From decidualization to pregnancy progression: An overview of immune and metabolic effects of VIP

Rosanna Ramhorst, Esteban Grasso, Daiana Vota, Soledad Gori, Vanesa Hauk, Daniel Paparini, Guillermina Calo, Claudia Pérez Leirós

Universidad de Buenos Aires (UBA). Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN-CONICET). Laboratorio de Inmunofarmacología. Buenos Aires, Argentina.

Corresponding Author:

Claudia Pérez Leirós

Laboratorio de Inmunofarmacología. Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), CONICET-Universidad de Buenos Aires, (1428) Buenos Aires, Argentina. Ciudad Universitaria, Pab. 2, piso 4.

1428, Buenos Aires, Argentina.

Email: cpleiros@qb.fcen.uba.ar

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Abstract

A tight immune and metabolic regulation underlies the early maternal-placental interaction to assist the energetic dynamic demands of the fetus throughout pregnancy. During decidualization, endometrial stromal cells undergo reticular stress and trigger unfolded protein response (UPR) that enable expansion of their endoplasmic reticulum and immunomodulatory factor synthesis. These processes appear differentially affected in recurrent abortion and *in vitro* fertilization failure suggesting their relevance in reproductive pathologies. Similarly, defective placentation associates

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with altered immune, vascular and trophoblast interaction resulting in complicated pregnancies that threaten maternal and neonatal health and underlie metabolic programming of adult life.

Here we overview critical aspects of embryo implantation and placental development with special focus on the immune and metabolic role of the vasoactive intestinal peptide (VIP) expressed by decidual and trophoblast cells. We discuss the most recent research on decidual, trophoblast and immune cell interaction on the light of VIP regulation. Its role in decidualization and UPR associated with a sterile inflammatory response and angiogenesis is discussed. Evidence on VIP modulation of cytotrophoblast cell function, metabolism and immune profile is revised as well as the shaping of decidual leukocyte phenotype and function from decidualization to term.

Key words decidualization, cytotrophoblast metabolism, maternal leukocytes, Vasoactive Intestinal Peptide, placental homeostasis.

Introduction

The maternal-fetal interaction is under tight immune and metabolic regulation in order to assist the energetic demands of the fetus and maintain immune homeostasis. During decidualization, endometrial stromal cells undergo reticular stress and trigger unfolded protein response (UPR) enabling the expansion of their endoplasmic reticulum and the synthesis of immunomodulatory factors. At placentation, concurrent processes such as extensive tissue remodeling, leucocyte infiltration and increasing metabolic demands of placental cells challenge immune and metabolic homeostasis.

Here we overview critical aspects of embryo implantation and placental development with special focus on the role of the vasoactive intestinal peptide (VIP), proposed as an immune and metabolic regulatory factor at the maternal-fetal interface. VIP is a 28 amino acid peptide that holds biochemical, metabolic and immune properties consistent with such a role in pregnancy: 1) it is synthesized by trophoblast and decidual cells; 2) it binds G protein seven-transmembrane receptors (GPCR) on trophoblast, stromal, vascular, glandular epithelial and immune cells. 3) it has immune and metabolic effects leading to anti-inflammatory responses in human and mouse models and to embryo growth in mice. Its broad spectrum of effects from implantation to term as well as its profile under pathological conditions is summarized to contribute to the identification of novel biomarkers for diagnosis and pharmacological targeting.

VIP: initially described as neurotransmitter emerges as an immune and metabolic regulatory peptide

VIP was first isolated by Sami Said and Viktor Mutt as a vasodilating polypeptide from porcine intestinal tissue and identified as a central and autonomic neurotransmitter [1–3]. Vasodilating effects of VIP were demonstrated in several tissues and particularly in the female genitourinary tract of different species where it increases myometrial blood flow and inhibits uterine muscle contractility [4]. In the central nervous system, VIP is critical for circadian rhythms synchrony as reported in mice [5,6].

Besides its nervous effects, VIP displays activity as a growth and metabolic regulatory factor in various settings. It supports neuronal survival with neuroprotective effects [3]. A notable embryotrophic and neurotrophic effect was demonstrated in mice [7]. Both VIP effects on tumor growth and evidence from human cancer are providing new clues for pharmacological targeting [8]. VIP modulates bone metabolism and remodeling [9] and stimulates bone marrow-mesenchymal stem cells osteogenesis differentiation [10]. Finally, VIP has potent anti-inflammatory and immunosuppressive effects in chronic and acute inflammatory disease models [11] and evidence accumulates on its role at immune homeostasis maintenance during pregnancy [12].

Most of the well-known effects of VIP on neuronal, vascular, intestinal, glandular and immune cells are mediated by high affinity receptors VPAC1 and VPAC2 (nomenclature as agreed by the NC-IUPHAR Subcommittee on Vasoactive Intestinal Peptide Receptors) and to a lesser extent by low affinity PAC1 receptors [2,13,14]. VIP binding to VPAC1 and VPAC2 receptors activates adenylyl cyclase and PKA-cAMP response element (CRE) signaling cascades, PI3K and ERK-mediated signals crosstalk and phospholipase C in some cells [11,13,15,16]. VPAC1 and VPAC2, as members of the large GPCR family, are subjected to desensitization upon sustained stimulation with VIP [13]. This is a noteworthy point when considering the effect of VIP as a growth factor at the maternal-placental interface. In fact, unlike the classical growth factors acting on protein kinase-linked family receptors, VIP displays a limited pulse-regulated response in, for example, trophoblast cells [17,18]. This uniqueness would enable the fine-tuning of highly regulated growth processes at the maternal-fetal interface.

Detection of VIP levels in human plasma [19] emerged as a valuable diagnostic and prognostic tool in rheumatic diseases [11,20,21], affective disorders [22,23] and to monitor neuroendocrine tumors [24]. In pregnant women, the concentration of VIP in peripheral blood is about half its concentration in median arterial and venous umbilical cord blood whereas it is higher in plasma at labor than during pregnancy [25].

VIP as one of the earliest inducers of the decidualization process

Embryo implantation requires a sequence of coordinated events between the competent blastocyst, the endometrial epithelial and stromal cells and the maternal immune system [26–28]. The endometrium undergoes a differentiation program that enables blastocyst attachment and invasion. Endometrial stromal cells differentiate into epithelioid decidual cells with secreted immunoregulatory molecules that support the nidation of a semiallogenic fetus [29–31]. To study decidualization and factors that underpin the transient endometrial receptive phenotype in humans is for ethical reasons inaccessible *in vivo.* Therefore, the understanding of early pregnancy events is based on knockout mice, gene expression studies in the human endometrium and models with *in vitro* designs.

The decidualization process depends on elevated and sustained intracellular cAMP levels in stromal cells [32,33]. Considering that it occurs around 10 days after ovulation in humans and depends on elevated cAMP levels, progesterone (P4) seems not to be the first stimulus to trigger this process. On the other hand, VIP regulates the steroidogenic activity of granulosa cell cultures by the stimulation of E2 and P4 production [34–36]. It decreases the activity of 20αHSD reducing P4 metabolism to inactive 20α-OH-progesterone thus sustaining P4 biological activity [34].

On this basis, we explored VIP contribution to the decidualization program in an *in vitro* model of decidualization with human endometrial stromal cells (HESC cell line). Endogenous VIP production increased from day 2 of differentiation supporting its role among the earliest mediators [37]. Regarding VIP receptors during decidualization in the HESC cell line, VPAC1 expression was not significantly modulated and VPAC2 was undetectable under these conditions. VIP triggered cAMP signaling and induced the expression of decidualization markers and chemokines whereas cells decidualized with VIP favored embryo implantation *in vitro* supporting its functional role [37,38]. Since decidualization depends on continuous P4 signaling and VIP induces P4 in trophoblast and stromal cells [18,39,40] it might also contribute to tissue integrity throughout this process.

VIP: an immunoregulatory factor during the decidualization process

Physiological inflammation associated with embryo implantation begins during the decidualization program indicating an active participation of decidualized cells [41–43]. In humans decidualization occurs in each menstrual cycle and does not require the presence of a blastocyst. In mice decidualization is triggered by the blastocysts [44]. Epithelial cells transduce signals from the attached trophectoderm to initiate a wave of cell differentiation that depends on gene activation downstream

the progesterone receptor [45,46]. It is proposed that cyclical decidualization with physiological inflammation conditions endometrium receptivity [47,48].

Physiological inflammation is controlled by immune cell subpopulations selectively recruited and/or expanded at early stages of gestation. Regulatory T lymphocytes (Tregs) and tolerogenic dendritic cells (DC) induction sustain uterus homeostasis [49,50]. Treg induction and recruitment to the uterus was demonstrated in murine and human cell designs [51–55]. Tregs stand for different subpopulations and clusters: natural Treg (CD4+CD25+FoxP3+) and Treg are induced from CD25- precursors in peripheral tissue and uterine draining lymph nodes by tolerogenic DC. Diverse clusters of Tregs-like cells found throughout pregnancy [56] include FOXP3- Tregs with a regulatory phenotype such as PD1highIL-10+ (Tr1-like cells), TIGIT+FOXP3dim [57] and CD4+HLA-G+ T cells identified in decidua [58,59]. Limited data are available on the mechanisms and the potential role of VIP at Treg induction or recruitment. We demonstrated that endogenous VIP production contributes to Tregs recruitment through a mechanism dependent on the β-chemokine RANTES (CCL5) [60].

Tolerogenic DC represent a complex subpopulation of highly specialized professional antigen-presenting cells that maintain the balance between tolerance and immunity [61,62]. Specialized subsets of DCs display functional plasticity depending on the microenvironment. In this sense, cells decidualized with VIP conditioned DC to a tolerogenic profile with lower expression of co-stimulatory molecules and a higher secretion of IL-10 compared to non-decidualized cells [38].

VIP at the reticular stress and unfolded protein response in decidual cells

During the decidualization program, endometrial stromal cells secrete pro-implantatory mediators [38,63]. Increased protein secretion saturates the protein folding machinery inside the endoplasmic reticulum (ER) leading to misfolded protein accumulation. In order to restore cellular homeostasis extra cargo proteins induce reticular stress (RS) which triggers the unfolded protein response (UPR) [64]. In non-stressed cells, the RS-membrane sensors, namely inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6α (ATF6α) are bound to the chaperone GRP78. When partially folded proteins accumulate, the RS triggers the dissociation of GRP78 from the sensors and then ATF6α, PERK or IRE1α activate different pathways leading the UPR cascade. It is interesting to note that ER stress and UPR are physiological processes and were linked to the induction of sterile inflammation and angiogenesis, both processes essential for the generation of the maternal-placental interface [38,65–67].

Experimental data indicate that decidualization induced by VIP increase ATF6α and favour UPR-pathway leading to inflammasome complex activation and increased IL-1β synthesis and secretion [38]. An inhibitor of the ATF6α-pathway not only prevented the production of IL-1β, but also the expansion of trophoblast cells on a monolayer of VIP decidualized cells in an *in vitro* model of implantation. The inflammasome is a multiprotein complex activated by stimuli from different sources, leading to the cleavage and secretion of the active forms of IL-1β and IL-18 [65]. This complex involves an inflammasome-sensor molecule, an adaptor protein ASC and caspase-1. In our *in vitro* model of decidualization with VIP, we observed higher expression of the NOD-like receptor (NLR) sensor molecule, NLRP3 [37].

IL-1β triggers the dissociation of actin filaments in human stromal cells [68] leading to cytoskeleton remodelling [69]. ATF6α mRNA and protein levels are highly expressed in the murine uterus at the implantation site contributing to blastocyst attachment and vascular remodeling [70]. Moreover, the relevance of ATF6α-pathway in the implantation process was confirmed by embryonic lethality in knockout mice [71]. A decreased expression of ATF6α in the placenta is associated with an incomplete attachment of the blastocyst and abnormal vascular remodelling as observed in late-onset pre-eclampsia [72].

In cells decidualized by VIP, the balance between pro- and anti-angiogenic secreted factors results in higher migration and tube formation by endothelial cells suggesting an active angiogenic process [73]. Conditioned media from VIP-decidualized cells enhanced the migration of human umbilical vein endothelial cells (HUVEC) cells and induced higher tube formation, increasing the core of the mesh and branching. Surprisingly, the pro-angiogenic effect was not associated to higher vascular endothelial growth factor A (VEGF-A) expression or secretion but to a decreased expression of the anti-angiogenic factor TSP-1 (thrombospondin type-1) by VIP-decidualized cells. Particularly, TSP-1 inhibits angiogenesis through direct effects on endothelial cell migration, proliferation, survival, and apoptosis and by antagonizing the activity of VEGF [74]. Taking together, the decidualization of stromal cells by VIP activates the ATF6α branch of UPR, and this might contribute to essential processes for embryo implantation as the generation of sterile inflammation and angiogenesis.

VIP in normal placentation

Normal placentation in humans relies on the orchestrating role of trophoblast cells that assist vascular transformation and immune homeostasis control as well as support nutrient and oxygen supply to the fetus [43,75,76]. Primarily in the first trimester, cytotrophoblast cells differentiate and

sprout out from cell columns of the anchoring villi (extravillous trophoblast cells), acquire an invasive phenotype and migrate through the decidual stroma, interact with vascular and endothelial cells and collaborate in vascular transformation [77]. In parallel, cytotrophoblast cells can syncytialize to settle a continuous layer (syncyciotrophoblast) for nutrient and waste exchange with the fetus [75].

VIP is expressed in the syncytium and in cytrophoblast cells of first- and third trimester human placenta [18,75,78,79]. In first-trimester placenta, VIP is involved in the progress of EVT invasion and vessel remodeling while it promotes an anti-inflammatory milieu [78]. VIP is highly expressed in trophoblast cells at the cell columns as well as in EVTs with invasive HLA-G+ and αVβ3 integrin+ phenotype [78]. In addition, VIP increased EVT outgrowth through a PKA-mediated pathway in 5-9 weeks human placental explants. Of note, EVTs localized to the lumen and the adventitia of arteries undergoing active transformation were positive for VIP immunostaining suggesting that VIP is involved in trophoblast-mediated vascular transformation. Besides trophoblast and vascular localization, glandular epithelial and stromal cells all express and release VIP under stimulated conditions. Regarding VIP levels in plasma, they are higher in pregnant vs. non-pregnant women and increase in cord blood and plasma at the end of pregnancy, however, due to its short half-life in serum the effects at the placenta are more probably dependent on VIP synthesized locally.

In mice, a maternal source of VIP at early stages of pregnancy is proposed. Mature VIP is expressed at implantation sites between gestation days 9 and 12 whereas its mRNA is not detected until day 12 in the embryonic structure [80,81]. In rats, VIP levels peak in serum at day 11 of pregnancy [82]. Regarding the role of VIP in circadian rhythms and gestation, VIP knockout (KO) mice exhibited a disrupted estrous cycle, longer periods between gestations and half the offspring of control females even when mated to the same males [83]. VIP appears to concentrate in invasive trophoblast giant cells at day 8.5 and it is released *ex vivo* into the supernatant of day 8.5-ectoplacental cone explants [84,85]. It induced trophic effects *in vitro* on post-implantation mouse embryos at day 9.5 if explanted with their placenta and yolk sac [86] whereas the pharmacological blockade of VPAC receptors during pregnancy reduced embryo weight gain and induced microcephaly [81,87]. In line with this, pregnancies with deficient VIP expression in trophoblast cells showed lower fetal weight at day 14.5 and 17.5 [85]. Similarly, neonates born to VIP deficient mothers presented a reduced birth weight that was restored by postnatal day 5 [88].

VIP synthesis and effects on human cytotrophoblast cells: focus on metabolic regulatory loops

VIP is expressed in the human trophoblast cell lines JEG-3, BeWo, HTR-8/SVNeo and Swan 71 [18,78,89–91]. VPAC1 and VPAC2 receptors are expressed in these cell lines [17,89,92]. Both receptors are involved in autocrine and paracrine regulation of cytotrophoblast cells HTR-8/SVNeo and Swan 71 function

through activating protein kinase A-cAMP response element (PKA-CRE) pathways and crosstalk signals [90]. VIP elicits a dose-dependent release of hormones and cytokines in trophoblast cells: Progesterone and human chorionic gonadotropin release as well as TGF-β and chemokine synthesis was reported in human trophoblast-derived cell lines upon VIP stimulation [17,18,89]. The PKA-CRE signaling pathway mediates migration and invasion [93] as well as hCG synthesis [17] in trophoblast cell stimulated by VIP.

The expression of the VIP gene is regulated by CRE and gp130 family cytokine element (CyRE) sites in its promoter in neuroblastoma cells [94,95]. Leukemia inhibitory factor (LIF) is among the most studied gp130 family cytokines in pregnancy [96,97]. VIP induced its own synthesis in Swan 71 cytotrophoblast cells and, interestingly, LIF induced the synthesis of VIP. Knocking down VIP expression in cytotrophoblast cells impaired not only VIP-elicited migration but also LIF-mediated migration pointing to VIP autocrine regulation [90].

Protein synthesis, cell migration and invasion are high energy requiring functions of trophoblast cells. Cytotrophoblast cells use glucose as a rapid available energy source early at placentation and beyond, as a support for the syncytium [98]. VIP stimulates both glucose and amino acid uptake in cytotrophoblast cell lines BeWo and Swan 71 [91] and knocking down VIP expression impaired glucose uptake in both cell lines. VIP induced the uptake of glucose through VPAC receptor-mediated phosphorylation pathways involving PI3K, ERK, PKA and the mammalian target of rapamycin (mTOR). The prominent role of PI3K compared to other protein kinases was evident in pharmacological inhibitors studies. mTOR integrates multiple hormonal, stress and energy signals involved in fetal and placental growth. A reciprocal regulation between VIP and mTOR was found in human cytotrophoblast cells: VIP induced mTOR expression and activated mTOR cascade phosphorylation. Silencing VIP expression down-regulated mTOR mRNA and protein expression. Conversely, inhibiting mTOR activity with rapamycin reduced VIP protein expression and VIP-induced phosphorylation of substrates downstream mTOR activation [91]. Ongoing studies are set to deepen into VIP/mTOR regulatory crosstalk for its potential in placental and fetal growth, with some recent results in VIP-deficient mice commented in the disease models section II below.

Trophoblast-immune interaction: VIP takes center stage

Cooperation between the mother and the placenta throughout pregnancy relies on the role of multifaceted trophoblast cells [43]. Cytotrophoblast cells integrate metabolic and immune signals and express a variety of soluble and contact factors that control the maternal local immune microenvironment and promote a tolerogenic response.

Treg cells and alternative M2 macrophages are central participants of tolerance induction in normal pregnancy [43]. Decidual macrophages (dMa) face varying, even opposite demands to sustain homeostasis and host defense against pathogens or excessive tissue injury. Efferocytosis and tissue repair in an immunosuppressive context characterizes dMa phenotype. Of note, novel approaches from the *Immunometabolism* standpoint showed differential metabolic reprogramming of human dMa in normal pregnancies and recurrent abortion [99,100]. The hypothesis of immunometabolic conditioning of maternal leukocytes by the trophoblast during placentation is attractive and needs further expansion to contribute to the Reproductive Immunology field in the future. Along with decidual NK cells (dNK), dMa have also been attributed a role in angiogenesis and spiral arteries transformation through the release of vasoactive factors such as VEGF, CXCL8 and angiopoietins [75,101,102]. A cooperation between dMa and dNK cells in placental vascular transformation that involves factors released by trophoblast cells has been proposed [103].

Regarding VIP, it induces the synthesis of leukocyte chemoattractant cytokines, namely CXCL8, CXCL10, CCL2, CCL3 and CCL5 [104], TGF-β1 [89] and IL-10 [78] in cytotrophoblast cells. VIP added to co-cultures of human trophoblast Swan 71 cells with human peripheral blood mononuclear cells (PBMCs) boosted a tolerogenic response *in vitro*. The effect was partly mediated by functional immunosuppressive Treg cell induction that involves TGF-β synthesized by trophoblast cells [89]. In normal mouse pregnancy, VIP favored Treg cell recruitment to the implantation sites and deficiency of VIP in trophoblast cells at the ectoplacental cones reduced Treg cell recruitment and Treg marker expression [85]. In parallel, Swan 71 and HTR8/SVNeo trophoblast cell lines primed with VIP favored the acquisition of anti-inflammatory and M2 alternative activation profiles in monocytes and macrophages in co-culture assays [104]. Likewise, conditioned media of cytotrophoblast cells primed with VIP enhanced immunosuppressive efferocytosis by human monocytes through a TSP-1 and αvβ3 integrin dependent mechanism. Both effects disappeared when VIP expression was silenced in trophoblast cells [90] pointing to the role of endogenous VIP in the trophoblast-immune interaction. This assumption was confirmed by further studies with human first trimester placenta: First, VIP is detected in the conditioned media of 5-9 weeks placental trophoblast cells [78]. Second, as shown with macrophages cocultured *in vitro* with trophoblast cells, dMa isolated from first trimester placental explants respond to VIP with higher expression of anti-inflammatory and vascular transformation mediators along with enhanced tissue repairing profiles [78]. Also dMa isolated from term placenta and circulating blood monocytes at term modulate their functional profile toward enhanced efferocytosis upon VIP and decidual factors stimulation [79]. Endogenous VIP-mediated indirect pathways are also demonstrated [78,90].

Placental neutrophils are less studied than T cells and macrophages. A low abundance proangiogenic phenotype was found in the decidua basalis and close to spiral arteries [105] in the second trimester, before the influx of proinflammatory neutrophils observed at term [49]. The balance of activation and deactivation states controls neutrophil lifespan. Besides apoptosis, another cell death mechanism with release of neutrophil extracellular traps (NETs) is elicited by pathogens, proinflammatory cytokines and chemical stimuli [106]. Enhanced NET formation was proposed as a pathogenic mechanism in preeclampsia [107] where massive presence of NETs localized within the intervillous space suggesting neutrophil excessive activation at the intervillous space [108,109]. Fetal membranes especially if pretreated with LPS can directly recruit neutrophils and induce inflammatory cytokine/chemokine secretion, NET release, ROS production and degranulation [110]. Similarly, trophoblast cells pretreated with gingival crevicular fluid from pregnant women with mild periodontal disease stimulate neutrophil recruitment [111]. An inhibitory effect on neutrophil oxidant production by contact and soluble factors of trophoblast cells was demonstrated using single-cell assays [112]. Trophoblast-mediated prevention of neutrophil activation was not seen if trophoblast cells had been exposed to common periodontal pathogens as P. gingivalis or F. nucleatum in gingival fluids from pregnant women [111]. A deactivating effect of VIP and trophoblast cells was also reported on human neutrophils stimulated with phorbol-myristate-acetate (PMA). Both trophoblast conditioned media and VIP inhibited PMA)-induced NET formation and ROS production [113]. VIP and trophoblast cells favored spontaneous apoptosis of LPS-stimulated neutrophils and the proapoptotic effect of trophoblast conditioned media was blunted if trophoblast cells were VIP-deficient expression, strongly supporting direct and indirect endogenous VIP-mediated pathways. The deactivating effect of VIP on NET formation is of particular relevance for pregnancy, since isolated NETs proved to be deleterious for trophoblast function [114,115]. Furthermore, VIP and trophoblast cells induced the expression of CCL2, arginase 1 and TGF-β mRNA, and protein and mRNA expression of VEGF-α in neutrophils. This proangiogenic phenotype had a functional correlate as trophoblast-conditioned neutrophils displayed proangiogenic effect in the chorioallantoic membrane assay [114,115].

An integrative view of the loops targeted or mediated by VIP during decidualization and placentation is summarized in figures 1 and 2.

VIP and implantation failure: Lessons from patients and disease models (I)

Endometrial receptivity implies several processes occurring in each menstrual cycle which are proposed to sustain embryo implantation and to favor placentation. Defective endometrial receptivity associates with recurrent implantation failures (RIF) [116–118], recurrent pregnancy loss (RPL)

[48] and preeclampsia (PE) [119]. Since RS response and UPR are connected with the onset of a sterile inflammatory response and angiogenesis [120], their impairment might compromise the balance of the immune response. In decidual cells, the activation of IRE1 pathway increases NLRP3 expression and inflammasome activation. Caspase-1 maturation in decidualized cells increased IL-1β production and *de novo* synthesis. Interestingly, the prevention of the IRE1-pathway in decidualized cells by STF-083010 (inhibitor of IRE1α endoribonuclease function without affecting its kinase activity), decreased trophoblast invasion as evaluated in an *in vitro* model of implantation, indicating that low levels of the inflammatory response are required to sustain embryo implantation.

Regarding ATF6α and VIP axis in the endometrium, samples from RIF patients displayed a reduced expression of ATF6α, VIP and VPAC2 compared to fertile women. Taking into account that VIP is an early inducer of the decidualization program through ATF6α-pathway leading to the attachment and invasion of the blastocyst, we propose ATF6α and VIP among other molecules as a suitable set of biomarkers to be studied for endometrial receptivity.

Immunoregulatory effects of VIP on uterine homeostasis were reported in VIP deficient female mice (knockout, KO and heterozygous, HT) and FOXP3-GFP-knock-in females in estrus or pregnant after mating with WT males. VIP deficient KO and HT females are prone to immune dysregulation [121–123]. They display a molecular profile indicative of an enhanced inflammatory and hostile microenvironment in the uterus associated with a reduction in Tregs cells that might interfere with nidation and embryo implantation [124]. VIP deficiency was associated with reduced number and diameter of the uterine glands, which promote uterine receptivity and stromal cell decidualization [125]. VIP contributes to the selective recruitment of maternal Tregs to the uterus and Treg enrichment by adoptive cell transfer improves uterine receptive microenvironment allowing a successful implantation. Particularly, FOXP3-GFP cells obtained from female inguinal and mesenteric lymph nodes injected to VIP KO females of three months of age resulted in a higher pregnancy rate compared to age matched not injected KO females [124]. Moreover, after embryo implantation at d5.5 VIP-deficient mice still presented a reduction in uterine FOXP3 expression. In WT pregnancies, mice treated intraperitoneally with VIP antagonist at d3.5 showed a reduction in the expression of FOXP3, IL-10, TGFβ and VEGFc as well as in Tregs recruitment highlighting the relevance of endogenous VIP in the peri-implantation period. Taken together, insufficient Tregs in the peri-implantation period in VIP deficient females might be impairing the resolution of inflammation that accompanies trophoblast invasion and maternal vessel remodeling [126].

VIP and pregnancy complications: Lessons from patients and disease models (II)

A defective invasion capacity of trophoblast cells with absent or incomplete vascular remodeling, deficient clearance of apoptotic trophoblast and vascular cells and a pro-inflammatory local environment characterize life threatening pregnancy complications such as PE and intrauterine growth restriction [43,75]. Deficient placentation risks maternal and fetal health during gestation, associates with metabolic disease programming in adult life and increase the susceptibility to cardiovascular disease in the mother [75,127].

The profile of VIP as pro-secretory in glandular cells, vasodilator, anti-inflammatory and as a growth factor is consistent with normal placenta development. Signaling activated by VIP is under a pulse-desensitization regulation that provides a fine-tuning of metabolic and growth effects. VIP treatment of resorption-prone mouse models and VIP-deficient pregnant mice support its role in placentation and pregnancy outcome. In the nonobese diabetic (NOD) and the CBA/J × DBA/2 mice [84,85,128], treatment with VIP at day 6.5 resulted in a higher number of viable implantation sites and enhanced expression of alternatively activated macrophages and Treg markers at the implantation sites.

In line with immune and growth effects of endogenous VIP, VIP-deficient pregnancies displayed impaired placentation and reduced fetal weight [85]. Fetal weight loss was restored by a single intraperitoneal injection of VIP at day 6.5 of pregnancy. Also, a disorganized layer of trophoblast giant cells (TGC), vascular defects, disruption of the endothelium and lower expression of VEGF-A and angiopoietin 1 was observed in implantation sites of wild type females crossed to VIP knockout males (WTxKO) [85]. Accordingly, in isolated TGC the expression of metalloproteinase-9 was reduced, paralleled by lower trophoblast migration and invasiveness that was restored upon VIP treatment *ex vivo*. A decreased frequency of Treg cells and reduced IL-10 and FOXP3 expression compared to WTxWT was also stated pointing to endogenous VIP as an immune tolerogenic peptide in pregnant mice.

Regarding placental metabolism and fetal growth, VIP deficiency in either maternal or embryo/placenta compartments resulted in placental metabolic adaptations [129]. An enhanced placental glucose uptake and transport to the fetus seemed to be a compensatory mechanism triggered by reduced local levels of VIP in the implantation sites with a normal maternal background. Moreover, in VIP deficient females crossed to heterozygous males (reduced VIP levels at the maternal side but normal or deficient VIP at placental/fetal compartments) metabolic alterations persisted regardless of fetal genotype suggesting that VIP sources in the mother and feto-placental unit work together to maintain metabolic homeostasis [129].

Lastly, evidence accounts for an anti-microbial effect of VIP or VIP analogs in certain infections [130]. VIP enhanced antimicrobial peptide secretion and antimicrobial efficacy of secretions in human primary serous cells [131] and also protected colonic epithelial cells against the negative effects of

Citrobacter rodentium-induced cytokines [132]. An antiviral role of VIP in HIV-1-infected primary human macrophages was proposed [133,134]. Regarding viral infections during pregnancy and VIP, we have recently reported on Zika virus infection of cytotrophoblast cells *in vitro* [135]. Cells infected in the presence of VIP were less susceptible to ZIKA virus infection displaying lower levels of viral RNA, TLR-3 and viperin mRNA expression. Moreover, in the presence of VIP, ZIKA virus-infected trophoblast cells presented normal cell migration and glucose uptake levels [135].

In addition to the VIP KO mouse model revised here, the VPAC1 and VPAC2 receptor KO animal models are valuable tools to study the role of endogenous ligands. Certainly, VPAC1 and VPAC2 receptors bind VIP with an affinity similar to that of the pituitary adenylyl cyclase-activating polypeptide (PACAP), another secretin-family neuropeptide [14]. VPAC2 KO mice and PACAP KO mice develop a comparable immune response to autoimmune encephalitis induction, different to VPAC1 KO animals, suggesting that some anti-inflammatory actions of endogenous PACAP are mediated by VPAC2 receptors [123]. On the other hand, studies in PAC1 receptor KO mice strongly support a role of endogenous PACAP in reproductive function [136]. This effect of PACAP had been primarily demonstrated at the gonads, in fertility rates and embryo implantation in mice treated with this neuropeptide [137,138] . On this basis, it is conjecturable that endogenous PACAP would only minimally counteract the placental deficiencies observed in the VIP KO mice model.

Perspectives and potential translational impact

Fine immune and metabolic regulation of the maternal-fetal interaction from decidualization until delivery is critical for pregnancy success. The VIP axis as a candidate in a set of potential biomarkers for endometrial receptivity is supported by presented data and it is attractive; however, more functional studies are required to identify the precise role of VIP during decidualization and its potential for the clinics. Similarly, a deeper understanding of human placentation broadens the set of molecular targets and biomarkers to prevent and treat pregnancy complications. In this scenario, VIP synthesized by the trophoblast appears to assist the development of the placenta and the maintenance of a metabolic and immunological milieu for adequate fetal growth. Its translational potential is part of ongoing clinical evaluation.

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Conflict of Interest

The authors declare no conflict of interest

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Figure legends

Figure 1: VIP participation in the decidualization process. VIP is depicted as an early inducer of the decidualization process that displays immunomodulatory and pro-tolerogenic effects contributing to endometrial receptivity. VPAC: high affinity seven-transmembrane VIP receptors. UPR: unfolded protein response. Tol DC: tolerogenic dendritic cells. cAMP: cyclic adenosine monophosphate. ATF6α: activating transcription factor 6α.

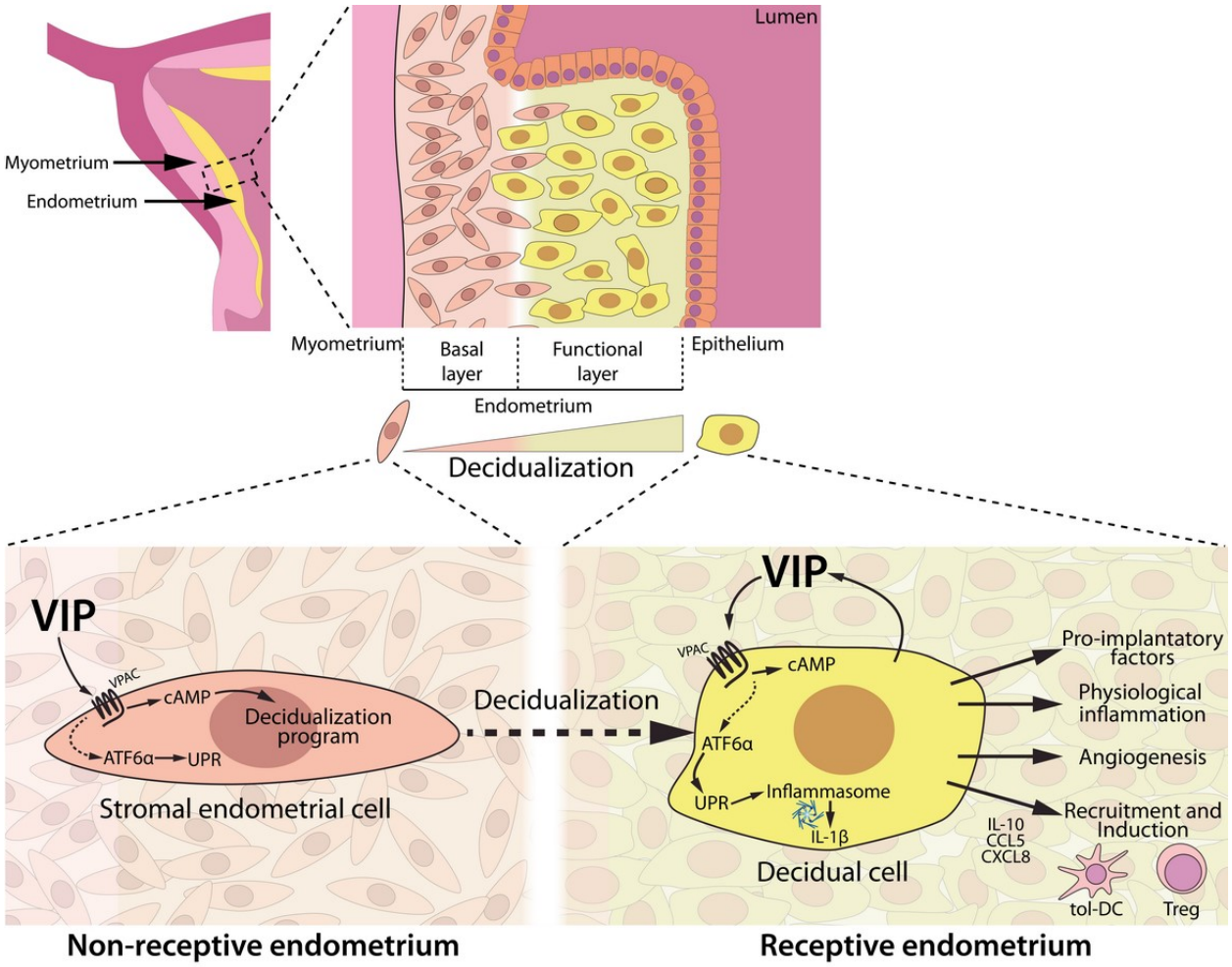

Figure 2: VIP-mediated immunoregulation during placentation. VIP is synthesized by trophoblast cells in the columns and extravillous trophoblast cells (EVT). It targets different immune cell populations present in the decidua promoting an anti-inflammatory and tolerogenic milieu along with vascular transformation signals. dMA: decidual macrophages. dNK: decidual natural killer cells. Neu: neutrophils. NETs: neutrophil extracellular traps. EVT: extravillous trophoblast. VEGF: vascular endothelial growth factor. Tol DC: tolerogenic dendritic cells.