptors reduced the basal invasiveness of normal EPC cells on an extracellular matrix extract (Geltrex). This is in line with previous reports on MMP9 in trophoblast cell lines (11, 12) as well as with the stimulating effect of VIP on MMP9 and MMP2 expression and activity in a human prostate cell line (54).

Preeclampsia and fetal growth retardation are associated with insufficient trophoblast invasion and vascular remodeling, with consequent reduced maternal blood supply to the placenta (7–10). In mouse pregnancy, the lack of adrenomedullin is associated with defects in trophoblast-cell invasion that underlie defects in fetal growth (17). The deletion of *BMPR2* triggered abnormal vascular development and trophoblast defects that led to fetal mouse growth restriction (15). However, corin or atrial natriuretic peptide KO pregnant mice presented impaired trophoblast invasion and uterine spiral artery remodeling and developed high blood pressure and proteinuria, characteristic of preeclampsia (18). Regarding VIP, homozygous VIP-deficient mice present normal mating behavior but a reduced pregnancy rate (46). Disruption of the estrus cycle, endocrine-altered regulation, and enlargement of the time elapsed from one pregnancy to the next underlie the subfertile phenotype of the VIP-KO female phenotype. Our present model avoids the effects of the VIP-deficient phenotype in the mothers and allows us to analyze the role of VIP synthesized by trophoblast cells in pregnancy outcome.

The trophoblast and placental abnormalities observed in this model, along with the reduction of fetal weight that is restored by a single injection of VIP early in pregnancy, strongly support the notion that trophoblast rather than maternal VIP is operating at this early stages *in vivo*. Results in a C57BL/6 background shown here and the previous work in BALB/c, CBA/J, NIH Swiss, and NOD mouse strains (40–43, 47, 50) also support the notion that the regulation of VIP synthesis at the maternal–placental interface varies within a narrow range of concentrations and with a temporal pattern peaking around gestational d 8.5. A failure in VIP regulation at the

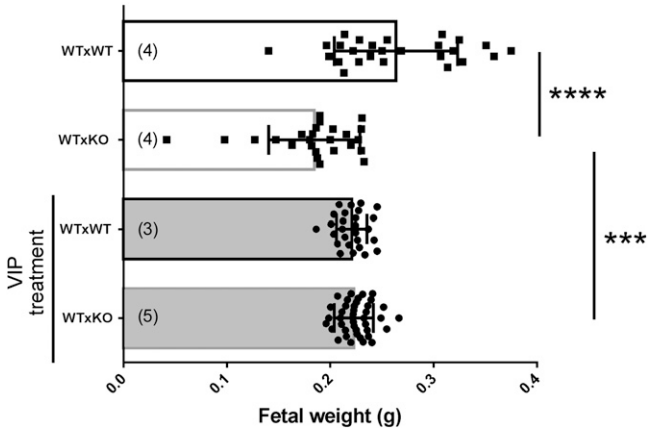

Figure 5. VIP treatment restores fetal weight loss in WTxKO pregnancies. Pregnant WT females crossed with WT or VIP KO males were injected intraperitoneally at gestational d 6.5 with 2 nmol VIP or with PBS. Fetal weight was evaluated at gestational d 14.5. Values are means ± SEM of number of individuals shown. $^{***}P < 0.001$, $^{****}P < 0.0001$. Number of litters for each group analyzed is depicted in brackets inside each bar.

maternal–placental interface would affect embryo growth and/or impair neurodevelopment without compromising maternal health. In line with this, the same dose of VIP that improved pregnancy outcome in VIP-deficient pregnancies here and in two resorption-prone mouse models induced embryo resorption when provided to normal BALB/c pregnant mice at d 6.5 (42, 43). Also, fetal growth retardation was observed after the blockade of VIP functions from E9.5 to E11.5 but not later with a VIP antagonist, consistent with a temporal expression of VIP activity (55). Interestingly, when the VIP antagonist was injected together with VIP, the effect of the antagonist was avoided, but if the same dose of VIP was injected alone to pregnant mice, it resulted in embryo loss, thus providing additional evidence of an optimal VIP local concentration required for placentation and fetal growth (55). Finally, VIP KO females become pregnant with a lower frequency and showed diverse limitations to get a normal litter size (46, 47, 49), indicating that although VIP would not be an absolute requirement, its presence at optimal levels entails relevant functions for normal mouse pregnancy.

Reduced levels of VEGF-α and angiopoietin and increased levels of sENG have been associated with poor angiogenesis, defective vascular transformation in deep placentation disorders associated with fetal growth retardation (52, 53, 56–58). VIP was shown to induce VEGF-α expression and enhance angiogenesis in murine ischemia as well as in human prostate and breast cancer cell lines (59–61). VIP has antiproliferative effects on smooth muscle vascular cells, and smooth muscle–cell apoptosis is involved in vascular transformation during early pregnancy (62). There are no reports on angiopoietin 1 or sENG and VIP in pregnancy in other cell types. Thus, the fact that VIP deficiency in trophoblast cells in our present model was associated with reduced expression of both VEGF-α and angiopoietin, increased expression of sENG, altered placental structure, and resulted in a reduction of fetal weight is consistent with a direct local effect of VIP on the interface cells as well as with an indirect VIP effect through the induction of growth factors, as previously reported (32, 47, 63, 64). In fact, the possibility that VIP might trigger downstream synthesis of growth factors like IGF-1, activity-dependent neurotrophic factor (ADNF), activity-dependent neuroprotector homeobox (ADNP), and nerve growth factor (NGF) (63, 64), and several cytokines that modulate trophoblast function (32) is also consistent with the observation reported here that a single VIP injection early in pregnancy was sufficient to reverse fetal growth retardation.

Finally, immune homeostasis is central to normal placentation and fetal growth (1–6). Trophoblast cells contribute to the enrichment of T_{reg} cells in the decidua. Consistently, a relationship between adverse pregnancy outcome and decreased number of T_{reg} cells in circulation and $FOXP3^+$ cells in the placenta has been reported (65–68). VIP induced T_{reg} cells *in vitro* and modulated the trophoblast–immune interaction with increased frequency of $CD4^+CD25^+FOXP3^+$ cells (32). The lower frequency of T_{reg} cells in the uterus of VIP-deficient pregnancies reported here as well as the lower FOXP3 and IL-10 expression is consistent with an *in vivo* role of the polypeptide to promote maternal T_{reg} recruitment to the uterus. It is likely that the level of local reduction of T_{reg} frequency determines the severity of the placentation disorder in mice. Moreover, the possibility that a differential abundance of natural and inducible T_{reg} populations in a trophoblast-regulated microenvironment appear in the WTxKO pregnancy cannot be ruled out.

Although leukocytes and trophoblast cells in mice have a different kinetics compared to human pregnancy, they contribute to vascular transformation, angiogenesis, and immune homeostasis maintenance from the post-implantation stage throughout placentation (14, 15, 69). VIP gains particular relevance at these early stages of pregnancy, acting as a multitarget polypeptide on immune, trophoblast, and vascular cells. Our results support its relevance *in vivo* and point to its potential as a candidate for therapeutic approaches in pregnancy complications associated with defective placentation. FJ

ACKNOWLEDGMENTS

The authors acknowledge P. Pomata (Instituto de Biología y Medicina Experimental–Consejo Nacional de Investigaciones Científicas y Técnicas (IByME–CONICET), for expert contribution to LCM assays, and M. Brahamian and Z. Naguila (both from Central Animal Facility of the School of Sciences, University of Buenos Aires, Argentina) for expert technical assistance with the VIP-deficient colony breeding. This work was funded by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) National Agency of Science and Technology Projects (PICT 2014-0657 to C.P.L., 2015-1281 to D.V., 2016-0464 to R.R., and 2016-1937 to V.H.), and the University of Buenos Aires Secretaría de Ciencia y Técnica (2014–2017 to C.P.L. and UBACyT 2016 to R.R.). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

V. Hauk, R. Ramhorst, J. Waschek, and C. P. Leirós designed the research; V. Hauk, D. Vota, L. Gallino, G. Calo,

D. Paparini, and F. Merech performed the experiments, cell cultures, and flow cytometry, and obtained and analyzed raw data; F. Ochoa and E. Zotta performed histologic studies; J. Waschek provided the VIP KO colony; and V. Hauk, R. Ramhorst, J. Waschek, and C. P. Leirós analyzed data, discussed the results, and wrote the manuscript.

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Received for publication April 3, 2018.
Accepted for publication August 6, 2018.