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Characterization of the interactions of pregnancy-specific glycoprotein 1 (PSG1) with extravillous trophoblasts and endothelial cells

by
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ABSTRACT

Title of Dissertation:	Characterization of the interactions of pregnancy-specific glycoprotein 1 (PSG1) with extravillous trophoblasts and endothelial cells
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Program and year:	Molecular and Cell Biology, 2020
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Pregnancy-specific glycoproteins are secreted predominantly by the syncytiotrophoblast of the placenta. Humans have ten protein-coding *PSG* genes (*PSG1-PSG9,* and *PSG11*), and *PSG1* is the most highly expressed throughout pregnancy. PSG1 has been previously reported to have immunomodulatory and pro-angiogenic functions. While the immunoregulatory role of PSG1 has been extensively studied, a potential role of this protein in the processes of placenta development has not been investigated. Our laboratory has previously determined that PSG1 binds to extravillous trophoblasts (EVTs) and endothelial cells (ECs) and we hypothesized that PSG1 could contribute to placental invasion and angiogenesis through the interaction with these cells. To characterize the interaction of PSG1 with EVTs, I focused my studies on the functions of EVTs that facilitate placenta formation, including adhesion, migration, and decidual invasion. PSG1 has four immunoglobulin-like domains, and I generated recombinant PSG1 and its single domains for my studies. I showed that PSG1 induces a dose-dependent adhesion of two immortalized human EVT cell lines (HTR8/SVneo and

Swan71). In addition, I showed that PSG1-mediated adhesion is integrin α5β1-dependent in both normoxic and low oxygen environments as observed in the first-trimester placenta. The interaction between PSG1 and α5β1 is direct and was confirmed using PSG1 purified from the serum of pregnant women. Furthermore, I showed that both the N and B2 domains of PSG1 interact with integrin α5β1. PSG1-mediated adhesion leads to the formation of focal adhesion structures and focal adhesion kinase phosphorylation and we observed that PSG1 simultaneously binds to heparin and to α5β1. Therefore, our current model predicts that PSG1 becomes incorporated into the decidual extracellular matrix and mediates the adhesion of EVTs. I also determined that the EVT cell lines had enhanced migratory activity when seeded on a PSG1-coated surface, although no differences were observed in their invasive capacity when compared to the control. Interestingly, the serum concentration of PSG1 is lower in African-American women diagnosed with early- and late-onset pre-eclampsia (PE), a pregnancy complication characterized by inadequate invasion by the EVTs, compared to their respective healthy-controls only when the fetus was a male, suggesting a complex association of PSG1 with PE.

PSG1 was shown to induce endothelial tube formation, which was abolished by treatment with heparinase. To elucidate the mechanism behind the pro-angiogenic activity of PSG1, I investigated the interaction between PSG1 and heparan sulfate (HS). I showed that the positively charged amino acids in the 43-59 region of the B2 domain of this protein interact with HS. In addition, the pro-angiogenic activity of PSG1 on ECs and EVTs *in vitro* and in mouse aortic ring assays *ex vivo* is dependent on the B2 domain of this protein. The multistep process of angiogenesis includes cell migration, proliferation, and

remodeling of the matrix through protease production. While PSG1 enhanced the migration of ECs, it did not affect the migration of EVTs. Interestingly, PSG1 enhanced matrix metalloproteinase (MMP)-2 secretion by EVTs but not by ECs. The observed effects on migration and MMP production suggest that there is a difference in the mechanism of PSG1-mediated pro-angiogenic action on EVTs and ECs. I observed that all human PSGs bind to heparan sulfate proteoglycans despite their differences in the amino acid sequence of the B2 domains. Furthermore, PSG9 and PSG6 induce endothelial tube formation as observed with PSG1, indicating that this activity may be conserved among all human PSGs. In conclusion, I have identified integrin α5β1 as a new receptor for PSG1, which mediates increased adhesion and migration of EVTs, and determined that the B2 domain of this protein interacts with HSPGs and mediates its pro-angiogenic functions. These studies indicate that PSG1 plays a significant role in placental biology and may have therapeutic potential for the treatment of pregnancy complications in which it is found at lower than normal levels, such as in PE.

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I would like to thank my parents for their encouragement and prayers. I would like to thank my husband and son for their sacrifices throughout my graduate study. Special thanks to my husband for his continuous support that lessen my difficulties during my PhD journey.

I also want to thank my lab members, James Warren and Dr. Mirian Mendoza, helping me in my experiments.

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DEDICATION

I dedicate this work to my younger sister for being my constant mental support, my husband for his continuous support and love, my parents for being the inspiration for the graduate study, and my son, who is the source of endless joy in my life.

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LIST OF ABBREVIATIONS

Ab: antibody

APC: allophycocyanin

ARA: aortic ring assay

CEA: carcinoembryonic antigen

CEACAM: carcinoembryonic antigen cell adhesion molecule

CM: conditioned media

CTB: cytotrophoblast

dpc: days post-conception

EC: endothelial cells

ECM: extracellular matrix

EGM: endothelial growth media

ELISA: enzyme-linked immunosorbent assay

eEVT: endovascular EVT

EVT: extravillous trophoblast

FACS: fluorescence activated cell sorting

FA: focal adhesion

FAK: focal adhesion kinase

GF: growth factor

GM: glycosylation mutant

GRADSP: glycine-arginine-alanine- aspartic acid-serine-proline

GRGDNP: glycine-arginine-glycine-aspartic acid-asparagine-proline

HBSS: hanks balanced salt solution

HDMEC: human dermal microvascular endothelial cells

HEEC: human endometrial endothelial cells

HIF: hypoxia inducible factor

HS: heparan sulfate

HSPG: heparan sulfate proteoglycans

iEVT: interstitial EVT

Ig: immunoglobulin

IgC: immunoglobulin -constant

IgV: immunoglobulin -variable

IVS: intervillous space

LAP: latency-associated peptide

mAb: monoclonal Ab

MW: molecular weight

PAE-YFP: porcine aortic endothelial cells-yellow fluorescence protein

PE: pre-eclampsia

PFA: paraformaldehyde

PSGs: pregnancy-specific glycoproteins

SP1: schwangerschafts protein 1

SPR: surface plasmon resonance

STB: syncytiotrophoblast

TGFβ: transforming growth factor beta

uNK: uterine natural killer

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

VS: vasculo-syncytial

Chapter 1:
DISSERTATION INTRODUCTION

1. INTRODUCTION

1.1 Importance of studying placenta development

Every year over 7 million women are reported worldwide with pregnancy complications that are partly due to the abnormalities in placenta development and functioning, referred to as placenta-related complications (78). These complications, including pregnancy-induced hypertension, pre-eclampsia (PE), intrauterine growth restriction, and preterm delivery, not only jeopardize the pregnancy but also predispose the mother with the risk for the recurrence of such complications in future pregnancies. In addition, some pregnancy complications increase the risk of developing cardiovascular disease in the mother and their children (36). A better understanding of the placenta development process is required to gain more insights into the pathophysiology of these complications and to develop strategies for early diagnosis and effective prevention.

1.2 What is Placenta?

The placenta is a temporary organ that forms during pregnancy and performs numerous functions to support fetal development, including the exchange of oxygen, the absorbance of nutrients, the removal of wastes, and the production of hormones (16). Also, the placenta serves as a barrier to protect the fetus from the maternal immune system (21; 55). Placentas in mammals are classified into three types, based on how the fetal and the maternal blood circulation are separated, as epitheliochorial, endotheliochorial, and hemochorial (Figure 1) (8; 19). In the epitheliochorial placenta, the fetal cells, which are called trophoblasts, appose to the uterine epithelium without invading it (8; 19). In the endotheliochorial placenta, the trophoblasts invade the uterine epithelium and reach the maternal capillaries in the endometrium but also do not invade

them (8; 19). On the other hand, in the hemochorial placenta, which is observed in humans and rodents, the trophoblasts invade the uterine epithelium and the maternal capillaries within the endometrium, resulting in the direct contact of fetal cells with maternal blood (8; 19).

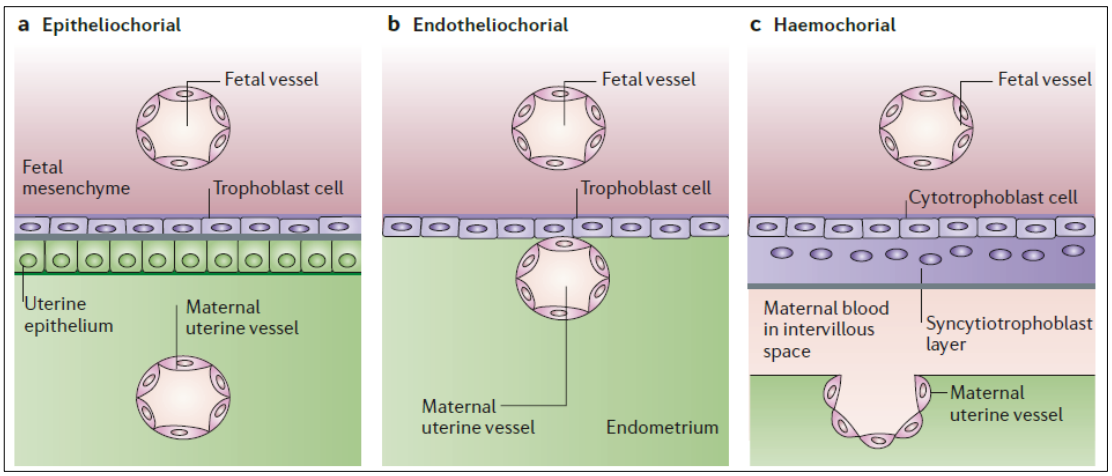


Figure 1: Three types of mammalian placentation, showing relative localization of the fetal trophoblasts and maternal blood. a. Epitheliochorial; apposition of the trophoblast cells to the epithelium surface of the uterus. b. Endotheliochorial; invasion of the uterine epithelium by trophoblasts that are in direct contact with the ECs of the maternal uterine blood vessels. c. Hemochorial; invasion of the maternal uterine blood vessels by trophoblasts causing rupture and release of maternal blood into the intervillous space of the placenta. Figure adapted from (67).

The mature placenta in humans is a discoid shaped organ with two sides. The side facing the fetus is called the chorionic plate connected to the fetus with the umbilical cord. The other side is attached to the decidua and is called the basal plate. The space between the plates is termed the intervillous space (IVS), into which there are a series of villous trees projected from the chorionic plate (Figure 2). Each villous tree is a globular lobule that contains repetitively branched villi originated from a stem villous attached to the chorionic plate. The lobule is centered over the opening of a maternal spiral artery through the basal plate. While each lobule serves as a maternal-fetal exchange unit in a

fully-formed placenta, this structure matures through sequential stages of development described below (16).

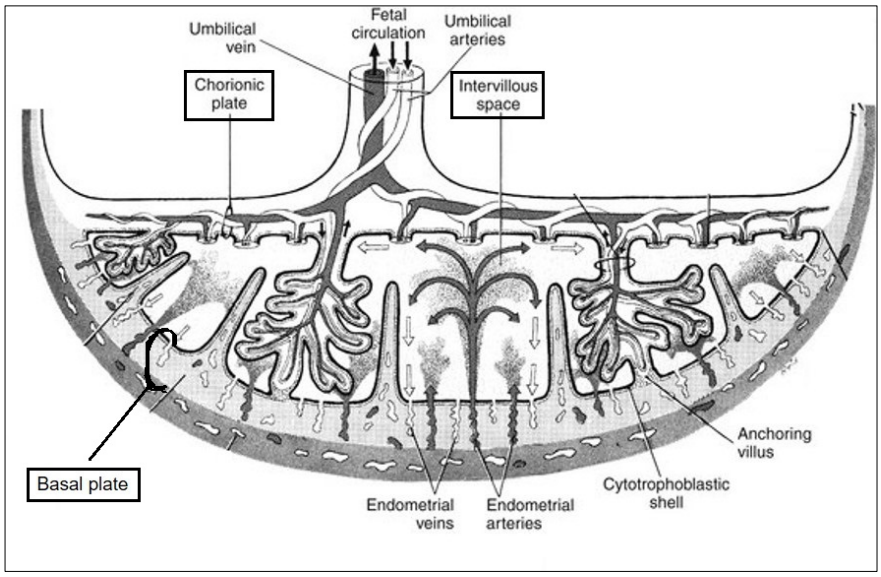


Figure 2: Diagram of a placenta showing two sides, designated as chorionic plate, and basal plate, separated with the intervillous space. Figure adapted from https://nursekey.com/fetal-and-placental-development-and-functioning/.

1.3 Stages and processes of placenta formation

1.3.1 Implantation

Placenta formation begins with the implantation of the blastocyst at around 6-7 days post-conception (dpc). At this stage, the blastocyst consists of an inner cell mass, which develops into the embryo, in a fluid-filled cavity surrounded by an outer wall, called the trophectoderm, comprising of a single layer of mononuclear trophoblast cells. Later during pregnancy, the trophectoderm gives rise to most parts of the placenta. The inner cell mass gives rise to the embryo, the umbilical cord, and the placental mesenchyme, which is derived from an extraembryonic mesoderm outgrowing from the early embryo. The trophoblasts overlying the inner cell mass are referred to as the polar

trophoblasts (Figure 3A), pointing the contact side of the blastocyst to the uterine epithelium that undergoes decidualization to support a pregnancy. Decidualization converts the endometrium into a well-vascularized receptive tissue called decidua that is temporarily formed as preparation for conception. Still it begins independently of the presence of the blastocyst. As soon as the implantation starts through the attachment of the blastocyst to the decidua, the polar trophoblasts undergo differentiation by syncytial fusion of mononuclear cells to generate the multinuclear syncytiotrophoblast (STB). The STB cells perform the initial invasion of the uterine epithelium. During the next few days, the embryo becomes embedded into the decidua with the STB forming a complete layer surrounding the conceptus (Figure 3B). The STB is the only fetal tissue at this stage that comes into direct contact with maternal cells and blood. The remaining mononuclear trophoblasts are referred to as cytotrophoblasts (CTBs) that act as the stem cells for subsequent differentiation into STB or extravillous trophoblasts (15; 44; 54).

1.3.2 Lacunar stage

The lacunar stage starts with the development of isolated small vacuoles or fluid-filled spaces, known as lacunae (Figure 3C), at around 8 dpc within the STB mass. Small lacunae gradually merge to form larger lacunae that are separated by the STB masses called trabeculae (Figure 3D). Three fundamental zones of the placenta can be defined at this stage: the primitive chorionic plate comprising of the extraembryonic mesoderm and the overlying CTBs, the lacunar system forming the IVS and the trabecular system developing later into the villous trees, and the primitive basal plate. The CTBs from the primitive chorionic plate enter the trabeculae at around 12 dpc, gradually break through the trabeculae, and extend laterally to join with the counterpart from the neighboring

trabeculae. These trabeculae start developing the villous structures of the placenta. The CTBs undergo further differentiation; they reach the endometrium by around 15 dpc, and invade the maternal tissue. The cells invading the endometrium are known as extravillous trophoblasts (EVTs) due to their location outside the villi (15; 44; 54).

1.3.3 Villous stages and the formation of different types of villi

At around 13 dpc, the CTBs proliferate within the trabeculae that begin to develop side branches known as **primary villi** (Figure 3D). These are the first villous structures in the developing villous tree and are composed of the STB entirely or of the STB with underlying CTBs. Shortly after the advent of primary villi, the extraembryonic mesodermal cells of the chorionic plate start entering into the trabeculae without penetrating the CTBs cores. These villi with the mesenchymal cores are called **secondary villi** (Figure 3E), containing columns of the CTBs in the distal part of the trabeculae and the mesodermal cells closer to the junction. These columns of CTBs are known as trophoblastic cell columns, which are the source of the EVTs (15; 44; 54).

Vasculogenesis starts within the mesenchyme of the secondary villi, giving rise to the first fetal capillaries. The villi with the vascular structures are designated as **tertiary villi** (Figure 3F). This classification of primary to tertiary villi represents how new villi generate and mature during the early gestation. In the mature placenta, the villi are seen as branches clustered in a series of lobules or villous trees that arise from individual trabeculae formed in the early stage. Each trabecula develops progressively into a thick villous stem, giving rise to the lobules by repeated branching. Although some branches in the lobules reach the basal plate, known as the anchoring villi, most ends freely float in the IVS. The free-floating villi are called the floating villi. They are divided into five

types based on their stromal characteristics, functional abilities, the type of vascular structures present, and the time of appearance during pregnancy as mesenchymal villi, stem villi, immature intermediate villi, mature intermediate villi, and terminal villi (Figure 4). Due to the presence of vascular structures in all these villi, these are technically subclasses of tertiary villi (54).

Mesenchymal villi:

Mesenchymal villi are composed of a weakly organized stromal core and covered by a CTB layer overlying with the STB layer. These villi are transitory populations of 100-250 μm in diameter, found predominantly during early pregnancy. They contain a large number of mesenchymal cells, possibly some Hofbauer cells (placental macrophages), and developing vasculature. These villi transform into primitive stem villi until the fifth-week post-conception, and they convert into immature intermediate villi later in the pregnancy. Although their number decreases after the first trimester due to their differentiation into intermediate immature villi, some mesenchymal villi persist until term (15; 44; 54).

Immature Intermediate villi:

Immature intermediate villi develop from mesenchymal villi and dominate the villous tree between weeks 8 and 22 of pregnancy. These are large, bulbous villi of a diameter between 100-400 μm, containing mesenchymal stromal cells with long processes, Hofbauer cells, smaller arterioles, venules, and capillaries. These immature villi are lined with a discontinuous CTB layer underneath the STB layer that remains continuous throughout development. These villi decrease in number after mid-gestation in parallel with the mesenchymal villi (15; 44; 54).

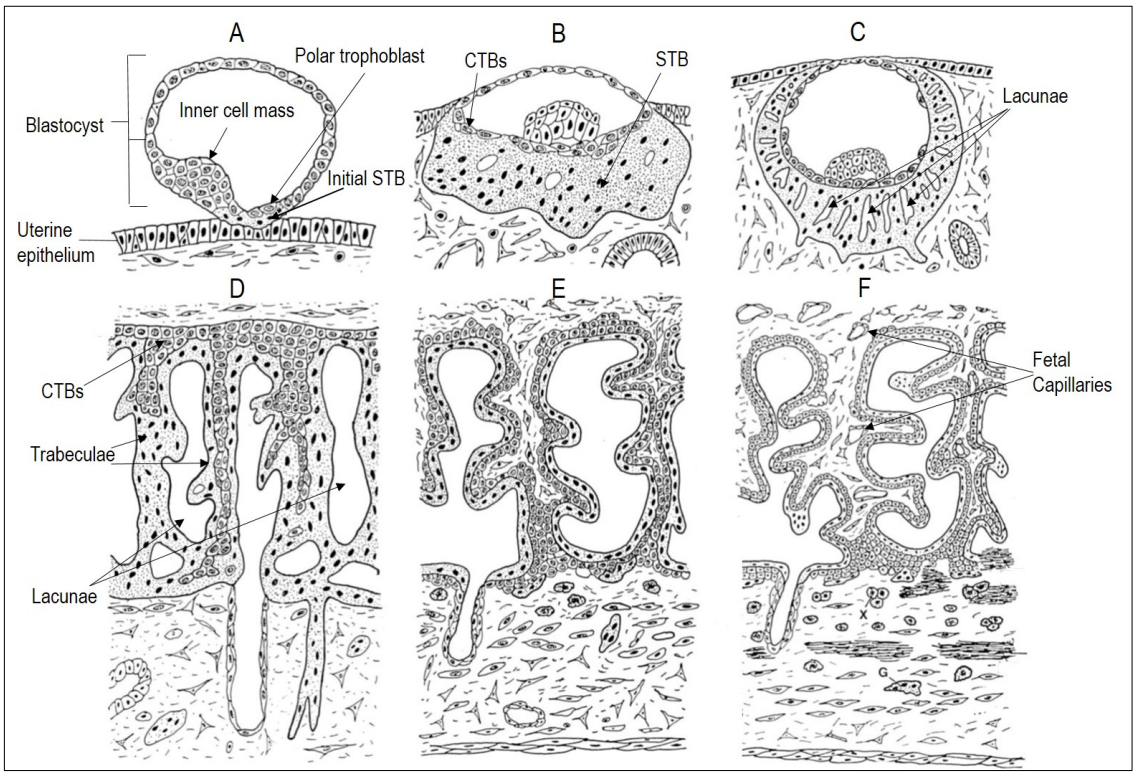


Figure 3: Early stages of placenta development. A, B. Implantation. C. Lacunar stage. D. Transition to primary villi formation. E. Secondary villi formation. F. Tertiary villi formation. CTBs = cytotrophoblast; STB = syncytiotrophoblast. Figure adapted from Burton, *et al*. (15).

Stem villi:

Stem villi differentiate from the immature intermediate villi through the deposition of fibrous stroma near the proximal end. These villi vary in diameter (100-3000 μm), contain centrally located arteries and veins in the dense fibrous stroma, consisting of collagen fibers enclosing fibroblasts, myofibroblasts and Hofbauer cells. The stroma is covered by trophoblast layers that become partially replaced with fibroids as the gestation progresses. Stem villi provide mechanical supports to the villous trees but play a minor role in placental exchange due to the rare presence of capillaries (14; 41: 51).

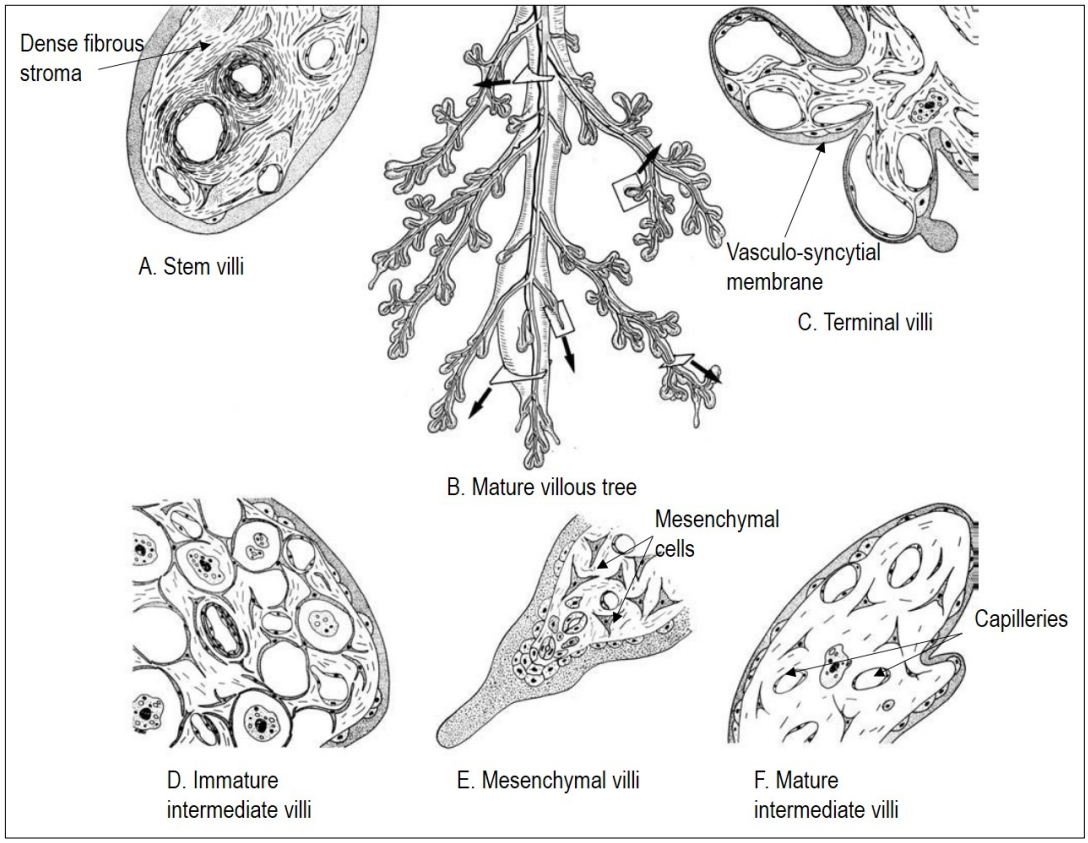


Figure 4: Diagram of the peripheral part of a mature placental villous tree and cross-section of different types of villi. A. Stem villi; B. Lobule of a mature villous tree; C. Terminal villi; D. Immature intermediate villi; E. Mesenchymal villi; F. Terminal villi. Figure adapted from Burton *et al*., 2006 (15).

Mature intermediate villi:

Mature intermediate villi are differentiated either from mesenchymal villi, up to the 24th week, or from stem villi, after the 24th week of gestation. These long slender villi of 80-120µm in diameter have loose stroma with scant fibers and cells, infrequent arterioles, and mostly capillaries (15; 44; 54).

Terminal villi:

Terminal villi, originated from the mature intermediate villi, are the final branches of the villous trees, and are characterized by a length of up to 100 µm, a diameter of

about 80 μm, a high number of capillaries containing sinusoids covered by vasculo-syncytial membranes. Having thin vasculo-syncytial (VS) membranes, consisting of the STB and the capillary endothelium separated by a joint basement membrane, makes the terminal villi very efficient in diffusional exchange. From 12 weeks onward, the VS membranes within the terminal villi become the major sites responsible for the fetal-maternal exchange of nutrients and gases (15; 44; 54).

1.3.4 Basic structure of placental villi and villous trophoblasts:

The fundamental structure of villi is a villous stroma covered with CTBs separated by a basement membrane and this arrangement is common among the various developmental stages from primary to tertiary villi (44). The differentiation pathway adapted by the CTBs in the villi is referred to as the villous pathway, through which the CTBs, the stem cell population attached to the basement membrane, proliferate and eventually fuse to the initial STB layer. The STB layer is a single continuous layer covering all villous trees and parts of the chorionic and basal plates that surround the IVS. This layer secretes pregnancy hormones and serves as an exchange station of nutrients and gases between the mother and the fetus.

1.3.5 EVT differentiation and invasion of the decidua

During the formation of villous trees, some villi become physically connected to the decidua and are known as the anchoring villi. The trophoblast differentiation pathway in the anchoring villi occurs through the formation of migratory cell columns, giving rise to EVTs that serve major invasive functions required for the establishment of the fetal-maternal interface (35). The migratory cells near the proximal cell column are proliferative; the cells near the distal cell column do not proliferate, eventually

differentiating into invasive EVTs, which further travel into the decidua and modify the maternal spiral arteries to sustain the pregnancy (35). The EVTs acquire extracellular matrix-degrading capability that permits these cells to migrate through and invade into the decidua. The EVTs invading the decidual stroma are called interstitial EVTs (iEVTs), and the EVTs invading the uterine spiral arteries are known as endovascular EVTs (eEVTs) (89). During the first trimester, plugs of the eEVTs block the opening of spiral arteries. At the end of the first trimester (week 10-12), the plugs disappear, and the eEVTs incorporate into the arterial walls by replacing endothelial cells (89). The low oxygen environment of the first-trimester placenta is one of the key regulators of trophoblast differentiation and invasion and is mandatory for the proper development of the placenta (99). The formation of cell columns occurs at the oxygen gradient between the low oxygen placenta and the high oxygen decidua. This oxygen gradient promotes the shift of the cells from a proliferative status at the proximal cell columns to an invasion status at the distal cell columns (51).

1.3.6 Remodeling of uterine spiral arteries

Due to the high metabolic demand of the feto-placental unit, the uterine spiral arteries, which supply blood to the placenta, undergo remodeling by converting from high resistance low flow to large dilated arteries with increased flow and low resistance. The spiral arteries before remodeling are 200 μm in diameter and are characterized by multiple circumferential layers of vascular smooth muscle cells surrounding a single intact layer of endothelial cells covering the lumen (67). The initial phase of remodeling involves the disruption, loss, and eventual replacement of vascular smooth muscle layer with fibrinoid materials, the endothelial cell vacuolation, and subsequent breaking of the

endothelium, all happening under the influence of maternal endocrine control. During this phase, the uterine natural killer cells and macrophages infiltrating into the arterial wall can induce matrix degradation, apoptosis of the smooth muscle cells, and the deposition of fibrinoid material. In the subsequent phase, the iEVTs and the eEVTs are the major players (67; 86). Either the iEVT invading through the vessel wall, or the eEVTs migrating retrograde intraluminally, replace the endothelial cells in the spiral arteries. The remodeled spiral arteries become dilated and measure approximately 2 mm in diameter near the opening into the IVS and have their endothelium replaced by the EVT-containing layers (Figure 5) (86).

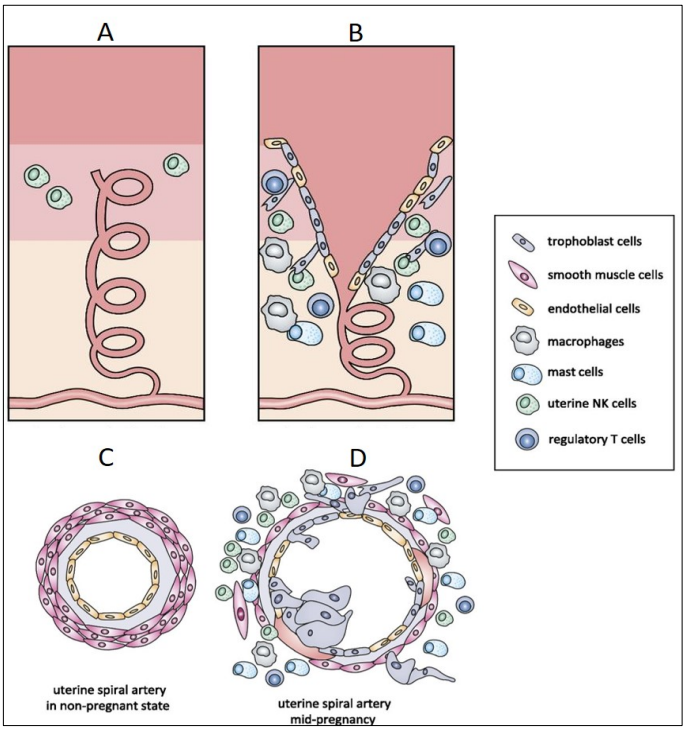


Figure 5: Uterine spiral artery remodeling. A, C. before remodeling. B, D. after remodeling. Top panels show general morphology, and bottom panels show detail cellular components. Adapted from (86).

1.3.7 Vasculogenesis and angiogenesis

Fetal-placental vascular network development occurs through two processes, namely vasculogenesis, and angiogenesis (7; 29). Vasculogenesis is the *de novo* blood vessel formation from precursor cells, whereas angiogenesis is the generation of new blood vessels from existing ones (22). Vasculogenesis involves a sequential differentiation of mesenchymal cells via hemangiogenic stem cells into angioblastic cells, the precursors of endothelial cells, and the subsequent formation of the primitive vascular structures (29; 108). In contrast, angiogenesis involves a switch of the resting endothelial cells in a preexisting blood vessel into an activated state, followed by the breakdown of extracellular matrix to facilitate endothelial cell proliferation and migration into an avascular tissue to sprout capillary structures (29; 34) Newly formed capillary structures become stabilized and matured with the recruitment of pericytes and smooth muscle cells (34). Vasculogenesis starts around 21 days post-conception (dpc) when the formation of the first vessels initiates with the differentiation of mesenchymal cells within the core of the secondary villi mesenchyme (7; 108). The CTBs overlying the mesenchyme core secrete angiogenic/vasculogenic growth factors that initiate the differentiation of the mesenchymal cells (16). Definite lumen structures in the tertiary villi are evident at about 28 dpc, and the erythrocytes within the vascular lumens are observed after 32 dpc (108). Although the development of villous capillaries through vasculogenesis continues until 10-12 weeks of gestation, angiogenesis begins around 32 dpc with the sprouting of new vessels at the distal ends of the existing capillaries. Angiogenesis continues until term to support placental vascularization via branching angiogenesis until about 24 weeks of gestation followed by, non-branching angiogenesis (22; 23; 52). Extensive expansion and adaptation of the endometrial vasculature occur through angiogenesis during

decidualization that continues throughout gestation to support the embryo and the placenta. Trophoblast cells and endothelial cells, together with decidual cells, macrophages, uterine natural killer (uNK) cells, and dendritic cells, participates in this vascular adaptation.

Numerous pro-angiogenic and anti-angiogenic factors, including the vascular endothelial growth factor (VEGF) family of proteins and their receptors, fibroblast growth factors and receptors, angiopoietins (angiopoietin-1/Ang-1 and angiopoietin-2/Ang-2) /Tie receptor system, and members of the TGF-β family, have been identified as important regulators of vasculogenesis and angiogenesis (Chen and Zheng, 2014; Wang and Zhao, 2010). Among them, the VEGF family ligands mediate their action primarily through two receptors, VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR. While VEGF binding to VEGFR-2 induces endothelial cell proliferation, binding to VEGFR-1 leads to endothelial tube formation (10). The secretion of VEGFs by the CTBs and Hofbauer cells and the presence of VEGF receptors on the hemangioblasts and angioblasts are essential for early placental vasculogenesis and subsequent angiogenesis (15; 52). Besides VEGF, the balance of placental growth factor (PlGF), a member of the VEGF family, and its inhibitor soluble Flt-1 (sFlt-1), produced by trophoblasts, is important for normal vascular development in the placenta (22; 52). Additionally, the ratio of Ang-1 and its antagonist Ang-2 and the timing of Ang-2 signaling through the Tie-2 receptor is critical for placental vascularization (52).

1.4 Integrins in placenta formation

Integrins are cell-surface adhesion molecules composed of α and β heterodimers (39). In mammals, 24 integrin heterodimers have been identified (39). Being capable of

mediating cell-cell and cell-extracellular matrix (ECM) interactions, integrins play vital roles in development, angiogenesis, metastasis, and immune functions (39). Integrin signaling can be pro-survival, pro-differentiation, proliferation- and migration-inducing (39). While integrins are devoid of intrinsic enzymatic activities, they are capable of recruiting a wide variety of intercellular signaling molecules and enzymes downstream to the sites of adhesion that lead to focal adhesion assembly (102). Integrin adhesions are stable but dynamic, and the composition of the assembly can rapidly change modifying adhesiveness and also internal signaling; by this means they can modulate cell activities, including migration and invasion (62). Given the influence of integrins in vital cellular processes, their involvement in different stages of human reproduction, such as fertilization, implantation, placentation, and embryogenesis, is widely acknowledged. Integrin signaling, including focal adhesion kinase phosphorylation, is required for the entry into the normal cell cycle post-fertilization (20). Integrins are involved in the implantation cross-talk between the endometrium and the blastocyst. The expression of numerous integrins in the uterine epithelium and the role in implantation are documented in several reviews (90; 97). Besides, spatial and temporal expression of specific integrins regulating some trophoblast activities is essential for proper placenta development. Integrin α6β4 expression at the basal surfaces of the CTBs and the STBs is important for their barrier functions. Also, the differentiation of CTBs into the EVTs, through the formation of cell columns in the anchoring villi, is accompanied by a sequential alteration of integrin expression referred to as integrin switching (27). The proliferative CTBs anchored to the basement membrane express integrin α6β4 (27). The non-polarized cell aggregates at the proximal cell-column downregulate integrin α6β4 while upregulating

integrin α5β1 expression. The trophoblasts at the distal cell-columns become non-proliferative and differentiate into invasive EVTs (55). Since acquiring a migratory phenotype is functionally related to integrin α5β1 expression, the α5β1-expressing EVTs migrate toward the uterine wall (27; 35). When these cells invade the uterine wall, they express integrin α1β1 and α5β1 (4; 27). Several members of the integrin family, including α5β1, α1β1, αVβ3, and αVβ5 are involved in modulating the adhesion, migration, and invasion of trophoblast, and in regulating trophoblast-endothelial cell interactions (40; 81). Furthermore, it has been reported that atypical integrin expression is linked to diseases of pregnancy, such as PE, and ectopic pregnancies (107).

1.5 Pregnancy-specific glycoproteins

Pregnancy-specific glycoproteins (PSGs) are a group of secreted proteins, predominantly expressed by the placenta (3; 105). PSGs are members of the carcinoembryonic antigen (CEA) family, which belongs to the immunoglobulin (Ig) superfamily (50; 105). Both PSGs and CEA cell adhesion molecules (CEACAMs) are encoded by multiple genes, and proteins of both groups contain Ig-variable (IgV)-like and Ig-constant (IgC)-like domains (25; 50; 65; 105). Although human PSGs have one IgV-like domain and two to three IgC-like domains, rodent PSGs have multiple IgV-like domains and one IgC-like domain (50). PSGs are expressed in species with hemochorial placentation including human and non-human primates, rodents, and some species of bats (49; 65). Several genes encoding PSGs are found in each of these species, for example, humans have 11 genes, bats have 42, mice 17 and rats have 8 PSG genes (49; 65). In humans, the 10 proteins are designated as PSG1-PSG9 and PSG11, and no protein is derived from *PSG10*, since it is a pseudogene (**Figure 6**) (68; 95). Splice variants have

been reported for many of these genes accounting for the potential of several PSG proteins (65; 66). Human PSGs are detected in the serum as early as 3-days post-fertilization (31). The maternal serum concentration of PSGs increases as gestation progresses, reaching its highest concentration close to term (87; 103). An investigation exploring the expression of human PSGs in a series of placental samples from two female and two male pregnancies showed that *PSG1* and *PSG3* comprised the majority of expression at the end of the first trimester, and *PSG1, PSG3, PSG4, PSG5,* and *PSG6* were nearly equally expressed with relatively lower expression of other *PSGs* at term (87). Human PSGs are detected predominantly in the STB, however, PSG expression has also been documented in tumors of trophoblastic and non-trophoblastic origin (6; 17; 18; 59; 82) . A low level of PSG expression has been reported in healthy colon and the squamous epithelium of the esophagus (42; 88). Although the role of PSGs in the gastrointestinal tract is still unidentified, PSGs may contribute to a tolerogenic response to commensal gut microbiota and food antigens and/or regulate the immune response to pathogens (42).

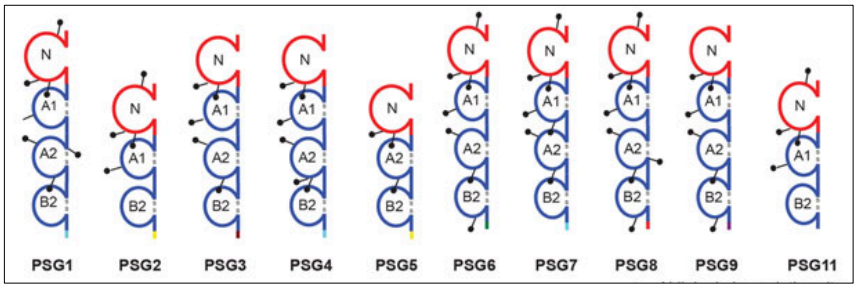


Figure 6: Domain organization of human PSGs. Ig variable-like domains are shown in red, Ig constant-like domains are shown in blue, and potential N-linked glycosylation sites are shown as black lollipops (101).

1.5.1 The role of PSGs' in pregnancy and the PSG receptors

The investigations of PSG-mediated functions have identified ligands of human PSGs, including heparan sulfate proteoglycans (HSPGs), latency associated peptide of TGFβ1, and integrin αIIbβ3 (9; 60; 87). Binding of PSGs to these ligands has been correlated to some of the proposed functions for these proteins. For example, inhibition of the PSG1-mediated pro-angiogenic function by the enzymatic removal of cell surface heparan sulfate proteoglycans (HSPGs) suggested that the pro-angiogenic activity of PSG1 is dependent on cell surface HSPGs (60). Specific binding assays showed that PSG1 does not bind to the cells lacking cell surface HSPGs expression and the binding is restored upon transfection with HSPG containing syndecans and glypicans, identifying HSPGs as a receptor for PSG1 (60). In addition to pro-angiogenic activity, PSG1 induces pro-angiogenic factors, including TGFβ1 and VEGF-A secretion by monocytes, macrophages, and EVTs, and PSG1 activates TGFβ1 by binding to the latency-associated peptide, which is bound to the activate form of the cytokine and precludes it from binding to its receptor (9; 38). Moreover, PSG1 and PSG9 were found to bind to platelet-specific integrin αIIbβ3 (84; 87). The inability of the PSG-αIIbβ3 interaction in triggering any signaling cascade suggests this interaction serves an antagonist function to the platelet-αIIbβ3 interaction (87). The presence of PSGs in high concentrations in the maternal circulation was suggested to be a mechanism to prevent thrombosis by an elevated level of fibrinogen during pregnancy (87). More than one domain of PSG1 was found to interact with αIIbβ3 and this interaction appears to be conserved in murine PSG23, indicating that despite their differences in amino acid sequence, PSGs in different species may have conserved functions (79). Besides, many studies have proposed that PSGs function as immune modulators. (37; 91). PSG1, PSG6, and PSG11 induce the secretion

of anti-inflammatory cytokines IL-6 and IL-10 by human monocytes (37; 91). IL-10 promotes a tolerogenic maternal environment required for the adaptation of the semi-allogenic fetus (91).

1.6 Central Hypothesis and Specific Aims:

Pre-eclampsia is a multi-system vascular syndrome of pregnancy defined by the gestational onset of hypertension and proteinuria that affects 2-8% of all pregnancies worldwide (96). Its exact etiology is not well understood but is currently believed to be driven by the dysregulation of a variety of molecular pathways leading to the failure of trophoblast migration and invasion, and endothelial dysfunction (15). The only available treatment for PE is the preterm delivery of the fetus. The significant morbidity and mortality associated with both preterm delivery and secondary postpartum complications necessitate a clear understanding of the processes that regulate placenta formation. Better elucidation of the molecular mechanisms regulating the key processes involved in placenta development, including trophoblast migration, invasion, and angiogenesis, could lead to novel strategies for the prevention and treatment of this pregnancy complication. Proteins secreted by trophoblasts and maternal cells, receptors on EVTs and ECs, and the components of extracellular matrices, all contribute to regulating the key processes of placenta development. While the immunoregulatory role of PSG1, the most highly expressed protein in the human PSG family based on the mRNA expression data, has been extensively investigated, a potential role of this protein in placenta development processes has not been studied (87). Therefore, the goal of my research project is to identify the roles of PSG1 in placenta formation. The central hypothesis of this study is

that **PSG1 plays a role in the regulation of EVT migratory and invasive behavior and in placental angiogenesis.**

The proposed hypothesis was addressed with the following specific aims.

Specific Aim 1: **Characterize the PSG1 integrin interaction that mediates cell adhesion**. Initially, I observed that only immobilized PSG1 induces adhesion of EVTs, and this function was blocked by the treatment of these cells with the chelating agent EDTA or the RGD peptide. This was suggestive of the involvement of an integrin molecule in the observed PSG1-mediated cell adhesion function. Therefore, I applied an array of function-blocking antibodies against integrins expressed in the EVTs to define the particular integrin that binds to PSG1. After the identification of the integrin α5β1 as the PSG1 ligand, I employed ELISA with the purified proteins to further investigate whether the interaction between them is direct.

Specific Aim 2: **Determine the functional consequences of PSG1-mediated cell adhesion**. An outcome of integrin-mediated signaling is the formation of focal adhesion (FA) structures (71). Therefore, I investigated FA structure formation in PSG1-adhered cells by examining the phosphorylation of focal adhesion kinase (FAK), a major downstream integrin-mediated signaling event. Because integrins play a vital role in cell migration and invasion, I further investigated whether PSG1 could influence EVT migration and invasion.

Specific Aim 3: **Determine the mechanism of PSG1-mediated pro-angiogenic activity.** Previously the Dveksler laboratory showed that PSG1 induces endothelial tube formation, which is dependent on cell surface heparan sulfate proteoglycan, as observed by the inhibition of PSG1-induced endothelial tube formation upon enzymatic removal of

heparan sulfate. By employing flow cytometry experiments and mutational analyses, I studied the interaction of HSPGs with individual domains of PSG1 and their mutants for the identification of the domain of this protein and the amino acids in that domain that are involved in the HSPG-interaction. In addition, individual domains and their mutants were also tested in tube formation assays. Since EVTs acquire an endothelial-like phenotype and replace endothelial lining during the remodeling of uterine spiral arteries in early gestation, as part of a collaboration, we investigated whether PSG1 is pro-angiogenic on EVTs. The effects of PSG1 on the migration and proliferation of EVTs and ECs and the expression of matrix metalloproteinase (MMP)-2 and MMP-9 by those cells were further investigated to explore the mechanism of PSG1-mediated angiogenic activity.

In summary, the experiments performed under specific aim 1 and 2 elucidate the molecular interaction of PSG1 with EVTs and describe a new functional role for this protein in placenta development. The discoveries presented in these two aims resulted in a manuscript presented in Chapter 3. The experiments performed under specific aim 2 describe the mechanism of PSG1-mediated pro-angiogenic function. The findings of the specific aim 3 have been submitted for publication and are presented in Chapter 3.

Chapter 2:
MANUSCRIPT 1

Interaction of Pregnancy-Specific Glycoprotein 1 With Integrin α5β1 is a Modulator of Extravillous Trophoblast Functions

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2.1 ABSTRACT

Human pregnancy-specific glycoproteins (PSGs) serve immunomodulatory and pro-angiogenic functions during pregnancy and are mainly expressed by syncytiotrophoblast cells. While PSG mRNA expression in extravillous trophoblasts (EVTs) was reported, the proteins were not previously detected. By immunohistochemistry and immunoblotting, we show that PSGs are expressed by invasive EVTs and co-localize with integrin α5. In addition, we determined that native and recombinant PSG1, the most highly expressed member of the family, binds to α5β1 and induces the formation of focal adhesion structures resulting in adhesion of primary EVTs and EVT-like cell lines under 21% oxygen and 1% oxygen conditions. Furthermore, we found that PSG1 can simultaneously bind to heparan sulfate in the extracellular matrix and to α5β1 on the cell membrane. Wound healing assays and single-cell movement tracking showed that immobilized PSG1 enhances EVT migration. Although PSG1 did not affect EVT invasion in the in vitro assays employed, we found that the serum PSG1 concentration is lower in African-American women diagnosed with early-onset and late-onset pre-eclampsia, a pregnancy pathology characterized by shallow trophoblast invasion, than in their respective healthy controls only when the fetus was a male; therefore, the reduced

expression of this molecule should be considered in the context of pre-eclampsia as a potential therapy.

Keywords: pregnancy-specific glycoproteins; extravillous trophoblasts; integrin α5β1; adhesion; migration; pre-eclampsia

2.2 INTRODUCTION

Pregnancy-specific glycoproteins (PSGs), also known as Schwangerschafts Protein 1 (SP1) and pregnancy-specific beta 1 glycoproteins, are secreted placental proteins found in the maternal circulation [1–4]. PSGs belong to the carcinoembryonic antigen cell adhesion molecules (CEACAM) family but while CEACAMs are mostly membrane-associated proteins, PSGs are secreted [5]. All species that express PSGs have multiple PSG-encoding genes [6]. In mice and rats, 17 genes (*Psg16–Psg32*) and eight genes (*Psg36–Psg43*) have been identified, respectively [7]. In humans, there are 11 *PSG* genes; while one of them has been reported to be a pseudogene, the expression of 10 PSGs designated as PSG1–PSG9 and PSG11 is predicted, although specific antibodies for each member of the family are not available [8]. The PSG1 messenger RNA (mRNA) is abundantly expressed throughout pregnancy and the protein concentration of PSGs reaches its maximum level in maternal plasma at term [9,10]. PSGs are found only in species with hemochorial placentation in which maternal blood comes into direct contact with fetal cells, posing a risk of rejection by the maternal immune system [11]. Interestingly, our group and others have shown that human PSGs and murine PSG23 have immune-regulatory activity consistent with the hypothesis that these proteins may participate in tolerance to the fetal semi-allograft [12–16]. In addition, we have reported that PSG1 has pro-angiogenic activity as it induces endothelial tubulogenesis [17,18].

Furthermore, human PSG1 and PSG9 and mouse PSG23 were shown to have anti-thrombotic activity [9].

PSGs are expressed predominantly, but not exclusively in trophoblasts as low levels of expression were detected in a healthy colon and in the squamous epithelium of the esophagus [19,20]. PSG expression has also been reported in tumors of trophoblastic and non-trophoblastic origin [21–23]. Similar to the presence of human PSGs in non-placental tissues, mouse PSG18 is expressed in the follicle-associated epithelium of Peyer's patches potentially playing a role in the interplay between epithelial cells and immune cells in mucosa-associated lymphoid tissue [24].

Trophoblasts are a specialized cell population in the placenta serving various functions ranging from attachment, migration and invasion to vascular remodeling [25]. In the human placenta, cytotrophoblasts (CTBs) proliferate and differentiate into spatially distinct populations [26]. In the floating villi, fusion of CTBs generates multinucleated syncytiotrophoblast (STB) [27–29]. STB produces pregnancy hormones, transport nutrients and oxygen from the mother to the fetus and remove fetal waste products [27,28]. In the anchoring villi that physically anchor the placenta to the uterine wall, differentiation of CTBs starts with the formation of trophoblast cell-columns, in which the proximal cell-column trophoblasts are highly proliferative, and the distal cell-column trophoblasts are non-proliferative, migratory and eventually differentiate into invasive extravillous trophoblasts (EVTs) [28]. EVTs migrate towards and invade into the maternal decidua to transform the uterine spiral arteries of the fetal-maternal interface [30,31]. In human pregnancy, exclusive expression of PSGs by STB was reported more than two decades ago [32]. More recent studies have indicated the presence of PSG mRNA in EVTs [33,34]. Therefore, we first

examined whether PSGs are expressed in EVTs using two PSG-specific antibodies and investigated the interaction of PSG1 with EVTs.

Some PSG1 ligands have been identified; human PSG1 and mouse PSG17, PSG22 and PSG23 bind to heparan sulfate proteoglycans (HSPGs) [17,35,36]. The interaction of PSG1 with HSPGs was shown to be required for the ability of PSG1 to induce endothelial tube formation [17]. Besides binding to HSPGs, PSG1, PSG9 and PSG23 bind to integrin αIIbβ3 and thereby inhibit fibrinogen binding to platelets [9]. As differentiation into EVTs is accompanied by a sequential alteration of integrin expression referred to as "integrin switching", which is regulated in a spatial and temporal manner, we next investigated the interaction of PSG1 with integrins. Proliferative CTBs anchored to the basement membrane express integrin α6β4 [37]. Their differentiation into EVTs near the distal cell column is accompanied by down-regulation of integrin α6β4 and up-regulation of integrin α5β1 expression, associating with acquisition of a migratory cell phenotype [31,38]. The cells that invade the uterine wall express integrin α1β1 along with α5β1 and loose α6β4 expression [37,39,40]. In this study we show via numerous functional assays that immobilized PSG1 induces adhesion of primary EVTs and EVT-like cell lines in an integrin α5β1-dependent manner and that PSG1 directly interacts with this integrin. Furthermore, EVTs seeded on PSG1 have increased migration when compared to cells seeded on a control protein as determined in wound healing assays and single-cell movement tracking experiments.

The spatial regulation of integrin expression is altered in various placental disorders involving either excessive invasion, such as in placenta accrete, or insufficient invasion, such as in pre-eclampsia (PE), suggesting that alteration of PSG levels may in part be connected to these defects [40–43]. In this study using a specific ELISA, we report reduced serum

concentrations of PSG1 in African American women diagnosed with early-onset and late-onset PE but only when they carried a male fetus. Altogether our results indicate that PSGs play important roles in several processes required for normal placentation.

2.3 MATERIALS AND METHODS

Protein Production and Purification

PSG1-Fc, PSG1-His, PSG1N-Fc and the proteins used interchangeably as negative controls, Fc and CEACAM9-Fc, were generated from the supernatant of stably transfected CHO-K1 single-cell clones established in our laboratory and grown in hollow fiber cartridge bioreactors (FiberCell Systems, Frederick, MD, USA), as previously described [44]. Plasmids encoding the complementary DNAs (cDNAs) of the single-domain proteins (PSG1A2-Fc and PSG1B2-Fc) were generated as previously reported [44]. For the generation of the PSG1A2 and PSG1B2-Fc single-domain proteins, the plasmids were transfected into ExpiCHO cells (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations and the supernatants were collected 5–6 days post-transfection based on cell viability. PSG1 from the serum of pregnant women and PSG1-His generated from the stable cell line in the bioreactor were purified using an anti-PSG1 mAb#4 column as described previously [16]. The identity of native PSG1 was confirmed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS). The anti-PSG1 mAb#4, which reacts with the N-terminal domain of PSG1, was obtained from Dr. S. Jonjic (University of Rijeka, Rijeka, Croatia). For the purification of Fc-tagged proteins, the supernatants were applied to protein A columns (GE Healthcare, Chicago, IL, USA) and the proteins were eluted with 0.1M glycine buffer pH 2.7, followed by immediate neutralization with 1 M Tris-HCl pH 8 (Thermo Fisher Scientific). The eluted fractions were

pooled, concentrated, and buffer-exchanged with phosphate-buffered saline (PBS) using an Amicon Ultra-10K centrifugal filter unit (MilliporeSigma, Burlington, MA, USA). For quantitation, the purified proteins were separated on NuPAGE 4%–12% Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) at different dilutions alongside known concentrations of bovine serum albumin (Thermo Fisher Scientific) used as standards. The proteins on the gel were stained with GelCode Blue (Thermo Fisher Scientific) and were quantified by densitometry. The identity of the proteins were confirmed by Western blot with specific Abs.

Cell Lines

The human first trimester trophoblast cell line HTR8/SVneo was provided by Dr. Caroline Dunk (Mount Sinai Hospital, Toronto, Ontario, Canada) and Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada), and was cultured in RPMI 1640 (Corning, Corning, NY, USA). The human first trimester trophoblast cell line Swan71 was provided by Dr. Gil Mor (Yale University School of Medicine, New Haven, Connecticut, USA) and CHO-K1 cells were obtained from American Type Culture Collection (Manassas, Virginia, USA), and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland). The cell line CHO-B2 (integrin α5 deficient) was a gift from Dr. Rudy L. Juliano (University of North Carolina, Chapel Hill, North Carolina, USA) and was cultured in Minimum Essential Medium (MEM) alpha modified with ribo- and deoxyribonucleosides (Thermo Fisher Scientific). All cell lines were maintained by using the media indicated above, supplemented with 10% fetal bovine serum (FBS) (Innovative Research Inc., Novi, MI, USA), 100µg/ml penicillin/streptomycin (Corning) and 100µg/ml normocin (InvivoGen, San Diego, CA, USA) in a 37 °C humidified incubator with 5% CO_2.

Placental Tissue Collection and Primary Cells Isolation

Placental and decidual tissues were obtained from women carrying a normal pregnancy after legal termination during the first- or second-trimester of pregnancy. All patients who donated a placenta provided their informed written consent prior to their inclusion in the study in accordance with the Declaration of Helsinki. Approvals on the use of human subject materials were obtained from the following institutions and their respective committees on human research: the Research Ethics Board (REB # 12-0007E) of Mount Sinai Hospital (Toronto, Ontario, Canada), the local ethics committee of the Maternity and Pediatrics Department (project code # Gyn 02-007) of the Geneva University Hospital (Switzerland) and the University of California San Francisco (California, USA)**.** Fresh tissue specimens were collected in ice-cold PBS and were washed several times in sterile Hanks balanced salt solution (HBSS). Placentas were microdissected to collect EVT columns at the tips of the villi. Approximately 30 EVT columns per placenta were digested with 1ml of an enzyme cocktail (0.125% trypsin, 100mM HEPES, 40mM $MgSO_4$, 0.15mg/ml DNase I and 100µg/ml normocin in HBSS) for 15 min in a shaking water bath at 37 °C. After neutralization with FBS, the cells were resuspended in DMEM and the suspension was filtered through 70µm mesh and counted. The cells were confirmed as >95% EVTs based on the expression of cytokeratin 8 (CK8), and human leukocyte antigen-G (HLA-G) and the absence of vimentin and then used for single-cell tracking experiments shown in Figure 8D–F. The EVTs utilized for the experiments shown in Figure 4B were purified on Percoll gradients as previously described [45].

CTBs were isolated from the chorionic villi of second-trimester placentas and purified on Percoll gradients according to previously described protocols [46,47]. After initial purification of CTBs, the remaining leukocytes were removed with anti-CD45-coupled magnetic beads. Purified CTBs were cultured in serum-free DMEM, with 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA) on a Matrigel-coated surface for 14–20h for differentiation into EVTs.

Fluorescence Immunohistochemistry for Analysis of PSGs Expression and Localization with Integrin α5 in First-Trimester Placentas

Tissues collected from the placenta at 5–8 weeks of gestation were fixed in paraformaldehyde (PFA) and processed to 5μm thick paraffin sections on Superfrost Plus glass slides (VWR, Mississauga, Ontario, Canada). Tissue sections were deparaffinized using xylene and rehydrated with a descending concentration gradient of ethanol. Heat-induced antigen retrieval with sodium citrate buffer (pH 6) was performed to avoid epitope masking. Autofluorescence was minimized with the treatment of 0.1% Sudan Black in 70% ethanol for 1 min. Blocking with a cocktail of 10% normal goat serum, 2% human serum, 2% donkey serum and 2% rabbit serum for 1h was done to prevent non-specific binding. Tissue sections were incubated overnight at 4 °C with the following primary Abs: 1:250 dilution of anti-PSG mouse mAb-BAP3 (cat#GM-0507; Aldevron, Fargo, North Dakota, USA) or anti-PSG mAb #4, which react with the B2 and N-domain of PSG1 respectively, and anti-cytokeratin 8 guinea pig polyclonal (cat# ab194130; Abcam, Cambridge, UK) at a 1:100 dilution. These Abs were applied at appropriate combination for dual immunofluorescence images shown in Figure 1; anti-PSG mouse mAb-BAP3 and anti-integrin α5 rabbit mAb (cat# ab112183; Abcam) at 1:500 dilution

were applied in combination for dual immunofluorescence images shown in Figure A1. Serial sections were incubated with anti-HLA-G (cat#11-499-c100; Exbio, Vestec, Czechia) to identify the EVT in the placental sections for Figure 1. As controls, mouse (cat#X0931, Agilient Technologies, Santa Clara, CA, USA), guinea pig (cat# NBP197036; Novus Biologicals, Centennial, CO, USA) and rabbit IgGs (cat#ab171870, Abcam) instead of specific primary Abs were applied at the same concentrations and combinations as the specific primary Abs. Secondary Abs used for Figure 1 were anti-mouse Alexa Fluor-488 (cat#A-28175; Thermo Fisher Scientific) at 1:200 dilution and anti-guinea pig biotin (cat#**106-066-003**; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at 1:1000 dilution and amplification was carried out using Streptavidin Alexa Fluor-546 (Thermo Fisher Scientific). Secondary Abs used for Figure A1 were anti-mouse Alexa Fluor-546 (at 1:300) (cat#A-11030; Thermo Fisher Scientific) in combination with anti-rabbit biotin (at 1:1000) (cat#ab6720; Abcam) and amplification was carried out using Streptavidin Alexa Fluor-488 at 1:1000 dilution (cat#S-32354; Thermo Fisher Scientific). Slides were washed in PBS and mounted in aqueous immune-mount (Thermo Fisher Scientific). Cells were counterstained with the nuclear dye DAPI (5µg/ml). Images were captured using the Quorum Wave FX spinning disc confocal system comprising a Leica DMI 6000B microscope (Leica Microsystems, Wetzlar, Germany) with a Yokogawa Spinning Head and Image EM Hamamatsu EMCCD camera and Velocity imaging software version 6.30 (Quorum Technologies Inc, Puslinch, Ontario, Canada). To ensure fair comparison across gestation, exposure time and laser intensity was kept constant across all images.

Immunoblot Analysis of PSG1 Expression in Second-Trimester Trophoblasts

Cell lysates were prepared from purified second-trimester CTBs following isolation (0h) or cultured on Matrigel for different periods of time (14 and 20h). Ten µg of cell lysate was loaded onto a 4%–12% SDS-PAGE gradient gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Anti-PSG mAb-BAP3 (Aldevron, Fargo, North Dakota, USA) and anti-PSG mAb#4 were used at 1µg/ml. The Anti-α tubulin mAb (mouse) was utilized to normalize protein loading (clone#B-5-1-2, cat#T5168; Sigma-Aldrich, St. Louis, MO). The signal was developed with horseradish peroxidase conjugated (HRP) anti-mouse secondary Abs (cat#715-0350150; Jackson ImmunoResearch Laboratories Inc.). Images were captured and analyzed with ImageJ software.

Cell Adhesion

Adhesion assays were performed in 96-well polystyrene high protein binding wells (Thermo Fisher Scientific). Wells were coated with 100µl of the purified proteins diluted in PBS at the indicated concentrations and incubated overnight at 4°C. Wells were then incubated with 300µl of 1% bovine serum albumin (BSA)/PBS for 1h at room temperature to block non-specific binding. After blocking, EVT-like cell lines, removed from tissue culture flasks with Accutase (Innovative Cell Technologies, Inc., San Diego, California, USA), were seeded in their corresponding serum-free media supplemented with 1% BSA-10mM HEPES at a final concentration of 2×10^4 cells/well and incubated for 2h at 37 °C with 5% CO_2. When purified primary EVTs were applied, 1×10^5 cells/well were seeded. After incubation, wells were washed three times with PBS to remove unbound cells and the remaining cells were incubated for 2h with CellTiter 96 AQueous One Solution (MTS; Promega, Madison, WI, USA), following the

manufacturer's recommendation, and absorbance at 490nm was recorded. When indicated, cells were pre-incubated with 500μM RGD-peptide (GRGDNP) or RGD-control peptide (GRADSP; Enzo Life Sciences, New York, NY, USA), or with echistatin (MilliporeSigma, Burlington, MA, USA) at 1ug/ml for 30 min at room temperature or with function-blocking mAbs: mouse anti-human integrin α1β1 (clone#5E8D9, cat# 05-246), mouse anti- human integrin αVβ3 (clone#LM609, cat#MAB1976) and mouse anti-human integrin αVβ5 (clone#P1F6, cat#1961Z; MilliporeSigma); rat anti-human integrin α5 mAb16 and rat anti-human integrin β1 mAb13 [48,49] at the indicated concentrations. Mouse isotype control and rat isotype control Abs were purchased from R&D Systems and BD Biosciences, respectively. To determine the effect of heparin on PSG1-mediated cell adhesion, cells were incubated with 50μg/ml heparin (MilliporeSigma), which was maintained during cell seeding. Experiments in hypoxic conditions with primary EVTs and EVT-like cell lines were performed using a hypoxic chamber in an incubator regulated to maintain 1% oxygen exposure. Hypoxic-like conditions for the EVT-like cell lines were chemically induced with 100μM $CoCl_2$ added for 4h prior and kept for the duration of the experiment.

Integrin Binding ELISA

Wells of a 96-well plate were coated overnight at 4 °C with 100μl of protein diluted in PBS at the concentrations indicated. After washing with tris buffered saline-tween (TBST), the wells were blocked with 300μL/well blocking buffer (1% BSA in TBS) for 2h at 37 °C. Recombinant human integrin α5β1 (R&D Systems, Minneapolis, MN, USA) prepared in 1% BSA in 1× TBS with 1mM $MnCl_2$ was added to the wells and incubated for 2h at 37°C. Following washes, 1μg/ml of biotin-conjugated anti-human integrin β1

Ab (cat#BAF1778, R&D Systems) was added to the wells for 2h at room temperature. The wells were washed again followed by the addition of Streptavidin-HRP and TMB substrate (R&D Systems). Reactions were stopped by addition of 2N of sulfuric acid and the absorbance at 450nm was recorded using a GloMax Discover multimode detection instrument (Promega, Madison, WI, USA). When indicated, the integrin was added to the wells together with either 5μM RGD peptide or the RGD-control peptide.

FAK Phosphorylation Analysis by Western Blot

Thirty-five mm poly-D-lysine dishes were coated with 30μg/ml PSG1-Fc or control protein overnight at 4°C. Cells maintained overnight in media with 2% FBS, were detached with Accutase and were resuspended in 1% BSA containing serum-free media prior to the addition at 3.5×10^5 cells/dish and incubated 60 min at 37°C. Dishes were placed on ice, washed with PBS and cells were lysed with 200μl LDS-sample buffer (Invitrogen, Carlsbad, CA, USA) supplemented with protease and phosphatase inhibitors (MilliporeSigma). Protein concentration was determined with the EZQ Protein Quantitation Kit (Invitrogen) and 50μg were loaded per lane and separated in a NuPAGE 3–8% tris-acetate gel (Thermo Fisher Scientific) before transferring to a PVDF membrane. The blot was probed sequentially with anti-FAK (cat#3285), anti-pFAK Y397 (clone#D20B1, cat#8556) and anti-α tubulin (clone#11H10, cat#2125; Cell Signaling Technology, Danvers, MA, USA) Abs, followed by HRP-conjugated bovine anti-rabbit IgG (cat#sc-2370, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and the West Pico Chemiluminescent Substrate (Thermo Scientific). Images were captured using Fujifilm laser imager (LAS 4000) (GE Healthcare, Chicago, IL, USA) and quantitated with the ImageJ software version 1.52r (NIH, Bethesda, MD, USA).

Focal Adhesion Structure Analysis by Fluorescence Microscopy

HTR8/SVneo cells were seeded in serum free media onto PSG1-Fc (20µg/ml) or poly-L-lysine (0.01%)-coated coverslips and allowed to adhere for 2h at 37°C. After removal of the media, cells were fixed in 4% PFA for 5 min at room temperature (RT), washed twice with TBS, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated with following primary mouse mAbs overnight at 4°C: anti-paxillin (cat#610051; BD Biosciences, San Jose, CA, USA) and anti-vinculin (cat#V9131, MilliporeSigma), followed by species-matching secondary Abs coupled to Alexa Fluor-488 (cat#A28175, Thermo Fisher Scientific) for 1h at room temperature. Co-staining was performed with Alexa-Fluor-555-Phalloidin (cat#A34055; Thermo Fisher Scientific) and mounting with Prolong (Invitrogen, Carlsbad, CA, USA) containing DAPI. Images were obtained with a Leica LSM510 inverted confocal microscope and analyzed with the Leica confocal LAS software version 3.8.0 (Leica Microsystems, Wetzlar, Germany).

PSG1-ECM Binding Assay

MRC-5 fibroblast cells were seeded at 2×10^5 cells/dish onto 60-mm dishes and grown for 7 days for extracellular matrix (ECM) deposition. After washing extensively with PBS, 1.5ml of decellularization solution (20mM NH_4OH with 0.5% Triton X-100) was applied four times to remove the cells followed by a second round of extensive washing with PBS. The deposited ECM was incubated with or without the addition of heparin (50µg/ml) for 3h at 37 °C. PSG1-Fc (10µg/ml) for 1h at room temperature. The ECM was washed with PBS for removal of unbound proteins and then lysed with 40µl 1% Triton X-100 solution supplemented with a protease inhibitor cocktail. Fifty µg of lysed ECM was loaded onto a 10% SDS-PAGE gel and transferred to a PVDF

membrane. The following Abs were used for Western blot analysis: goat anti-human Fc at 1:1000 dilution (cat#H10500, Thermo Fisher Scientific), rabbit anti-fibronectin at 1:2000 dilution (cat#F3648, MilliporeSigma), mouse anti-GAPDH and appropriate secondary Abs (1:1000; LI-COR Biosciences, Lincoln, NE, USA).

Wound-Healing Assay

Cell migration was measured in a wound-healing assay using a 2-chamber culture-insert with a middle gap (Ibidi GmbH, Munich, Germany). The culture-inserts were placed in each well of a 24-well poly-D-lysine plate previously coated overnight with PSG1-His or control protein. Then, either 6.5×10^4 HTR8/SVneo or 6×10^4 Swan71 in 80µl of serum-free media (SFM) with 1% BSA was seeded in each of the two chambers of the insert and incubated at 37°C with 5% CO_2 for 3h. Cells that did not attach to the plate were gently washed off and the inserts were removed 6–7h post-cell seeding. The wells were washed with SFM and the inserts filled with 1.5ml 1% BSA in SFM. Cell migration into the 500 µm gap was recorded by time-lapse microscopy (Leica AF6000 DMI6000B with built in camera DFC 365FX-566903911) for 20h. Images were captured every 20 min. Movies were edited using the LAS X software version 3.3.2 (Leica Microsystems, Wetzlar, Germany), analyzed with the "Wound Healing ACAS Image Analysis" (MetaVi Labs Inc., Bottrop, Germany) and expressed as gap closure area (measurement of difference between gap area at t = 0 and t = 20h) and gap closure speed (measurement of gap area covered per unit time). To mimic hypoxic conditions, cells were pre-treated for 4h with 100µM $CoCl_2$ in serum-containing media. The wound healing assays were carried out with 100µM $CoCl_2$, in SFM with B-27 Supplement (Thermo Fisher Scientific) or using a hypoxic chamber in an incubator regulated to

maintain 1% oxygen exposure. Results shown are mean ± S.D. of seven fields of four wells from one representative of three independent experiments.

Tracking of Single Cell Migration

Wells of a 24-well poly-D-lysine plate were coated with 20µg/ml PSG1-His or control protein overnight at 4 °C. Wells were blocked with 1% BSA/PBS, and HTR8/SVneo and Swan71 cells were seeded in Opti-MEM (Thermo Fisher Scientific) at a density of 5000 cells/well and allowed to adhere overnight at 37°C in a humidified 5% CO_2 incubator. For experiments with primary EVTs, a 48-well plate was used and the cells were seeded with 2% FBS containing Opti-MEM at a density of 2500 cells/well. Imaging was performed using a Zeiss Axiovert (Carl Xeiss Microscopy, Jena, Germany) 135 microscope with a temperature-controlled chamber maintained at 37°C with 5.0% CO_2. Images were captured at 10min intervals for a total of 100 frames and were analyzed with ImageJ using the plug-in MTrackJ, which in the 'Measure' option provided output as track length, velocity, and distance to previous point for the individual cells that were tracked. Results are shown as mean ± S.D. of four experiments consisting of $n = 10$ for control protein, and $n = 22$ for PSG1-His in each experiment.

HIF1α Stabilization Analysis by Western Blot

Swan71 and HTR8/SVneo cells were plated in standard growth medium at a density of 2.5×10^6 cells or 2.2×10^6 cells per 100mm dish, respectively. The following day, cells were washed and treated with 100µM $CoCl_2$ (MilliporeSigma) in 0.1% BSA containing media for 4h. Cells were lysed with 400µl ice-cold LDS sample buffer (Invitrogen) containing protease inhibitors (MilliporeSigma). The lysates were centrifuged at 15,000 rpm for 30 min and the protein concentration of the soluble

material was determined with the EZQ Protein Quantitation Kit (Invitrogen). Fifty µg of protein were loaded per lane and separated on a NuPAGE 4%–12% Bis-Tris gel (Invitrogen). The proteins were transferred to a PVDF membrane and, after blocking, incubated with mouse anti-HIF1α (clone#241809, cat#MAB1536; R&D Systems) and rabbit anti-α-tubulin (clone#11H10, cat#2125; Cell Signaling), followed by the respective HRP-conjugated secondary antibodies. The signal was developed with the SuperSignal West Pico Chemiluminescence substrate (Thermo Fisher Scientific) and visualized in a Fujifilm laser imager (LAS 4000).

Flow Cytometry

Expression of integrin α5β1 in HTR8/SVneo and Swan71 cells was studied by flow cytometry. For flow cytometry, cells were detached with Accutase, and 1×10^6 cells in 100µl of Fluorescence Activated Cell Sorting (FACS) analysis buffer (PBS with 2% BSA and 0.05% NaN3) were incubated with 1µg of anti-human α5β1 mAb (clone#HA5, cat#MAB1999; MilliporeSigma) or mouse isotype control (clone#11711, cat#MAB002; R&D systems) for 1h on ice, followed by 0.25µg allophycocyanin (APC) conjugated anti-mouse Ig (BD Biosciences) for 30min. Excess antibodies were removed by washing with the FACS buffer between steps. Cells were analyzed using the BD LSR II (BD Biosciences). A total of 50,000 events were collected for each treatment using the FACS Diva software (BD Biosciences), and the FlowJo software version 10.0.8 (BD Biosciences, San Jose, CA, USA) was used for post-acquisition analysis.

Invasion Assays

Cell invasion was examined using a transwell invasion assay and a Biogel cell invasion assay. For the transwell invasion assay, 24-well format cell culture inserts (BD

Biosciences) were used. Insert membranes were coated with Matrigel (Corning) or Geltrex (Thermo Fisher) diluted at 0.1 or 0.2mg/ml with culture media. PSG1-His or control protein at 60µg/ml was added to the diluted matrix before coating. Coating was performed by incubating at 37 °C for 2h followed by drying under laminar air flow for 5h. After rehydrating the dried matrix-coated insert for 2h with 100µl SFM, HTR8/SVneo and Swan71 cells in SFM were plated in the inserts at 8×10^4/insert and 6×10^4/inserts, respectively. Serum containing (10% FBS) media was added into the lower wells and plates were incubated at 37°C for 24h. Non-invaded cells from the upper surface of the insert were removed by scrubbing with cotton swabs and the cells on the under surface were fixed with 5% glutaraldehyde (Electron Microscopy Sciences) for 10 min, followed by staining with 2% crystal violet (Sigma Aldrich) for 5 min. For each experiment, cells in 10 randomly chosen fields of each filter were counted manually and the experiment was repeated three times. The collagen I coated, 96-well cell migration Biogel assay plates (Enzo Life Sciences) were used for cell invasion assay according to the manufacturer's instruction with some modifications. HTR8/SVneo and Swan71 cell suspensions prepared with 2% serum containing media were plated at 2.5×10^4/well and 2.2×10^4/well, respectively. Plates were incubated at 37°C for 2h in which the cell-free detection zone in the center of the wells became available after the Biogel dissolved. After gentle washes with SFM, 50µl of Geltrex at 0.1 or 0.2mg/ml mixed with 60µg/ml PSG1-His or control protein was added onto the seeded cells in the wells and incubated for 2h to allow for gel formation. Some wells were used as reference for pre-invasion conditions and fixed immediately without adding Geltrex. After adding 100µl of media with 1% FBS to each well with the gelled matrix, plates were further incubated for 24h.

After that time, cells were fixed with 5% glutaraldehyde for 10 min and stained with TRITC-phalloidin (Thermo Scientific) for 40 min followed by DAPI (Thermo Scientific) for 5 min. Images were captured using LAX AF600 software compatible with a Leica AF600 fluorescence imaging system with a built in camera (DFC 365FX-566903911). All images were analyzed using ImageJ software version 1.52r (NIH, Bethesda, MD, USA) and the number of cells invaded into the detection zones was determined by comparison to the reference wells.

Determination of PSG1 Concentration in Serum of Pregnant Women

Plasma samples were collected at the time of a prenatal visit and all patients provided written informed consent prior to sample collection. Women were enrolled as participants of a study conducted at the Center for Advanced Obstetrical Care and Research of the Perinatology Research Branch, NICHD/NIH/DHHS, the Detroit Medical Center and Wayne State University (Detroit, Michigan, USA). Women with multiple gestations, severe chronic maternal morbidity (i.e., renal insufficiency, congestive heart disease and/or chronic respiratory insufficiency), acute maternal morbidity (i.e., asthma exacerbation requiring systemic steroids and/or active hepatitis) or fetal chromosomal abnormalities and congenital anomalies were excluded from the study. PE was defined as new-onset hypertension that developed after 20 weeks of gestation (systolic or diastolic blood pressure ≥140 mm Hg and/or ≥90 mm Hg, respectively, measured on at least two occasions, 4h to 1 week apart) and proteinuria (≥300mg in a 24 h urine collection, or two random urine specimens obtained 4h to 1 week apart containing ≥1+ by dipstick or one dipstick demonstrating ≥2+ protein) [50]. Early-onset PE was defined as PE diagnosed

and delivery ≤34 weeks of gestation, and late-onset PE was defined as PE delivered and delivery ≥34 weeks of gestation [51].

We determined the concentration of PSG1 with the PSG1 Quantikine ELISA kit following the manufacturer's recommendations (R&D Systems). Serum samples were tested at 1:100 diltion for the early-onset control and early-onset PE groups (obtained at weeks 20.0–33.3 of gestation) and at 1:200 dilution for the late-onset PE and control (obtained between 37 to 41.7 weeks of gestation). For the few samples in which the values of PSG1 obtained were lower than the lowest standard, samples were diluted 1:50 or 1:13 and retested. All samples, obtained from the study population of African American women, were analyzed in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) followed by pairwise comparisons using Tukey's adjustment for multiple comparisons. Two-way ANOVA, followed by pairwise tests of simple main effects, was used for comparisons stratified by fetal gender.

2.4 RESULTS

Expression of PSGs in EVTs

While PSG mRNA expression for different members of the family has been reported in EVTs, previous studies using immunohistochemistry identified the STBs of the placenta as the sole site for PSG expression [32,33]. Therefore, we performed dual immunofluorescence staining of tissues obtained from the first-trimester placental villi and decidua basalis to examine PSGs expression employing more sensitive techniques by using two anti-PSG mAbs. EVTs were identified in the 5-week and 7-week old placentas (1A, 1F), and 6.5-week old decidua (1J) with anti-CK-8, and HLA-G antibodies (1D, 1I, 1M). Expression of PSGs was examined with either anti-PSG BAP3 (1B) or anti-PSG

mAb#4 (1G, 1K). Co-staining of 5-week and 7-week-old placentas with anti-PSG and anti-CK8 Abs shows that PSGs are localized to the EVT cell edges. Stronger staining was seen in the more distal fully invasive EVTs (Figure 1C and 1H). As shown in panel 1L, PSG expression is also observed in the EVTs actively invading the decidua and localizing to points possibly associated with focal adhesions (arrows). Only minimal background was observed with control IgGs (1E). In addition, we observed substantial co-localization of PSGs and integrin α5 in the first-trimester EVTs (Figure A1a). PSG expression was also detected in the EVTs that were differentiated in vitro from second-trimester CTBs (Figure A1b) and in second-trimester EVTs as examined by immunohistochemistry (data not shown). Altogether, these results indicate that besides the high expression by STBs, PSGs are expressed by EVTs in the cell column where they colocalize with integrin α5, and by EVTs invading the decidua. In addition, PSGs are expressed by EVTs differentiated in vitro from CTBs.

Immobilized PSG1 Induces Adhesion of EVTs by Binding to Integrin α5β1

To investigate whether PSG1 interacts with EVTs and affects their adhesive capacity, Swan71 cells were seeded onto non-tissue culture wells that had been pre-coated with either PSG1-Fc or the Fc-tag as control protein. As shown in the pictures taken 2h post-seeding, cells adhered to the PSG1-coated wells (Figure 2A, top), but not to the control protein-coated wells (Figure 2A, bottom). Similar results were obtained with the HTR8/SVneo cell line (data not shown). We also determined that this PSG1-mediated effect was observed with PSG1 with a His tag and was dose-dependent with a maximal response observed at a PSG1 concentration of 60μg/ml. (Figure 2B).

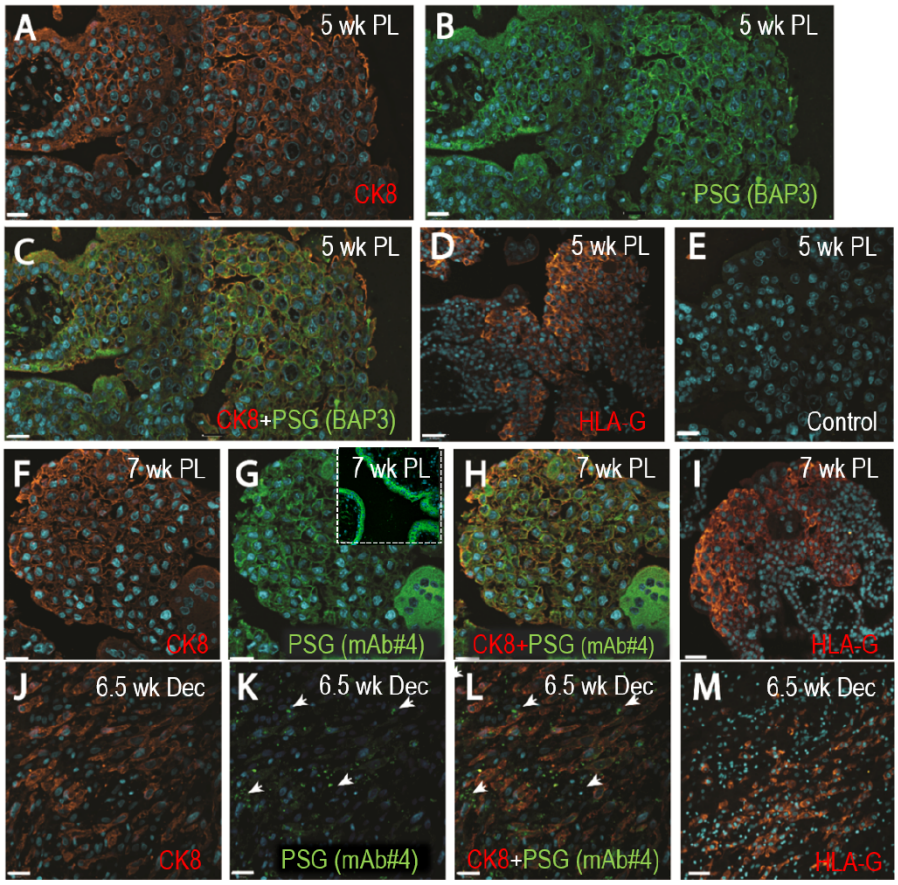


Figure 1. Pregnancy-specific glycoproteins (PSGs) localize to extravillous trophoblasts (EVTs) in first-trimester placenta and interstitial EVTs in the decidua. Representative photographs of dual fluorescent immunohistochemistry localizing cytokeratin 8 (red) and PSGs (green with both BAP3 and #4 mAbs) are shown in both individual and dual channels in sections of first-trimester placenta and decidua basalis. Serial sections stained with a mAb against HLA-G are shown in (**D**), (**I**) and (**M**) (red) to confirm localization in the EVTs. (**A**–**E**) 5-week old placenta. **A**: CK8, **B**: PSG (BAP3), **C**: CK8 and PSG (BAP3), **D**: HLA-G and **E**: guinea pig and mouse IgG control. (**F**–**I**) 7-week old placenta. **F**: CK8, **G**: PSG (mAb#4) with the insert showing the STB layer, **H**: CK8 and PSG (mAb#4) and **I**: HLA-G. (**J**–**M**) 6.5-week old decidua. **J**: CK8, **K:** PSG (mAb#4), **L:** CK8 and PSG (mAb#4) and **M:** HLA-G. Scale bars = 25 μm for (**A**–**C**), (**E**–**H**) and (**J**–**K**), and 45 μm for (**D**), (**I**) and (**M**).

The PSG1-mediated cell adhesion was inhibited when the cells were pre-treated with the chelating agent EDTA or with the disintegrin echistatin, indicating a requirement for a divalent-cation dependent receptor and a potential involvement of integrins (data not shown). To confirm the requirement of an integrin receptor for the adhesion of cells to PSG1, the cells were incubated with an RGD or control peptide prior to seeding onto the PSG1-

coated wells. As shown in Figure 2C, pre-incubation with the RGD peptide prevented adhesion of the cells to PSG1 while the control peptide did not. Studies were then undertaken to define which of the integrins on EVTs were involved in this interaction. EVTs have been reported to express several integrins including α1β1, α4β1, αVβ3, αVβ5 and α5β1 [52]. We found that anti-αVβ3, anti-αVβ5 and anti-α1β1 neutralizing Abs did not inhibit PSG1-mediated cell adhesion (Figure 2D). On the other hand, the adhesion of Swan71 (Figure 2F, left) and HTR8/SVneo (Figure A1c) cells to immobilized PSG1 were significantly reduced in the presence of neutralizing Abs to the integrin α5 and integrin β1 subunits, in ambient oxygen conditions (21%), $CoCl_2$-induced hypoxia-like conditions (Figure A1d) and under low oxygen (1%) conditions (data not shown). Hypoxia more closely reflects the conditions during the first-trimester of pregnancy and has been shown to modulate integrin expression [53–56]. Importantly, expression of α5β1 on the membrane of these cells was confirmed by flow cytometry (Figure A1e).

Given the limited availability of native PSG1 protein, we performed cell adhesion experiments with PSG1 purified from the serum of pregnant women only in 21% oxygen conditions and demonstrated that the adhesion of HTR8/SVneo cells to native PSG1 was reduced by 45% and 90% in the presence of neutralizing antibodies to integrin α5 and integrin β1, respectively (Figure 2E). To determine whether the interaction between PSG1 and α5β1 was also observed in primary cells, we performed adhesion experiments with primary EVTs maintained in 21% and 1% oxygen. Blocking of integrin α5 and integrin β1 in primary EVTs resulted in 70%–90% reduction of the adhesion mediated by PSG1 (Figure 2F, right). Moreover, we observed that PSG1 induced adhesion of CHO-K1 cells, which express integrin α5, but did not induce adhesion of CHO-B2 cells, which

lack expression of this integrin [57] (Figure 2G). Thus, these results indicate that immobilized

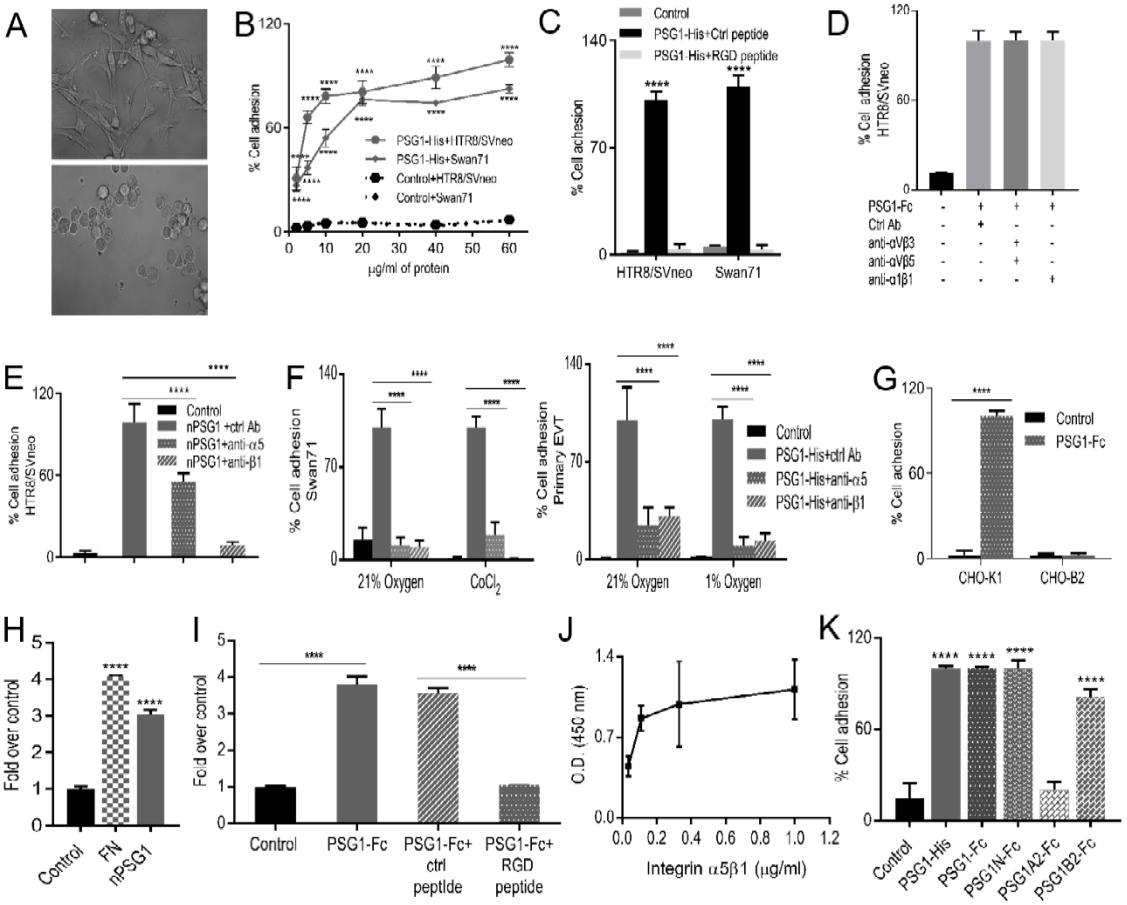


Figure 2. PSG1-mediated adhesion of the EVTs is dependent on a direct interaction with integrin α5β1. Micrographs of Swan71 cells on wells coated with 30µg/ml PSG1-Fc for 1.5 h in serum-free media (**A**, top) or a protein consisting of just the Fc-tag used as control (**B**, bottom). Images were taken at 40× magnification. (**B**) HTR8/SVneo and Swan71 cells were seeded in wells coated with PSG1-His or control protein at various concentrations. Cells were incubated for 2h at 37°C and the wells were washed to remove non-adherent cells. Cells remaining in the wells were incubated with cell titer aqueous solution and cell adhesion was quantified as described in Materials and Methods. The adhesion of Swan71 cells to wells coated with 60µg/ml PSG1-His is considered as 100%. (**C**) HTR8/SVneo and Swan71 cells, pre-incubated with 500µM RGD peptide or control (ctrl) peptide for 30 min at room temperature were seeded in wells coated with 30µg/ml PSG1-His or control protein. The adhesion of control peptide-treated cells to PSG1-His is considered as 100%. (**D**) HTR8/SVneo cells were pre-incubated with the indicated mAbs for 30 min at RT, after which they were seeded in wells coated with 30µg/ml PSG1-Fc or

control protein. (**E**) HTR8/SVneo cells were pre-incubated with the indicated mAbs for 30 min at RT and seeded on wells coated with 30μg/ml of native PSG1 (nPSG1) or control protein. (**F**) Swan71 cells and purified primary EVTs, pre-incubated with the indicated mAbs were seeded on wells coated with 30μg/ml PSG1-His or control protein and adhesion assays were performed in 21% oxygen and $CoCl_2$-induced hypoxic-like condition or in 1% oxygen conditions as described in Materials and Methods. The adhesion of control Ab-treated cells to PSG1 is considered as 100% in (**D**), (**E**) and (**F**). (**G**) CHO-K1 (integrin α5-expressing) or CHO-B2 (integrin α5-deficient) cells were seeded on wells coated with 30μg/ml PSG1-Fc or control protein. The adhesion of CHO-K1 cells to PSG1-Fc is considered as 100%. (**H**) nPSG1 or protein control at 20μg/ml or bovine fibronectin (FN) at 2μg/ml was coated on wells. After blocking, 1μg/ml of α5β1 in 1× TBS/1mM $MnCl_2$ was added and binding of the integrin was detected with biotin-labeled anti-β1 mAb followed by Streptavidin-HRP. **I.** Wells were coated with PSG1-Fc or protein control (20μg/ml). After blocking, 1μg/ml of α5β1 in 1× TBS/1 mM $MnCl_2$ was added in combination with 0.5 μM RGD or control peptides and binding of the integrin was detected as indicated in (**H**). The binding of the integrin to the control protein is considered as 1 in (**H**) and (**I**). (**J**) Wells were coated with 20μg/ml PSG1-Fc or CEACAM9-Fc. After blocking, α5β1 in 1× TBS/1 mM $MnCl_2$ was added at various concentrations and binding of the integrin was detected as described above. The graph shows values for PSG1 obtained after subtracting the average of the control values. (**K**). HTR8/SVneo cells were seeded on wells coated with the indicated proteins (20μg/ml) and adhesion experiment was carried out as described in Figure 2. Cell adhesion to PSG1-His is considered as 100%. Results shown are mean ± S.D. of triplicates from one representative of three independent experiments. *p* values were obtained by a one-way ANOVA followed by Sidak's multiple comparison tests for (**H**), (**I**) and (**K**), by a two-way ANOVA followed by Tukey's multiple comparison tests for (**B**), (**C**), (**E**) and (**F**), or followed by Sidak's multiple comparison tests for (**G**) (****$p < 0.0001$).

native and recombinant PSG1 induce cell adhesion in an integrin α5β1-dependent manner in primary and EVT-like cell lines under 21% oxygen and 1% oxygen conditions.To determine whether PSG1 interacts directly with integrin α5β1, we performed an ELISA with purified proteins. Figure 2H–J shows that binding of native and recombinant PSG1 to α5β1 is direct and dose-dependent and can be specifically blocked by the RGD-peptide. Altogether, these results indicate that PSG1 is a new ligand for integrin α5β1.

More Than One Domain of PSG1 Mediates EVT Adhesion

PSG1 is comprised of four Ig-like domains designated as N, A1, A2 and B2 and a short cytoplasmic tail of varying length depending on the splice variant [6]. The PSG1-Fc

utilized in our initial studies is a natural splice variant that lacks the A1 domain; therefore, we concluded that this domain is not essential for the ability of PSG1 to bind to α5β1 and induce cell adhesion [6]. We generated recombinant single-domain proteins with an Fc tag and tested them for their ability to induce EVT adhesion. We found that the single-domain proteins composed of either the N- or the B2- domain induced adhesion of HTR8/SVneo while the A2 domain did not (Figure 2K). Similar results were obtained with the Swan71 cell line (data not shown).

PSG1 Induces Focal Adhesion (FA) Structures and Focal Adhesion Kinase (FAK) Phosphorylation

Ligand binding to integrins initiates the recruitment of adaptor proteins and signaling molecules, followed by cytoskeleton reorganization leading to the assembly of focal adhesion (FA) structures [58]. When EVTs were seeded on PSG1-coated covered slips, we observed the assembly of FA structures as determined by the detection of the adaptor proteins vinculin and paxillin associated with F-actin in HTR8/SVneo cells (Figure 3A). In addition, HTR8/SVneo adhesion to PSG1-coated wells induced phosphorylation of the Tyr-397 of FAK, which was not observed in the poly-D-lysine-coated wells (Figure 3B). Similarly, phosphorylation of FAK at Tyr-397 was detected in Swan71 cells when adhered to PSG1-coated wells but not in the cells adhered to poly-D-lysine-coated wells (data not shown).

PSG1 Binds to HSPGs in the ECM and can Concurrently Bind to Integrin α5β1 on the Cell Surface

We observed PSG1-mediated cell adhesion only when PSG1 was immobilized on a solid surface but not when the protein was added to the media (data not shown). Previous results from our group showed that PSG1 binds to the ECM and to HSPGs [13,17]. To

confirm these results and to determine whether the interaction of PSG1 with the ECM is mediated by binding to HSPGs, we tested whether PSG1-Fc could bind to the ECM

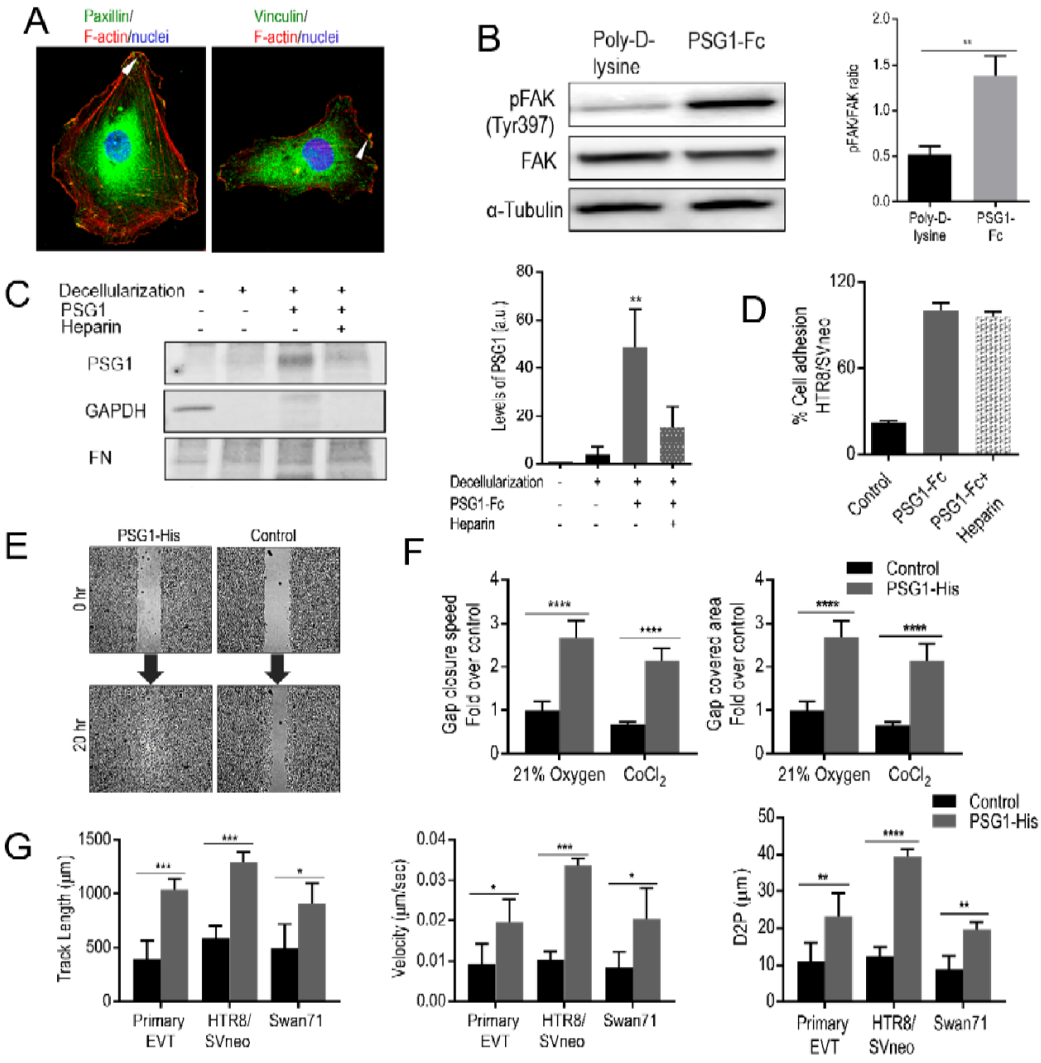


Figure 3. Binding of PSG1 leads to focal adhesion formation and PSG1 can concurrently bind to heparan sulfate proteoglycans (HSPGs) affecting EVT migration. (**A**) Representative immunofluorescence images of HTR8/SVneo cells on PSG1-Fc coated cover slips co-stained with Abs to vinculin, paxillin, and F-actin. DAPI was used to stain the nuclei. Arrowheads show the presence of paxillin (left) and vinculin (right) in the FA structures. Scale bars = 25µm. (**B**) Representative immunoblot (left) of lysates of HTR8/SVneo cells seeded on poly-D-lysine or PSG1-Fc-coated wells with the corresponding densitometric analysis (right) normalized to total FAK are shown. (**C**) Fibroblast-deposited ECM was decellularized and PSG1-Fc (10µg/ml) was added in the presence or absence of heparin for 1h at RT. After several washes, the ECM was lysed and 5µg was loaded on an SDS-PAGE gel and probed with Abs to human Fc (for PSG1-

Fc detection), FN (for ECM detection) and GAPDH (to demonstrate successful cell removal; left). Densitometric analyses of the results are presented in arbitrary units (a.u.; right). (**D**) HTR8/SVneo cells were seeded in wells coated with PSG1-Fc or protein control (30μg/ml) in the presence or absence of 50μg/ml heparin. The cell adhesion experiment was carried out as described in Figure 2. The adhesion of untreated cells to PSG1-Fc is considered as 100%. (**E**) PSG1-His or protein control were immobilized on poly-D-lysine-coated wells. Swan71 cells were seeded inside the 2-chamber inserts placed on the protein-coated wells and grown until confluent. Inserts were removed (0h) to create a cell-free gap area. Cell migration was recorded periodically for 20h and representative images are shown. (**F**) Quantitative analyses of a representative wound healing assay performed in 21% O_2 and in $CoCl_2$-induced hypoxia-like conditions with Swan71 cells seeded on PSG1-His or control protein-coated wells. Gap closure speed and gap covered area were calculated as described in Materials and Methods. The average of the replicates obtained with the cells seeded on the control protein-coated wells in 21% O_2 is considered as 1. Results shown are mean ± S.D. of triplicates in (**B**–**D**). (**G**) Quantitative analyses of migration of single cells (primary EVTs, HTR8/SVneo and Swan71) seeded on PSG1-His or control protein-coated wells measured as track length in μm (**G**, left), velocity in μm/sec (**G**, middle) and distance to previous point (D2P) in μm (**G**, right). *p* values were obtained by Student's *t*-test for **B** (**$p < 0.003$), by a one-way ANOVA followed by Sidak's multiple comparison tests for **C** (**$p < 0.002$) and by a two-way ANOVA followed by Sidak's multiple comparison tests for (**F**–**G**) (****$p < 0.0001$, ***$p < 0.0002$, **$p < 0.002$ and *$p < 0.03$).

deposited by a fibroblast cell line in the presence or absence of excess heparin, a highly sulfated form of HSPGs [59,60].MRC-5 fibroblasts were cultured for 7 days for ECM deposition after which the cells were removed as confirmed by the absence of reactivity with the anti-GAPDH Ab (Figure 3C). We observed that binding of PSG1-Fc to the ECM was inhibited in the presence of heparin (Figure 3C). We also examined whether binding of PSG1 to heparin could interfere with PSG1′s ability to induce adhesion of EVTs. We observed that the presence of added heparin did not interfere with PSG1-Fc-mediated adhesion of HTR8/SVneo cells (Figure 3D). These results strongly suggest that PSG1 can concurrently bind to HSPGs in the ECM and to integrin α5β1 on the cell surface.

PSG1 Increases EVT Migration

Integrin α5β1 expression on EVTs is required for their migratory activity [61,62]. Therefore, we explored whether PSG1 can modulate migration of EVTs. We performed

wound healing assays and found that Swan71 cells on the PSG1-His-coated wells migrated faster and covered the wound area almost completely in 20 h in comparison to the cells on the control protein-coated wells (Figure 3E). Cells on the PSG1-coated wells showed a faster gap closure speed and covered a larger area, which was 2–2.5 times over that of cells on the control protein-coated wells both in an oxygen concentration of 21% and in the presence of $CoCl_2$ (Figure 3F). We observed similar results when the wound-healing assay was performed with HTR8/SVneo cells in 21% or 1% oxygen (data not shown). The observed increase in cell migration was not the result of increased proliferation or viability induced by PSG1 as HTR8/SVneo and Swan71 did not proliferate differently or differ in viability on PSG1 when compared to poly-D-lysine coated wells as determined with the MTS assay (data not shown). We also studied cell migration at the single cell level, which allowed us to investigate the effect of PSG1 on the migration of purified first-trimester EVTs (Figure 3G). We observed higher migration of primary EVTs, HTR8/SVneo and Swan71 on the PSG1-coated wells when compared to the control protein-coated wells as determined by measurements of track length, velocity and distance from previous point (D2P) of the tracked cells (Figure 3G). The average track length obtained for primary EVTs, HTR8/SVneo and Swan71 cells on the PSG1-coated wells were 908–1292 μm, which were more than twice of that of the cells on the control protein-coated wells (394–498μm; 3G, left). The velocities of single cells on the PSG1-coated wells were 0.019–0.033μm/sec, which were also more than two times higher than the values obtained for the cells on the control protein-coated wells (0.0083–0.01μm/s; Figure 3G, middle). Similarly, D2P values of single cells on the PSG1-coated wells (19.6–39.4μm) were almost three times higher than the values

obtained for the cells on the wells coated with control protein (8.9–12.4μm; Figure 3G, right). Altogether, the results indicate that PSG1 induces increased migration of EVTs.

The PSG1 Concentration is Significantly Reduced in Pregnant Women Diagnosed with Early-Onset and Late-Onset PE

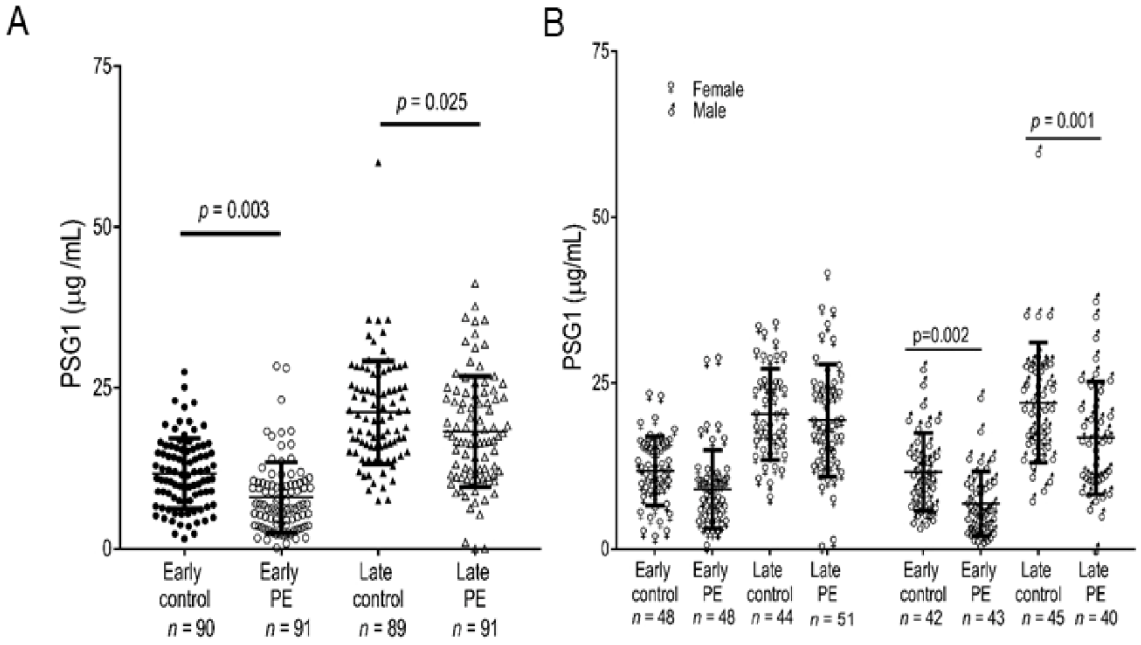


Figure 4. Serum PSG1 concentration is lower in women diagnosed with early-onset and late-onset pre-eclampsia (PE) carrying a male fetus compared to gestational age-matched controls. (**A**) The PSG1 concentration was determined in triplicate in each serum sample using the Quantikine PSG1 ELISA kit. (**B**) PSG1 concentrations shown in A were analyzed considering the gender of the baby. *p* values were obtained by one-way ANOVA followed by pairwise comparisons using Tukey's adjustment for **A** and by a two-way ANOVA followed by pairwise comparison using Holm-Sidak's adjustment for **B**.

We explored whether PSG1 can modulate the invasive capacity of HTR8/SVneo and Swan71 cells on Matrigel in transwell and Biogel cell invasion assays. We were unable to detect any significant differences in the invasive capacity of these cells in the presence of PSG1 (data not shown). Invasion of EVTs into the endometrium is an essential process in placenta formation, and shallow invasion of the decidua and incomplete transformation of the

spiral arteries by EVTs is believed to be an initiating cause of PE [28,63–66]. Although we did not observe any direct effect of PSG1 on EVT invasion, these in vitro studies have limitations [67]. Previous reports suggest that lower than normal levels of PSGs are found in some placental pathologies, including PE [68]; therefore, we investigated the PSG1 levels in PE with a validated specific PSG1 ELISA. We measured PSG1 in serum samples obtained from African American women diagnosed with early-onset and late-onset PE and the respective gestational age-matched controls with no pregnancy complications. We found that the concentration of PSG1 was significantly lower in women diagnosed with early-onset and late-onset PE compared to their respective controls (Figure 4A). The data was then analyzed taking into consideration the gender of the fetus. Interestingly, we found no statistical difference in the PSG1 concentration between early-onset or late-onset PE and the respective controls when the fetuses were female. On the other hand, when the fetuses were male, there was a significant difference for early-onset ($p = 0.002$) and late-onset ($p = 0.001$) PE and their respective gestational age-matched controls (Figure 4B).

2.5 DISCUSSION

PSGs were previously shown to be expressed exclusively by STBs by immunohistochemistry; however, some recent studies have reported expression of PSG mRNAs in EVTs [32–34,69]. Therefore, to determine whether PSGs are present in EVTs at the protein level, we utilized two specific anti-PSG mAbs that do not react with any member of the CEACAM family, previously shown to be expressed in the placenta (data not shown) [70,71]. At present, there are no available Abs to a specific PSG family member that could be employed for immunohistochemistry. Characterization of the two mAbs utilized in this study showed that mAb#4 binds to the N-terminal domain of PSG1,

PSG6, PSG7 and PSG8, and BAP3 binds to the B2 domain of PSG1, PSG3, PSG4, PSG6, PSG7 and PSG8 (data not shown). We observed positive reactivity with both anti-PSG mAbs in EVTs and STB of 5-week and 7-week-old placentas, with more intense staining in STBs as anticipated based on previously published RNA-seq data [34]. Analysis of staining in EVTs showed stronger staining in the more distal areas, suggesting increased expression of PSGs in the EVTs with an increased invasive cell phenotype. PSG expression in the EVTs invading into the decidua was mostly seen as localized puncta, possibly associated with FA structures. The discrepancy in our results from those of Zhou and co-workers, using the BAP3 mAb, is likely due to the difference in the tissue processing; as for these studies, we utilized antigen retrieval [32]. In addition, we showed co-localized expression of PSGs and integrin α5 in the first-trimester EVTs. EVTs differentiated from second-trimester CTBs also showed expression of PSGs as determined by the immunoblot analysis [46,47]. Although our study reports for the first time the presence of human PSGs in invasive EVTs, expression of mouse PSGs has been reported in trophoblast giant cells, which are similar to human EVTs, as well as in spongiotrophoblasts [35,72,73].

Here we show that immobilized PSG1 induces adhesion of two EVT-like cell lines, HTR8/SVneo and Swan71, and primary EVTs. Inhibition of PSG1-mediated adhesion by EDTA, an RGD-containing peptide, or the disintegrin echistatin pointed to the involvement of an integrin receptor [74,75]. Among the RGD-binding integrins, αVβ3, αVβ5 and α5β1 are expressed on trophoblast cells at various stages of differentiation [52]. Integrin switching from α6β4 in villous CTB, to α5β1 in cell-column trophoblasts, and to α1β1 in mature invasive EVTs has been reported in normal placental development

[37]. Using function-blocking Abs to different integrins, we determined that PSG1-mediated adhesion was dependent on integrin α5β1, a major fibronectin receptor [76]. The first-trimester placenta grows in a low oxygen or environment required for successful placenta development [77,78]. Since the differentiation of EVTs occurs most extensively in the first-trimester, the developing EVTs in the growing cell-columns of anchoring villi arise within an oxygen gradient with a shift from proliferative to non-proliferative phenotypes and changes in integrin expression, including that of integrin α5 [26]. Integrin α5β1 has been shown to be up-regulated in low oxygen conditions via HIF1α stabilization, which is only expressed in EVTs of the early placenta [28,56]. We observed PSG1-mediated adhesion of EVTs in ambient (21%) and in low (1%) oxygen conditions. In both cases, cell adhesion was dependent on the interaction of PSG1 with α5β1. The requirement for α5β1 integrin expression was further confirmed using a CHO cell line lacking integrin α5 as these cells did not attach to PSG1-coated wells while wild type cells did. Furthermore, we determined that the interaction of α5β1 and native or recombinant PSG1 is direct by ELISA using purified proteins.

An interaction of PSGs with a different integrin, platelet αIIbβ3, has been previously reported by Shanley and co-workers [9]. They observed that PSG1, PSG9 and mouse PSG23 bind to αIIbβ3 and inhibit the interaction of platelets with fibrinogen. Similar to their observation, we show that more than one domain of the protein mediates the interaction with α5β1 integrin as we observed that both the N and B2 domains of PSG1 individually induce EVT adhesion.

PSG1 induced adhesion of EVTs only when immobilized on a well; therefore, as PSG1 was previously shown to bind to HSPGs, we hypothesized that PSG1 may bind

simultaneously to the ECM and to α5β1 on the cell membrane [17]. We observed that PSG1 binds to the ECM deposited by fibroblast and that this binding can be inhibited in the presence of excess heparin. On the other hand, PSG1-induced cell adhesion was not inhibited by heparin indicating that cells can potentially bind to PSG1 anchored to the decidual ECM via its interaction with HSPGs, facilitating the attachment of EVTs to the decidua.

Clustering of ligand-engaged integrins leads to FA assembly that involves the recruitment of signaling molecules similar to FAK and cytoskeletal adaptor proteins, such as vinculin and paxillin, to the integrin cytoplasmic tails [79]. FAK, a mediator of integrin-mediated signaling pathways, has been shown to regulate anchorage-dependent behaviors such as cell proliferation, migration and anoikis suppression [79]. The observed localization of vinculin and paxillin only in the EVTs bound to PSG1 and not to poly-D-lysine is consistent with the PSG1-induced FAK phosphorylation [80]. Integrin α5β1-dependent anchorage of trophoblast to the uterine ECM and FAK signaling are well documented as important regulators of EVT migration during early placental development [31,80]. Integrin α5β1 expression in EVTs has been shown to be essential for their migration and IGF-stimulated enhancement in EVT migration is α5β1 dependent [31,61,62]. In accordance with these previous findings, we show that adhesion to PSG1 increased the migratory capacity of primary EVTs and EVT-like cell lines.

The influence of integrin α5 on EVT invasion is controversial with some groups proposing that binding to fibronectin via this integrin has invasion-restraining and others proposing an invasion-promoting effect [37,81]. In our studies using two different invasion assays, we did not observe differences in invasion between cells incubated with

PSG1 and control protein. While the interaction of α5β1 with fibronectin likely differs from the interaction of α5β1 with PSG1, the inherent limitations of the in vitro assays employed does not properly address the complexity of the in vivo EVT invasion process including the composition of the ECM and the presence of PSG1 in the matrix rather than in the solution.

Several studies conducted to investigate the correlation of serum PSG levels with various pregnancy complications reported conflicting results [68,82]. However, inherent limitations of the assays used to measure PSG concentration as well as significant inter- and intra- patient variability may explain some of these discrepancies [83–85]. Earlier studies reporting on the association between the PSG level and pregnancy complications did not take into consideration the difference in incidence of PE across different ethnicities and have generally involved small numbers of patients insufficient to produce reproducible and generalizable conclusions [86–88]. However, some recent studies identified some risk factors that are differentially associated with certain forms of PE, such as African American race for early-onset PE [89]. Recent reports have revisited the association of PE with PSG levels. A proteomic study identified some PSGs as potential predictive markers of early-onset PE [90]. Another study using global RNA profiling has reported down-regulated PSG expression in patients with severe PE [33]. Down-regulation of mRNA coding for some PSG family members and of PSG-derived peptides, had yet to be validated at the protein level [33,90,91]. We measured the concentration of PSG1, the most highly expressed member of the family based on mRNA expression data, to determine whether prior observations that PSGs are found at lower concentrations in pregnant women suffering from PE could be replicated with an ELISA using a validated

anti-PSG1 antibody pair [9]. We observed significant differences in PSG1 concentration in African American women diagnosed with early-onset and late-onset PE compared to their gestational age-matched controls. It is likely that a major fraction of PSG1 measured in maternal serum is derived from the multinucleated STB layer of the placenta. Therefore, both the STBs and EVTs can contribute to the circulating levels of PSG1 in the mother. Abnormally low levels of PSG1 in women with PE may reflect dysfunctional or stressed STBs, which may in turn contribute to the pathogenesis of this placental syndrome [92]. In contrast, in this study we show that staining of PSG in EVT is located at the membrane where its functional interaction with integrin α5 and focal adhesions mediate migration. Due to the limited availability of placental bed biopsies from pre-eclamptic women we were unable to assess levels of PSG1 in this EVT context in this study. Whether PSGs also affect the initial penetration of the uterine epithelium by blastocytic STBs that occurs in the earliest stages of implantation requires further investigation. Fetal gender-specific differences in gene expression during pregnancy have been reported for cytokines, pro-angiogenic factors, and some galectins [93,94]. In addition, carrying of a male fetus has been identified as an increased risk factor for adverse pregnancy outcomes [95,96]. Therefore, we also analyzed the expression of PSG1 considering a fetal gender. Interestingly, our results indicate that samples obtained from women carrying a male fetus are the major contributors to the observed differences in PSG1 concentration. At present, the mechanisms that regulate the expression of PSGs in the placenta are not completely understood, and whether fetal sex influences their expression has never been investigated. Additional studies using well-validated reagents, which do not cross-react with members of the CEACAM family and can detect only PSG

family members, are required to determine whether the concentration of PSGs as a whole are diminished in PE and are also lower than normal in women of different ethnicities.

Author contributions: C.E.D., S.M.B., K.M.Y., and G.D. conceptualized and designed the research. S.R., C.E.D., M.I., O.G., Y.Z., M.C., and M.Y-M. performed the research; S.R., C.E.D., M.I., Y.Z., M.Y-M., K.M.Y., O.E., N.G-L., B.H., S.J.L., R.R., and G.D. contributed in data interpretation. C.E.D., M.C., K.M.Y., O.E., N.G-L., and R.R. contributed with essential reagents, primary cells and human serum samples. All authors revised the manuscript; S.R. and G.D. wrote the manuscript and S.M.B. helped in generating figures.

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2.6 APPENDIX A

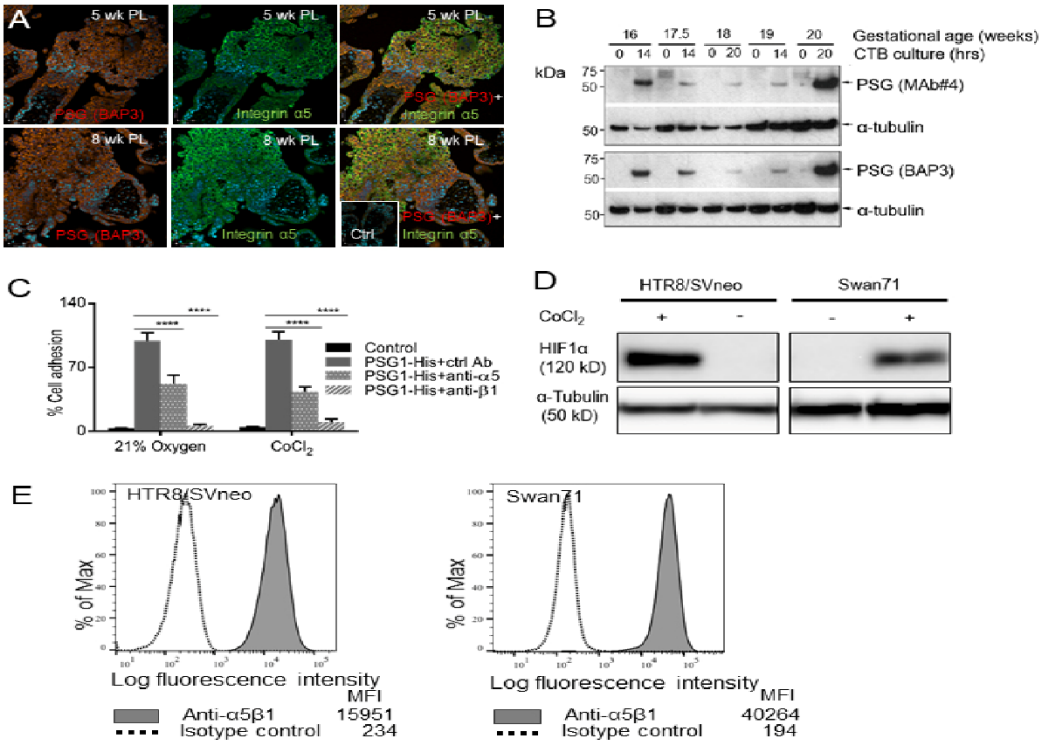


Figure A1. Co-localization of PSGs with integrin α5, PSG1 expression in EVTs differentiated in vitro, PSG1-mediated adhesion of HTR8/SVneo cells, HIF1α stabilization by $CoCl_2$ and integrin α5β1 expression in EVT-like cell lines. (**a**) Representative photographs of dual fluorescent immunohistochemistry localizing PSGs (red) and integrin α5 (green) are shown in both individual and dual channels in sections of first-trimester placenta. Scale bars = 45μm. ctrl = control Ab. (**b**) Immunoblot of cytotrophoblast (CTB) lysates with two different anti-PSG mAbs shows no PSG expression in undifferentiated (0h) second-trimester CTBs but high expression in the EVTs that were differentiated in vitro (14h and 20h) from second-trimester CTBs. (**c**) HTR8/SVneo cells that were pre-incubated with the indicated Abs were seeded on wells coated with 30 μg/ml PSG1-His or control protein. When indicated, cells were pre-treated 4h with 100μM $CoCl_2$ and the treatment was maintained during the experiment. Cell adhesion experiments were carried out as described in Figure 2. (**d**) Representative image of Western blot showing HIF1α stabilization in HTR8/SVneo and Swan71 cells treated with 100μM $CoCl_2$ for 4h. (**e**) HTR8/SVneo and Swan71 were incubated with anti-integrin α5β1 or isotype control mAb followed by APC-conjugated anti-mouse Ig. MFI = mean fluorescence intensity.

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Chapter 3:
MANUSCRIPT 2

Pro-angiogenic effects of pregnancy-specific glycoproteins in endothelial and extravillous trophoblasts

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3.1 Abstract

We previously reported that binding to heparan sulfate (HS) is required for the ability of the placentally-secreted pregnancy-specific glycoprotein 1 (PSG1) to induce endothelial tubulogenesis. PSG1 is composed of four immunoglobulin-like domains but which domains of the protein bind to HS remains unknown. To analyze the interaction of PSG1

with HS, we generated several recombinant proteins, including the individual domains, chimeric proteins between two PSG1 domains, and mutants. Using flow cytometric and surface plasmon resonance studies, we determined that the B2 domain of PSG1 binds to HS and that the positively charged amino acids encompassed between amino acids 43-59 are required for this interaction. Furthermore, we showed that the B2 domain of PSG1 is required for the increase in the formation of tubes by endothelial cells including a human endometrial EC line and two extravillous trophoblast (EVT) cell lines and for the pro-angiogenic activity of PSG1 observed in an aortic ring assay. PSG1 enhanced the migration of ECs while it increased the expression of matrix metalloproteinase-2 in EVTs, indicating that the pro-angiogenic effect of PSG1 on these two cell types may be mediated by different mechanisms. Despite differences in amino acid sequence, we observed that all human PSGs bound to heparan sulfate proteoglycans and confirmed that at least two other members of the family, PSG6 and PSG9, induce tube formation. These findings contribute to a better understanding of the pro-angiogenic activity of human PSGs and strongly suggest conservation of this function among all PSG family members.

3.2 Introduction

A successful pregnancy requires proper placental angiogenesis to expand the capacity for blood flow to ensure fetal growth (Chen, *et al*. 2017, Reynolds and Redmer 2001). An aberrant placental vasculature is associated with various pregnancy complications, including fetal growth restriction and pre-eclampsia (Mayhew, *et al*. 2004, Redman and Sargent 2005, Reynolds, *et al*. 2006). Therefore, it is important to understand the factors that modulate placental angiogenesis, as some could be candidates for therapeutic intervention (Belyakova, *et al*. 2019, Sokolov, *et al*. 2017, Torry, *et al*. 2007).

Pregnancy-specific glycoproteins (PSGs) are secreted by syncytiotrophoblasts and extravillous trophoblasts (EVTs) into the maternal circulation in species with hemochorial placentation (Aleksic, *et al*. 2016, Bohn 1971, Kammerer, *et al*. 2017, Rattila, *et al*. 2019, Roberts, *et al*. 2016, Sorensen 1984). All PSG-expressing species have multiple PSG-encoding genes; for example, there are eleven genes in humans, fifteen in baboons, and seventeen in mice (Kammerer, *et al*. 2017, McLellan, *et al*. 2005). PSG1 is the product of one of ten PSG-protein encoding genes designated as PSG1-PSG9 and PSG11 and the best studied (Moore and Dveksler 2014, Shanley, *et al*. 2013, Teglund, *et al*. 1994, Wurz, *et al*. 1981, Zhou and Hammarstrom 2001). To date, several functions and ligands have been described for PSG1 (Martinez, *et al*. 2013, Moore and Dveksler 2014, Rattila, *et al*. 2019, Shanley, *et al*. 2013). PSG1 binds to the latency-associated peptide of TGF-β1, inducing the activation of the latent form of this cytokine (Blois, *et al*. 2014). In addition, PSG1 binds to integrin αIIbβ3 and inhibits the platelet-fibrinogen interaction (Shanley, *et al*. 2013). Furthermore, PSG1 plays a role in modulating the adhesion and migration of EVTs by binding to integrin α5β1 *(Rattila, et al*. 2019).

A potential role of murine and human PSGs in the regulation of angiogenesis was previously reported (Lisboa, *et al*. 2011). PSG1 binds to heparan sulfate (HS) and this interaction was involved in its ability to induce endothelial tube formation (Lisboa, *et al*. 2011b). Heparan sulfate proteoglycans (HSPGs) contribute to various biological processes, including cell growth, adhesion, migration, and angiogenesis (Chiodelli, *et al*. 2015, Julian, *et al*. 2001). The HS moiety of HSPGs interacts with growth factors (GFs), GF-binding proteins, extracellular proteases, protease inhibitors, chemokines, cytokines,

morphogens, and adhesive proteins. The interaction with HS regulates the activity, stability, kinetics, and dynamics of many ligand-receptor complexes functioning as a co-receptor, and it is also implicated in the formation of GFs gradients (Fuster and Wang 2010, Nagarajan, *et al*. 2018, Ruoslahti 1991, Yanagishita and Hascall 1992).

PSG1 is comprised of four Ig-like domains designated as N, A1, A2, and B2, and different ligands have been shown to interact with more than one domain of the protein (Ballesteros, *et al*. 2015, Rattila, *et al*. 2019, Shanley, *et al*. 2013). Here, we found that only the B2 domain of PSG1 binds to HS, and the importance of this domain in the pro-angiogenic activity of this protein was confirmed in *in vitro* tube formation assays using endothelial cells (ECs) and EVTs and in an aortic ring assay. The effects of addition of PSG1 to ECs and EVTs in their migration and metalloproteinase (MMP)-2 and -9 expression were also investigated. In addition, we determined whether the ability to bind to HS and induce endothelial tubulogenesis is restricted to PSG1 or if it is shared by other members of the human PSG family.

3.3 Materials and Methods

Cell lines

The human endometrial endothelial cell (HEEC) line and the human trophoblast cell line Swan71 were provided by Dr. Gil Mor (Wayne State University, Detroit, MI, USA) and were cultured as previously described (Krikun, *et al*. 2004, Straszewski-Chavez, *et al*. 2009). The trophoblast cell line HTR8/SVneo was obtained from Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada) (Graham, *et al*. 1993). Porcine aortic ECs expressing the yellow fluorescent protein (PAE-YFP) were provided by Dr. Enrique Zudaire (Janssen, Pharmaceutical Companies of Johnson & Johnson, Spring House, PA,

USA) and were cultured in DMEM/F12 medium (Corning) supplemented with 10% fetal bovine serum (FBS) and 100U/ml penicillin/streptomycin (PS) (Corning) (Lisboa, *et al*. 2011, Ronnstrand, *et al*. 1992). The Namalwa cell line and Namalwa cells expressing syndecan-1 (Namalwa-SD1) or syndecan-2 (Namalwa-SD2) were provided by Dr. Guido David (Department of Human Genetics, KU Leuven, Leuven, Belgium) and were cultured in RPMI 1640 medium with 7.5%FBS, PS, 2mM glutamine, and 0.5mg/ml G418 (Zhang, *et al*. 2001). All cells were maintained in a 37°C humidified incubator with 5%CO_2.

Protein production

PSG1-Fc, PSG1-His, PSG1N-Fc, PSG9-Fc, and a protein containing the Fc portion of human IgG1 (designated as Fc) were generated from the supernatant of stably transfected CHO-K1 cells and grown in a hollow fiber cartridge (FiberCell Systems) as previously described (Ballesteros, *et al*. 2015). The cDNAs encoding the PSGs with a V5His tag were provided by Dr. Tom Moore (University College Cork, Cork, Ireland) and the proteins were generated and purified as previously published (Warren, *et al*. 2018). Plasmids encoding the cDNAs for the chimeric constructs, the chimeric mutant, PSG1 lacking the B2 (PSG1ΔB2) domain, and the B2 domain of PSG6 (PSG6B2) were synthesized by Genscript and were cloned in the pFuse-IgG e3-Fc1 vector (InvivoGen). PSG1ΔB2-Fc, PSG6B2-Fc, PSG1A1-Fc, PSG1A2-Fc, and PSG1B2-Fc were generated by transient transfection of CHO-K1 or ExpiCHO cells (Thermo Fisher Scientific) (Ballesteros, *et al*. 2015, Warren, *et al*. 2018). All Fc-tagged proteins were purified on protein A columns as described (Jones, *et al*. 2018, Rattila, *et al*. 2019).

Tube formation assay

Tube formation assays were performed as previously described (Beltrame, *et al*. 2018). Briefly, 96-well plates were coated with 50µl/well of Geltrex (Life Technologies) and incubated for 30 min at 37°C to allow for matrix solidification. Cell suspensions prepared in serum-free media (SFM) containing the indicated proteins or an equal volume of PBS were seeded (HTR8/SVneo and Swan71 cells at 15,000cells/well, PAE-YFP cells at 18,000cells/well, and HEECs at 20,000cells/well) on top of the solidified Geltrex and incubated at 37°C with 5%CO2. PBS and Fc were used interchangeably as controls due to their similar effect in the assay. Images of tubules were captured using Olympus C-5060 digital camera connected to an inverted light microscope (10x, IMT2 Olympus) (at 6h post-seeding for EVTs) or using a Leica AF600 DMI600B microscope with a built-in DFC 365FX-566903911 camera (at 4h and 12h post- for PAE-YFP and HEECs, respectively). Each treatment was performed in triplicate wells and five different fields per well were analyzed using the Wimtube image analysis software (Wimasis GmbH). To determine whether PSG1 binding to the cell surface was sufficient to induce tube formation, HEECs were incubated with PSG1-His or PBS for 1h. After washing with SFM, the cells were seeded on the matrix, as described above. To define whether PSG1-mediated tube formation in HEECs is dependent on MMPs, the cells were pre-treated with the pan-MMP inhibitor, GM6001 (cat#BML-EI300, Enzo Life Sciences) for 1h before seeding.

Determination of protein binding to cells by flow cytometric analysis

Cell suspension was prepared at 1×10^6cells/100µl of fluorescence-activated cell sorting (FACS) buffer (PBS with 2%BSA and 0.05%NaN3) and kept on ice. After adding FcR blocking reagent (Miltenyi Biotec), the cells were incubated with the indicated proteins.

Binding of the Fc-tagged proteins was detected after an incubation of 30 min with Alexa Fluor 488-conjugated anti-human IgG1 Ab (cat#A10631, Invitrogen). Binding of the proteins containing the V5His tags was detected with the anti-human PSG mAb (cat#MAB6799, R&D Systems), followed by allophycocyanin (APC) conjugated anti-mouse IgG (cat#550826, BD Biosciences). Washes between steps were performed with FACS buffer. BD LSR II flow cytometer (BD Biosciences) was used for cell analysis and fifty thousand events were collected per treatment using the FACS Diva software (BD Biosciences). FlowJo software V10.0.8 (BD Biosciences) was used for analysis.

Surface Plasmon Resonance (SPR) Studies

The SPR experiments were performed in a BIAcore 3000 (GE Healthcare) at 25°C with HBS-EP (0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, and 0.005%v/v Surfactant P20) as running buffer. Between 50 and 250 resonance units (RU) of biotinylated heparin (Cat#375054, Millipore Sigma) were immobilized on a streptavidin-coated sensor chip (SA chip, cat#BR100032; GE Healthcare). Before the immobilization to the sensor surface, the biotinylated heparin (cat# 375054: Millipore Sigma) was cleaned in a PD SpinTrap G-25 desalting column (GE Healthcare). The chip surface was first washed three times with one-minute injections of 1M NaCl/50mM NaOH. We next performed two 2 min injections of 5μg/ml biotinylated heparin in HBS-EP buffer at a 5μl/min flow rate. A surface where no heparin was immobilized was used as control. Proteins were diluted in HBS-EP buffer and injected over the immobilized heparin at a flow of 30μl/min. A sensor SA surface with 200 RU of immobilized heparin was used to test the binding of PSG1-Fc, PSG1A2-Fc, and the chimeric proteins at a 1μM. Chip surface was

regenerated with a one min injection of 2M $MgCl_2$ in 10mM Glycine pH2.0. The real-time sensorgrams were aligned using the BIAevaluation 4.1 software (GE Healthcare).

Gelatin zymography

HTR8/SVneo and Swan71cells and HEECs were seeded at 1.2×10^6cells/ml in wells of a 24-well plate in RPMI and DMEM supplemented with 10%FBS and endothelial growth media-2, respectively, and incubated until they reached 80-90% confluency. The cells were washed with PBS and incubated with PSG1-V5His or control protein diluted in SFM for the indicated time. Conditioned media were collected, span down and concentrated ten-fold in a Nanosep centrifugal device (Pall Corporation) prior to determination of protein concentration using the EZQ protein reagent (Invitrogen). One hundred micrograms of protein from each sample were mixed 1:1 with Tris-Glycine SDS 2X Sample buffer (Life Technologies) and resolved in a 10% Zymogram Plus (Gelatin) protein gel (Life Technologies). The gels were incubated three times for 30 min each with Zymogen Renaturing Buffer (Life Technologies) followed by addition of the Zymogen Developing Buffer (Life Technologies) for 30 min at RT. Afterwards, the gels were incubated with fresh developing buffer for 24h at 37°C, stained with GelCode Blue (Thermo Fisher Scientific) for 30 min and de-stained with water. Densitometry was performed using the ImageJ software version 1.52r (NIH).

Wound healing assay

HEECs in 2%FBS containing media were seeded at 1.3×10^5cells in 300µl/well in a 48-well plate. Swan71 at 1.2×10^5cells/well and PAE-YFP at 1.3×10^5cells/well were seeded in 300µl of 10%FBS containing media, respectively. Cells were incubated overnight to form confluent monolayers. The wells were washed with SFM after scratching with

200µl pipette tips. The proteins to be tested were added in 0.2%FBS containing media for HEECs and in SFM for PAE-YFP and Swan71 cells. Cell migration was recorded by time-lapse microscopy (Leica AF6000 DMI6000B with a built in camara DFC 365FX-566903900) for 4h, 6h, and 12h, for PAE-YFP, Swan71, and HEECs, respectively. Images were captured every 20 min using the LASX software version 3.3.2 (Leica Microsystems), analyzed with the "Wound Healing ACAS Image Analysis" (MetaVi Labs Inc) and results were analyzed as gap closure area (the measurement of the difference between gap area at t = 0 and t = 12h (for HEECs)/ t=4h (for PAE-YFP)/ t=6h (for Swan71)). Results shown are the mean ± S.D. of nine fields of three wells per treatment from one representative experiment.

Proliferation assay

HEECs were seeded on wells of a 96-well plate at 2×10^4cells/well with EBM-2 medium with 0.2%FBS and treated in triplicate with PSG1-V5His or PBS. At 6h or 24h post-seeding, the cells were incubated with 20µl CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA) and incubated for an additional 2h. The absorbance at 490nm was recorded in a GloMax Discover multimode detection instrument (Promega).

Mice and aortic ring assays (ARA)

Eight-week-old C57BL/6J mice (Jackson Laboratory) were housed in specific pathogen-free conditions on a 12:12-h dark-light cycle and fed a standard chow ad libitum. All experiments were performed according to the local animal protection laws (Regierung von Unterfranken, Germany). The mouse aorta was removed for the ARA post-cervical dislocation, therefore no specific approval for these experiments was required according to the Guideline 2010/63/EU of the European Parliament. Aortic ring segments from the

mice were prepared and embedded in a collagen I gel (cat#08-115, Millipore Sigma) containing the factors indicated following the protocol described previously and were cultured in Opti-MEM (Life Technologies) supplemented with GlutaMAX (Thermo Fisher Scientific) and incubated at 37°C in 5%CO_2 (Baker, *et al*. 2011). The medium was changed at day three of culture and sprouting was documented using a phase-contrast microscope (Leica® DFC 3000G, Leica Microsystems) at day four. Maximum sprouting distance was analyzed using ImageJ 1.52a.

3.4 Results

Binding of PSG1 to HSPGs on the EC surface mediates tube formation

We previously showed that PSG1 induces the formation of tubular structures by primary dermal human ECs and PAE-YFP cells and that PSG1 no longer induced tubes following treatment with heparinase I and chondroitinase ABC (Ha, *et al*. 2010, Lisboa, *et al*. 2011). To further understand the involvement of HS in PSG1-mediated tube formation, we performed additional studies to determine whether binding of PSG1 to HS on the EC surface is sufficient. In addition, we studied the effect of PSG1 in a more relevant EC to pregnancy, the HEECs. To this end, PSG1 or a control protein were first added to the cells, followed by washes prior to seeding on the matrix (Fig. 1A). Fig. 1B shows that PSG1 induced higher numbers of branching points, tube length, and tubes compared to the control protein.

The B2 domain of PSG1 binds to HSPGs and induces endothelial tube formation

PSG1 does not bind to Namalwa cells, which lack HS expression while it binds to Namalwa cells transfected with any of the four HSPG-containing syndecans (Alexopoulou, *et al*. 2007, Lisboa, *et al*. 2011). To define which domain(s) of PSG1

interact with HS, we utilized Fc-tagged single-domain proteins (N, A1, A2 and B2) (Ballesteros, *et al*. 2015), and also generated a protein comprised of the N, A1, A2 domains of PSG1, designated as PSG1ΔB2-Fc. We tested whether these proteins bind to syndecan 1-expressing Namalwa cells (Namalwa-SD1) by flow cytometry (FC) and found that only the protein comprised of the B2 domain bound to Namalwa-SD1 cells significantly over the control (Fig. 1C). In addition, PSG1ΔB2-Fc did not bind to the Namalwa-SD1 cells (Fig. 1C) and as expected, none of these proteins bound to wild type Namalwa cells (Fig. 1D).

We next tested which domains of PSG1 induce tube formation by HEECs. As shown in Fig. 1E-F, tube structures were only formed over what was observed in the control-treated cells with the B2 domain. In accordance with the lack of binding to HSPG, the N, A1, A2 single domains, and the PSG1ΔB2 did not induce tube formation by the HEECs (Fig. 1E-F). Similar results were obtained with PAE-YFP cells (Fig 2A). We also examined whether the B2 domain was required for endothelial tube formation when the proteins were mixed with Geltrex and found that PSG1 induced the formation of tubes over the control when mixed with the matrix while PSG1 lacking the B2 domain did not (Fig 2B).

Amino acids 43-59 in the B2 domain of PSG1 interact with HSPGs and heparin

To identify the region in the B2 domain that interacts with HSPGs, we generated chimeric proteins composed of parts of the A2 domain, which lacks the ability to interact with HS, and parts of the B2 domain. The chimeric constructs were designed based on a model of the B2 domain to replace entire β-sheets and loop structures indicated by different colors in Fig. 3A. The amino acid sequences of the resulting chimeric proteins

are shown in Fig. 3B and were designated as A2/B2(1-20), A2/B2(21-42), A2/B2(43-59), and A2/B2(60-84). These proteins were tested for binding to heparin by SPR. We observed that the A2/B2(43-59) bound considerably better to heparin when compared to the other chimeras and the control protein (Fig. 3C). In addition, we analyzed the binding of the chimeric proteins to Namalwa-SD1 cells by FC. (Fig. 3D). Among the four chimeras, A2/B2(43-59) and A2/B2(1-20) bound to Namalwa-SD1 over the control protein, with the A2/B2(43-59) showing the highest binding activity. As expected, none of the chimeras bound to the untransfected Namalwa cells (data not shown). These experiments strongly suggest that the 43-59 region of the B2 domain is required for the interaction of this domain with HS.

Positively charged amino acids in the 43-59 region of the B2 domain of PSG1 are required for binding to HSPGs and the induction of endothelial tubulogenesis

The negative charges of the HS chains are believed to interact with positively charged patches of proteins often delineated by clusters of arginine, lysine, and histidines (Meneghetti, *et al*. 2015, Smock and Meijers 2018). Therefore, we hypothesized that mutation of these amino acids in the 43-59 region of the B2 would abolish the interaction with HSPGs (Fig. 3E). To test this hypothesis, we generated a mutant of the A2/B2(43-59) where K48, R52, H53, K57, and H58, in the B2 portion were replaced by glutamic acids and designated this mutated protein as A2/B2(43-59)E_{mut} (Fig. 3E). We observed that A2/B2(43-59)E_{mut} did not bind to syndecan-expressing Namalwa cells (Fig. 3F) and that it bound significantly less than the A2/B2(43-59) to heparin (Fig. 3G). We next tested the ability of A2/B2(43-59) and A2/B2(43-59)E_{mut} to induce endothelial tube formation. As shown in Fig. 3H and 3I, PAE-YFP cells formed tube networks in the

presence of A2/B2(43-59) but not when incubated with A2/B2(43-59)E_{mut}. Altogether, the results indicate that the positively charged amino acids in the 43-59 region of B2 domain are involved in the binding to HS and are required for the induction of endothelial tube formation.

PSG1 induces endothelial-like tube formation by EVT cell lines

During remodeling of the uterine spiral arteries, EVTs adopt an EC-like phenotype and replace the EC lining (Ashton, *et al*. 2005). Therefore, we tested whether PSG1 could induce endothelial-like tube formation by two EVT cell lines: HTR8/SVneo and Swan71. The cells were incubated with PSG1 at 15, 30 and 60µg/ml and PSG1 induced tube formation by HTR8/SVneo and Swan71 cells over the control protein starting at 30µg/ml, which is equivalent to 500nM (Fig. 4A and data not shown). In accordance with our observations in ECs, the B2 domain was required for the PSG1-mediated tube formation by EVTs, as the cells treated with PSG1ΔB2-Fc at all three concentrations tested did not form tubes when compared to the control (data not shown).

Effect of PSG1 in cell migration and matrix metalloproteinase (MMP)-2 and -9 expression:

The process of angiogenesis involves cell migration, proliferation, and remodeling of extracellular matrix through proteases (Conway, *et al*. 2001, Fuster and Wang 2010). Hence, we investigated whether PSG1 plays a role in any of these processes. Because the expression of MMP-2 and MMP-9 by EVTs is important for their extracellular matrix-remodeling function, we examined whether PSG1 modulates the expression of these enzymes (Chen and Khalil 2017, Isaka, *et al*. 2003). As shown in Fig. 4B and C, PSG1 induced higher expression of MMP-2 by HTR8/SVneo and Swan71 (data not shown)

while the expression of MMP-9 was not changed. Interestingly, we did not observe an effect of PSG1 on the expression of MMP-2 and MMP-9 by HEECs (data not shown). We next performed wound healing assays with HEECs and analyzed the migration of the cells incubated with PSG1 or PBS. Cells incubated with PSG1 covered an area within the gap that was larger than the area covered by the cells in the presence of PBS (Fig. 5A). Similar results were obtained with PAE-YFP cells (data not shown). The PSG1 migration-enhancing effect was not due to changes in cell proliferation as cells did not proliferate differentially in the presence or absence of PSG1 (Fig. 5B). On the other hand, we did not observe differences in migration of the EVT cell lines as compared to the control-treated cells when PSG1 was added to the media (data not shown). Overall, these results indicate that PSG1 differentially regulated the MMP secretion and migratory activity of ECs and EVTs.

Binding of other members of the human PSG family to HSPGs

We explored whether other human PSGs can bind to HS to determine if this is a conserved function of these glycoproteins. The B2 domains of human PSGs share 72 to 95% amino acid sequence identity (Fig. 6A), and two family members (PSG9 and PSG6) have a potential N-linked glycosylation site that could interfere with HS binding (Warren, *et al*. 2018). We tested binding of PSG2, PSG4, PSG5, PSG7, PSG8, PSG9 and PSG11 to Namalwa-SD1 cells and found that all bound to these syndecan-expressing cells (Fig. 6B). Unfortunately, PSG3 was not tested due to an insufficient amount of protein resulting from very low yield following transfection. Then we selected PSG9 and PSG6 to test whether like PSG1, they induced endothelial tube formation. As shown in Fig. 6C,

PSG9 induced tube formation. Furthermore, we determined that the B2 domain of PSG6 is sufficient to bind to HS and to elicit the formation of tubes (Fig. 6A and 6D).

The B2 domain is required for the PSG1-mediated pro-angiogenic effect in the aortic ring assay

We performed experiments to determine whether PSG1 has pro-angiogenic activity in a different assay, in which non-ECs are also present (Baker, *et al*. 2011). To this end, aortic rings were seeded on collagen I containing PSG1, PSG1ΔB2-Fc, or equal volumes of PBS. As shown in Fig. 6E and 6F, at four days post-treatment, PSG1 showed higher maximum capillary sprouting distance when compared to the control-treated rings. In addition, there was no significant difference in capillary sprouting distance from the rings treated with PSG1ΔB2 and the control. These results indicate that the PSG1-mediated angiogenic activity in this assay is also dependent on the B2 domain of the protein.

3.5 Discussion

PSG1 induces the formation of tubes by primary human ECs and the porcine aortic ECs, which lack the VEGF kinase insert domain (KDR) receptor, when seeded in collagen I and Geltrex, respectively (Ha, *et al*. 2010, Lisboa, *et al*. 2011b). PSG1-mediated tube formation was significantly reduced in ECs in which HS was enzymatically removed (Lisboa *et al.*, 2011). In addition, as stated above, PSG1 induced tube formation on collagen I, which does not contain HS (Ha, *et al*. 2010, Lisboa, *et al*. 2011b). We extended our investigation of PSG1's pro-angiogenic activity with HEECs and confirmed that PSG1 binding to HSPG on the cell membrane rather than the matrix is required for this activity. Cell-surface HSPGs include syndecans 1-4, GPI-tethered glypicans, CD44, and betaglycan (Ambalavanan, *et al*. 2008, Sarrazin, *et al*. 2011, Teng, *et al*. 2012). In

addition, all four SDs have been implicated in EC biology (De Rossi and Whiteford 2014, Teng, *et al*. 2012). We observed expression of syndecan (SD)-2 and SD4 in HEECs and of all four SDs in PAE-YFP cells (data not shown). We also showed that PSG1 induces tube formation in a B2-dependent manner even when mixed with Geltrex. This observation indicates that PSG1 remains in a dynamic equilibrium between the matrix, which contains HSPGs, and the membrane of the cells.

Our studies show that the B2 is the only domain of PSG1 that binds to HS and induces tube formation. This domain was previously shown to bind to the latency-associated peptide of TGF-β1 and to integrin α5β1, although which amino acids are involved in these interactions remain unknown (Ballesteros, *et al*. 2015, Rattila, *et al*. 2019). We showed that a region between amino acids 43-59 of the B2 domain are involved in the interaction with HS. Basic amino acids are considered key contributors in the interaction of proteins with HS, therefore it is not surprising that mutations of K48, R52, H52, K57, and H58 reduced the interaction with HS as demonstrated in the SPR and flow cytometric analysis (Esko and Linhardt 2009, Rudd, *et al*. 2017). We also observed binding of the chimeric protein which included amino acids 1-20 of the B2 domain to SD-expressing cells over the control protein. Therefore, a possible participation of these amino acids in HS-binding cannot be ruled out.

EVT cell lines have been shown to form tubes on Matrigel in response to pro-angiogenic factors, such as activin A (Belyakova, *et al*. 2019, Li, *et al*. 2015). PSG1 bound to HTR8/SVneo and Swan71 (data not shown) in a HS-dependent manner therefore we hypothesized that PSG1 could induce the formation of tubes in these cells (Lisboa, *et al*.

2011). We found that PSG1 but not PSG1 lacking the B2 domain induced the formation of tube structures in these cells.

Interestingly, when added to the media, PSG1 increased the migration of ECs while having no effect on the migration of Swan71. However, we had previously shown that when coated on a well, PSG1 increased the migration of primary and EVT cell lines including Swan71, indicating that soluble and immobilized PSG1 differently affect the migration of these cells (Rattila, *et al*. 2019). On the other hand, PSG1 did not increase the secretion of MMP-2 by ECs while it increased the secretion of MMP-2 by EVTs, which correlates with the invasive capacity of these cells (Peng, *et al*. 2016). While we did not detect any PSG1-mediated effect in MMP-2/9 expression in HEECs, we observed a reduction in the PSG1-mediated tube formation in the presence of a pan-MMP inhibitor (data not shown). Therefore, a possible role of PSG1 in the regulation MMPs other than MMP-2 and 9 in HEECs should be further investigated.

The B2 domains of human PSGs differ both in number and position of positively charged residues. Nevertheless, we found that the HS-binding ability is conserved among all the nine PSGs tested. In the analysis of the HS interaction with the PSGs with the V5His tags, we utilized the anti-PSG MAb6799, which binds to an epitope in the A1 and A2 domains (Warren, *et al*. 2018). PSG2, PSG11, and PSG5 lack either the A1 or A2 domain and the amino acids in the epitope recognized by the Ab in the A1 domain is not fully conserved in all PSGs. Therefore, while different concentrations of each PSG were required to observe binding to the SD-transfectants in these studies, SPR analysis should be performed to determine whether the protein bind to HS with different affinity.

We found that PSG6 and PSG9, which have fewer positiveIy charged amino acids in the B2 domain than PSG1, also induced the formation of tubes. Occasionally, polar residues, such as glutamine, nearby positively charged amino acids or hydrophobic residues, e.g. tyrosine, have been found to contribute to HS-binding (Hascall and Esko 2015, Sarkar and Desai 2015). Whether some polar residues in the other PSGs contribute to their ability to bind to HS requires further investigation. Some murine PSGs including PSG17, 22, and 23 were shown to bind to HS through their N1 domain, and PSG22 and PSG23 (unpublished results) induced endothelial tubulogenesis highlighting the conservation of this function of PSGs in different species (Blois, *et al*. 2012, Sulkowski, *et al*. 2011).

The PSG1-mediated angiogenic activity in the *ex vivo* aortic ring assay (ARA) was also dependent of the B2 domain of PSG1. Cultivation of the entire vascular wall in the ARA enables to investigate not only the role of ECs in new vessel formation but also the impact of smooth muscle cells, pericytes of the vasa vasorum, progenitor cells of the aortic adventitia as well as local inflammatory cells that are generated from these resident progenitor cells, e.g. macrophages (Baker, *et al*. 2011, Worsdorfer, *et al*. 2017). Furthermore, the ARA evaluates the important initiation step of activating the ECs in the vessels and their sprouting for creating new capillaries (Nakatsu, *et al*. 2003, Nowak-Sliwinska, *et al*. 2018).

Since HSPGs serve mostly as co-receptors, PSGs may engage a very low-affinity receptor, which requires HS. Another possibility is that PSGs might be involved in the competitive binding of HSPGs and displaces a bound molecule, undefined in our current study, for promoting angiogenesis as observed in the competitive glycosaminoglycan binding for chemokine cooperativity (Verkaar, *et al*. 2014). Surprisingly, we found that

PSG1 without the B2 domain increased EC sprouting over the control in the ARA when it was performed in the presence of 2.5% serum. Similarly, we observed that both PSG1 and PSG1 lacking B2 domain induced the formation of tube by PAE-YFP on collagen I over the control, although the number of tubes and branching point and the length of tubes induced by PSG1 lacking the B2 were significantly reduced when compared to the effect of full-length PSG1 on these cells. This suggests that either serum may up-regulate an unknown binding partner on the cell surface, which is absent under serum-free conditions and independent of the B2 domain, or a serum component indirectly serves as an adaptor protein for cellular binding.

In summary, our findings indicate that PSG1 is proangiogenic in ECs and EVTs. Also, the B2 domain of PSG1 is important for this activity and exerts this function through an interaction with HSPGs. Since all human PSGs tested bind to HSPGs, the proangiogenic activity might be a conserved function of human PSGs.

Conflict of interest:

The authors report no financial or other conflict of interest relevant to this study. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Author contribution statement:

GD, SR, and FK conceptualized and designed the research. SR, FK, AB, and JSB performed the research; SR, FK, AB, JSB, MLR, SE, and GD contributed in data interpretation. SR and GD wrote the manuscript, which was revised by all authors.

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3.7 Figure legends:

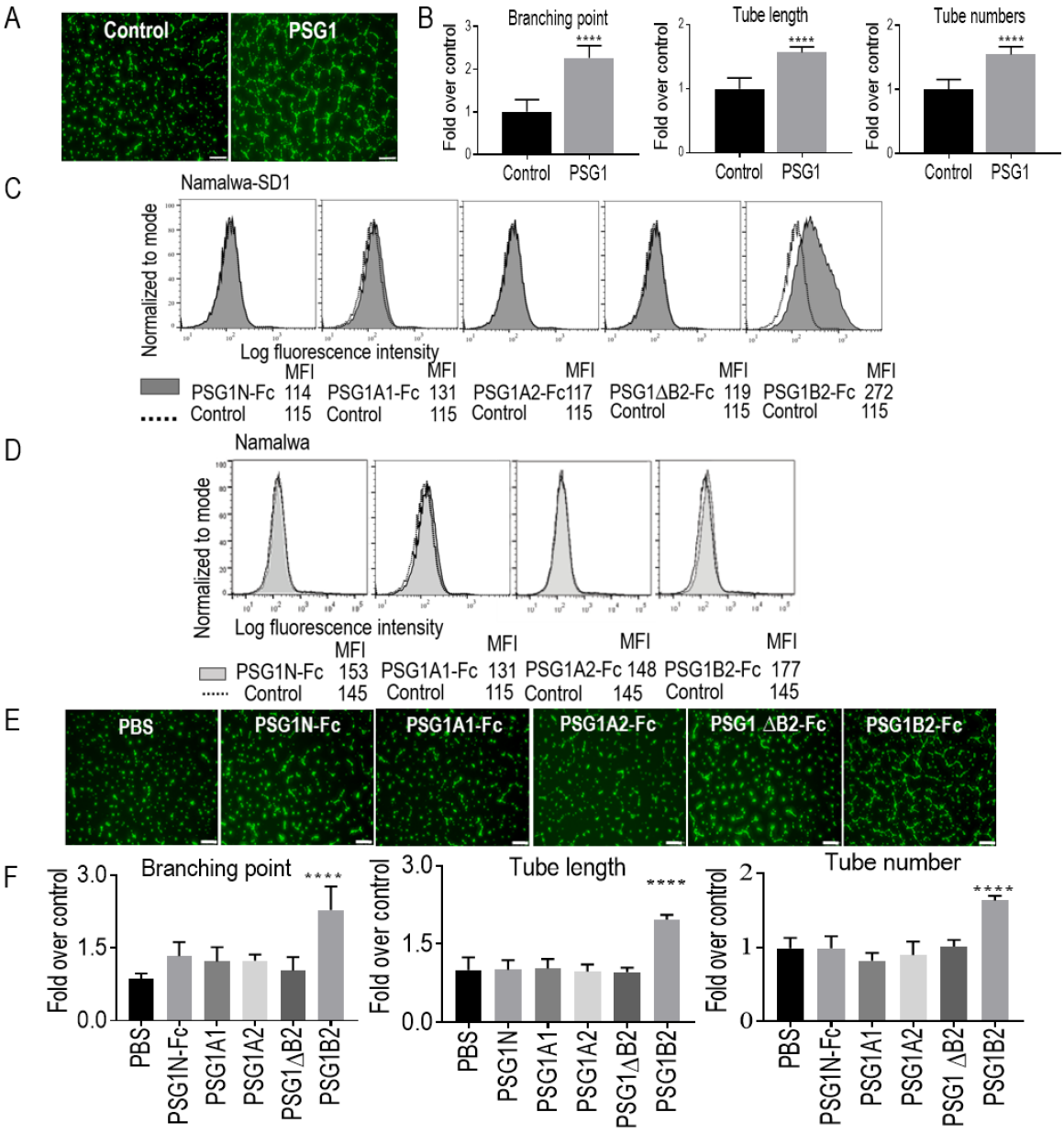


Figure 1. **Binding of PSG1 to heparan sulfate proteoglycans (HSPGs) on the cell surface is sufficient for PSG1-mediated endothelial tube formation and identification of the domain of PSG1 required for HS binding and pro-angiogenic activity of this protein**. **A**. HEECs were incubated with 1500nM PSG1-His or a protein containing the Fc-tag as control for 1h on ice. After washing, cells were seeded on the gelled growth factor-reduced Geltrex (GFR-Geltrex). Representative images of the endothelial tube structures formed with PSG1 or control protein are shown. **B**. Image analyses of tube structures formed are expressed as branching point, tube length, and tube numbers. **C**. Namalwa cells stably transfected with syndecans-1 (SD1) were treated with PSG1N-Fc, PSG1A1-Fc, PSG1A2-Fc, and a protein consisting of an Fc-tag (control) at

10µg/ml, PSG1ΔB2-Fc at 20µg/ml, and PSG1B2-Fc at 5µg/ml, followed by an Alexa Fluor 488-conjugated anti-human Fc Ab. The median fluorescence intensity (MFI) of each treatment is indicated. **D**. Namalwa cells were treated with PSG1N-Fc, PSG1A1-Fc, PSG1A2-Fc, and a protein consisting of the Fc-tag (control) at 10µg/ml, and PSG1B2-Fc at 5µg/ml, followed by Alexa Fluor 488-conjugated anti-human Fc Ab. The median fluorescence intensity (MFI) of each treatment is shown. **E**. HEECs were seeded on the gelled GFR-Geltrex and treated with the indicated proteins at 1000nM or with equal volume of PBS (control); representative images of the endothelial tube structures formed in response to the treatments are shown. **F**. Image analyses of the tube structures shown in **E**. The average of the replicate wells with the cells incubated with the Fc control (in B) or with PBS (in F) is considered as 1. Magnification = 5x; Scale bar = 250µm. Quantitative results are expressed as the mean ± S.D. of replicates; one representative of three individual experiments is shown. Statistical significance was obtained by Student's *t*-test (****P<0.0001) (in B) or by one-way ANOVA, followed by Sidak's multiple comparison tests (****P<0.0001) (in F).

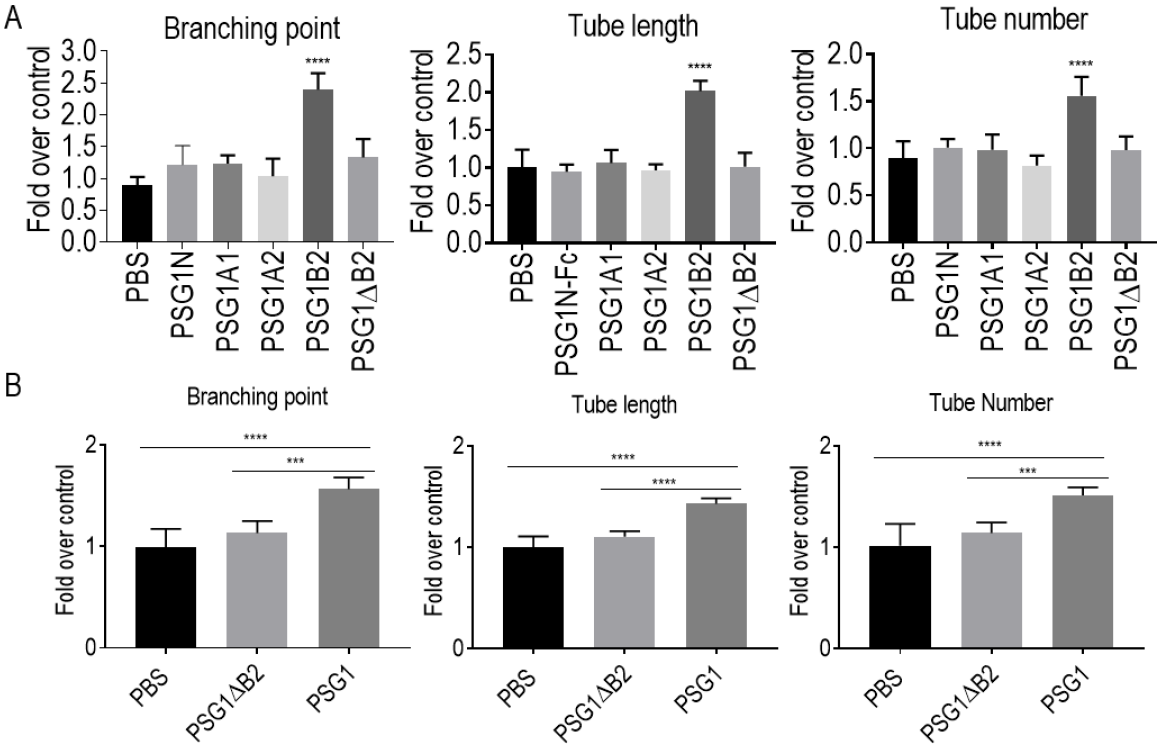


Figure 2. **The B2 domain of PSG1 is required for endothelial tube formation when either added in media or mixed with GFR-Geltrex**. **A**. Quantitation of tube structures formed by PAE-YFP cells in response to various domains of PSG1 added in the media is shown. **B**. HEECs were seeded on the gelled GFR-Geltrex pre-mixed with 1000nM of PSG1-V5His, PSG1ΔB2-Fc, or with equal volume of PBS (control). Quantitation of tube structures formed by HEECs are shown as described in Fig. 1B. The average of the replicate wells with the Geltrex mixed with PBS is considered as 1. Results are expressed as the mean ± S.D. of replicates; one representative of three individual experiments is

shown. Statistical significance was obtained by one-way ANOVA, followed by Sidak's multiple comparison tests (****P<0.0001, ***P<0.0002).

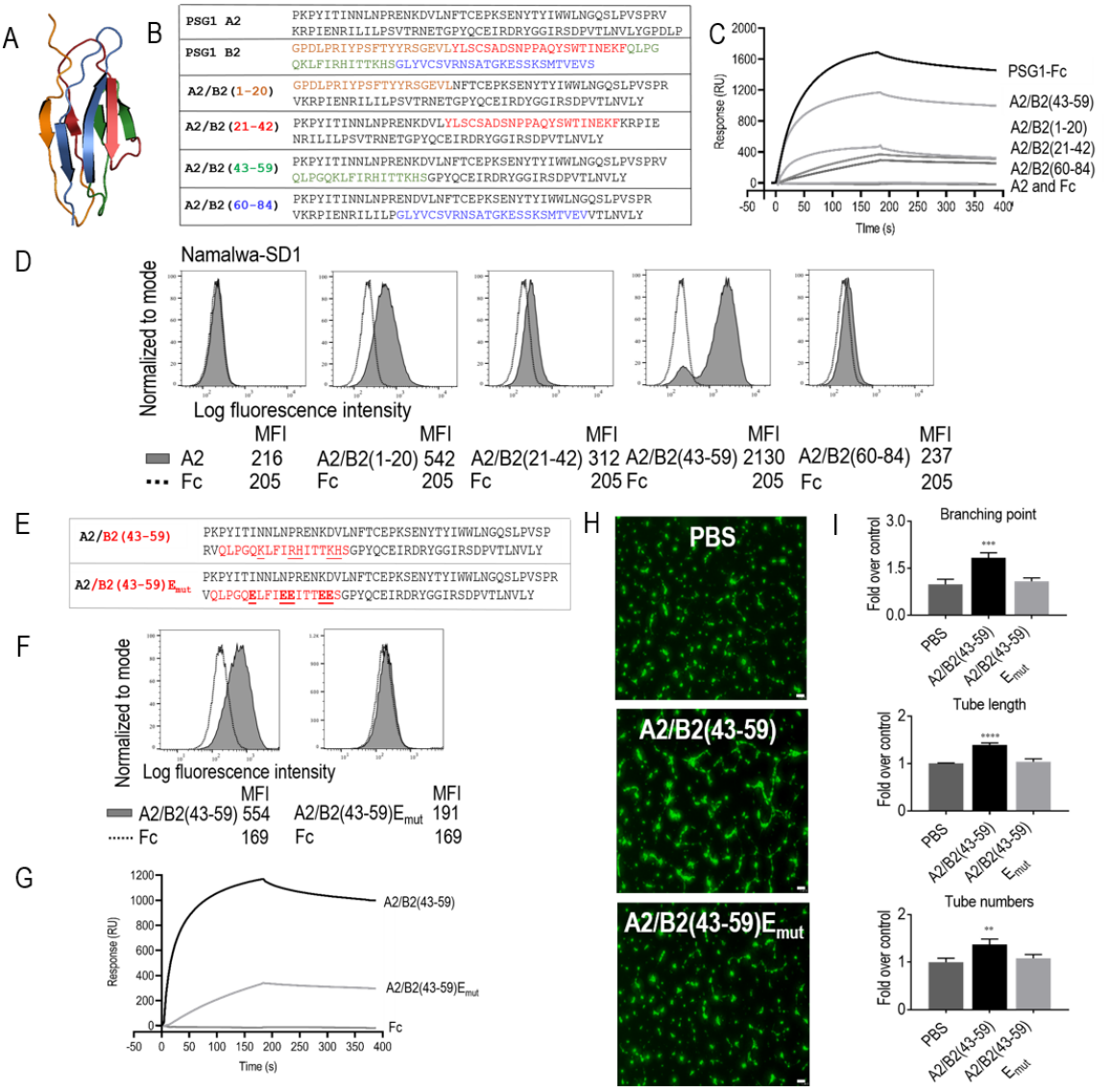


Figure 3: Amino acids 43-59 in the B2 domain of PSG1 interact with HSPGs and heparin, and the positively charged amino acids in that region of the B2 domain are required for HSPG-binding and for endothelial tube formation. **A**. A predicted model of the B2 domain of PSG1 with the theoretical demarcation of the four regions is shown in different colors. **B**. Amino acid sequences of the A2 and B2 domains of PSG1 and the chimeric proteins. **C**. SPR sensorgram of the interaction of the indicated proteins with heparin. All the proteins were injected at 1μM in HBS-EP buffer over a sensor surface with immobilized heparin during a 3 min association and 3 min dissociation phase. A protein consisting of an Fc-tag was used as control. **D**. Namalwa-SD1 cells were treated with the indicated proteins, followed by Alexa Fluor 488-conjugated anti-human Fc Ab. **E**. Amino acid sequences of the A2/B2(43-59) and its mutant with glutamic acid

replacing the K48, R52, H53, K57, and H58 in the 43-59 region, designated as A2/B2(43-59)E_{mut}. **F**. Namalwa-SD2 cells were incubated with the indicated proteins at 10μg/ml, followed by Alexa Fluor 488-conjugated anti-human Fc Ab. **G**. A2/B2(43-59) and A2/B2(43-59)E_{mut} were injected at 1μM in HBS-EP buffer over a heparin surface as described in part C. **H**. PAE-YFP cells were seeded on the gelled GFR-Geltrex and treated with the indicated proteins at 1000nM or equal volume of PBS (control). Representative images of the endothelial tube structures formed are shown. Magnification = 5x; Scale bar = 250μm. I. Image analyses of the tube structures are expressed as described in Fig. 1B. The average of replicate wells with the cells treated with PBS is considered as 1. Quantitative results are expressed as the mean ± S.D. of replicates; one representative of three individual experiments is shown. Statistical significance was obtained by one-way ANOVA, followed by Sidak's multiple comparison tests (****P<0.0001).

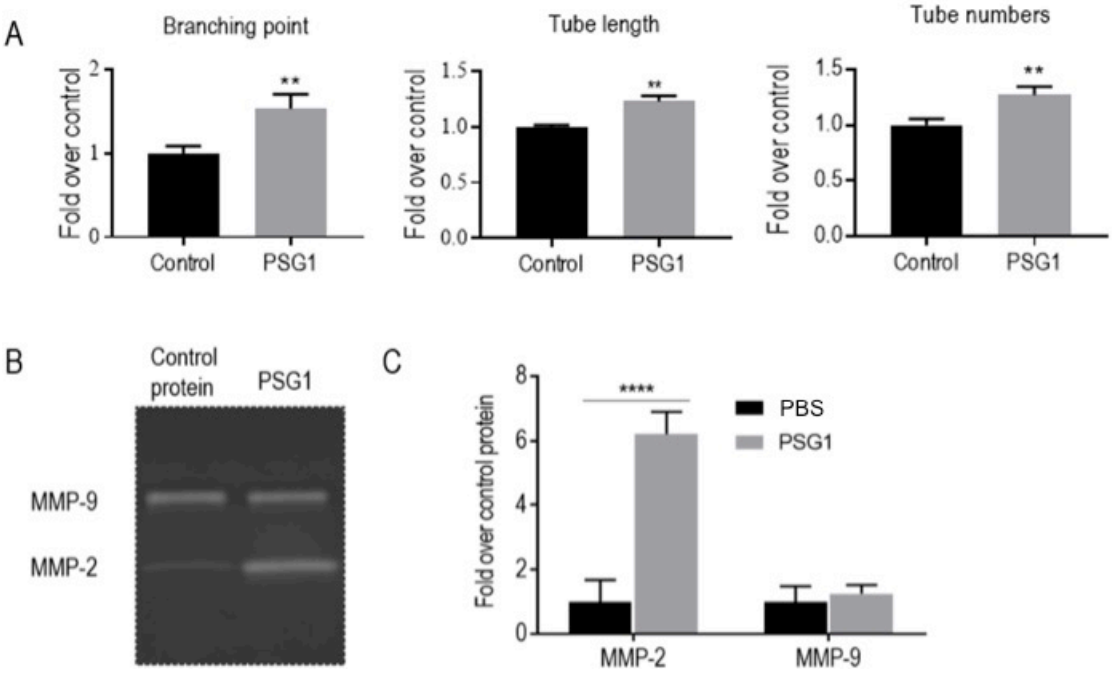


Figure 4. **PSG1 induces endothelial-like tube formation and an increased expression of MMP-2 by HTR/SVneo cells.** **A**. HTR/SVneo cells were seeded on the gelled GFR-Geltrex and treated with PSG1-His at 500nM or equal volume of PBS (control). Quantitative analyses of the images taken 6h post-seeding are expressed as described in Fig. 1B. **B-C**. An image of gelatin zymograph and the related quantification of MMP-2 and MMP-9 secretion by HTR8/SVneo in response to PSG1-treatment. The average of the replicate wells with the cells seeded in the presence of PBS is considered as 1 in parts A and C. Quantitative results are expressed as the mean ± S.D. of replicates; one representative of three individual experiments is shown. Statistical significance was obtained by Student's *t*-test (in A) or by two-way ANOVA, followed by Sidak's multiple comparison test (in C) (****P<0.0001, **P<0.002).

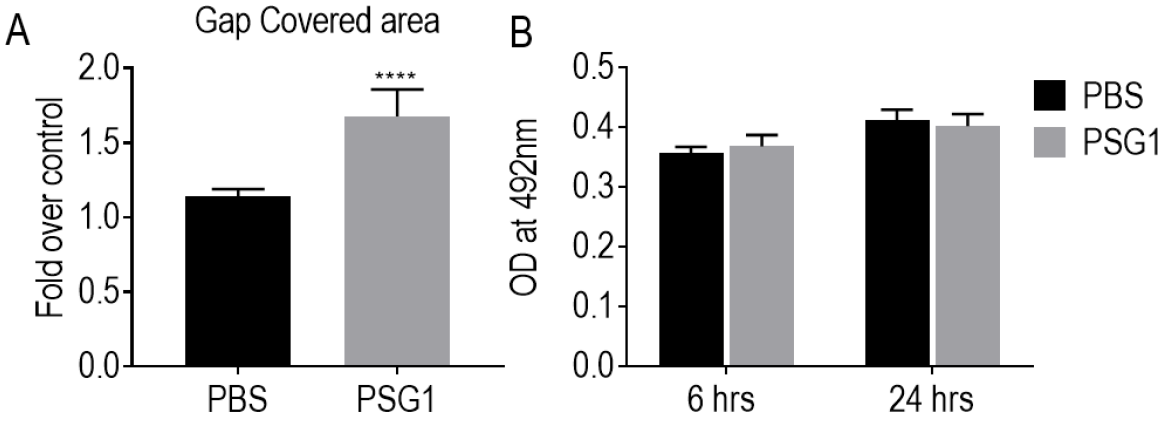


Figure 5: PSG1 enhances migration of HEECs without affecting their proliferation. **A**. Quantitative analyses of a representative wound healing assay performed with HEECs in the presence of PSG1-V5His or PBS control. Gap covered area were calculated as described in the 'Materials and Methods' section. **B**. HEECs were seeded in the presence of PSG1-V5His, PSG1ΔB2-Fc, or PBS in 0.2% serum-containing media. Cell proliferation was analyzed at 6h and 24h as described in the 'Materials and Methods' section. Quantitative results are expressed as the mean ± S.D. of replicates; one representative of three individual experiments is shown. Statistical significance was obtained by Student's *t*-test (in A) or by two-way ANOVA, followed by Sidak's multiple comparison test (in B) (****P<0.0001).

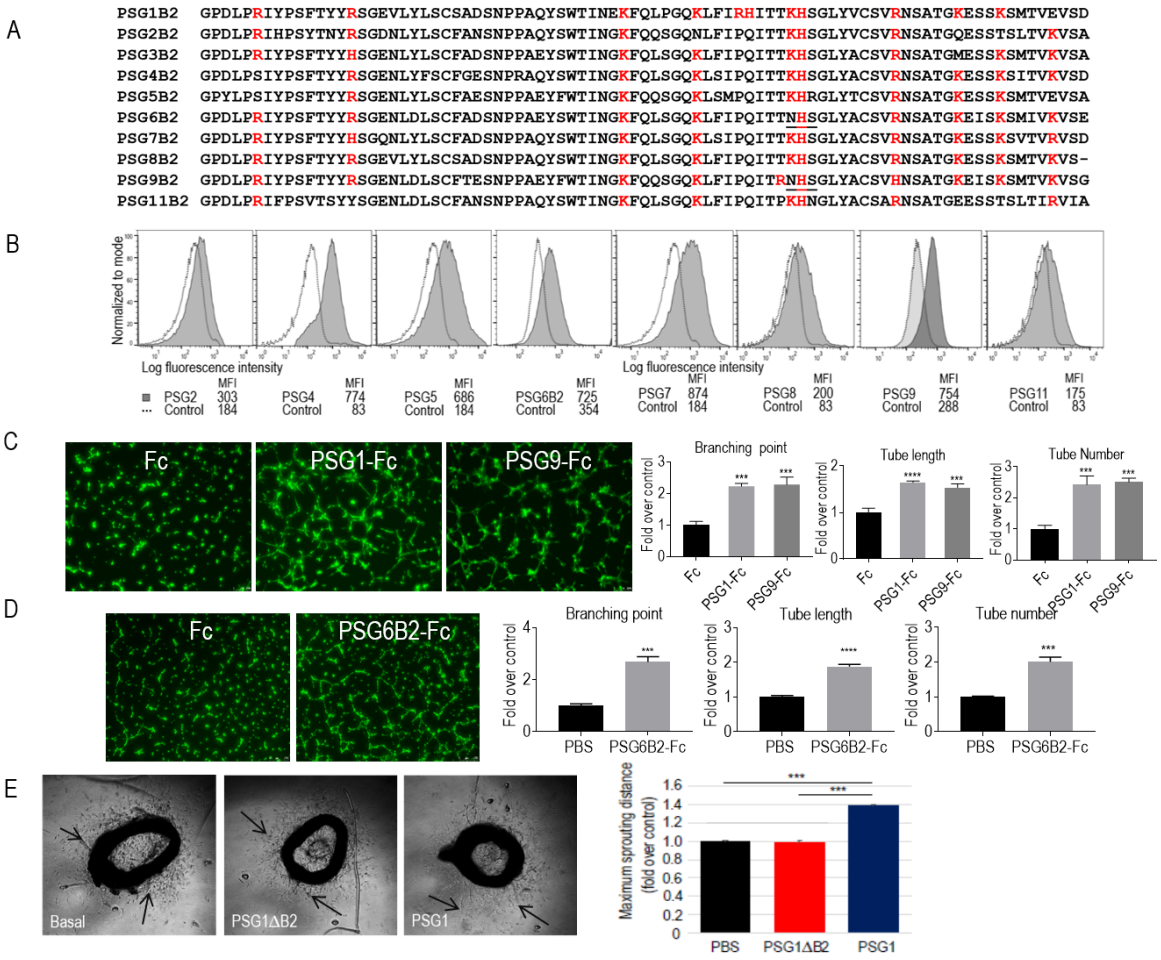


Figure 6: **All human PSGs bind to HSPGs and PSG1 induces capillary sprouting in mouse aortic rings in a B2 domain-dependent manner**. **A**. The positively charged amino acids in the B2 domains of all human PSGs are shown in red and the potential glycosylation sites in the B2 domains of PSG6 and PSG9 are underlined. **B.** Namalwa-SD1 cells were treated with PSG2-V5His (50 µg/ml), PSG4-V5His (30µg/ml), PSG5-V5His (80µg/ml), PSG6B2-Fc (5µg/ml), PSG7-V5His (50µg/ml), PSG8-V5His (50µg/ml), PSG9-Fc (20µg/ml), PSG11-V5His (50µg/ml), and a protein consisting of the Fc tag (5 and 20µg/ml) used as control. Binding of the proteins containing V5His tags was detected with the anti-PSG MAb6799 (anti-PSG), followed by APC-conjugated anti-mouse IgG (anti-mIgG-APC). Cells directly incubated with the anti-PSG Ab, followed by anti-mIgG-APC were included as a control for the V5His-tagged PSGs. Binding of the Fc-tagged proteins was detected with an Alexa Fluor 488-conjugated anti-human Fc Ab. The median fluorescence intensity (MFI) of each treatment is shown. **C** and **D**. PAE-YFP cells were seeded on the gelled GFR-Geltrex in the presence of the indicated proteins. Quantitative analyses of the tube structures are expressed as described in Fig. 1B. Results are shown as the mean ± S.D. of replicates; one representative of three individual experiments is shown. **E**. Micrographs showing capillary sprouting of mouse aortic rings in the presence of PSG1, PSG1ΔB2, or PBS (control), four days post-treatment. Quantitative analyses of maximum sprouting distance per well (n≥15 of N=3 experiments) are expressed after normalization to control. Statistical significance was

obtained by Student's *t*-test (in C) or by one-way ANOVA, followed by Sidak's multiple comparison test (in E) (****$P<0.0001$, ***$P<0.0002$).

Chapter 4:
DISSERTATION DISCUSSION
&
FUTURE DIRRECTION

4.1 DISCUSSION

The work of this dissertation was intended to extend our current knowledge about the role of PSG1 in placenta formation during pregnancy. The purpose of these studies was to determine how PSG1 may have a role in the regulation of EVT migratory and invasive behavior and in placental angiogenesis; we were also interested in determining the underlying functional mechanisms for these interactions. My findings in this dissertation support our hypothesis that PSG1 has a regulatory role in the integrin-dependent enhancement of EVT adhesion and migration as well as the pro-angiogenic role in both EVTs and ECs. Our work has some novel findings that ultimately help us to gain an understanding of how PSG1 works in several aspects of placental biology. These are discussed below.

PSG1 facilitates EVT adhesion

During placenta formation, EVTs originate in the anchoring villi that physically attaches the placenta to the uterus (89). The uterine wall undergoes decidualization, which involves extensive remodeling of the extracellular matrix, and EVT migration and invasion into the uterus starts with the attachment of EVTs into the decidualized uterine wall (70; 99). Based on our results, we propose that binding of PSG1 to heparan-sulfate proteoglycans in the decidual ECM contributes to the adhesion of EVTs in an integrin-dependent manner.

Integrin α5β1 is a new receptor for PSG1

Shanley and co-workers reported that the PSG-integrin αIIbβ3 interaction exerts an inhibitory function by blocking the platelet-fibrinogen interaction (87). Besides the KGD-tripeptide motif, which is recognized by integrin αIIb3, human PSGs have

conserved RGD or RGD-like tripeptide motifs. Therefore, it has been proposed that PSGs are candidates as potential ligands for the RGD-binding integrins and that this interaction might mediate diverse PSG-mediated functions (87). Because integrin switching into integrin α5β1 is linked to the migratory and invasive behaviors of EVTs, PSG1's ability to induce EVT adhesion through α5β1 suggests that PSG1 may play an important regulatory role in EVT migration in the decidua (4; 27). Importantly, placenta development progresses in a low oxygen environment in the first trimester when the differentiation of CTBs into EVTs occurs extensively in an oxygen gradient (46). Moreover, integrin α5β1 is upregulated in the low oxygen condition via HIF1α stabilization in EVTs in the first-trimester placenta (5; 55). Here, we showed that PSG1 induces the adhesion of EVTs in an integrin α5β1-dependent manner in both normal oxygen and low oxygen environments (76).

PSG1 becomes incorporated in the ECM through HSPG binding and facilitates anchoring of the EVTs

Our results indicate that even though PSG1 is a secreted protein, it has a role in EVT adhesion only when this protein is immobilized. As stated above, we suggest that EVTs could potentially bind to PSG1 anchored to the decidual ECM via its interaction of the B2 domain of the protein with HSPGs. Here we showed that PSG1 binds to the ECM deposited by fibroblasts in a HS-dependent manner (69). The findings presented in this dissertation under Aim 1 are represented in a model shown in Figure 1. In this model, PSG1 becomes incorporated into the extracellular matrix through HS binding and subsequently interacts with integrin α5β1 to facilitate the adhesion of EVTs during placenta formation.

PSG1-integrin α5β1 leads to downstream signaling

Integrin clustering initiated by ligand binding results in focal adhesion (FA) formation that involves the recruitment of signaling molecules, such as focal adhesion kinase (FAK) and cytoskeletal adaptor proteins, such as vinculin and paxillin, to the integrin cytoplasmic tails (75). FAK and paxillin are expressed in the first-trimester EVTs (63). Moreover, expression of activated-FAK has been colocalized with integrin α5 in EVTs (63). Therefore, our observation of activated FAK and the detection of FA-adaptor proteins in the PSG1-adhered cells by our collaborators strongly suggest that PSG1-mediated adhesion activates downstream integrin-mediated signaling (48).

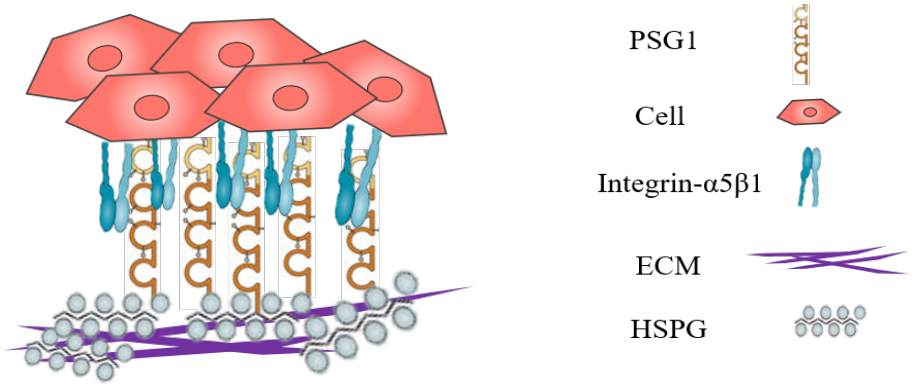


Figure 1. **The proposed model of how PSG1 may facilitate adhesion of EVTs during placenta formation**. In this model, PSG1 is incorporated into the extracellular matrix and interacts with integrin α5β1 to facilitate the adhesion of EVTs during placental formation (left). The labels of the objects drawn in the model are shown (right).

The PSG1-mediated adhesion enhances EVT migration

Integrin-mediated signaling regulates anchorage-dependent cell behaviors, including proliferation, migration, and invasion. Integrin α5β1-dependent attachment of EVTs to the uterine ECM and FAK-mediated signaling are important regulators of their migration during early placental development (35; 63). Our observation of enhanced

migration of primary EVTs and EVT cell lines on PSG1-coated wells suggests that PSG1 also facilitates EVT migration during placenta formation. This finding, together with the finding of FAK-activation and FA formation in the PSG1-mediated adhesion, constitutes the second aim.

PSG1 is pro-angiogenic on ECs and EVTs

Vascular adaptations through remodeling of decidual spiral arteries are required during pregnancy to ensure that nutrient and oxygen supply from the mother is passed on to the growing fetus. Therefore, non-pathological angiogenesis is normally observed in the decidua and the placenta (92). During placental vascular remodeling, EVTs come in direct contact with decidual cells, including natural killer cells and macrophages, and ECs to contribute toward decidual angiogenesis (11; 24). Previously we observed that the interaction of PSG1 with HSPG was required for *in vitro* endothelial tube formation (60). Our current studies show that PSG1 has a pro-angiogenic effect in both ECs and EVTs.

The B2 domain of PSG1 interacts with cell surface HSPGs and exerts the pro-angiogenic effect on the EVTs and ECs

We report here that as previously observed in ECs, PSG1 binds to HS-expressed on the membrane of EVTs as this interaction is competed by the presence of excess heparin. However, the domain(s) of PSG1 that bind to HS had not been identified. Experiments shown as part of this dissertation show that the B2 domain is required for the interaction with HS and for the PSG1pro-angiogenic activity demonstrated in ECs and EVT cell lines.

Conservation of the ability to bind to HSPG by members of the human PSG family

We investigated all human PSGs, except PSG3, and observed that all PSGs tested bind to HSPGs. Moreover, we reported that like PSG1, PSG6 and PSG9 induce endothelial tube formation. Because all tested human PSGs bind to HSPG, and PSG1, PSG6, and PSG9 are pro-angiogenic; therefore, all human PSG may have pro-angiogenic activity. Interestingly, our laboratory has previously shown that a murine member of the PSG family induced endothelial tube formation. We speculate that while binding to HS via different domains (the B2 domain in human PSGs and the N1 domain in murine PSGs), human and murine PSGs, which have significant differences in amino acid composition, have retained this function. Whether PSGs from all species share this function should be further investigated.

Clinical Significance

Several studies indicate that pregnancy disorders, including PE, have long-lasting health effects predisposing both mother and child to heart disease, obesity, and insulin resistance (1; 28; 53; 77). Although PE is a complex disorder with a poorly understood pathogenesis, most studies suggest that it is related to abnormalities in the placenta (45). Mechanistic studies are thus needed to define the processes involved in placental biology in order to determine the pathophysiology of PE. Among PSGs, PSG1 is one of the few PSGs expressed during early placental development in which dysregulation may result in the PE (87). The results presented here suggest an important role of PSG1 in processes important in placental development. These include the adhesion and migration of EVTs and the angiogenic activities of EVTs and ECs. In the present study, we reported reduced serum concentrations of PSG1 in African American women diagnosed with early- and late-onset PE (12; 72; 83). Interestingly, this observation occurs only when the women

diagnosed with PE carried a male fetus. The overall clinical observation suggests that PSG1 is important for normal placenta development, albeit its functions may affect placenta formation differently based on fetal gender. Nevertheless, the current studies indicate that a therapeutic potential of PSGs for the treatment of PE should be further investigated.

4.2 FUTURE DIRECTIONS

4.2.1 Investigation of the correlation between fetal gender and the expression of PSG1 and other human PSGs

African-American ethnicity is considered an increased risk of developing PE, therefore we investigated PSG1 levels in pregnant women of this ethnicity (61). We found that the serum PSG1 concentration was significantly lower in women diagnosed with early- and late-onset PE compared to the gestational age-matched controls only when the fetus was a male (76). While fetal sex has been correlated with PE, there are conflicting reports regarding this observation (12; 72). Some reports propose a male fetus as an increased risk factor, some indicating no relationship with fetal gender, and others suggesting a greater risk in female fetus-bearing pregnancies (2; 30; 43; 64; 94). It was also observed that a male fetus posed an increased risk of PE only in certain ethnicities, such as non-Asian and African-American populations (47; 61). Further studies should be conducted to determine whether lower levels of PSGs correlate with PE only in male babies in women of other ethnicities. In addition, future studies should be designed to address the following questions.

a) Why PSG concentration is lower in pre-eclamptic women only when carrying a male fetus; is there a difference in the function or requirement of PSG1 for a successful pregnancy based on fetal gender?
b) What is the expression level of other PSGs in normal and pre-eclamptic pregnancies?
c) Could there be elevated expression of other PSGs that potentially compensate for a lower PSG1 level?

4.2.2. Investigation of PSG1-mediated effect on the invasion of EVTs using a placental-decidual explant co-culture technique

Although PSG1 has migration-enhancing effects on EVTs, we were unable to detect any differential effect of PSG1 on the invasive capacity of these cells (76). During placenta formation, EVTs invade the decidua where other cell types, such as the decidual fibroblasts, uterine natural killer cells, and macrophages, modulate this process (73). While we used standard methods (Transwell invasion and Biogel cell invasion assays) in our invasion study with EVT cell lines, we could not recapitulate the complex nature of the cell-cell interaction that happens during placenta development (76). Since the invasion of the uterus by EVTs is controlled by autocrine and paracrine factors, the potential invasion-modulating effect of PSG1 may require the presence of both factors (13). Therefore, future studies using complex models that more closely simulate the process *in vivo,* such as placental-decidual explant co-culture systems, are warranted to investigate the potential role of PSG1 in EVT invasion (32).

4.2.3. Investigation of the effect of PSG1 on the STB invasion

Our collaborator has demonstrated that EVTs are an additional source of PSGs along with the STB, which is the major producer of PSGs (76). While EVTs are the major invasive cells in the placenta, the STB is responsible for the initial invasion of the decidua during implantation (44). The secretion of PSGs starts as early as 3-days post-fertilization, which precedes the implantation window (31). Moreover, the uterine wall undergoing decidualization expresses several integrins, including the integrin α5β1, the receptor of PSG1 for its adhesion inducing function (74; 76; 104). Therefore, PSGs secreted by the STB may remain bound to the primary syncytiotrophoblast cell surface and facilitate the interaction of the syncytial layer as it attaches to the uterus, thereby assisting the initial placental invasion process. Therefore, studies of PSG1 interactions with STB are also warranted.

Chapter 5
APPENDIX

5.1 Modulatory role of PSG1 glycans in its interaction with integrin α5β1

To characterize the interaction of PSG1 and integrin α5β1, recombinant PSG1 and the N-domain of PSG1 (PSG1N) were generated using the following expression systems: 1) PSG1 was generated in stably transfected CHO-K1 cells ($PSG1_{stb}$), 2) PSG1 was generated by transient transfection of CHO-K1 cells ($PSG1_{transCHO}$), 3) PSG1N was generated in stably transfected CHO-K1 cells ($PSG1N_{stb}$), and 4) PSG1N was generated by transient transfection of CHO-K1 cells ($PSG1N_{transCHO}$). While PSG1 purified from serum of pregnant women induced cell adhesion in an integrin α5β1-dependent manner, the cell adhesion-inducing ability of recombinant PSG1 varies depending on the methods utilized for protein generation. I found that $PSG1_{stb}$ induced cell adhesion while $PSG1_{transCHO}$ did not (Figure 1). Interestingly, the ability of PSG1 to activate TGF-β was observed for all tested PSG1 preparations, irrespective of the cell line utilized for its generation (14; 101).

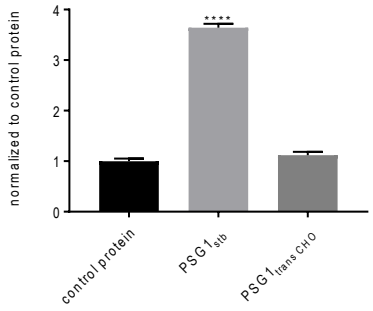


Figure 1. **The cell adhesion ability of PSG1 varies depending on the protein expression system utilized for its generation**. Indicated proteins were coated at 30μg/ml on wells of a 96-well plate. HEECs were seeded on the wells at 2×10^4cells/well and incubated for 2h at 37°C. After washing off the unbound cells, the cells remaining on the wells were incubated with cell titer aqueous solution for 2h at 37°C, and the absorbance at 490nm was recorded. The absorbance values of the wells coated with the different recombinant PSG1s were normalized to the control protein-coated wells. Statistical significance was obtained by one-way ANOVA followed by Sidak's multiple comparison test (****p <0.0001).

A similar phenomenon was observed when comparing the ability of the N-domain of PSG1 to induce cell adhesion. $PSG1N_{stb}$ induced cell adhesion while $PSG1N_{transCHO,}$ did not (Figure 2). Like PSG1, the expression methods utilized to generate the recombinant PSG1N influences the ability of the protein to bind to integrin α5β1.

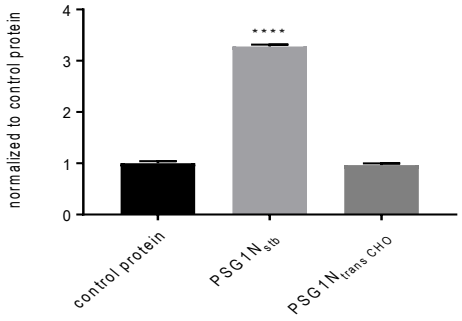


Figure 2. **The ability of PSG1N to induce cell adhesion is dependent on the expression system-utilized to generate the protein**. Indicated proteins were coated at 30μg/ml on wells of a 96-well plate. The cell adhesion assay with HEECs was performed as described in Fig. 1. The absorbance values of the wells coated with the two recombinant PSG1N were normalized to the control protein-coated wells. Statistical significance was obtained by one-way ANOVA followed by Sidak's multiple comparison test (****p <0.0001).

To understand the difference between the recombinant proteins, they were resolved on an SDS-PAGE gel and visualized with Gel Code Blue. The PSG1N protein generated in stably transfected CHO-K1 cells ($PSG1N_{stb}$) has a larger molecular weight (MW) than $PSG1N_{transCHO}$ (Figure 3), suggesting differences in post-translational modification of PSG1N. Importantly, PSG1 has seven potential glycosylation sites; three of them are in the N-domain at positions 61, 104, and 111. Therefore, the observed MW difference between $PSG1N_{stb}$ and $PSG1N_{transCHO}$ could be due to differences in glycan composition, which may influence their activity.

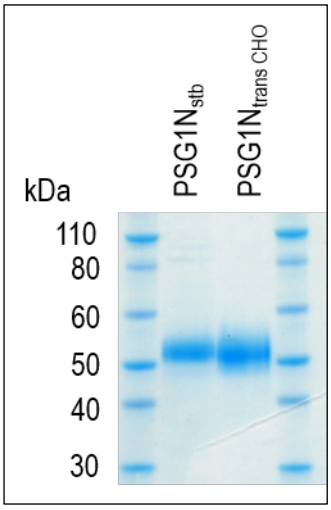


Figure 3. **Recombinant PSG1N generated in different expression systems.** Fc-tagged PSG1N generated in the stably transfected CHO-K1 cells ($PSG1N_{stb}$) and by transient transfection of CHO-K1 cells ($PSG1N_{transCHO}$) were purified using protein A columns. Purified $PSG1N_{stb}$ and $PSG1N_{trans\ CHO}$ were separated on a 4-12% NuPAGE gel and were stained with GelCode Blue Safe protein stain.

To investigate the potential effects of glycosylation, I designed a glycosylation mutant (GM) of PSG1N in which all three potential N-glycosylation sites were destroyed by mutating the asparagines to alanines (Asn61, 104, and 111→Ala). I generated the GM protein by transient transfection and compared its MW to the wild type protein. As predicted, PSG1N $GM_{transCHO}$ has a smaller MW than $PSG1N_{transCHO,}$ as shown in Figure 4, confirming the presence of glycans on at least one of the three-glycosylation sites in $PSG1N_{transCHO}$. This conclusion is supported by a glycoproteomic analysis performed by Dr. Anne Dell (Imperial College of London), showing that Asn61 is occupied with glycans in native PSG1 (manuscript in press).

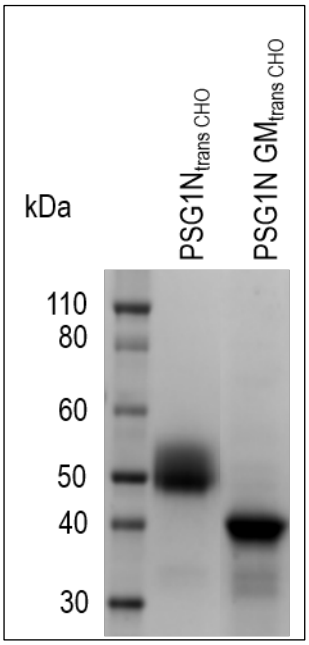


Figure 4. **Recombinant PSG1N and its glycosylation site mutant.** Fc-tagged PSG1N and its glycosylation mutant were generated by the transient transfection of CHO-K1 cells ($PSG1N_{transCHO}$ and PSG1N $GM_{transCHO}$, respectively). The proteins were purified using protein A columns and were separated on a 4-12% NuPAGE gel, followed by staining with GelCode Blue Safe protein stain.

Next, I tested PSG1N to determine the importance of gycosylation for the ability to induce cell adhesion. PSG1N GM generated either by transient transfection of CHO-K1 or ExpiCHO cells induced cell adhesion (Figure 5). This result indicates that the presence of glycans is not required for the cell adhesion-inducing ability of PSG1N. It is important to note that $PSG1N_{trans\ ExpiCHO}$ induced cell adhesion, while $PSG1N_{trans\ CHO}$ did not (Figure 5). In addition, PSG1N generated in the Expi293 cells ($PSG1N_{trans\ Expi293}$) did not induce cell adhesion (data not shown). The differential activity of $PSG1N_{trans\ ExpiCHO}$ and $PSG1N_{trans\ CHO}$/ $PSG1N_{trans\ Expi293}$ is likely due to the differences in glycosylation of the proteins. Therefore, I suggest that the presence of specific glycans (like the ones present in $PSG1_{transCHO}$ and $PSG1_{transExpi293}$) can have an inhibitory effect on the cell adhesion function of PSG1N. Interestingly, published reports indicated the importance of

glycosylation in the function of several pregnancy-related proteins. For example, a variation in glycan composition of glycodelin, a protein secreted in the early placental development by the decidua, greatly affects its function and receptor binding ability (26; 56; 58; 85). Also, the glycosylation of human chorionic gonadotropin varies with the stages of pregnancy, the sites of synthesis, and the pathological state (33). Moreover, recent reports indicate that pregnancy pathologies, including spontaneous miscarriage, intra-uterine growth restriction, and PE, are linked to glycosylation alterations (57; 80; 100; 106). While initial studies indicate that there are changes at the level of the entire N-glycome in the placenta in early-onset PE, the specific glycoproteins affected have not been identified (79). Therefore, glycoproteomic studies of PSG1 purified in a defined times of gestation in normal and pre-eclamptic pregnancies could reveal glycan differences that could be utilized as a potential biomarker for PE diagnosis.

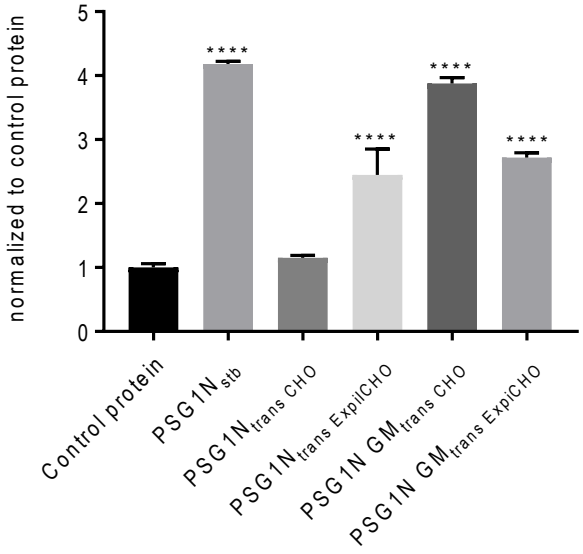


Figure 5. **The ability of PSG1N to induce cell adhesion is modulated by glycans, although glycans are not required**. Indicated proteins were coated at 30µg/ml on wells of a 96-well plate. The cell adhesion assay with HEECs was performed as described in

Fig. 1. The absorbance values of the wells coated with PSG1N and PSG1N GM were normalized to the control protein-coated wells. Statistical significance was obtained by one-way ANOVA followed by Sidak's multiple comparison test (****p <0.0001).

I extended the investigation of PSG1N-mediated cell adhesion to identify, which amino acids in the N-domain bind to α5β1 integrin. Our laboratory has previously generated two mutants of the N-domain based on a model created by Dr. Ballesteros (NIH, Bethesda, USA) in which two likely exposed loops, C-C' and C'-C", were mutated to the corresponding sequences in CEACAM5, a member of the CEA family (Figure 6A) (9). The mutants were designated as PSG1N C-C' mutant and PSG1N C'-C" mutant (Figure 6B). Since the ExpiCHO-expressed PSG1N induced cell adhesion, I generated both mutants using the ExpiCHO expression system. I compared the cell adhesion ability of the two mutants with the wild type N-domain protein.

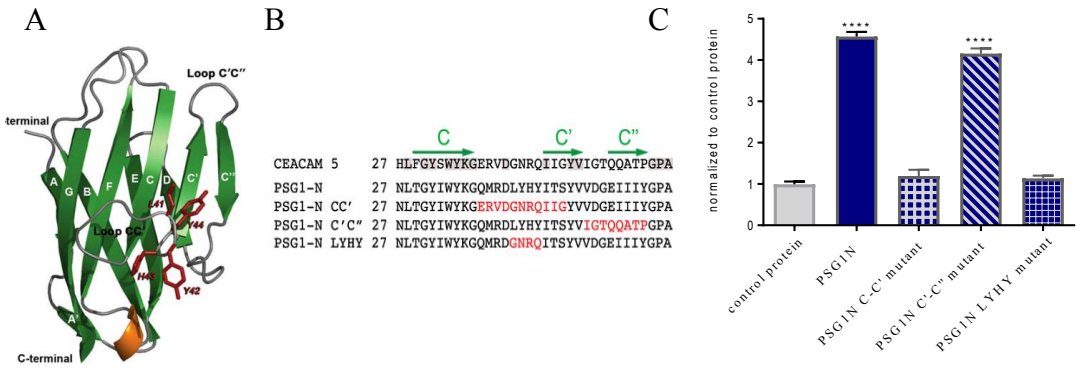


Figure 6. **The amino acids LYHY in the N-domain of PSG1 are required for its cell adhesion activity.** A. A predicted model of the N-domain of PSG1 (9). B. Sequence alignment of the C-C' and C-C" loops of CEACAM5, PSG1N, and its mutants described (9). C. Indicated proteins were coated at 30µg/ml on wells of a 96-well plate. The cell adhesion assay with HEECs was performed as described in Fig. 1. The absorbance values of the wells coated with PSG1N and its mutants were normalized to the control protein-coated wells.

While the PSG1N C'-C'' mutant induced adhesion, the PSG1N C-C' mutant did not. We generated another mutant by changing the $L_{41}Y_{42}H_{43}Y_{44}$ amino acids in the C-C'

loop to GNQR (PSG1N LYHY mutant), the amino acids present in the similar position of CEACAM5. PSG1N LYHY mutant did not induce cell adhesion (Figure 7). Therefore, I concluded that amino acid LYHY in the N-domain is required for the ability of the N-domain of PSG1 to induce cell adhesion.

Besides the N-domain, the B2-domain of PSG1 also induces cell adhesion. Therefore, it will be interesting to determine which amino acids in the B2-domain bind to integrin α5β1 to induce cell adhesion.

5.2 Identification of the integrin α subunit that interacts with the A1 domain of PSG1 to mediate cell adhesion

I showed that two domains of PSG1 (N and B2) individually induce cell adhesion, which is the result of the PSG1-integrin α5β1 interaction (76). Also, I observed that the A1 domain of PSG1 induces cell adhesion, which was inhibited with a peptide (GRGDNP) containing an RGD tripeptide motif, a minimal integrin recognition sequence conserved among certain extracellular matrix proteins, but not with a control peptide (GRADSP) (Figure 7A). Besides, the A1-mediated adhesion was inhibited with an integrin β1-neutralizing antibody (Figure 7B), as we observed for the full-length PSG1 containing four domains (N, A1, A2, and B2). As opposed to the full-length protein and the two other domains of PSG1, I was unable to inhibit the A1-induced adhesion with an anti-α5 neutralizing antibody. A knockdown of integrin-α5 in EVTs may be required to rule out the involvement of this integrin in the A1 domain-mediated cell adhesion.

In addition, I could not inhibit the A1-induced adhesion with a neutralizing antibody to integrin-α1β1, an integrin expressed by EVTs [34]. Further experiments are needed to identify the integrin α subunit, which, together with the β1 subunit, is involved in the A1-

mediated cell adhesion. Besides α5β1, other β1-containing and the RGD motif recognizing integrins expressed on the EVTs are αvβ1, and α8β1, which should be considered for the search of the A1-domain interacting integrin α subunit (41; 69).

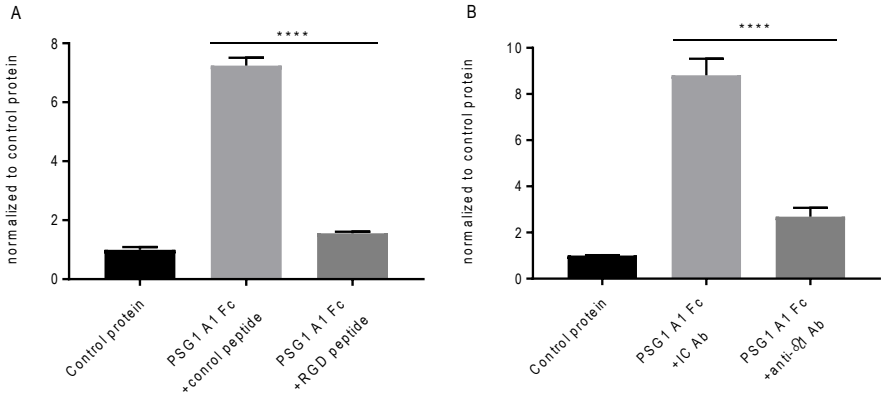


Figure 7. The PSG1A1-mediated adhesion is inhibited by the RGD-peptide and by a neutralizing anti-integrin β1 Ab. Indicated proteins were coated at 30μg/ml on wells of a 96-well plate. HEECs were treated with control peptide or RGD peptide (A) and with isotype control antibody (IC Ab) or anti-β1 Ab (B) for 30min at RT. The cells were seeded on the wells at 2×10^4cells/well and incubated for 2h at 37°C. After washing off the unbound cells, cells remaining on the wells were incubated with cell titer aqueous solution, and cell adhesion was quantified as the absorbance at 490nm. The absorbance values of the wells coated with PSG1A1 were normalized to that of the control protein-coated wells. Statistical significance was obtained by one-way ANOVA followed by Sidak's multiple comparison tests (****p <0.0001).

5.3 Investigation of the cell adhesion-inducing ability of other PSGs and the receptors involved in that function

I observed that PSG4, PSG9, and a mouse PSG, PSG23, induce adhesion of EVTs and ECs. Moreover, PSG4 (Figure 8A) and PSG23-mediated adhesion (Figure 8B) are inhibited with an RGD peptide (GRGDNP) but not with the control peptide (GRADSP). In addition, the PSG9-mediated adhesion is blocked with anti-β1 Ab (Figure 8C). Further experiments are needed to characterize the PSG4, PSG9, and PSG23-mediated

trophoblast adhesion and a potential involvement of integrin α5β1. In addition, other human and murine PSGs should be tested for integrin interaction and functions.

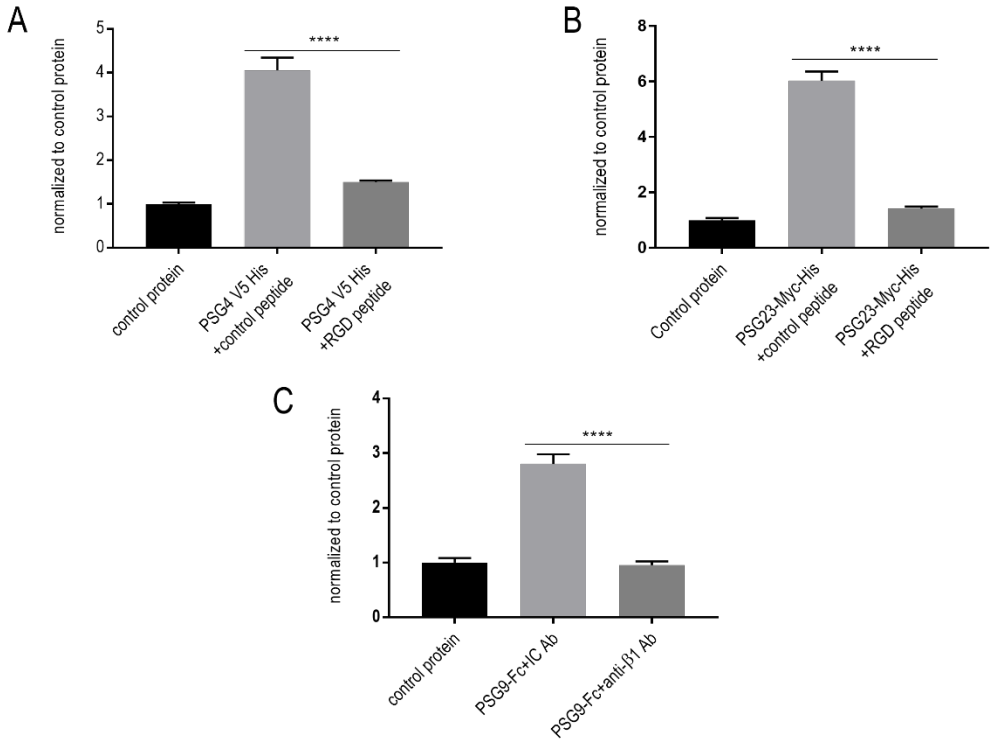


Figure 8. The PSG4, PSG9, and PSG23-induced adhesion of the HEECs, human dermal microvascular ECs (HDMECs), and the trophoblast cells (Swan71), respectively. Indicated proteins were coated at 30μg/ml on 96-well plates. HEECs (A) or Swan71 cells (B) without treatment or treated with a control peptide (GRADSP) or the RGD peptide (GRGDNP) at 500μM, or HMVECs (C) without treatment or treated with indicated Abs were seeded on the wells at 2×10^4cells/well, and the adhesion experiment was carried out as described in Fig. 1. The absorbance values of the wells coated with PSG4 or PSG23 were normalized to that of the control protein-coated wells. Statistical significance was obtained by one-way ANOVA followed by Sidak's multiple comparison test (****p <0.0001). IC= isotype control.

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