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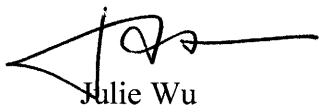

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ABSTRACT

Title of dissertation:	Induction of Pro-Angiogenic Factors by Pregnancy-Specific Glycoproteins and Studies on Receptor Usage
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Pregnancy-specific glycoproteins (PSGs), also referred to as Schwangerschafts-protein 1 (Sp1) or pregnancy-specific β1 glycoproteins, are a family of proteins secreted by the syncytiotrophoblast whose expression increases as pregnancy progresses and correlates with pregnancy success. Although PSGs have been identified for more than 30 years their functions have yet to be fully elucidated. PSGs belong to the carcinoembryonic antigen (CEA) family of proteins whose members have been implicated in the processes of angiogenesis, cell adhesion, immunomodulation and cell migration. The ability of human PSG1, 6 and 11 and murine PSG17, 18 and 19 to induce anti-inflammatory cytokines has led to the hypothesis that these proteins may function to protect the fetus from a potential maternal immune attack. Recent reports demonstrated positive staining for murine PSGs lining the capillaries within the decidua at the implantation site, suggesting a possible role for PSGs in maternal vasculature remodeling. To determine if PSGs have a functional role in angiogenesis, we generated three new recombinant PSGs, murine PSG22 and 23 and human PSG1d, and studied the ability of these proteins to up-regulate the pro-angiogenic factors

transforming growth factor (TGF)-β_1 and vascular endothelial growth factor (VEGF) in a murine macrophage cell line (RAW 264.7), primary human and mouse monocytes/macrophages, a murine and human endothelial cell line (C166 and uterine microvascular endothelial cells, respectively) and immortalized murine and human (HTR-8/SVneo) trophoblast cells. We observed up-regulation of TGF-β_1 and VEGF-A after PSG treatment in the cell types we tested which suggests that PSGs are involved in remodeling of the vasculature. Previously, our lab identified the tetraspanin CD9 as the receptor for murine PSG17 and 19 and reported that CD9 is not the receptor for human PSG1 and 11. Tetraspanins are a family of transmembrane proteins which are involved in numerous cellular processes, including angiogenesis. We determined that the effects on macrophages which PSG22 and 23 elicit were not dependent on CD9; however, initial binding studies revealed that PSG23 may bind to the tetraspanin CD151. CD151 is closely associated with the laminin-binding integrins, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$, and has been observed to play a role in vessel formation. The possibility that CD151 serves as a receptor for PSG23 may imply a putative function for PSGs in the development and maintenance of the placental vasculature. In addition, we screened a human spleen and RAW cell cDNA library to clone and identify a receptor for human PSG1d and 11. Although we have not currently identified a receptor for a human PSG, our data presents important first steps towards isolating a receptor for human PSGs. In conclusion, we have shown that PSGs up-regulate pro-angiogenic factors and identified novel targets for PSG binding and activity. Together these results support a role for PSGs in placentation.

INDUCTION OF PRO-ANGIOGENIC FACTORS BY PREGNANCY-SPECIFIC GLYCOPROTEINS AND STUDIES ON RECEPTOR USAGE

by

Julie A. Wu

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TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY

A431 Rx:	human CD151-silenced epithelial carcinoma cell line stably transduced to re-express CD151
A431 sh3:	stable human CD151-silenced epithelial carcinoma cell line
Ang1:	angiopoietin 1
Ang2:	angiopoietin 2
CEA:	carcinoembryonic antigen
CEACAM:	CEA-related cell-cell adhesion molecule
CTB:	cytotrophoblast
DC:	dendritic cell
dNK:	decidual natural killer
EC1:	extracellular loop 1
EC2 (LEL):	extracellular loop 2 (large extracellular loop)
EVT:	extravillous cytotrophoblast
FITC:	fluorescein isothiocyanate
GFP:	green fluorescent protein
GST:	glutathione S-transferase
HLA:	human leukocyte antigen
HTR-8/SVneo:	human trophoblast cell line
IL:	interleukin
IUGR:	intrauterine growth retardation
KIR:	killer immunoglobulin-like receptor
MMP:	matrix metalloprotease

NK:	natural killer
PE:	phycoerythrin
PI3K:	phosphatidylinositol-3-kinase
PI4K:	phosphatidylinositol-4-kinase
PKC:	protein kinase C
PlGF:	placental growth factor
PSG:	pregnancy-specific glycoprotein
RGD:	arginine-glycine-asparagine
STB:	syncitiotrophoblast
TGF-β:	transforming growth factor beta
Th1 or 2:	T helper-type 1 or 2
Treg:	regulatory T cell
VEGF:	vascular endothelial growth factor
uNK:	uterine natural killer
UtMVEC:	uterine microvascular endothelial cell line

PART ONE:

INTRODUCTION

INTRODUCTION

Significance

Importance of proper placental development

Human pregnancy is a unique physiologic state, in which fetal cells come into direct contact with maternal tissue. The development of the placenta, which is crucial to the successful establishment of pregnancy, requires synchronization of several important processes. The intimate association between the placenta and the endometrium, or decidua, results in different levels of immune interaction between mother and fetus throughout gestation. As the fetal trophoblast cells of the placenta migrate through the decidua they come into contact with maternal leukocytes, which do not normally interfere with the process of placentation.

The human placenta is classified as haemochorial because trophoblast cells are highly invasive [1]. The process of implantation is tightly regulated. Uncontrolled invasion of the trophoblast cells is associated with choriocarcinoma; conversely, incomplete invasion of the maternal spiral arteries during implantation can lead to various pregnancy complications, including intrauterine growth retardation (IUGR), pre-eclampsia and, in certain cases, spontaneous abortion [2]. Importantly, pre-eclampsia is the leading cause of maternal and fetal morbidity and mortality associated with pregnancy world-wide [2]. In the U.S. alone there are approximately 1 million fetal losses each year, 70% of which occur during the very early stages of pregnancy [3]. The link between insufficient trophoblast invasion and immune dysfunction has been known for some time, and 30% of early spontaneous abortions are believed to be due to immune dysfunction [4]. It is speculated that the immune cells at the

maternofetal interface are vital to modulating the maternal immune system to tolerate the developing fetus and to regulating placentation. At present, the factors that influence the leukocytes at the site of maternal and fetal cell interaction are currently unclear.

Invasion and placentation

Process of placentation

The blastocyst is comprised of an inner cell mass that will give rise to the embryo proper and a surrounding outer cell mass that will form the trophoblast and give rise to the placenta [5]. Implantation is an invasive process. Once the blastocyst attaches to the uterine epithelium, the outer cells differentiate into the mitotically active cytotrophoblast (CTB) cells surrounded by the multinucleated syncytiotrophoblast (STB) cells that erode the tissues of the uterine lumen at the attachment site [6]. Approximately day 14 of pregnancy invasive cytotrophoblast cells, referred to as extravillious cytotrophoblast (EVT) cells, migrate down the uterine spiral arteries and replace the maternal vascular endothelium [7]. EVTs remodel the uterine spiral arteries to initiate the uteroplacental blood flow [8-10]. The maternal blood fills the intervillous space of the placenta by week 11 of pregnancy, bathing fetal tissues with essential nutrients. Pre-eclampsia occurs when the invasion and replacement of maternal vascular tissue by the EVT cells is incomplete, constricting the flow of maternal blood to the developing fetus and causing an ischemic and hypertensive condition in the mother [8]. (Figure 1)

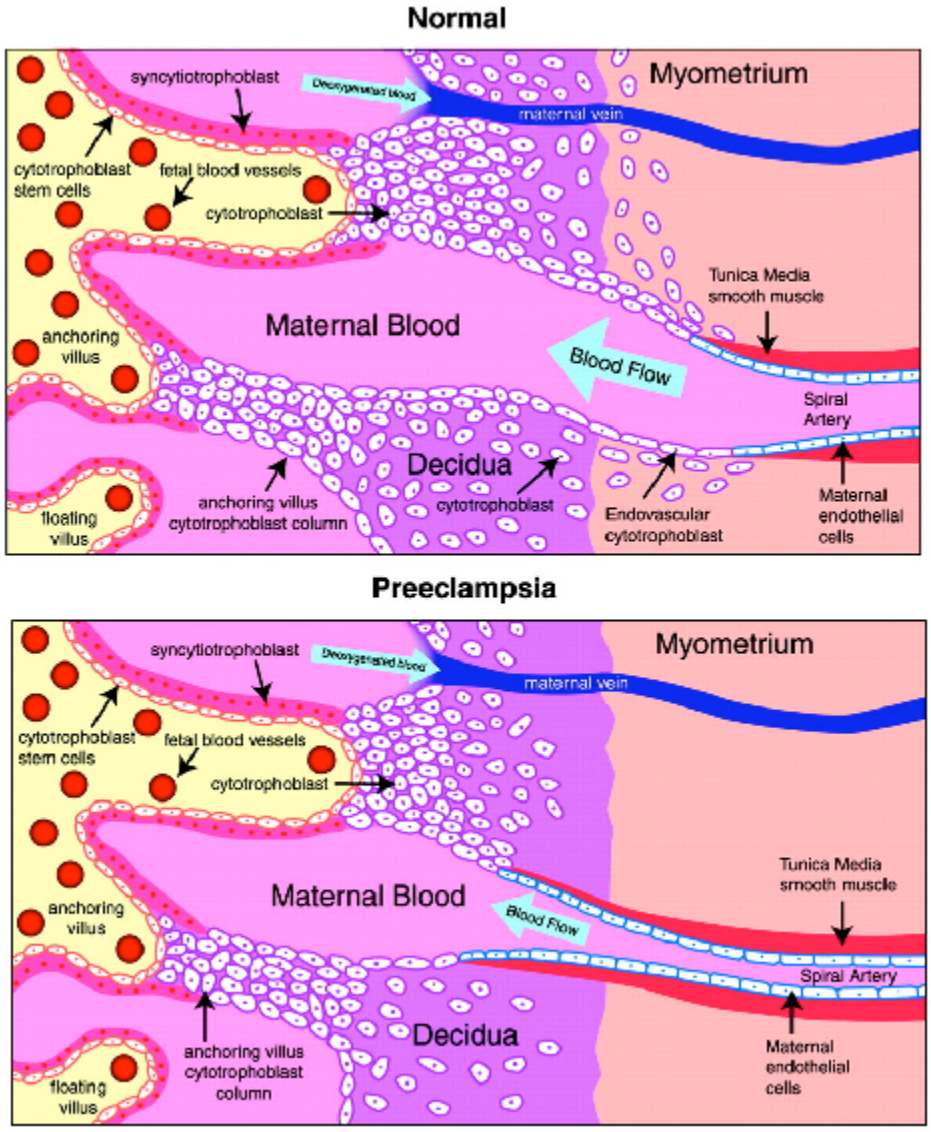


Figure 1: Normal and abnormal placentation. In normal placental development (top panel), EVTs invade and remodel the decidual spiral arteries to allow adequate placental perfusion to sustain the developing fetus. In pre-eclamptic pregnancies (bottom panel) incomplete invasion of the spiral arteries results in severely reduced blood flow leading to the development of fetal hypoxia, ischemia and hypertension. [9]

Due to the ethical restrictions on testing in pregnant women, our current understanding of trophoblast invasion and placentation comes from studies that rely on terminated pathological pregnancies and animal models [10]. Non-human primates provide the closest animal models of human pregnancy; however, rodents are more practical subjects due to availability of transgenic cell lines, brevity of their gestation times and large litter sizes [11]. Although the process of placentation in rodents is not as invasive as it is in humans [12], many of the cell types, growth factors, cytokines and hormones involved in human pregnancy are identical to those found in rodent pregnancy.

Vascular endothelial growth factor

Blood vessels develop in two sequential processes: (1) formation of primitive capillaries by in situ differentiation of hemangiogenic stem cells, known as vasculogenesis, and (2) formation of new blood vessels from the existing vessels, known as angiogenesis [13]. Members of the vascular endothelial growth factor (VEGF) family are primary players during vasculogenesis and angiogenesis [14]. The VEGF family targets numerous cell types, including endothelial cells, monocytes and trophoblasts [15-17]. VEGFs are also reported to work in conjunction with angiopoietin (Ang) 1 and Ang2 whose actions are tied to the formation of new blood vessels [18].

The VEGF family includes VEGF-A, -B, -C, -D and placental growth factor (PlGF). VEGF-A is the primary family member expressed during placental vasculogenesis which occurs approximately 21 days post-conception [13], though small amounts of VEGF-B, -C and –D have also been detected [19]. VEGF is expressed by trophoblast cells throughout gestation

and is thought to regulate vasculogenesis and angiogenesis at the beginning of pregnancy and to help maintain vessel integrity and permeability at the end of pregnancy [19]. Importantly, lowered amounts of VEGF and PlGF have been found in the serum of women with pre-eclampsia [20].

The five mammalian VEGF ligands, VEGF-A, -B, -C, -D and PlGF, bind to one of three VEGF receptors (VEGFRs): VEGFR1, also known as fms-related tyrosine kinase 1 (Flt1), is utilized by VEGF-A, -B and PlGF; VEGFR2, also known as fms-like tyrosine kinase 1 or kinase domain region (Flk1/KDR), is utilized by VEGF-A; and VEGFR3, also known as fms-related tyrosine kinase 4 (Flt4), is utilized by VEGF-C and –D [21]. VEGFRs are type III receptor tyrosine kinases (RTKs) that belong to the same subclass as the receptors for platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [22]. Clinical observations of pre-eclamptic patients revealed that their serum levels of soluble Flt1 (sFlt1), a naturally occurring VEGF antagonist that binds to free VEGF and PlGF [9], are increased compared to patients with normal pregnancies [23].

VEGF expression is seen in the trophoblast cells within the first few days after implantation [24] and deficiencies in VEGF is often embryonic lethal [25]. In the context of pregnancy, VEGF-A is particularly important as it is abundantly expressed at the maternofetal interface [26]. VEGF-A was first discovered as a rapid inducer of vascular permeability in humans, and, therefore, is sometimes referred to as vascular permeability factor (VPF) [21]. Inactivation of a single *Vegfa* allele in mice leads to embryonic death around embryonic (E) day E11-12 due to a lack of vessel formation and deficient endothelial cell development [25].

Conversely, overexpression of VEGF-A can result in aberrant heart development and embryonic death around day E12.5-14 [27].

Transforming growth factor β

VEGF transcription is induced by transforming growth factor (TGF) β in both human [22] and murine macrophages [23]. Along with other molecular constituents, the TGF-β family of proteins is involved in regulating trophoblast invasion [24]. The TGF-β family consists of greater than 40 members, including TGF-β_1, -β_2 and –β_3, which are expressed by mammals, and have been identified in all the cell types of the endometrium [25, 26]. Several studies have implicated TGF-β_1, -β_2 and –β_3 in all aspects of reproduction, including apoptosis, angiogenesis, cell proliferation, differentiation and tissue remodeling [28]. For example, TGF-β_1 is expressed by the uterine epithelium in early pregnancy and is believed to enhance trophoblast attachment to the endometrium by inducing the production of fibronectin [29]. In addition, TGF-β_1 has also been shown to inhibit trophoblast invasion, in part by the indirect inhibition of matrix metalloproteinases (MMPs) and plasmin [30]. Moreover, neutralizing anti-TGF-β antibodies injected into mice blastocoels were shown to reduce the rate of implantation [31].

The first in vivo evidence that TGF-β is involved in angiogenesis came from the observation that mice injected with TGF-β formed new capillaries [32]. Additional evidence for the role of TGF-β in angiogenesis comes from studies with TGF-β-null mice that are 50% embryonic lethal or die as neonates due to defective vasculogenesis and haematopoiesis [33]. Recent investigations have reported elevated levels of a soluble TGF-β coreceptor, endoglin (sEng),

in the serum of pre-eclamptic patients [34-36], and the level of sEng in the serum of patients correlated with the severity of their disease [37]. Perhaps the strongest evidence that TGF-β is implicated in angiogenesis is that it positively regulates VEGF expression in numerous cell types, including tumor cells [38], mouse fibroblasts [39], epithelial cells [40], first trimester trophoblast cell lines [24] and macrophages [41].

TGF-β expression is tightly regulated. TGF-β is synthesized as an inactive precursor comprised of the active TGF-β protein associated with the propeptide latency associated protein [42]. Under physiological conditions proteolysis of latent TGF-β involves the actions of enzymes such as plasmin, generated by urokinase plasminogen activator [43], and MMPs [44]. Proteins that bind directly to TGF-β, including the proteoglycans decorin, fibromodulin and biglycan, can also control its activity [45].

There are two functional classes of receptors for the TGF-β superfamily ligands, type I and II, which have a conserved intracellular serine-threonine kinase domain [46]. The type II receptor kinase is thought to be constitutively active and can phosphorylate the type I receptor at serine and threonine residues located within a domain which is also rich in glycine and serine residues [47]. Phosphorylation of the type I receptor leads to recruitment of receptor-regulated (r) Smad (small mothers against decapentaplegic, rSmad) 1, 2, 3, 5 and 8 [47]. The type I receptor phosphorylates the rSmads allowing them to complex with the common mediator Smad4 [46]. The heterodimeric Smads translocate to the nucleus and bind to specific Smad-binding elements in the promoter regions of target genes [48].

Leukocytes at the maternofetal interface

The process of implantation and placentation involves immune interactions at the maternofetal interface. As EVT cells migrate through the maternal decidua they come into direct contact with a variety of maternal leukocytes, which include dendritic cells (DCs), monocytes/macrophages and uterine natural killer (uNK) cells [8] (Figure 2).

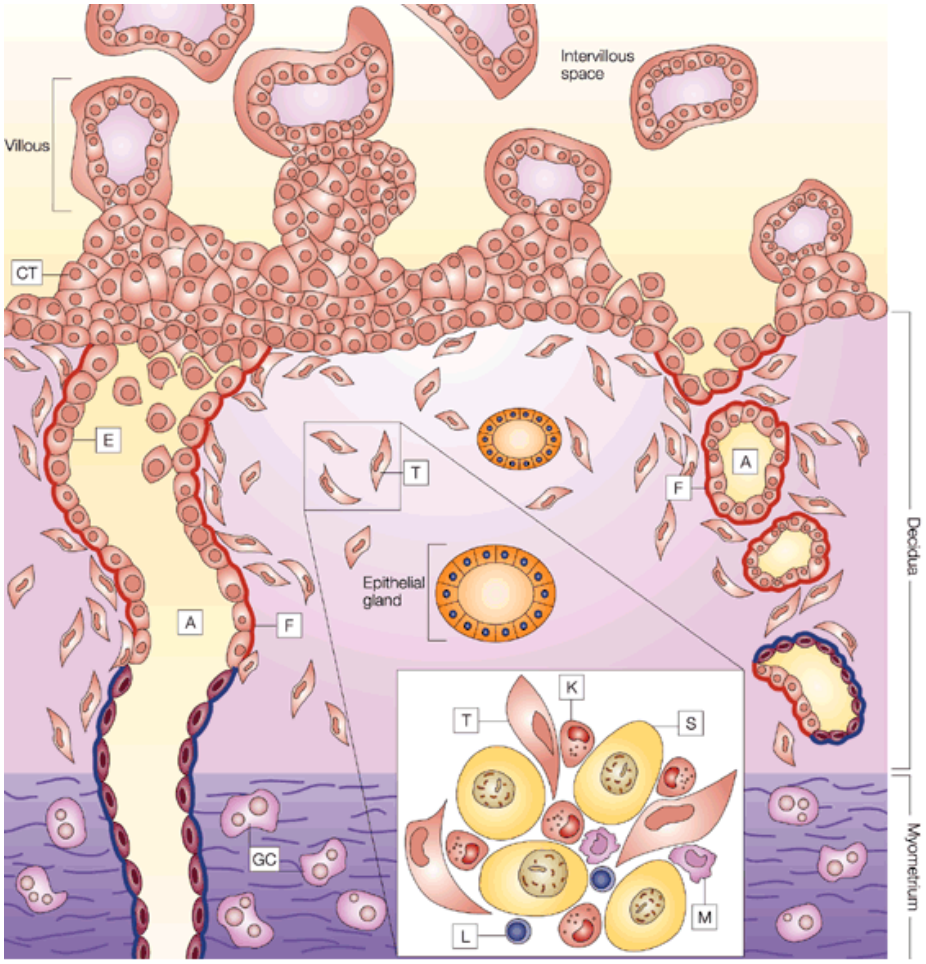


Figure 2: Leukocytes at the maternofetal interface. Resident immune cells in the decidua include macrophages (M) and uNK cells (K) which come into direct contact with fetal tissue. Key: uterine artery (A), CTB (CT), EVT (E), fibrinoid material (F), giant cells (GC), T lymphocytes (L), stromal cells (S) and interstitial trophoblasts (T). [8]

Macrophages have been shown to be involved in vasculogenesis and angiogenesis, but only after stimulation by activating factors [49]. In the pregnant uterus, macrophages account for approximately 20% of the leukocyte population [50]. Recently, it has been reported that macrophages might positively regulate trophoblast invasion by production of interleukin (IL)-10 during pregnancy [51]. Macrophages have been reported to participate in angiogenesis and produce a variety of pro-angiogenic factors, including VEGF [52], after activation [53].

DCs help regulate immune responses at mucosal surfaces, including the uterine lining that gives rise to the decidua during pregnancy. It is hypothesized that DCs in the decidua act in sampling, processing and presenting fetal antigens to the maternal immune system [54]. Although the specific functions of decidual DCs remains unclear, it is possible that they may influence the activation of NK cells at the maternofetal interface [54]. More recent evidence suggests that DCs in the decidua display decreased ability to stimulate proliferation of allogenic, potentially anti-fetal T cells [55].

The primary leukocyte population at the maternofetal interface is uNK cells [8]. Croy et al. showed that uNK cells regulate uterine vascular remodeling by secreting inflammatory cytokines [56]. In human and murine pregnancy, uNK cells are only present during early and mid-pregnancy and are absent at birth [57]. During early pregnancy, uNK cells have been shown to mediate trophoblast invasion [37] and produce pro-angiogenic factors such as VEGF and PlGF [58]. The murine homologue of human uNK cells are a subpopulation of decidual NK (dNK) cells and have been shown to direct trophoblast invasion [37].

Pregnancy-specific glycoproteins in pregnancy

Historical context of the role of PSGs in pregnancy

The immunological paradox of fetal survival amidst the maternal immune background was first articulated in 1953 by Peter Medawar, a transplantation immunologist [59, 60]. His work on the rejection of skin grafts based on antigen recognition in mice (reviewed in [61]) lead him to ask the question of how the fetus, an antigenically foreign body, could exist in and be nourished by the pregnant mother [59]. Medawar compared the fetus to an allograft that was tolerized by the maternal immune system [62]. According to Medawar's theory the fetus did not evoke an immunological reaction (i.e., rejection) from the mother because (1) the fetus was anatomically separated from the mother by the placenta, (2) the fetus did not present any mature antigens, (3) the mother's immune system was inert during gestation, or (4) the uterus was an immune privileged site [30, 34, 35]. It is now widely accepted that the fetus expresses antigens which the maternal immune system recognizes as foreign [63]. It has also been determined that fetal tissue is not sequestered from maternal tissue by the placenta, and by week 4 of gestation fetal cells have been found in the maternal circulation [64]. Although the maternal immune system is not suppressed during pregnancy, it does not attack the fetal tissue in normal pregnancy.

In 1993 Thomas Wegmann proposed the theory that successful pregnancy depended upon maternal production of T helper (Th) type 2 (Th2)-based immune responses [65]. Predominantly Th2 cytokines, including IL-10, -4, -5, and -6, and TGF-β, were observed in the serum of normal pregnant mice, and Th1-type cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF), were up-regulated in pregnancies complicated by IUGR, pre-

eclampsia, spontaneous abortion and thrombocytopenia [66]. These observations led to the hypothesis that Th1-type immunity is suppressed during the majority of pregnancy as elevated levels of inflammatory cytokines, such as IFN-γ and TNF-α, are seen in the serum of women who have had spontaneous abortions when compared to serum of women who have normal pregnancies [67].

During pregnancy, Th2-type responses appear to be favored [45, 50], however, pregnancy-specific factors produced by placenta are postulated to counterbalance possible maternal immune responses to the antigenically distinct fetus [41, 42]. A family of secreted proteins found at the maternofetal interface and in the maternal circulation are pregnancy-specific glycoproteins (PSGs), also referred to as Schwangerschafts-protein 1 (Sp1) or pregnancy-specific β1 glycoproteins [68, 69]. PSGs, produced by the STB cells of the placenta, are the most abundant proteins in the maternal serum, increasing to 200-400 μg/ml at term [55, 56]. Although PSGs have been identified for more than 30 years, their biological function is still being elucidated. PSG expression has been linked to embryogenesis, maternal tolerance to the developing fetus and trophoblastic invasion and restructuring of the uterus [70]. In this dissertation, we describe a role for PSGs in establishing and maintaining the fetoplacental blood supply. In addition our initial experiments designed to discover novel receptors for human and murine PSGs will be discussed.

Carcinoembryonic antigen (CEA) family

PSGs are members of the carcinoembryonic antigen (CEA) family which was first described in 1965 as antigens associated with intestinal malignancies [71]. The CEA family of

evolutionarily conserved members has been identified, including CEA-related cell-cell adhesion molecule (CEACAM) 5, nonspecific cross-reacting antigen (NCA or CEACAM6), biliary glycoprotein (BGP or CEACAM1), human CEA gene family member (CGM) and PSGs [72]. The CEA family belongs to the immunoglobulin (Ig) superfamily and its members have an IgV-like N-terminal domain, followed by a variable number of IgC-like A and B domains [73]. CEACAM5 and -6 are tethered to the membrane by a GPI anchor, CEACAM1 is a transmembrane protein and PSGs, which lack a hydrophobic C-terminus, are secreted proteins [72].

The functional properties of the CEA family members are still being explored; however, a role for CEA proteins in angiogenesis, cell adhesion and immune regulation has been suggested. CEACAM1 has been observed to stimulate angiogenesis in endothelial cells through its N-domain in a calcium-dependent manner [74]. In addition, several members of the CEA family have also been implicated in immune regulation and may act on CD8+ T lymphocytes (CTLs) at muscosal surfaces, including at the maternofetal interface [72].

Human PSG expression in pregnancy

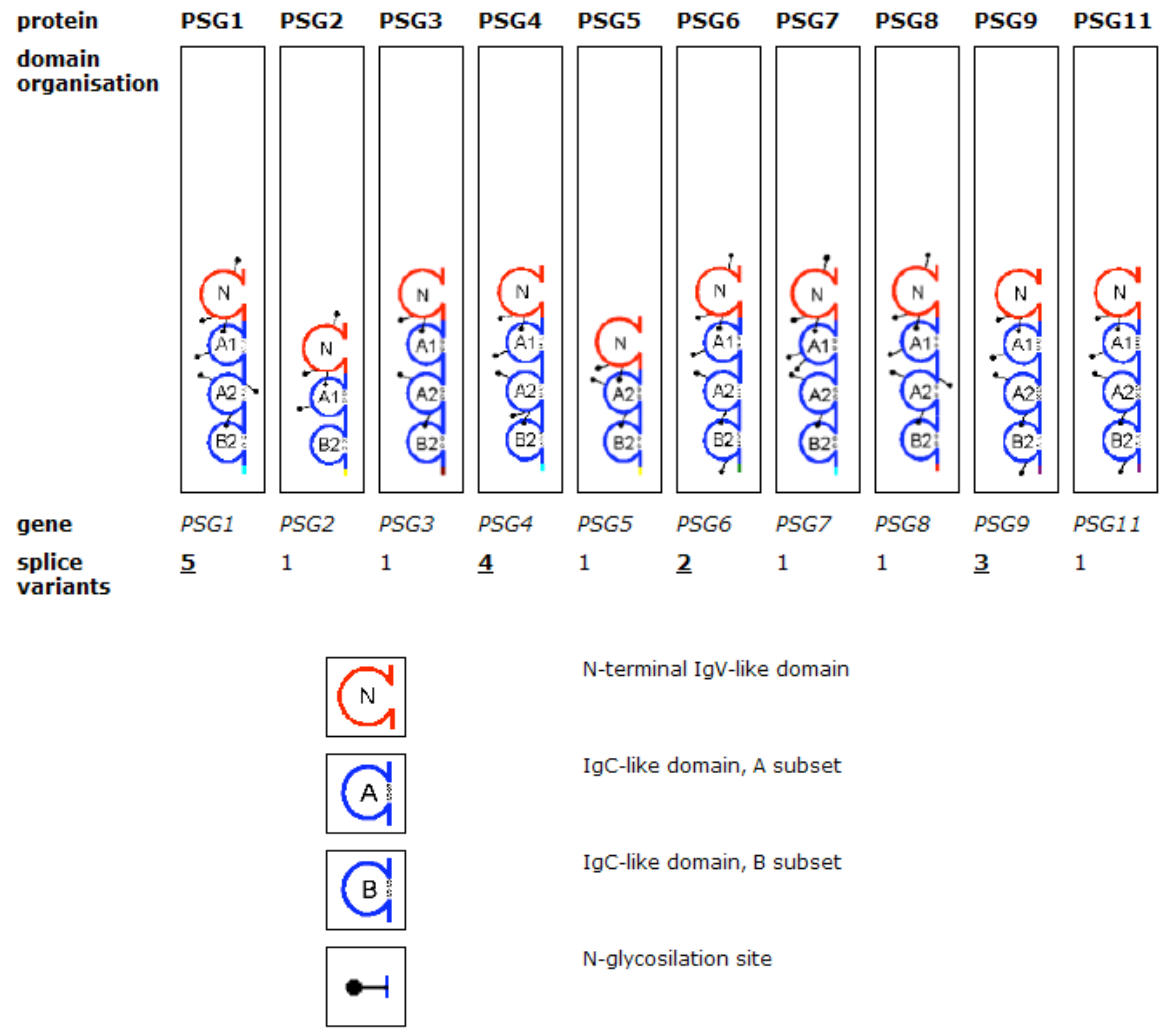
Currently, there are 11 human PSG genes (designated *PSG1-11*) which are detected in the maternal serum as early as 3-4 days post fertilization [75]. PSGs have been identified at low levels in non-pregnant serum, as well as in bone marrow plasma, breast tumors, endometrial tissue, intestinal tissue, salivary glands, testes and uterus [51-53]. Using a specific antibody to the B2 domain of human PSGs, PSG expression was identified in the STB cells of the placenta [10]. The domain structure of human PSG1-11 are shown in Figure 3a. The half-life

of PSGs in maternal serum after parturition is 20-40 hours and PSGs are the only known pregnancy-related protein that increases in concentration as pregnancy progresses [76].

Glycosylation accounts for approximately 30% of the mass of PSGs, and PSGs are predicted to have N-glycoside linked carbohydrate chains [77]. Glycosylation can enhance protein stability, affect protein conformation, improve protein solubility, determine protein immunogenicity and protect proteins from degradation [78]. Recently, it was demonstrated that PSGs produced in bacteria and lacking their carbohydrate groups failed to bind to their known receptors [79], providing experimental evidence supporting the importance of glycosylation to PSG binding. Additionally, members of the CEA family are known to interact, via their carbohydrate groups, to dendritic cell–specific intercellular adhesion molecule (ICAM)-3–grabbing nonintegrin (DC-SIGN) on DCs, promoting DC immune tolerance to tumor cells [80]. Therefore, glycosylation of PSGs may mediate both binding to their receptors and modulate their immune function.

The PSG gene transcript encodes a 34 amino acid leader peptide (L), followed by a 108-110 amino acid IgV-like N-domain (N), two to three IgC-like A and B domains, comprised of 93 and 85 amino acids, respectively, and a short C-terminus of 2-13 hydrophilic amino acids [81]. As a result of alternate splicing, there are currently 83 possible PSG cDNAs that are classified as type I (L/N-A1-A2-B2-C), type IIA (L/N-A1-B2-C), type IIB (L/N-A2-B2-C), type III (L/N-B2-C) and type IV (L/A1-B2-C) (Figure 3b) [62, 66]. The 11 human PSG genes share approximately 90% sequence homology [82].

A



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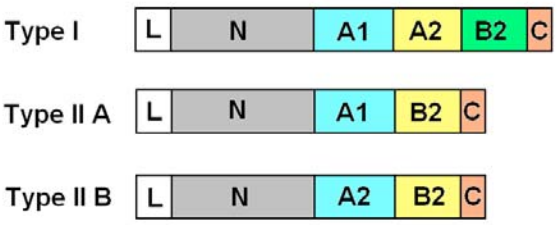

Figure 3: Structure of the human PSG subgroup of the CEA family. (A) Human PSGs contain one IgV-like N-terminal domain followed by multiple IgC-like A and B domains [83]. (B) The structure of the type I, type IIA and type IIB PSG transcripts [84].

Human PSG2, 3, 5, 6, 7, 9 and 11 have an arginine (Arg)-glycine (Gly)-aspartic acid (Asp) motif (RGD) in their N-domains that might contribute to their interaction with integrins and aid in their regulation of adhesion [85]. Many integrin ligands utilize RGD sequences as key structural components of their receptor-binding domains [68]. The RGD motif in PSG11 has been proposed to aid in its binding and activity [86]; however, PSG1 does not contain an RGD motif and shares many of the same biological functions with PSG11 [70]. Therefore, the significance of the RGD sequence in some of the human PSGs remains unclear.

The importance of PSGs to successful pregnancy is highlighted by the fact that reduced PSG levels in maternal serum correlates with fetal hypoxia, IUGR, pre-eclampsia, and spontaneous abortion [87]. Specifically, women with recurrent spontaneous abortion were found to have lower levels of PSG11 in their serum when compared to women with normal pregnancy [88]. In addition, the injection of anti-human PSG antibodies into non-human primates reduced fertility and increased spontaneous abortion [73]. Elevated PSG serum concentration has also been implicated in molar pregnancies and choriocarcinoma [74, 75].

Potential functions for human PSGs in immunomodulation have been reported. Studies with recombinant human PSGs have demonstrated their ability to induce anti-inflammatory cytokines, such as IL-10, IL-6 and TGF-β_1, and not pro-inflammatory cytokines, such as IFN-γ, IL-1β and TNF-α [79, 89-94]. Moreover, recombinant human PSGs were shown to induce alternative activation of monocytes [90] and regulate T cell function [95], potentially contributing to a Th2 environment that is advantageous for successful pregnancy [96]. In

addition, we have reported that the binding and actions of human PSGs occur through the N-domain [80].

Murine PSG expression in pregnancy

Human PSG homologues have been identified in non-human primates, mice and rats. There are 17 murine PSG genes, designated *Psg16-32* (Figure 4a) [69]. Murine PSGs are synthesized by the giant cells and spongiotrophoblasts of the mouse placenta which are analogous to the human STB [84]. In general, rodent PSGs are comprised of at least 3 and as many as 7 IgV-like N-domains followed by a single IgC-like A-domain [97]. The murine N1-domain and C-terminal A domain correspond to the human N-domain and B2 domain, respectively [71]. Additionally, human and rodent PSGs share a 60% homology in their N-domains; therefore, it is possible that the primate and rodent PSG gene families underwent convergent evolution with conserved PSG function (Figure 4b) [72].

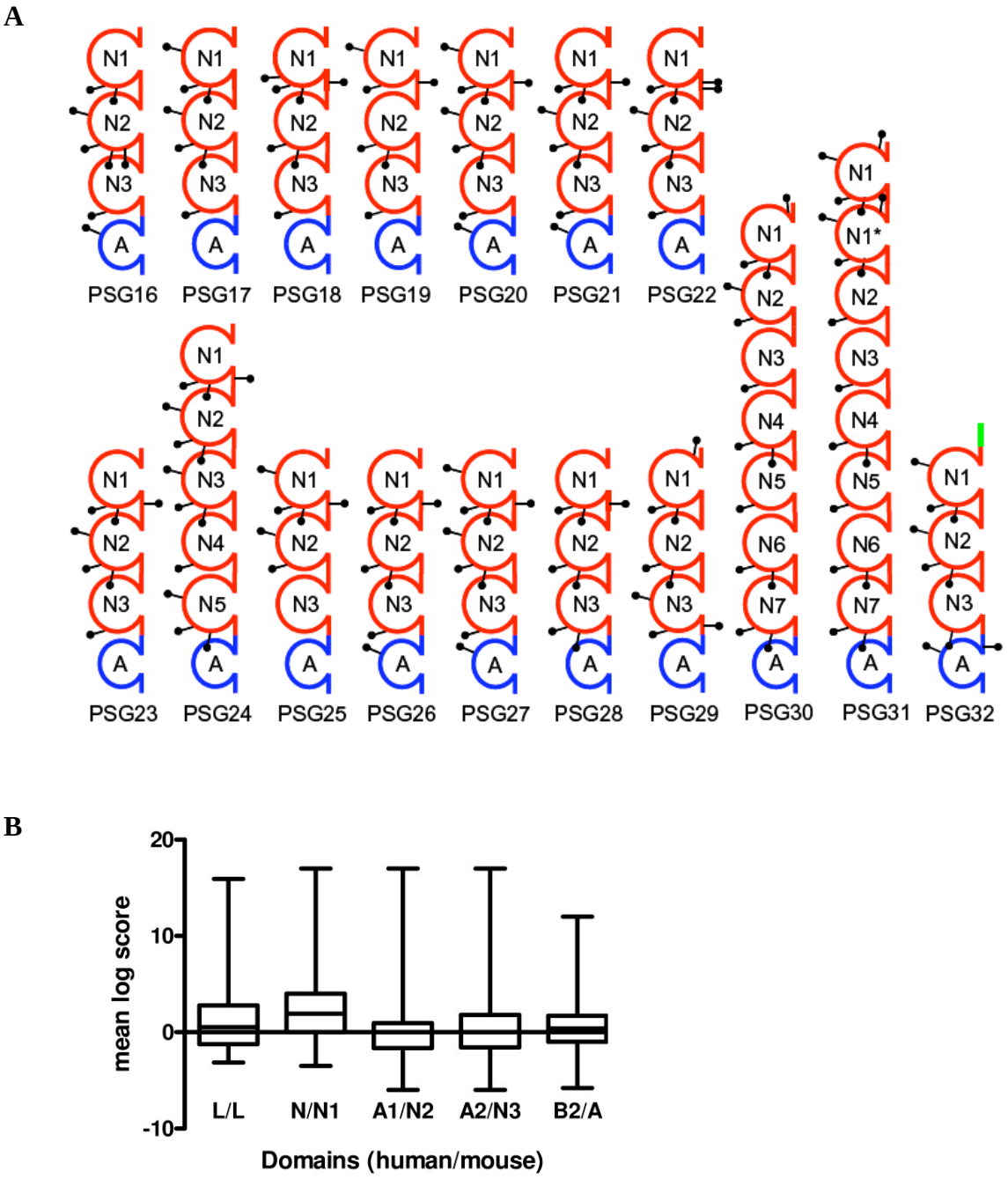

Figure 4. Structure of the murine PSG family and homology to human PSGs. (A) Murine PSGs are comprised of multiple IgV-like N-domains (in red), followed by a single IgC-like A domain (in blue) and are highly glycosylated (indicated by black lollipops) [98]. (B) Dayhoff PAM 250 scores for ClustalX alignment of full-length human and mouse PSGs represented as box and whisker plots. Domain name abbreviations correspond to: human leader peptide (L) versus mouse L, human N versus mouse N1, human A1 versus mouse N2, human A2 versus mouse N3 and human B2 versus mouse A. Positive scores indicate conservation, and the human N and mouse N1 domains have significantly higher scores. [99]

The physiologic roles of murine PSGs are still being elucidated. Similar to human PSGs, mouse PSGs have been shown to induce anti-inflammtory cytokines (Table 1). The N1 domain of PSG17 was shown to induce the secretion of IL-10, TGF-β_1 and prostaglandin E_2 (PGE_2) in macrophages [77]. It was also demonstrated that N1 domain of PSG18 induced IL-10 in human monocytes [70]. Recently, Kawano et al. identified PSG18 in the follicle-associated epithelium of the Peyer's patches which suggests a more generalized role for PSGs in the immunology of mucosal linings [87]. Wynne et al. also found PSG21 lining the capillaries within the decidua, implicating PSG involvement in implantation and vasculature remodeling [73].

Table 1. Anti-inflammatory cytokines induced by PSGs

PSG family member	Target cell type	Cytokines induced	References
Murine PSGs			
17	murine peritoneal macrophages	IL-6, IL-10, TGF-β_1	[79, 89, 93]
	RAW 264.7 murine macrophage cell line	IL6, IL-10, TGF-β_1	[79, 89, 93]
18	human monocytes	IL-6, IL-10, TGF-β_1	[94]
19	RAW 264.7 murine macrophage cell line	TGF-β_1	[79]
Human PSGs			
1a	murine splenocytes	IL-4, IL-10	[91]
	murine peritoneal macrophages	IL-10, TGF-β_1	[91]
1d	human monocytes	IL-6, IL-10, TGF-β_1	[92]
	murine peritoneal macrophages	IL-6, TGF-β_1	[89]
	RAW 264.7 murine macrophage cell line	IL-6, IL-10, TGF-β_1	[89]
6	human monocytes	IL-6, IL-10, TGF-β_1	[92]
	RAW 264.7 murine macrophage cell line	IL-6, IL-10, TGF-β_1	[92]
11	human monocytes	IL-6, IL-10, TGF-β_1	[92]
	murine peritoneal macrophages	IL-6, TGF-β_1	[89]
	RAW 264.7 murine macrophage cell line	IL-6, TGF-β_1	[89]

Previously, our laboratory identified the tetraspanin CD9 as the receptor for PSG17 and PSG19 [76, 81]. Currently, this is the only known PSG receptor and is not used by human PSGs [62]. The murine PSG family proteins are 59-70% homologous in their N1 domains [84]; therefore, it is possible that the other murine PSGs utilize a different tetraspanin as their receptor.

Tetraspanins

Background

The tetraspanins are a family of highly homologous transmembrane proteins which span the plasma membrane four times [100]. Tetraspanins have two extracellular (EC) domains, a small EC loop (EC1) and larger EC loop (EC2 or LEL) [101]. Within the EC2 of all the tetraspanins, there is a cysteine-cysteine-glycine (CCG) amino acid sequence, and in 94% of the tetraspanins there is also a proline-Xaa-Xaa-cysteine (PXXC) sequence in the EC2 and the presence of these cysteine residues have been shown to form disulfide bonds essential to correct folding of the EC2 (Figure 5) [101]. Tetraspanins are found in all cell types, excluding erythrocytes [102] and have been implicated in adhesion, migration, co-stimulation, signal transduction and sperm-egg fusion [92, 93].

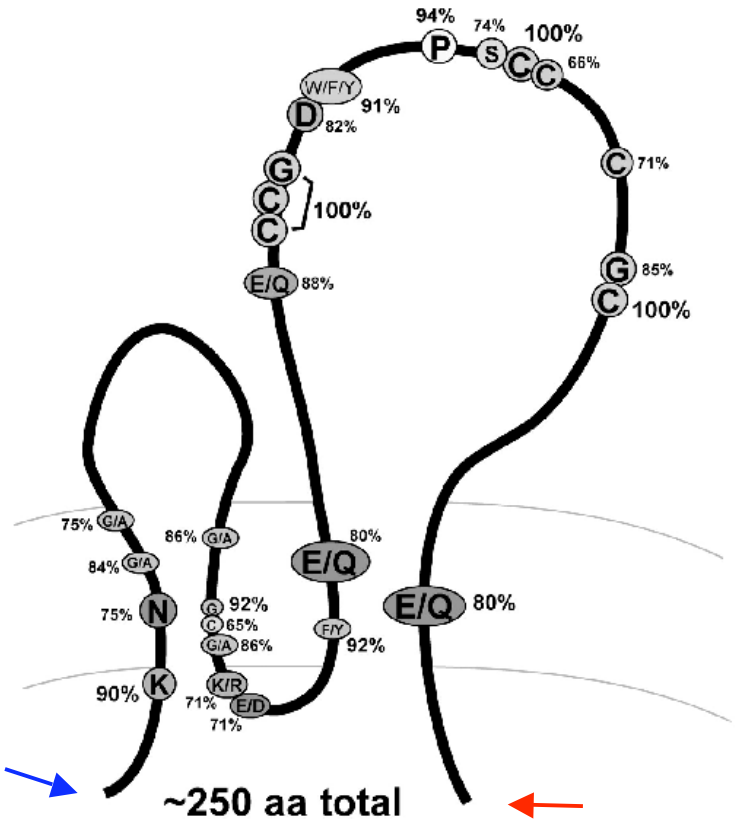

Figure 5: General structure of tetraspanins. Tetraspanins cross the plasma membrane four times resulting in the formation of two extracellular loops, one small (EC1) and one small (EC2) and three short cytoplasmic regions. Key residues and the percentage of conservation at each among the human tetraspanins are indicated. Blue arrow indicates the N-terminal domain and the red arrow indicates the C-terminus. [103]

A unique feature of tetraspanins is their ability to form lateral associations with each other and multiple other proteins, known as a tetraspanin web [104]. Well-known tetraspanin web interactions include the CD19/CD21/CD81/Leu-13 in the B lymphoid activation complex [105], the CD9/precursor of heparin-binding-epidermal growth factor (proHB-EGF) interaction for binding of diphtheria toxin [96], and the CD151/$\alpha_3\beta_1$ integrin interaction on the surface of endothelial cells [106]. In addition, it has been speculated that the palmitoylation of the tetraspanins contributes to their protein interactions, as its loss was shown to result in decreased lateral associations of tetraspanins [80, 81].

Tetraspanins in pregnancy

Currently, the only tetraspanins directly associated with pregnancy are CD9 and CD81. Both CD9 and CD81 have been associated with successful egg-sperm fusion [107], and mice deficient in these proteins have decreased fertility [101]. In addition, CD9 protein expression has been detected on trophoblast cells suggesting that it might play a role in the migration of invasive trophoblasts during placentation [102].

After the discovery that CD9 acts as the cell surface receptor for PSG17, the immunological consequences of this interaction were explored. Binding of PSG17 to CD9 occurs through serine-phenylalanine-glutamine (SFQ) residues (amino acids 173-175) in the EC2 of CD9 [103]. This interaction leads to up-regulation of anti-inflammatory cytokines IL-10, TGF-β_1 and prostaglandin E_2 (PGE_2) on macrophages [77], thus establishing a role for tetraspanins in reproductive immunology.

Tetraspanins and angiogenesis

Tetraspanins are involved in cell migration and adhesion. The mechanism by which tetraspanins regulate these processes is still unknown, but is thought to be regulated by a tetraspanin-integrin interaction [100]. In studies using tumor cells, the tetraspanins CD9, CD81 and CD151 were found to localize at the tumor cell—endothelial cell junctions [104]. Recently, the expression of the tetraspanin CO-029 was implicated in angiogenesis of gastrointestinal tumors [108].

The tetraspanin CD151, or platelet-endothelial cell tetraspanin antigen-3 (PETA-3/SFA-1), is closely associated with integrins [109] and has been implicated in cell adhesion, cell migration, platelet aggregation, vasculogenesis and angiogenesis [110]. CD151 might play a role in pregnancy as it has been detected in the placenta [86]. In addition, CD151 is expressed by uNK cells and thought to play a role in their migration to the maternofetal interface [109].

The importance of the tetraspanins and PSGs in normal pregnancy suggests that these two families may have co-evolved to guarantee pregnancy success. The conserved functions of PSGs across species [79, 89-92, 94] and their high homology [99] suggest that they may utlize the same receptors. Although we determined that human PSG1d and 11 do not require the tetraspanin CD9 for binding and activity in macrophages [89], we cannot exclude the possibility that another member of the tetraspanin family serves as the receptor for human PSGs.

Research

Central hypothesis and specific aims

Previous work from our laboratory and others demonstrates the function of human and murine PSGs in regulating maternal immune tolerance to the developing fetus. The ***central hypothesis*** of this study is that PSGs, which are potent inducers of TGF-β_1, also induce pro-angiogenic factors during pregancy. To address this, the ***specific aims*** of this study are to:

1. Examine the cytokine expression of monocytes/macrophages treated with PSG23, the most abundant murine PSG in mid-pregnancy.
2. Identify new target cell types for PSG binding and activity.
3. Analyze the functions of human PSG1d and murine PSG22.
4. Clone the receptor for murine and human PSGs.

Experimental approach

Based on evidence that murine PSG17 and PSG18 and human PSG1, PSG6 and PSG11 induce TGF-β_1 in monocytes/macrophages, we will investigate the effects of truncated forms of PSG22 and 23, both containing the N1 and A domains, on cytokine profiles of a murine macrophage cell line and peritoneal macrophages isolated from Balb/c and C57Bl/6 mice. In addition, we hypothesize that murine PSG22 and 23 will induce anti-inflammatory and not pro-inflammatory cytokines in human monocytes based on our previous observations that murine PSGs show cross-reactivity in human cells.

Based on the findings that TGF-β_1 acts as a pro-angiogenic factor, we will also investigate whether murine PSG22 and 23 and human PSG1d induce VEGF proteins which are other

angiogenic factors. We will also test the effects of PSGs on murine and human endothelial cells and trophoblasts, which are also involved in placentation.

Previously, we identified the tetraspanin CD9 as the receptor for PSG17 and 19. In this dissertation we will examine whether CD9 also acts as the receptor for PSG22 and 23. We previously reported that CD9 does not act as the receptor for human PSGs; therefore, we will screen a cDNA library containing genes of the target cell type, macrophages, for a possible human PSG receptor. Cloning the receptors for these additional murine PSGs and human PSGs will allow us to determine if all murine PSGs utilize the same receptor.

Results

Murine PSG22 and 23 induced TGF-β_1 up-regulation in both the RAW 264.7 cell line and peritoneal macrophages. Despite the fact that the constructs for PSG22 and 23 were the same, the concentrations of PSG23 required to induce cytokines in both RAW cells and primary cells were less than that of PSG22. We also determined that PSG23 induces TGF-β_1 in bone marrow-derived dendritic cells, a novel target leukocyte for PSG activity. Additionally, PSG22 and 23 induce TGF-β_1 in human monocytes.

Murine PSG22 and 23 and human PSG1d induced VEGF-A in mouse macrophages and human monocytes, but not other VEGF family members. PSG23 was also shown to induce VEGF-A in murine dendritic cells. In addition, the PSGs we studied induced TGF-β_1 and VEGF-A in murine and human endothelial cell lines and immortalized trophoblasts. PSG23-

induced VEGF-A expression in the RAW murine macrophage and the murine endothelial cell lines we tested was shown to be TGF-β-dependent.

In the receptor identification studies, it was determined that CD9 is not required for PSG23 binding and activity in macrophages. Therefore, we explored the possibility that PSG23 utilized another tetraspanin, CD151, as a receptor. Preliminary binding studies showed a CD151/PSG23 interaction. Putative ligand-receptor interaction between PSG23 and CD151, which has been implicated in the process of angiogenesis, would further the hypothesis that PSGs play a role in vasculogenesis and angiogenesis during pregnancy. We also determined that PSG22 does not require CD9 for binding and activity in macrophages; however, a candidate receptor for PSG22 has yet to be identified.

Finally, while our efforts to conclusively identify the receptor for the human PSGs have proven difficult, our data provides a foundation for future studies to isolate the receptor(s) for human PSGs.

PART TWO:

MANUSCRIPT 1

Pregnancy-specific glycoprotein (PSG) 23 induces the pro-angiogenic factors transforming growth factor (TGF)-β_1 and vascular endothelial growth factor (VEGF)-A in cell types involved in vascular remodeling in pregnancy

In preparation for submission to *Biology of Reproduction*

Pregnancy-specific glycoprotein (PSG) 23 induces the pro-angiogenic factors transforming growth factor (TGF)-β_1 and vascular endothelial growth factor (VEGF)-A in cells types involved in vascular remodeling in pregnancy

Short title: PSG23 induces angiogenic factors in pregnancy

Summary sentence: Murine PSG23 induces TGF-β_1 and VEGF-A in macrophages, dendritic cells, endothelial cells and trophoblasts.

Key words: angiogenesis, mouse pregnancy specific glycoproteins.

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ABSTRACT

Haemochorial placentation is a unique physiological process where the fetal trophoblast cells remodel the maternal decidual spiral arteries to establish the fetoplacental blood supply. Pregnancy-specific glycoproteins (PSGs) are members of the carcinoembryonic antigen family. PSGs are produced by the placenta of rodents and primates and are secreted into the bloodstream. PSG23 is one of 17 members of the murine PSG family (designated PSG16 to PSG32). Previous studies determined that PSGs have immunoregulatory functions due to their ability to modulate macrophage cytokine secretion. Here we show that recombinant PSG23 induces TGF-β_1 and VEGF-A in primary murine macrophages and the macrophage cell line RAW 264.7 cells. In addition, we identified new cell types that responded to PSG23 treatment. Dendritic cells, endothelial cells and trophoblasts, which are involved in maternal vasculature remodeling during pregnancy, secreted TGF-β_1 and VEGF-A in response to PSG23. PSG23 showed cross-reactivity with human cells, including human monocytes and the trophoblast cell line, HTR-8/SVneo cells. We analyzed binding of PSG23 to the tetraspanin CD9, the receptor for PSG17, and found that CD9 is not required for PSG23 binding and activity in macrophages. These results indicate that not all PSGs appear to use the same receptor. Overall these studies show that PSGs can modulate the secretion of important pro-angiogenic factors, TGF-β_1 and VEGF-A, by different cell types involved in the development of the placenta.

INTRODUCTION

Haemachorial placentation in mammals represents a unique physiological state in which intimate contact between maternal and fetal tissues must occur. One of the leading causes of pregnancy-associated maternal and fetal morbidity and mortality world-wide is pre-

eclampsia, which is characterized by insufficient invasion and remodeling of the maternal vasculature during implantation [111]. The factors that promote or hamper proper trophoblast invasion of the maternal spiral arteries are still unknown; however, the involvement of the pro-angiogenic factors transforming growth factor (TGF)-β [36] and vascular endothelial growth factor (VEGF) [112] has been implicated in the process of proper implantation. Thus, signals responsible for regulating these two molecules during pregnancy warrants further investigation.

Pregnancy-specific-glycoproteins (PSGs) are among the most abundant fetally-derived proteins, reaching concentrations of 200-400 μg/ml in the serum of pregnant women at term [70]. PSGs are members of the carcinoembryonic antigen (CEA) family of immunoglobulin (Ig)-like genes [113]. Similar to other members of the CEA family, PSGs are highly glycosylated, with N-linked glycosylation comprising approximately 30% of their molecular weight [114]. While the exact function of PSGs is still unknown, previous studies have focused on their role as immunomodulators and have defined monocytes/macrophages as their target cells [89, 91, 92, 94].

To date, 17 murine *Psg* genes (*Psg16-32*) have been identified. PSG protein and mRNA have been detected in trophoblast giant cells and spongiotrophoblasts of the mouse as early as day E6.5 [114]. Murine PSGs consist of a varying number of immunoglobulin (Ig) variable (V)-like N-domains and one Ig constant (IgC)-like A-domain [114]. Murine PSGs have a 59-72% homology in their N-domains and they likely arose by duplication from a single ancestral mouse *Psg* gene [114]. While individual PSGs are expressed at different times and levels during pregnancy [114] and may play unique roles in gestation, it is also likely that they have some overlapping functions. *Psg23* is one of the most abundantly

expressed murine PSGs during mid-gestation as determined by RT-PCR [115] but its functions have never been investigated.

We have demonstrated that the N1 domain of recombinant murine PSG17, PSG18 and PSG19 as well of human PSG6, is sufficient for binding and induction of anti-inflammatory cytokines in monocytes and macrophages [92, 93]. Monocytes and macrophages work in numerous processes during pregnancy, including at the sites of fetal trophoblast invasion and migration [115]. Macrophages have been shown to promote angiogenesis after stimulation with concanavalin A or endotoxin [53], or when changes in metabolic conditions, such as low oxygen tension [116], occur. In this report we show that PSG23 may be another stimulation factor for macrophages to become angiogenic.

During pregnancy TGF-β plays a number of key roles at the maternofetal interface, including the regulation of trophoblast invasion [30, 118], angiogenesis [36], immunomodulation [117], and cell growth and remodeling of the endometrium [120]. TGF-β-null mice die during mid-gestation or shortly after birth due to both immunologic [121] and vascular [33] defects. It has also been reported that TGF-β deficient embryos do not implant properly into the uterus [122].

Previous studies suggest that TGF-β-mediated angiogenesis occurs through the induction of VEGF in a variety of cell types, including fibroblasts and epithelial cells [39], trophoblasts [24], monocytes and macrophages[41]. The VEGF family of growth factors, which includes VEGF-A, -B, -C and –D and placental growth factor (PlGF), promotes vasculogenesis and angiogenesis [123] and are believed to guide the early development of the placenta [13]. VEGF has also been reported to increase during gestation, implying that angiogenesis is a dynamic process important throughout normal pregnancy [124].

Accumulating evidence suggests that a decrease in pro-angiogenic factors, such as VEGF and TGF-β, may participate in the pathophysiology of HELLP (hemolysis, elevated liver enzyme levels, and low platelet count) syndrome leading to pre-eclampsia [34, 110]. The signals that induce cells of the placenta and decidua to produce and secrete these pro-angiogenic factors are not well characterized. Wynne and coworkers recently showed that murine PSG21 is associated predominantly with endothelial cells lining the vascular channels in the decidua [125]. Therefore, we hypothesized that PSGs could bind and regulate the function of endothelial cells.

We have previously shown that the tetraspanin CD9 is the receptor for murine PSG17 and PSG19 [79, 93]. Tetraspanins are a family of membrane proteins expressed in most cell types and many different tetraspanins are co-expressed in the same cell [102]. While we cannot exclude the possibility that one or more tetraspanin family members could serve as the PSG23 receptor, we determined that expression of CD9 is not required for the PSG23-induced secretion of TGF-β_1 and VEGF in macrophages.

Here we report that murine PSG23 induces TGF-β_1 and VEGF in monocytes, macrophages and dendritic cells, immune cells at the implantation site that have been shown to secrete angiogenic factors [116, 126]. In addition, we demonstrate that treatment of endothelial and trophoblast cell lines with PSG23 leads to induction of TGF-β_1 and VEGF in a dose-dependent manner. Our results strongly suggest a new function for PSGs during maternal vasculature remodeling, as well as identify novel targets, endothelial and trophoblast cells, for PSG activity. In addition, we determined that not all PSGs utilize the same receptor. Further studies are required to establish whether human PSGs share these

newly described properties and to further dissect the role of the different members of this family in the establishment and maintenance of pregnancy.

MATERIALS AND METHODS

Antibodies

For flow cytometry analysis the following antibodies were used: streptavidin-fluorescein isothiocyanate (FITC), streptavidin–phycoerythrin (PE), and phycoerythrin Rat IgG2b isotype control (eBioscience, Inc., San Diego, CA), anti-human PSG Ab BAP3 (Genovac, Freiburg, Germany), anti-Flag BioM2 (Sigma, St. Louis, MO), rat anti-mouse IgG1 phycoerythrin and CD16/CD32 (Fcγ III/II receptor, BD Biosciences, San Jose, CA), GST (B-14) antibody (clone sc-138, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and LIVE/DEAD Fixable Red and Blue Dead Cell Stain kits (Molecular Probes, Carlsbad, CA).

For immunoblotting, the following Antibodies were employed: His-probe (H-15) - (clone sc-803, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Flag M2 and anti-Flag M2-peroxidase (HRP) (Sigma, St. Louis, MO).

Neutralization of TGF-β_1 was performed by adding the anti-human TGF-β_1 neutralizing antibody (R & D Systems, Minneapolis, MN) and normal chicken Ig antibody was used as control (R & D Systems).

Protein production and purification

Recombinant murine PSG23N1A-His-Flag (hereafter referred to as PSG23N_1A) was synthesized by the GenScript Corporation (Piscatawy, NJ). The cDNA encoding for the leader peptide, N1 and A domains of *Psg23*, along with the nucleotide sequence for six histidines (6X His) and a Flag tag (Sigma) were inserted into the *Sma*I site of pUC57 (EZBiolab, Inc., Westfield, IN). *Psg23N1A*-His-Flag was subcloned into the *Hind*III and

*Eco*RI sites of the pEAK10CV vector (Edge Biosystems, Gaithersburg, MD) and transfected into dihydrofolate reductase-negative (dhfr-) CHO cells, along with the pDCHIP plasmid (obtained from Dr Kaplan, FDA, MD), which encodes the *dhfr* minigene. Positive transformants were obtained by methotrexate selection, propagated and seeded in a 5 kDa molecular weight (MW) cutoff hollow fiber cartridge (FiberCell Systems, Inc., Frederick, MD). The supernatant from the cartridge was harvested daily and kept frozen until it was processed as described below.

PSG23N1A was dialyzed into a 20 mM sodium phosphate buffer containing 50 mM imidazole and purified from the harvested supernantant using a HisTrap column on the ÄKTAprime Plus system (GE Healthcare, Piscataway, NJ). Positive fractions eluted from the HisTrap column were identified by immunoblotting, pooled, buffer-exchanged into PBS and applied to a column packed with anti-Flag M2 agarose (Sigma, St. Louis, MO), and eluted with 3X Flag peptide (Sigma). Fractions, which reacted with the anti-Flag mAb, were collected, pooled, and buffer-exchanged with PBS. The purified protein was run on a SDS-PAGE gel, stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL), and quantitated against BSA standards.

Recombinant murine PSG17N-myc-His was generated and purified as previously described [127].

Cell lines

For overexpression experiments HEK 293T cells and BHK-21 cells (ATCC, Manassas, VA) were used. For protein activity assays we tested, RAW 264.7 (ATCC), C166 (ATCC), human invasive trophoblast (HTR-8/SVneo) cells [118] (kind gift from Charles Graham, Queen's University, Kingston, ON, Canada), and a murine trophoblast cell line

derived from C57Bl/6 x 129 placenta [119] (kind gift from Joan Hunt, University of Kansas Medical Center, Kansas City, KS) were used.

HEK 293T and BHK-21 cells were maintained in Dulbeccos Modified Eagles Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT), 10 mM Hepes (Cellgro), and antibiotics. RAW 264.7 and C166 cells were cultured in DMEM supplemented with 10% fetal clone III serum alternative (Hyclone), 10 mM HEPES (Mediatech, Inc., Manassas, VA), and penicillin-streptomycin (PS, Mediatech, Inc.). HTR-8/SVneo cells were maintained in HyQ RPMI 1640-RS (Hyclone) media. Human monocytes were maintained in RPMI 1640 (Gibco) supplemented with 2 mM glutamine.

Primary cells

Six- to eight-week-old C57Bl/6 mice deficient in CD9 were bred from a CD9+/- breeding pair obtained from Dr. Claude Boucheix (Hôpital Paul Brousse, Villejuif, France). Six- to eight-week-old Balb/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Briefly, mice were injected i.p .with 3 mls of sterile 3% thioglycollate broth (BD Biosciences). Three days after i.p. injection, macrophages were obtained by peritoneal lavage. Cells were seeded at 1.5 x 10^6 per well in a 24-well plate. Twenty-four hours after seeding the cells, macrophages attached to wells and non-adherent cells were removed. Plating media (RPMI supplemented with 2% FBS and 0.5X PS) was replaced with treatment media (RPMI supplement with 2% delipidized FBS and 0.5X PS, sterile filtered through a 0.2 μm membrane) prior to addition of protein or PBS control.

Murine bone marrow-derived dendritic cells (BMDCs) were isolated and differentiated as previously described [129]. Human monocytes were isolated from the peripheral blood of healthy adult donors and purified as previously described [92].

Plasmids and cell transfection

For cell transfection, HEK 293T or BHK cells were seeded at a density of 4.5 x 10^6 in a 60 mm poly-L-lysine culture dish (BD Biosciences). After cells attached, culture media was removed and 20 ml of plating media was added to each plate. Plasmids were incubated with FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switerzland) as per manufacturers instructions and added to the cells. Forty-eight hours post-transfection, cells were removed from the culture dishes using CellStripper (MediaTech, Inc.) and used for binding experiments. The transfection efficiency was approximately 80% for the plasmids tested. The green fluorescent protein (GFP) murine CD9 fusion cDNA was constructed as previously reported [127].

ELISAs

For enzyme-linked immunosorbant assays (ELISAs), cells were plated in triplicate wells for each treatment in 24-well plates (BD Biosciences) and incubated in a 37°C humidified incubator with 10% CO_2. RAW 264.7 cells were seeded at a density of 1 x 10^6 cells per well; C166 cells were seeded at a density of 0.25 x 10^6 cells per well; HTR-8/SVneo cells were seeded at 0.4 x 10^6 cells per well; human monocytes were seeded at a density of 1.2 x 10^6 cells per well; and peritoneal macrophages were seeded at a density of 1.5 x 10^6 per well. Cells were incubated overnight and treated on the following day with recombinant proteins or PBS in 300 µl of fresh media for treatment times less than 6 hours long or 1 ml of fresh media for treatment times greater than six hours. In some experiments, RAW cells were treated with PSG23N1A protein after it had been boiled for 15 minutes or endotoxin-free BSA (Sigma) as controls.

After treatment, the supernatants were collected and centrifuged at 3,000 rpm for 5 minutes to remove cell debris. For TGF-β_1 ELISA, supernatant or media alone was activated as per the manufacturer's instructions (R & D Systems). Murine VEGF-A and PlGF expression were measured by ELISA (R & D Systems). Murine VEGF-C ELISA was performed using a rat VEGF-C ELISA kit, based on cross-reactivity with mouse cells, from Bender MedSystems (Burlingame, CA). Human VEGF-A and VEGF–C were also measured by ELISA (R& D Systems). TNF-α in the supernatants from both human and murine cells was determined using the BD OptEIA Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences). At least three different protein preparations were tested and experiments were repeated a minimum of three times.

Quantitative relative real-time polymerase chain reaction (QRT-PCR)

For quantitative relative real-time PCR (QRT-PCR) reactions, RAW 264.7 or murine peritoneal macrophages were seeded at densities of 1×10^6 cells per well or 1.5×10^6 cells per well in a 24-well plate, respectively. Cells were treated with varying concentrations of protein or control. Cell lysates were harvested after 45 minutes to up to 24 hours post-treatment, and RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). All primers for QRT-PCR reactions were purchased from Applied Biosystems. The following murine cytokines and growth factors were measured by QRT-PCR, using hypoxanthine guanine phosphoribosyl transferase (HPRT) 1 (Applied Biosystems) as an endogenous control: *IL-6, IL-12p35, IL-23p19, Vegf-a* and *Vegf-c* and *Ang-1* and *Ang-2*. The QRT-PCR reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems).

Binding experiments

For FACS binding experiments, 1 x 10^6 cells in 100 µl were treated with Multiple Tag, which contains a Flag tag, (GenScript Corporation) employed as control protein or PSG23N1A - for one hour on ice. Cells were incubated with 0.5 µg of biotinylated anti-Flag antibody (Sigma) to detect protein binding, followed by incubation with 0.5 µg FITC- or PE-conjugated streptavidin. Excess protein and antibodies were removed by washing with FACS wash buffer (PBS supplemented with 3% FBS and 0.05% NaN_3). In the experiments indicated, a live/dead cell discriminate dye was used (LIVE/DEAD Fixable Dead Cell Stain Kit, Molecular Probes). Cells were fixed with BD Cytofix (BD Bioscences) and analyzed using the Coulter Epics XL Flow Cytometer (Beckman Coulter, Fullerton, CA). For flow cytometry analysis of peritoneal macrophages, cells were first blocked with anti-mouse CD16/CD32 (Fcγ III/II receptor, BD Biosciences) antibody prior to incubation with the control or PSG23N1A proteins.

Statistical analysis

SPSS (SPSS, Inc., Chicago, IL) and Microsoft EXCEL were used for data statistical analysis. The two-tailed Student's *t*-test was used to determine statistical significance in experiments comparing protein treated versus untreated cells, with a p-value of <0.05 as a cut-off. One-way ANOVA was used to determine statistical significance of dose-response assays with a p-value of <0.001 as a cut-off. QRT-PCR results were analyzed using the 7500 System Sequence Detection Software version 1.2.3 (Applied Biosystems). FACS data were analyzed using WinList 6.0 (Verity Software House, Topsham, ME). All data is representative of at least three independent experiments.

RESULTS

Generation of recombinant PSG23N1A

The murine *Psg* gene family is comprised of seventeen members consisting of varying numbers of IgV-like N-domains and only one IgC-like A-domain [109]. Reverse transcriptase-PCR data identified *Psg23* as one of the most abundant murine *Psg*s expressed in the placenta during mid-pregnancy [114] but the function of this protein has never been studied. We generated recombinant PSG23N1A protein that consists of the leader peptide, the N1 and A domains, followed by six histidines and a C-terminal Flag tag. The 36 kDa recombinant protein was harvested from the supernatant of stably transfected CHO cells generated as described in the Materials and Methods section, and was sequentially purified on a HisTrap and anti-Flag affinity column. These two purification steps rendered a glycoprotein that was approximately 90% pure based on Coomassie Blue staining (Figure 1). In addition, the protein was determined to be endotoxin-free by the Limulus Amebocyte Lysate (LAL) assay and its inability to induce TNF-α in macrophages.

Recombinant PSG23N1A binds to macrophages and dendritic cells

Previously, it was demonstrated that murine PSGs elicit an anti-inflammatory cytokine response in monocytes and macrophages [89, 91-94] and that the N1 domain of three different murine PSGs is sufficient for binding to target cells [92, 93]. We first tested the response of the murine macrophage cell line, RAW 264.7, to different concentrations of PSG23N1A .RAW cells secreted TGF-β_1 in response to PSG23N1A starting at 5 μg/ml as determined by ELISA (Figure 2a). This response was observed as early as 4 hours post-treatment (data not shown). While we could observe up-regulation of TGF-β_1 by ELISA in response to PSG23N1A treatment, there was no increase of TGF-β_1 mRNA at 0.5, 1, 2, and 4

hours (data not shown). Therefore, PSG23N1A, as previously observed for other murine PSGs [92], did not up-regulate TGF-β_1 at the transcriptional level.

We also tested primary macrophages, obtained by peritoneal lavage of C57Bl/6 and Balb/c mice, and found that PSG23N1A induced TGF-β_1 in these cells at concentrations ranging from 5-20 μg/ml (Figure 2b) with a peak expression at 8 hours post-treatment.

Dendritic cells (DCs) have also been implicated in maintaining maternal immune tolerance to the developing fetus [55] because of their ability to influence the differentiation of T cells to a T helper type 2 (Th2) subset [130] and their cross-talk with the most abundant leukocyte population at the maternofetal interface, uterine natural killer (uNK) cells [120]. Therefore, we investigated whether DCs could respond to PSG treatment. We harvested DCs from the bone marrow (BMDCs) of C57Bl/6 mice as decribed [129] and after 7 days in culture we treated the cells with PSG23N1A at 15-30 μg/ml for 24 hours. We found that PSG23N1A induces TGF-β_1 in BMDCs (Figure 2c).

We also measured the production of pro-inflammatory cytokines and of IL-6 in RAW cells in response to PSG23N1A treatment. PSG23N1A did not up-regulate *TNF-α*, *IL-12p35*, *IL-23p19* or *IL-6* mRNA in RAW cells as determined by quantitative real-time PCR (data not shown). Analysis of the supernatants using specific ELISAs for TNF-α, IL-12 and IL-6 at 24 hours post-PSG23N1A treatment demonstrated a lack of up-regulation of these cytokines at the protein level.

PSG23N1A induces VEGF-A in -macrophages and dendritic cells

In mammals with haemochorial placentation, fetal tissue comes into direct contact with maternal tissue and invasive trophoblast cells migrate down and remodel uterine spiral arteries to initiate blood flow to the developing fetus [121]. Trophoblasts, endothelial cells

and monocytes/macrophages are all involved in the establishment and maintenance of the fetal blood supply during placentation.

TGF-β is a pro-angiogenic factor which plays an important role in the development of the foetoplacental capillary system during implantation [36]. TGF-β secretion has been correlated to up-regulation of VEGF family members [36, 41] and both angiogenic factors are abundant in pregnancy. Upon the observation that PSG23N1A treatment of macrophages and BMDCs induced TGF-β_1, we investigated the possibility that PSG23N1A might regulate VEGF-A, which is expressed at the site of trophoblast invasion [133].

RAW cells secreted VEGF-A in response to PSG23N1A treatment, in doses ranging from 10-30 μg/ml (Figure 3a) after 24 hours of treatment, as determined by ELISA. Primary macrophages from Balb/c and C57Bl/6 mice also secreted VEGF-A after 4 hours of PSG23N1A treatment, and showed peak expression of VEGF-A after 8 hours of treatment (Figure 3b). We also measured induction of *Vegf-a* message after RAW and peritoneal macrophages were treated with PSG23N1A at 45 minutes up to 8 hours post-treatment. We did not see induction of *Vegf-a* mRNA (data not shown). As observed in macrophages, PSG23N1A treatment resulted in the up-regulation of VEGF-A secretion in BMDCs isolated from C57Bl/6 mice measured at 24 hours (Figure 3c).

VEGF induction in macrophages has been associated with the up-regulation of TGF-β [41]. To test whether PSG23N1A induction of VEGF-A in macrophages was TGF-β dependent, we pretreated RAW cells with 20 μg/ml of neutralizing anti-TGF-β antibody for 30 minutes prior to the addition of PSG23N1A. The anti-TGF-β antibody, but not the antibody control, effectively inhibited PSG23N1A-induced VEGF-A secretion (Figure 3d).

VEGF-C is another VEGF family member important in pregnancy and is expressed by trophoblasts, endothelial cells and uNK cells during pregnancy [124, 134]. The factors that promote VEGF-C secretion remain to be elucidated. We investigated whether PSG23N1A induced VEGF-C in macrophages both at the mRNA and protein levels. We did not see *Vegf-c* mRNA, measured by QRT-PCR, or VEGF-C protein, measured by ELISA, in RAW cells or peritoneal macrophages (data not shown).

The VEGF family member placental growth factor, PlGF, is expressed during and regulates differentiation of vascular endothelia. It is expressed by uNK cells of the mouse pregnant uterus and promotes uNK cell proliferation and differentiation [124, 134]. PlGF has also been shown to be a chemotactic and survival factor for macrophages involved in tumor angiogenesis [122], therefore we investigated whether PSG23N1A induced PlGF in macrophages. In our studies, however, PlGF was not induced in macrophages after PSG23N1A treatment as measured by ELISA (data not shown). The angiopoetins, Ang-1 and Ang-2, have been shown to synergize with VEGF family members during angiogenesis, and Ang-2 production by macrophages was previously reported [136]. We measured *Ang-1* and *Ang-2* in peritoneal macrophages and RAW cells by QRT-PCR at different times post PSG23N1A treatment and found that Ang-1 was not expressed in these cells and Ang-2 , while expressed,was not induced by PSG23N1A treatment (data not shown). These results could not be verified at the protein level due to the lack of commercially available reagents to detect murine Ang-1 and Ang-2 proteins.

Identification of endothelial cells and trophoblasts as targets for PSG23N1A

The cell types involved in the spiral artery remodeling and establishment of the foetoplacental blood supply include macrophages, endothelial cells, uNK cells and

trophoblasts. As PSG23N1A induced TGF-β_1 and VEGF-A in macrophages, we hypothesized that PSGs could affect the functions of other cell types other than macrophages and dendritic cells, which are involved in angiogenesis. Therefore, we included in these studies an endothelial and a trophoblast cell line to determine if these cells responded to PSG23N1A treatment.

The murine endothelial cell line (C166) was derived from a mouse yolk sac and has a normal endothelial cell phenotype [123]. The C166 cells secreted TGF-β_1 in response to PSG23N1A in a dose-dependent manner (Figure 4a). Induction of TGF-β_1 in these cells was seen as early as one hour post-treatment (data not shown). VEGF-A was also induced in C166 cells after 24 hours of treatment in a dose-dependent manner, determined by ELISA (Figure 4b). As observed in macrophages, the secretion of VEGF-A in C166 cells was dependent on TGF-β_1 induction; upon addition of an anti-TGF-β antibody, PSG23N1A-induced VEGF-A expression was greatly amoerilated (Figure 4c).

A murine trophoblast cell line, provided to us by Dr. Joan Hunt [128], responded to PSG23N1A treatment as shown by induction of TGF-β_1 and VEGF-A (data not shown). These trophoblast cells were isolated from mouse placentae and shown to exhibit markers similar to those of labyrinthine trophoblast cells, such as cytokeratin intermediate filaments, transferrin receptors and alkaline phosphatase. The cell line was differentiated into spongiotrophoblast cells [119].

Similar to our results using murine macrophages, PSG23N1A did not induce secretion of mRNA for *Vegf-a*, *Vegf-c*, *Ang-1* or *Ang-2* or the cytokines for VEGF-C and PlGF, measured by ELISA, in C166 cells or the murine trophoblast cells (data not shown).

PSG23N1A shows cross-reactivity with human cells

Murine and human PSGs share a 60% homology in their N-domains [109], and previous studies showed that murine PSGs induce anti-inflammatory cytokine secretion in human monocytes [92]. We therefore tested the effects of PSG23N1A on human monocytes isolated from the peripheral blood of healthy adult donors. Upon PSG23N1A treatment, human monocytes secrete TGF-β_1 and VEGF-A after 24 hours of treatment as measured by ELISA (Figure 5a and b). PSG23N1A did not induce VEGF-C in human monocytes (data not shown).

The invasive trophoblast cell line, HTR-8/SVneo, has proved an important tool in understanding some of the mechanisms involved in trophoblast invasion and migration during pregnancy [138]. We tested the effect of PSG23N1A treatment on secretion of pro-angiogenic factors by HTR-8/SVneo cells. Cells were treated for 24 hours with 10-30 μg/ml of PSG23N1A. TGF-β_1, VEGF-A and VEGF-C secretion in the supernatants of the cells were determined by ELISA. We observed that PSG23N1A induced TGF-β_1 and VEGF-A in HTR-8/SVneo cells (Figure 5c & d) in a dose-dependent manner but that VEGF-C was not up-regulated in these cells (data not shown).

PSG23 activity does not depend on CD9

Murine PSGs share a 59-70% homology [114]. Previously, murine PSG17 was shown to induce TGF-β_1, IL-10 and IL-6 secretion in both murine macrophages and human monocytes [89, 92]. We also determined that PSG17 elicits an anti-inflammatory cytokine response in these cells through binding to its receptor CD9 [89], a member of the tetraspanin superfamily. The binding of PSG17 to murine CD9 was determined by panning, ELISA,

FACS and in functional assays with CD9 deficient mice [93]. Because PSG23 also targets monocytes/macrophages we sought to determine whether PSG23 also utilizes CD9 as its receptor.

We transfected BHK-21 cells with a plasmid encoding the murine CD9 cDNA or control plasmid. By FACS, we did not observe any differences in PSG23N1A binding to the CD9-expressing cells over the control-transfected cells (data not shown). In an ELISA binding assay, we incubated HEK293T cells transfected with a murine CD9-GFP fusion or a GFP only expressing plasmid with PSG23N1A or PSG17. We did not observe binding of PSG23N1A to the CD9-transfected cells over the control-transfected cells, whereas PSG17 bound preferentially to the CD9-transfected cells as previously reported [93] (data not shown). After panning murine CD9-transfected HEK293T cells on PSG23N1A-coated or uncoated plates, we also did not see a difference in binding to the PSG23N1A-coated plates by the cells over-expressing CD9 (data not shown). In addition, PSG23N1A bound to the same degree to peritoneal macrophages isolated from wild-type and CD9 deficient mice, as determined by FACS (Figure 5a).

In functional assays, peritoneal macrophages isolated from wild-type and CD9-null mice were treated with PSG23N1A. We measured expression of TGF-β_1 and VEGF-A in the wild-type and CD9-null peritoneal macrophages by ELISA. Cells from wild-type and CD9 knockout mice responded to PSG23N1A by secreting TGF-β_1 after 24 hours of treatment (Figure 5b). The primary macrophages from both mice also secreted VEGF-A in response to PSG23N1A treatment after 4 hours of treatment (data not shown). In conclusion, both binding and functional assays showed that PSG23 does not depend on the presence of CD9 to bind to macrophages or elicit a response.

DISCUSSION

Haemochorial placentation is a unique physiological phenomenon of viviparity. During placentation, the fetal cells come into direct contact with maternal tissue. The invasion of the maternal tissue by fetal trophoblasts and the growth of new blood vessels are imperative for successful pregnancy [13]. Detrimental conditions, such as intrauterine growth retardation for the fetus and pre-eclampsia for the pregnant mother, can develop without proper invasion of the uterus and maintenance of the fetal blood supply [111].

PSGs are among the most abundant proteins in the maternal serum yet their function during pregnancy is still being explored [70]. Previously, the role of PSGs centered on their functions as immunoregulators during pregnancy [91]. Indeed, treatment of monocytes/macrophages with recombinant human and murine PSGs leads to induction of anti-inflammatory cytokines, including IL-10 and TGF-β_1 while there is no induction of pro-inflammatory cytokines, such as TNF-α, IL-1β and IFN-γ [92]. In addition, human PSG1 was shown to induce alternative activation of monocytes and to enhance Th2-type immune responses [91]. PSGs belong to the carcinoembryonic gene family (CEA) [124]. Interestingly, other CEA family members which, unlike PSGs, are membrane-bound, have been shown to be involved in angiogenesis and immunoregulation [72]. For example, CEACAM1 has angiogenic properties [140, 141] and inhibits activated decidual lymphocytes [125] and CEACAM6, expressed on placental trophoblast, has been shown to activate a distinct subset of regulatory CD8+ T cells [72].

Recent studies have identified PSG expression at a unique site in non-pregnant mice. Kawano et al., reported expression of murine PSG18 in the follicle-associated epithelium of the Peyer's patches [143], and suggested a role for this murine PSG in the interplay between

epithelial cells and immune cells in mucosa-associated lymphoid tissue. While the role of PSGs appears to be broader than just as immunomodulators strictly in pregnancy, further investigation is required to confirm this hypothesis.

The important players in placentation include macrophages, DCs, uNK cells, endothelial cells and trophoblasts [8]. Previous work with murine and human PSGs identified monocyte/macrophages as the target cells for protein binding and activity [89, 93]. Therefore, we first explored the effect of PSG23 treatment on macrophages. We found that macrophages up-regulate TGF-β_1 secretion in response to PSG23 in a dose-dependent manner. In addition, treatment of dendritic cells (BMDC) with PSG23 resulted in up-regulation of TGF-β_1. TGF-β_1 has multiple roles during pregnancy, including regulation of extravillous trophoblast migration and proliferation [118] and regulation of NK cell function [144, 145]. TGF-β_1 and VEGF-A have additive effects on vasodilation [36] and have been implicated in immunosuppression [146, 147]. Based on the observation that TGF-β_1 activated macrophages to express VEGF [41], we explored the possible induction of angiogenic factors in response to PSG23 treatment. VEGF-A, but not VEGF-C, PlGF, Ang-1 or Ang-2, was strongly induced in macrophages and BMDCs upon PSG23 treatment. The induction of VEGF-A was found to be TGF-β_1-dependent, as a neutralizing antibody against TGF-β negated the induction of VEGF-A in macrophages.

To extend our hypothesis that PSG23 is involved in angiogenesis, we investigated the effect of PSG23 on additional cell types involved in this process, such as endothelial cells and trophoblasts [126]. Mouse PSG21 was found to be associated with the maternal vasculature in the pregnant uterus [125] indicating a possible interaction between PSGs and endothelial cells. PSG23 treatment of a murine endothelial cell line led to induction of both

TGF-β_1 and VEGF-A in a dose-dependent manner, and the induction of VEGF-A was TGF-β-dependent. In a murine trophoblast cell line we saw that PSG23 treatment induced both TGF-β_1 and VEGF-A. Similar to our results in murine macrophages, treatment with PSG23 did not result in up-regulation of other pro-angiogenic factors commonly associated with pregnancy.

We previously reported cross-reactivity between human cells and murine PSGs [89], hence we treated human monocytes and a human trophoblast cell line with PSG23. In the two cell types we tested, PSG23 induced TGF-β_1 and VEGF-A. Induction of pro-angiogenic factors in human cells with the mouse protein suggests that human PSGs may also play a role in placentation, although this has yet to be explored.

Our laboratory previously identified the tetraspanin CD9 as the receptor for murine PSG17 [93]. We therefore examined whether PSG23 binds to CD9. We could not demonstrate binding of PSG23 to CD9 by ELISA and FACS analysis. While we cannot conclusively rule out the possibility that PSG23 binds to CD9, this interaction, if happens, may be of low affinity. Importantly, expression of CD9 in peritoneal macrophages was not required for induction of TGF-β_1 and VEGF. Therefore, we conclude that CD9 is not essential for PSG23 function. Tetraspanins are often organized together in a tetraspanin web on the cell surface and many tetraspanin family members are expressed in the cell types shown to respond to PSG23 in this report [102]. Therefore, the possibility that PSG23 may use another tetraspanin family member as its receptor should be explored.

Here we report on a novel function for another murine PSG, PSG23. Our studies indicate that PSG23 is as an inducer of two pro-angiogenic factors, TGF-β_1 and VEGF-A, in several cell types important in the maintenance of the fetal blood supply. Therefore these

findings suggest that PSGs, besides their immunoregulatory role, may be important in the processes of vasculogenesis and angiogenesis required for the establishement and mantainance of the fetoplacental blood supply. We are currently investigating whether other murine PSGs or any human PSGs share the ability with PSG23 to induce pro-angiogenic factors. Further studies could elucidate whether PSGs have an effect on uNK cells, which are the most abundant leukocytes in pregnancy [149] and are involved in angiogenesis and trophoblast chemoattraction [150].

ACKNOWLEDGEMENTS

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FIGURE LEGEND

Figure 1: Recombinant PSG23N1A protein produced in CHO cells. PSG23N1A secreted by CHO cells was analyzed by western using an anti-Flag antibody followed by a horseradish peroxidase-conjugated secondary antibody (Lane 1). Lane 2 shows the same protein after two rounds of purification in a Coomassie stained gel. Arrows indicate the size of the purified protein, approximately 36 kDa.

Figure 2: PSG23N1A induces TGF-β_1 in macrophages and BMDCs. (a) RAW 264.7 cells were treated with 5-20 μg/ml of PSG23N1A for 24 hours. (b) Peritoneal macrophages isolated from Balb/c or C57Bl/6 mice were treated with PSG23N1A at 5-20 μg/ml of protein for 4 hours. (c) Following differentiation BMDCs isolated from C57Bl/6 mice were treated with 30 μg/ml of PSG23N1A for 24 hours. TGF-β_1 in the supernatants was analyzed by ELISA. For Figure 2a and 2b statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.001$. For Figure 2c statistical significance between the protein-treated and untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. All cells were treated in triplicate wells and supernatants were analyzed as separate samples in each ELISA. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 3: PSG23N1A induces VEGF-A in macrophages and BMDCs. (a) RAW 264.7 cells were treated with 10-30 μg/ml PSG23N1A protein for 24 hours. Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.001$. (b) Peritoneal macrophages isolated from Balb/c mice were treated with 20 μg/ml of PSG23N1A

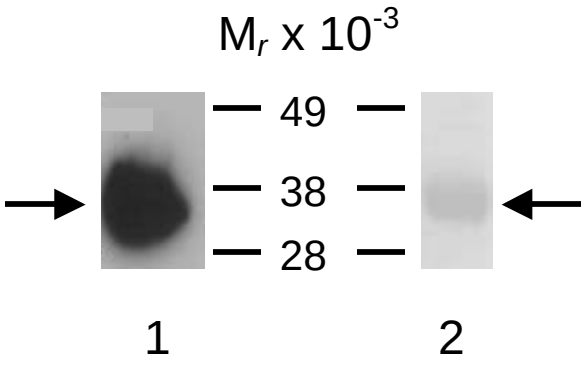
in a time-course study. (c) Following differentiation, BMDCs isolated from C57Bl/6 mice were treated with 20 μg/ml of PSG23N1A for 24 hours. (d) RAW 264.7 cells were pre-treated with 20 μg/ml control antibody or neutralizing anti-TGF-β antibody. After 30 minutes of incubation with antibodies, cells were treated with 20 μg/ml PSG23N1A in the indicated wells and incubated for 24 hours. VEGF-A in the supernatants was analyzed by ELISA. For Figure 3b and 3c statistical significance between the protein treated and untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. For Figure 3d statistical significance between protein-treated or antibody-treated samples versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 4: PSG23N1A induces pro-angiogenic factors in murine endothelial cells. (a & b) C166 cells were treated with PSG23N1A at concentrations of 10-30 μg/ml for 24 hours. Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.001$. (c) C166 cells were pre-treated with 20 μg/ml of control antibody or neutralizing anti-TGF-β antibody. Following 30-minute incubation with the antibodies, 20 μg/ml of PSG23N1A was added to the indicated wells for 24 hours. For Figure 4c statistical significance between protein-treated or antibody-treated samples versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. TGF-β_1 and VEGF-A in the supernatants were analyzed by ELISA. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 5: PSG23N1A induces pro-angiogenic factors in human monocytes and trophoblast cells. (a & b) Human monocytes were treated with 30 μg/ml PSG23N1A for 24 hours. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. (c & d) HTR-8/SVneo cells were treated with PSG23N1A in concentrations of 10-30 μg/ml for 24 hours. Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.001$. TGF-β_1 and VEGF-A in the supernatants were analyzed by ELISA. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 6: PSG23N1A binding and activity is not CD9-dependent. (a) Peritoneal macrophages from wild-type and CD9-null mice were incubated with 5 μg of PSG23N1A or Multiple Tag control protein, followed by subsequent incubation with biotinylated anti-Flag antibody, and stained with FITC-conjugated streptavidin and a live/dead cell discriminator. Cells were fixed and samples processed with the BD LSRII cell analyzer. Data was analyzed using WinList 3.0 software. (b) PSG23N1A induced TGF-β_1 secretion in wild-type and CD9-null peritoneal macrophages after 24 hours of treatment as measured by ELISA. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 1



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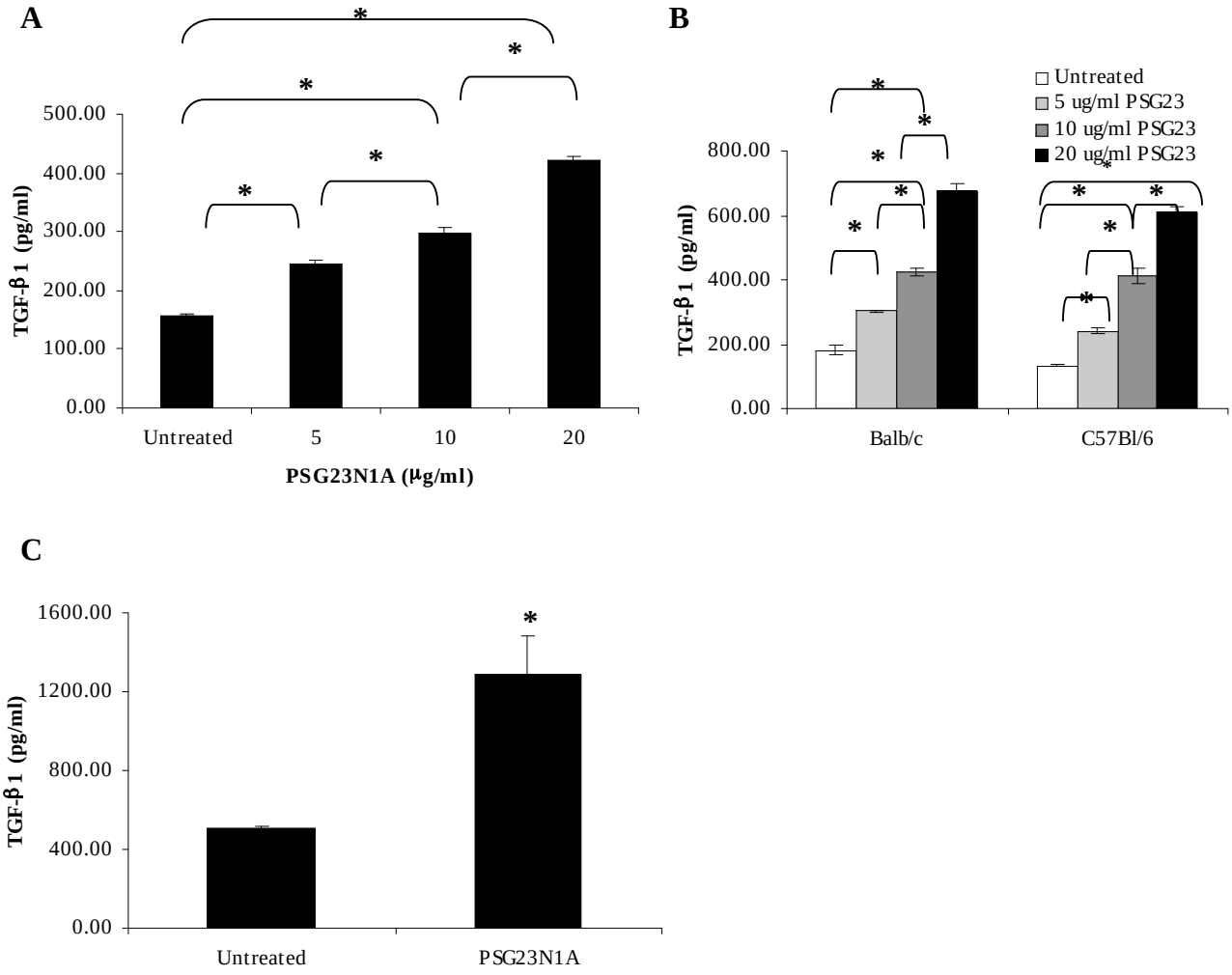
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Figure 3

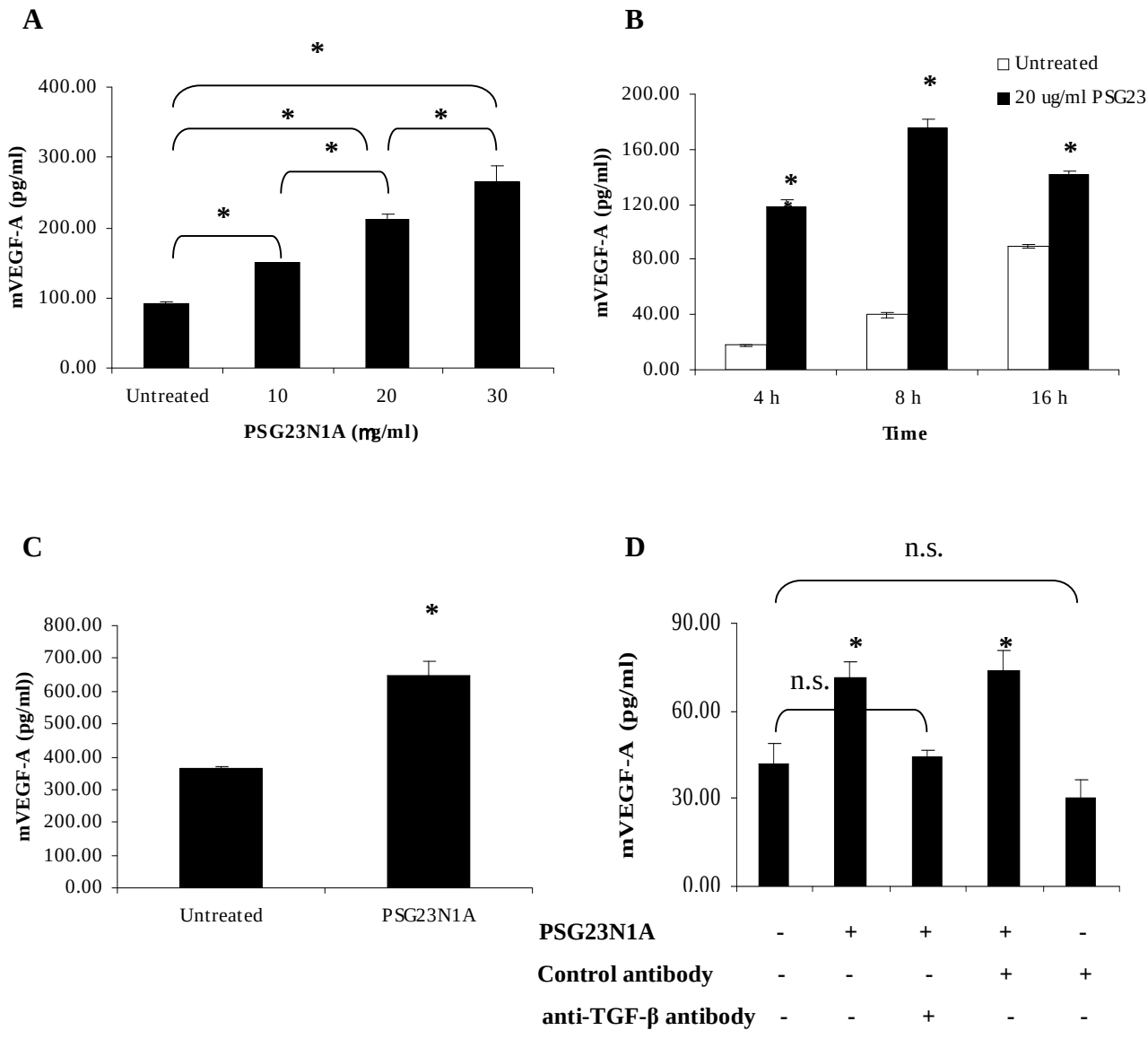
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Figure 4

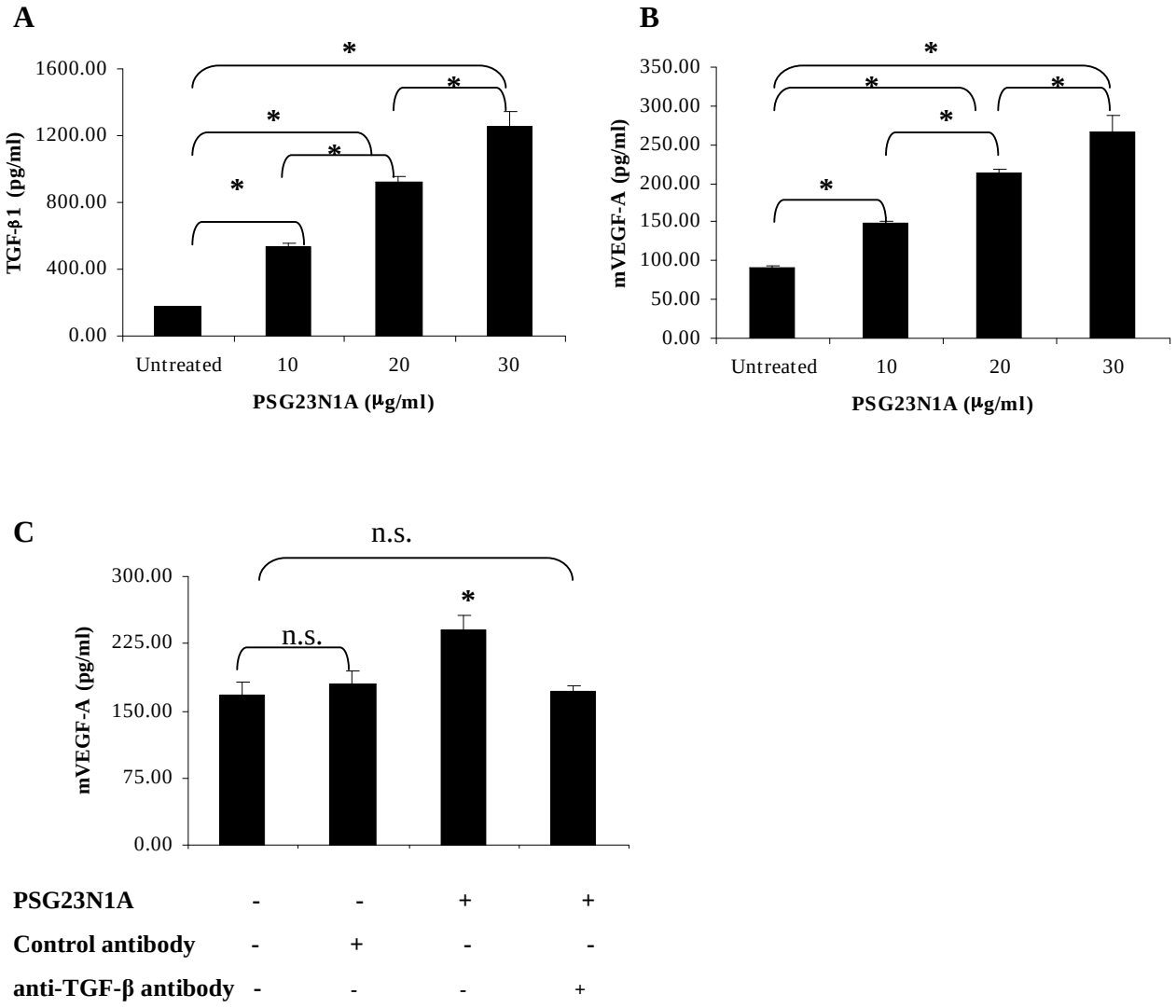
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Figure 5

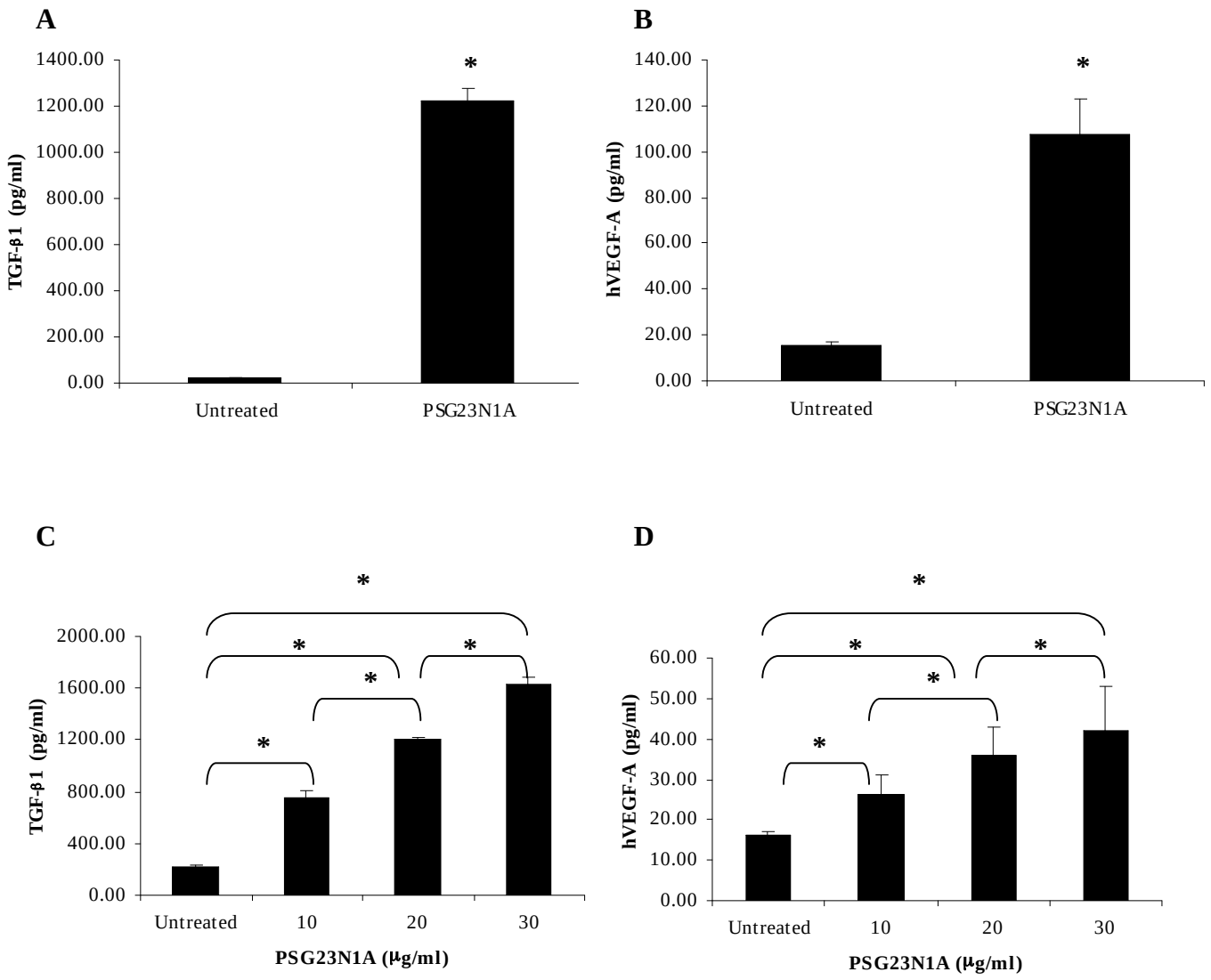
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Figure 6

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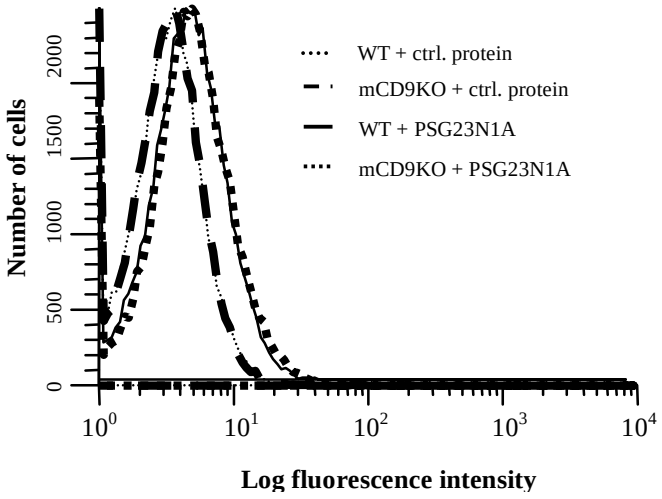
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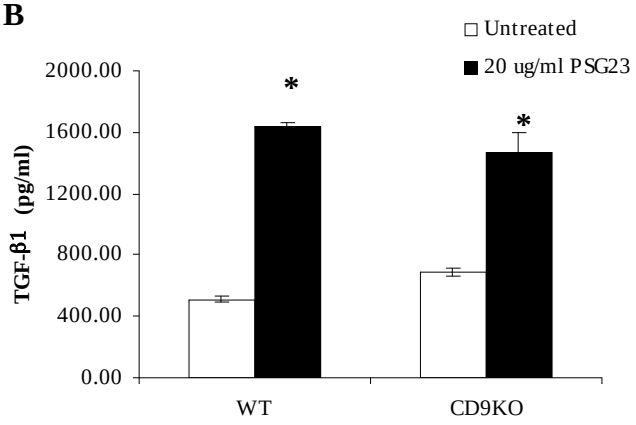
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PART THREE:

PROJECT 2

Proposed manuscript 2:

Human PSG1d and murine PSG22 induce pro-angiogenic factors

PROJECT 2: Human PSG1d and murine PSG22 induce pro-angiogenic factors

Introduction

Implantation is a very important first process in successful pregnancy for humans and rodents. The proper attachment and invasion of the uterus by the blastocyst establishes the first contact between mother and fetus and also initiates the process of placentation [127]. Problems that arise in the beginning stages of pregnancy can have damaging effects that can lead to pathologies associated with pregnancy such as pre-eclampsia or even early spontaneous abortion [2]. Many factors are produced in early pregnancy that are vital to the maintenance of pregnancy, among them are pregnancy-specific glycoproteins (PSGs).

PSGs are members of the carcinoembryonic antigen (CEA) family and are highly glycosylated, secreted proteins [113]. PSG homologues have been identified in primates, rats and mice [99]. Human and murine PSGs share a 60% homology [99] and the functions of human and murine PSGs reported to date are often similar [89, 93, 94]. There are 11 human PSGs and 17 murine PSGs all of which contain N-terminal IgV-like domains [98, 128, 129]. Human PSGs have one N-domain followed by two or three A and B IgC-like domains [77, 130]. Murine PSGs have multiple N-domains, followed by a single IgC-like A domain [98]. The N1 domain of murine PSGs is homologous to the single N-domain of human PSGs, and for some of the murine and human PSGs studied to date the N1 or N domains, respectively, are sufficient for binding to and activity in target cell types [92, 93].

In human pregnancy, PSGs have been detected as early as 3-4 days post-fertilization coinciding with the attachment of the blastocyst to the uterine wall [75]. Although the

function of PSGs is still being explored, previous studies focused on their role of potent immunomodulators during pregnancy [75, 89, 91, 92, 94]. Murine PSGs have been more difficult to detect in the mouse placenta due to the lack of specific antibodies; however, murine PSG transcripts have been detected as early as E6.5 [131]. The function of murine PSGs as more than just immunomodulators has been suggested [132, 133] (Dveksler lab, unpublished results), though their role as inducers of anti-inflammatory cytokines in macrophages has been established [79, 89, 93, 134].

Previous studies with human and murine PSGs identified monocytes/macrophages as target cells for their binding and activity. Treatment of these cell types with recombinant human and murine PSGs resulted in upregulation of anti-inflammatory cytokines, such as IL-10, TGF-β_1 and IL-6 [94] and T-helper type 2 (Th2) factors, including PGE_2, cyclooxygenase (COX)-2 and arginase I [89, 90] which are thought to aid in pregnancy success [135].

The pro-angiogenic properties of monocytes/macrophages have been reported [136-138]. During pregnancy, macrophages are thought to regulate angiogenesis by acting on invasive trophoblast cells necessary for uterine spiral artery remodeling and establishment of the fetoplacental blood supply [51], as well as secreting a number of pro-angiogenic factors [36, 41]. Due to the findings that human and murine PSGs target monocytes/macrophages, it was conceivable that PSGs might also play a role in angiogenesis.

During early pregnancy TGF-β_1 plays an important role in implantation and placenta development [139, 140]. TGF-β_1 is a pro-angiogenic factor [141] which not only aids in

vascular development during placentation [33] but also regulates the major family of proteins involved in vasculogenesis and angiogenesis: the VEGF family [24, 39, 41, 142].

The VEGF family of growth factors includes VEGF-A, -B, -C, -D and PlGF [143], and is believed to guide the early development of the placenta [13]. Transgenic mice with VEGF mutations exhibit impaired vascular and lymphatic development [144, 145] and murine *Vegfa* knockouts are embryonic lethal [25]. Although evidence suggests that decreased expression of VEGF and TGF-β may lead to the development of pre-eclampsia [34], the signals that promote secretion of these pro-angiogenic factors are still being explored. Based on the recent observation that murine PSG21 is found in association with maternal vasculature [133], and the fact that PSGs induce TGF-β_1 in monocytes/macrophages, we hypothesized that human and murine PSGs expressed during early pregnancy may play a role in implantation and placentation.

Our studies show that human PSG1d, one of the most abundant human PSGs in pregnancy [146], and murine PSG22, which is expressed in early mouse pregnancy [98] induce TGF-β_1 and VEGF in monocytes/macrophages, immune cells important to the process of implantation [51, 147]. We also demonstrate that treatment of human and murine endothelial cells and human trophoblast cells with PSG1d and PSG22 leads to induction of TGF-β_1 and VEGF in a dose-dependent manner.

Previously, it was reported that murine PSG17 uses the tetraspanin CD9 as its receptor [93], but that PSG1d does not require CD9 for binding or to elicit activity in macrophages [89]. In

this report, we show that PSG22 also does not require CD9 for induction of TGF-β_1 and VEGF in macrophages. Tetraspanins are membrane-bound proteins that are found in nearly every cell type and have been shown to play a role in angiogenesis [103, 108]; therefore, while the presence of CD9 does not appear necessary for the activities of PSG1d and PSG22, we cannot excluded the possibility that one or more tetraspanins could serve as the receptors for these proteins.

Here we report on a possible function for human and a murine PSG expressed in giant cells on implantation and placentation as well as identify new targets, endothelial cells and trophoblasts, for PSG activity.

Materials and Methods

Antibodies

For flow cytometry analysis the following antibodies were used: streptavidin-fluorescein isothiocyanate (FITC), streptavidin–phycoerythrin (PE), and phycoerythrin Rat IgG2b isotype control (eBioscience, Inc., San Diego, CA), BAP3 (Genovac, Freiburg, Germany), purified anti-Flag BioM2 (Sigma, St. Louis, MO), rat anti-mouse IgG1 phycoerythrin and CD16/CD32 (Fcγ III/II receptor, BD Biosciences, San Jose, CA), GST (B-14) antibody (clone sc-138, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and LIVE/DEAD Fixable Red and Blue Dead Cell Stain kits (Molecular Probes, Carlsbad, CA).

For immunoblotting, His-probe (H-15) antibody (clone sc-803, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Flag M2 antibody and anti-Flag M2-peroxidase (HRP) antibody (Sigma, St. Louis, MO) were used.

Protein production and purification

Recombinant PSG1d-His-V5-Flag (hereafter referred to as PSG1d-Flag) protein was generated by the GenScript Corporation (Piscatawy, NJ) and contained the leader (L), N, A2 and B2 domains of the *PSG1d* cDNA and a Flag tag (Sigma). *PSG1d-Flag* was subcloned into the *Bgl*I and *Not*I sites of the pEF1/V5-His (version C) vector (Invitrogen) resulting in the addition of the V5 and His tags at the C-terminus. We transfected the plasmid into dihydrofolate reductase-negative (DHFR-) CHO cells, along with the pDCHIP plasmid, which encodes the *dhfr* minigene (kind gift from Dr. G. Kaplan, FDA). Positive transformants were obtained by methotrexate selection and cells were seeded into a 5 kDa

molecular weight cutoff hollow fiber cartridge (FiberCell Systems, Inc., Frederick, MD). The supernatant from the cartridge was harvested daily and kept frozen until it was processed as described below.

Recombinant PSG22N1A-His-Flag (hereafter referred to as PSG22N1A) was generated by the GenScript Corporation (Piscatawy, NJ) by inserting the leader peptide, N1 and A domains of *Psg22*, along with the nucleotide sequence for six histidines (6X His) and a Flag tag (Sigma) into the *Sma*I site of pUC57 (EZBiolab, Inc., Westfield, IN). *Psg22*N1A-His-Flag was subcloned into the *Eco*RI and *Xba*I sites of the pEF1/V5-His expression vector (Invitrogen, Carlsbad, CA) and transfected into dihydrofolate reductase-negative (DHFR-) CHO cells, along with the pDCHIP plasmid, which encodes the *dhfr* minigene. Prior to methotrexate selection, single cell colonies were obtained by limiting dilution in a 96 well plate. Cell tracker (Molecular Probes) was used to identify the single colonies by fluorescent microscopy. The single colonies were then seeded in a 24-well plate, and upon confluency were transferred to a 6-well plate, followed by a T25 tissue culture flask, at which point methotrexate selection began. Four stably transfected single cell clones were obtained, and the protein was isolated from the supernatants of the single cell clones by concanavalin (Con) A bead (GE Life Sciences) purification. The purified protein was run on a SDS-PAGE gel and analyzed for relative expression by immunoblot analysis. Colony #1 was found to express the highest level of PSG22N1A and was expanded and seeded into a 5 kDa molecular weight cutoff hollow fiber cartridge (FiberCell Systems, Frederick, MD). The supernatant from the cartridge was harvested daily and kept frozen until it was processed as described below.

PSG1d-Flag and PSG22N1A were dialyzed into a 20 mM sodium phosphate buffer containing 20 mM imidazole (Company, City, State) and purified from the harvested supernatant using a HisTrap column on the ÄKTAprime Plus system (GE Healthcare, Piscataway, NJ). Positive fractions eluted from the HisTrap column were pooled, buffer-exchanged into PBS and applied to a column packed with anti-Flag M2 agarose (Sigma), and eluted with 3X Flag peptide (Sigma). Positive fractions from the anti-Flag column were collected, pooled concentrated and buffer-exchanged with PBS. The purified protein was run on a SDS-PAGE gel, stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) and quantitated against BSA standards.

Recombinant PSG17N-myc-His was generated as previously described [134].

Cell lines

For overexpression experiments HEK 293T cells and BHK cells (ATCC, Manassas, VA) were used. For protein activity assays we tested, RAW 264.7 (ATCC), C166 (ATCC), human invasive trophoblast (HTR-8/SVneo) cells [118] (kind gift from Charles Graham, Queen's University, Kingston, ON, Canada), and uterine microvascular endothelial cells (UtMVECs, Lonza, Basel, Switzerland).

HEK 293T and BHK cells were maintained in Dulbeccos Modified Eagles Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT), 10 mM Hepes (Cellgro), and antibiotics. RAW 264.7 and C166 cells were cultured in DMEM supplemented

with 10% fetal clone III serum alternative (Hyclone), 10 mM HEPES (Mediatech, Inc., Manassas, VA), and penicillin-streptomycin (PS, Mediatech, Inc.). HTR-8/SVneo cells were maintained in HyQ RPMI 1640-RS (Hyclone) media. UtMVECs were maintained in endothelial cell basal medium-2 supplemented with human recombinant epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin, amphotercin, and 5% fetal bovine serum (EGM-2MV media, Lonza) and were used after passage 2 or 3 for each experiment.

All cells were cultured in a 37ºC humidified incubator with 10% CO_2.

Primary cells

Five- to six-week-old C57Bl/6 mice deficient in CD9 were bred from a CD9+/- breeding pair obtained from Dr. Claude Boucheix (Hôpital Paul Brousse, Villejuif, France). Five- to six-week-old Balb/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Briefly, mice were injected i.p. with thioglycollate (BD Biosciences). Three days after i.p. injection, macrophages were obtained by peritoneal lavage. Cells were seeded at 1.5×10^6 per well in a 24-well plate. Twenty-four hours after seeding the cells, macrophages attached to wells and non-adherent cells were removed. Plating media (RPMI supplemented with 2% FBS and 0.5X PS) was replaced with treatment media (RPMI supplement with 2% delipidized FBS and 0.5X PS, sterile filtered through a 0.2 μm membrane) prior to addition of protein or PBS control.

Human monocytes were isolated from the peripheral blood of healthy adult donors and purified by centrifugal elutriation as previously described [92]. Human monocytes were maintained in RPMI 1640 (Gibco) supplemented with 2 mM glutamine.

Plasmids and cell transfection

For cell transfection, HEK 293T or BHK cells were seeded at a density of 4.5 x 10^6 in a 60 mm poly-L-lysine culture dish (BD Biosciences). After cells attached, culture media was removed and 20 ml of plating media was added to each plate. Plasmids were incubated with FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switerzland) as per manufacturers instructions and added to the cells. Forty-eight hours post-transfection, cells were removed from the culture dishes using CellStripper (MediaTech, Inc.) and used for binding experiments. Transfection efficiency was approximately 80% with the plasmids used. Green fluorescent protein (GFP)-labeled murine CD9 were constructed as previously reported [134].

ELISAs

For enzyme-linked immunosorbant assays (ELISAs), cells were plated in triplicate wells for each treatment in 24-well plates (BD Biosciences) and incubated in a 37°C humidified incubator with 10% CO_2. RAW 264.7 cells were seeded at a density of 1 x 10^6 cells per well; C166 cells were seeded at a density of 0.25 x 10^6 cells per well; HTR-8/SVneo cells were seeded at 0.4 x 10^6 cells per well; human monocytes were seeded at a density of 1.2 x 10^6 cells per well; peritoneal macrophages were seeded at a density of 1.5 x 10^6 per well; and UtMVECs were seeded at 0.4 x 10^6 cells per well. Cells were incubated overnight and treated

on the following day with recombinant proteins or PBS in 300 µl of fresh media for treatment times less than 6 hours long or 1 ml of fresh media for treatment times greater than six hours. In some experiments, endotoxin-free BSA was used as the protein control and did not induce TGF-β_1. When UtMVECs were used in experiments measuring VEGF, cells were incubated in supplement-free EGM media (Lonza) two hours prior to treatment due to the presence of VEGF in the EGM-2MV media.

After treatment, the supernatants were collected and centrifuged at 3000 rpm for 5 minutes to remove cell debris. For TGF-β_1 ELISA, supernatant or media alone was activated as per the manufacturer's instructions (R & D Systems). Murine VEGF-A and PlGF expression were measured by ELISA (R & D Systems). Human VEGF-A and VEGF–C were also measured by ELISA (R& D Systems). TNF-α in the supernatants from both human and murine cells was determined using the BD OptEIA Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences). At least three different protein preparations were tested and experiments were repeated a minimum of three times.

Quantitative relative real-time polymerase chain reaction (QRT-PCR)

For quantitative relative real-time PCR (QRT-PCR) reactions, RAW 264.7 or murine peritoneal macrophages were seeded at densities of 1×10^6 cells per well or 1.5×10^6 cells per well in a 24-well plate, respectively. Cells were treated with varying concentrations of protein or control. Cell lysates were harvested after 45 minutes to up to 24 hours post-treatment, and RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems,

Foster City, CA). All primers for QRT-PCR reactions were purchased from Applied Biosystems. The following murine *Vegfa* and *Vegfc* were measured by QRT-PCR, using hypoxanthine guanine phosphoribosyl transferase (HPRT) 1 (Applied Biosystems) as an endogenous control. The QRT-PCR reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems).

Binding experiments

For FACS binding experiments, 1 x 10^6 cells in 100 μl were treated with Multiple Tag (GenScript Corporation) control protein or PSG22N_1A protein for one hour on ice. Cells were incubated with 0.5 μg of anti-Flag antibody (Sigma) to detect protein binding, followed by incubation with 0.5 μg of biotinylated goat anti-mouse antibody (BD Biosciences) 0.5 μg FITC- or PE-conjugated streptavidin. Excess protein and antibodies were removed by washing with FACS wash buffer (PBS supplemented with 3% FBS and 0.05% NaN_3). Cells were fixed with BD Cytofix (BD Bioscences) and analyzed using the Coulter Epics XL Flow Cytometer (Beckman Coulter, Fullerton, CA).

Statistical analysis

SPSS (SPSS, Inc., Chicago, IL) and Microsoft EXCEL were used for data statistical analysis. The two-tailed Student's *t*-test was used to determine statistical significance in experiments comparing protein treated versus untreated cells, with a p-value of <0.05 as a cut-off. One-way ANOVA was used to determine statistical significance of dose-response assays with a p-value of <0.001 as a cut-off. QRT-PCR results were analyzed using the 7500 System Sequence Detection Software version 1.2.3 (Applied Biosystems). FACS data were

analyzed using WinList 6.0 (Verity Software House, Topsham, ME). All data is representative of at least three independent experiments.

Results

Generation of recombinant PSG1d-Flag and PSG22N1A

There are 11 members of the human *PSG* family and *PSG1* is the most abundant family member [146]. We generated recombinant PSG1d-Flag that consists of the leader peptide followed by the N, A2 and B2 domains followed by a Flag, V5 and 6X His tags. The 51 kDa recombinant protein was harvested from the supernatant of stably transfected CHO cells as described in the Materials and Methods section.

Murine *Psg*s are 60% homologous to human *PSG*s [98], and *Psg22* has been identified as an early expressing gene in the mouse placenta [98, 133]. We generated a recombinant PSG22N1A protein that consists of the leader peptide, N1 and A domains, followed by six histidines and a C-terminal Flag tag in CHO cells. We isolated a single cell clone that expressed high levels of recombinant PSG22N1A as described in the Material and Methods section.

Both the recombinant PSG1d-Flag and PSG22N1A proteins were sequentially purified on a HisTrap and anti-Flag affinity column. These two purification steps rendered glycoproteins that were approximately 90% pure based on Coomassie Blue staining (Figure 1a and b). In addition, the protein preparations were determined endotoxin-free by their inability to induce TNF-α in macrophages.

Recombinant PSG1d-Flag induces VEGF-A in monocytes/macrophages

It was previously reported that PSG1d induces TGF-β_1 in human monocytes, the murine macrophage RAW 264.7 cell line and murine peritoneal macrophages [79, 89-92, 94]. TGF-β_1 has been shown to induce the pro-angiogenic factor VEGF in macrophages [41] and VEGF-A and -C are expressed at the site of trophoblast invasion [148]. We, therefore, wanted to investigate the possibility that PSG1d-Flag might regulate VEGF expression.

Human monocytes (Figure 2a) and RAW 264.7 cells (Figure 2b) secreted VEGF-A in response to 10 μg/ml of PSG1d-Flag at least 10-fold over control-treated cells after 24 hours of treatment, as determined by ELISA. We also tested the induction of VEGF-C, which is important in placentation [143], by ELISA and did not see induction of this growth factor in human monocytes after PSG1d-Flag treatment (data not shown).

Human endothelial cells and trophoblasts as novel targets for PSG1d-Flag

Numerous cell types are involved at the site of implantation, among which are monocytes/macrophages, endothelial cells, uterine natural killer (uNK) cells, and trophoblasts [149]. Based on the observations that PSG1d-Flag induces TGF-β_1 in monocytes/macrophages [92] and VEGF-A in monocytes, we hypothesized that human PSG1d could affect the functions of other cell types involved in implantation. We explored the possibility that endothelial cells and a trophoblast cell line respond similarly to PSG1d-Flag treatment.

The human endothelial cell line (UtMVEC) is derived from normal human myometrial uterine microvascular endothelial cells and its use in co-culture experiments to mimic the uterine endothelial lining is established [150, 151]. UtMVECs secreted TGF-β_1 in response to 24 hours of PSG1d-Flag treatment (Figure 3a). VEGF-A was also induced in UtMVECs after 24 hours of protein treatment (Figure 3b). We did not see induction of VEGF-C, measured by ELISA, in UtMVECs after PSG1d-Flag treatment (data not shown).

The invasive human trophoblast cell line (HTR-8/SVneo) has aided in the understanding of some of the mechanisms involved in trophoblast invasion and migration during pregnancy [118]. The effects of PSG1d-Flag treatment on the secretion of TGF-β_1 and VEGF-A and –C by HTR-8/SVneo was investigated. Cells were treated for 24 hours with 20-40 μg/ml of PSG1d-Flag, and up-regulation of TGF-β_1, VEGF-A and VEGF-C secretion in HTR-8/SVneo cells (Figures 3c, d and e) by ELISA were observed.

Recombinant PSG22N1A targets macrophages

It was previously demonstrated that the N1 domain of murine PSG17, 18 and 19 elicits an anti-inflammatory cytokine response in monocytes/macrophages [79, 89, 94]. We tested the response of the murine RAW 264.7 macrophage cell line to various concentrations of PSG22N1A treatment. RAW cells secreted TGF-β_1 in response to PSG22N1A in a dose-dependent manner starting at 30 μg/ml as determined by ELISA (Figure 4a).

The response of primary macrophages, isolated by peritoneal lavage of Balb/c mice, was also examined and it was found that PSG22N1A induced TGF-β_1 in these cells after 4 hours of treatment (Figure 4b) and up to 24 hours post-treatment.

We examined the production of pro-inflammatory cytokines in response to PSG22N1A. PSG22N1A did not up-regulate TNF-α and IL-6 at 24 hours post-PSG22N1A, even up to treatment concentrations of 50 μg/ml (data not shown), as measured by ELISA.

PSG22N1A induces VEGF-A in macrophages

Previous studies inducated that VEGF-A is up-regulated in macrophages in response to PSG23N1A treatment (Dveksler lab, unpublished results). Therefore, we tested whether PSG22 had the same effect in macrophages. The secretion of VEGF-A was up-regulated in response to PSG22N1A treatment in a dose-dependent manner, starting at 20 μg/ml (Figure 5a), and measured 24 hours post-treatment by ELISA. We also measured *Vegfa* mRNA by QRT-PCR in RAW cells after 2 and 4 hours of PSG22N1A treatment and did not see induction of *Vegfa* mRNA (data not shown).

Peritoneal macrophages isolated from Balb/c mice were treated with 40 μg/ml of PSG22N1A for 4 and 24 hours (Figure 5b). VEGF-A was induced in peritoneal macrophages at both time points.

Similar to our results with PSG23N1A (Dveksler lab, unpublished results), we did not see induction of *Vegfc* mRNA after 2 and 4 hours of treatment with PSG22N1A (data not

shown). In addition, we did not observe induction of the VEGF family member PlGF in RAW cells after 24 hours of PSG22N1A treatment, measured by ELISA (data not shown).

PSG22N1A targets murine endothelial cells

Previously, we observed that PSG23N1A induces the secretion of pro-angiogenic factors in the C166 murine endothelial cell line (Dveksler lab, unpublished results). Endothelial cells are involved in the process of implantation and placentation [152]. The C166 cells secreted higher levels of TGF-β_1 in response to PSG22N1A in a dose-dependent manner (Figure 6a) after 24 hours of treatment, as measured by ELISA. VEGF-A was also induced in C166 cells after 24 hours of treatment, as measured by ELISA (Figure 6b). We also did not observe a significant difference between 20 μg/ml of PSG22N1A treatment and 40 μg/ml PSG22N1A in C166 cells (Figure 6b). Similar to our results with PSG23N1A, we did not observe induction of *Vegfa* or *Vegfc* mRNA after PSG22N1A treatment, measured by QRT-PCR, or PlGF, measured by ELISA (data not shown).

PSG22N1A shows cross-reactivity with human cells

Based on the homology between human and murine PSGs and previous studies that indicated cross-reactivity between human PSGs and murine macrophages [89, 91, 92], we investigated the possibility that PSG22N1A may induce TGF-β_1 and VEGF-A in human monocytes and invasive human trophoblast cell line (HTR-8/SVneo). Human monocytes secreted TGF-β_1 24 hours post-treatment in response to 30-40 μg/ml of PSG22N1A (Figure 7a) and secreted VEGF-A 24 hours post-treatment in response to 50 μg/ml of PSG22N1A (Figure 7b).

We also measured TGF-β_1 and VEGF-A up-regulation in HTR-8/SVneo cells after PSG22N1A treatment. By ELISA analysis of the cell supernatants 24 hours post-treatment, we found that 30 μg/ml of PSG22N1A induced secretion of TGF-β_1 in HTR-8/SVneo cells (Figure 7c) and that VEGF-A was induced in the trophoblast cell line in a dose-dependent manner (Figure 7d). As seen for PSG23N1A treatment of human monocytes and HTR-8/SVneo cells (Dveksler lab, unpublished results), we did not observe up-regulation of VEGF-C after PSG22N1A treatment as measured by ELISA 24 hours post-treatment (data not shown).

PSG22N1A activity does not require CD9

Murine PSGs share a 59-70% homology [98], and previous studies with murine PSG17 demonstrated that PSG17-induced TGF-β_1 in macrophages occurs through the binding of PSG17 to the tetraspanin CD9 [93]. Our recent data shows that PSG22 also targets macrophages and induces anti-inflammatory cytokines in these cell types; therefore, we sought to determine if PSG22 utilizes CD9 as its receptor.

In functional assays, we isolated peritoneal macrophages from wild-type and CD9-null mice and treated the cells with PSG22N1A. We measured expression of TGF-β_1 in the wild-type and CD9-null peritoneal macrophages by ELISA. Cells from wild-type and knockout animals secreted TGF-β_1 after 24 hours of treatment (Figure 8). In conclusion, the functional studies showed that PSG22 does not depend on the presence of CD9 to bind to macrophages and elicit a response.

Discussion

Successful pregnancies begin with attachment and implantation of the blastocyst into the uterine decidua [153]. Without proper initialization of the placenta, the mother and fetus can experience vascular stress leading to pregnancy complications, such as fetal hypoxia and maternal pre-eclampsia [2]. The molecular mechanisms involved in the process of implantation and placentation are still not well characterized; however, placentally-derived factors may be crucial at the beginning stages of pregnancy [154-156].

PSGs are among the most abundant fetal proteins in pregnancy and yet their roles during gestation are still being explored [70]. PSGs belong to the CEA family whose other members have been implicated in the processes of immunoregulation and angiogenesis [72]. CEACAM1 has been shown to be an effector of VEGF, one of the key growth factors involved in vasculogenesis and angiogenesis [14, 157], and CEACAM1 and 6 have been shown to act on lymphocytes [72, 125]. While the role of PSGs as inducers of anti-inflammatory cytokines, such as IL-10 and TGF-β_1, which have been implicated in promoting pregnancy success [51, 158-160] has been demonstrated [89-92, 94], their roles in other process vital to pregnancy success have not.

Recent reports have suggested a putative role for PSGs in angiogenesis. Wynne et al. identified murine PSG21 expression in the endothelial cells lining the maternal vasculature [133]. Previous studies from our laboratory also defined a new role for murine PSG23 in the induction of VEGF in monocytes/macrophages and identified novel targets, dendritic cells, endothelial cells and trophoblasts, for PSG binding and activity (Dveksler lab, unpublished

results). The functional significance of PSG association with the maternal vasculature and induction of pro-angiogenic factors is still unknown. However, accumulating evidence suggests that PSGs may play a role in placentation.

The expression of human PSGs in pregnancy coincides with the early stages of implantation [70]. We hypothesized that the expression of PSGs at this point in pregnancy may be important in tolerizing the maternal immune system to the developing fetus and aiding in the establishment of the placenta. Based on our recent observation that murine PSG23 induces VEGF-A in macrophages (Dveksler lab, unpublished results) and the fact that human PSGs induce TGF-β_1 in monocytes/macrophages [89, 92], we explored the possibility that human PSGs also induce pro-angiogenic factors in monocytes. VEGF-A, but not VEGF-C, was strongly induced in human monocytes in response to PSG1d treatement.

We also treated human endothelial cells (UtMVECs) and a human invasive trophoblast cell line (HTR-8/SVneo) with PSG1d to determine if these cell types, which are involved in maternal vasculature remodeling [121], responded to PSG treatment. PSG1d induced TGF-β_1 and VEGF-A in both UtMVECs and HTR-8/SVneo cells. We also saw induction of VEGF-C in HTR-8/SVneo cells in response to PSG1d treatment, but did not see VEGF-C induction in UtMVECs. As endothelial cells are involved in angiogenesis during early pregnancy, and VEGF-C is reported to be important in placentation [143], endothelial cell secretion of VEGF family members in response to human PSGs warrants further investigation.

Murine PSG23 is expressed in mid-gestation [98]and its induction of TGF-β_1 and VEGF-A (Dveksler lab, unpublished results) may be important in maintaining the placenta during pregnancy; however, the role of murine PSGs in the early stages of placentation is still unknown. We generated a recombinant murine PSG22, which is expressed in early mouse pregnancy [98], in order to explore possible pro-angiogenic properties of early mouse PSGs.

Recombinant PSG22 induced TGF-β_1 and VEGF-A in RAW cells and peritoneal macrophages. We also observed induction of both pro-angiogenic factors in a mouse endothelial cell line, human monocytes and HTR-8/SVneo cells. Therefore, it is possible that murine PSGs also play a role in implantation and the establishment of the mouse placenta.

Our laboratory previously identified the tetraspanin CD9 as the receptor for murine PSG17 [93] and 19 [79], and we also demonstrated that CD9 is not the receptor for human PSG1d [89] or murine PSG23 (Dveksler lab, unpublished results). We therefore examined the possibility that murine PSG22 bind to CD9, but found that PSG22 does not require the presence of CD9 for successful binding to mouse macrophages. Tetraspanins have been implicated in angiogenesis and are expressed at the maternofetal interface in pregnancy [100]. Due to the homology between human and murine PSGs and the similarity amongst the murine PSGs, we cannot rule out the possibility that human PSG1d and murine PSG22 and PSG23 utilize another tetraspanin as their receptor and are in the process of exploring this hypothesis.

Here we report on a novel function for human PSGs and an early murine PSG, PSG22, in the process of implantation and vasculature remodeling. Our studies indicate that PSG1d and PSG22 are inducers of the pro-angiogenic factors TGF-β_1 and VEGF-A in several of the cell types involved in spiral artery remodeling during early pregnancy. We are currently investigating the possibility that PSG1d and PSG22 induce other pro-angiogenic factors, specifically, the angiopoetins (Ang-1 and Ang-2) and other members of the VEGF family involved in vasculogenesis and angiogenesis (PlGF). The effects, if any, that PSGs have on uNK cells, the most abundant leukocyte population at the maternofetal interface [149] will also be examined. Receptor identification studies for PSG1d, PSG22 and PSG23 are also on-going in our laboratory the results from this study along with these future studies will aid us in elucidating the full functions of PSGs in pregnancy.

Figure legend

Figure 1: Generation of recombinant PSG1d-Flag and PSG22N1A. (A) The 51 kDa PSG1d-Flag (arrows) secreted by CHO cells was analyzed by western using anti-V5 (left blot) and anti-Flag (right blot) antibodies. (B) Single cell clones which secreted PSG22N1A were analyzed by immunoblot after ConA purification (right blot) and colony #1 was determined to be the best producer (Lane 2). Lane 1 shows the same protein after two rounds of purification in a Coomassie stained gel. Arrows indicate the size of the protein, approximately 36 kDa.

Figure 2: PSG1d-Flag induces VEGF-A in human monocytes and murine RAW 264.7 cells. Human monocytes (A) or RAW cells (B) were treated for 24 hours with 10 μg/ml PSG1d-Flag and supernatants were analyzed by ELISA for VEGF-A induction. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 3: PSG1d induces TGF-β_1 and VEGF-A in UtMVECs and TGF-β_1, VEGF-A and C in HTR-8/SVneo cells. UtMVECs were treated for 24 hours in unsupplemented media with 10 or 30 μg/ml of PSG1d-Flag. Supernatants were analyzed by ELISA for TGF-β_1 (A) and VEGF-A (B) induction. HTR-8/SVneo cells were treated with 30 μg/ml PSG1d-Flag for 24 hours and up-regulation of TGF-β_1 and VEGF-A and –C was measured in cell supernatants by ELISA. Statistical significance between protein-treated versus the untreated

samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 4: PSG22N1A induces TGF-β_1 in RAW 264.7 cells and peritoneal macrophages from Balb/c mice. (A) RAW 264.7 cells were treated with 30-50 μg/ml PSG22N1A for 24 hours and supernatants were analyzed by ELISA. Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.001$. (B) Peritoneal macrophages isolated from Balb/c mice were treated with 30 μg/ml of PSG22N1A for 4 or 24 hours. Supernatants were collected at the specified time points and analyzed for TGF-β_1 up-regulation by ELISA. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 5: PSG22N1A induces VEGF-A in RAW 264.7 cells and peritoneal macrophages from Balb/c mice. (A) RAW 264.7 cells were treated with 20-40 μg/ml PSG22N1A for 24 hours. Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.05$. (B) Peritoneal macrophages isolated from Balb/c mice were treated with 40 μg/ml of PSG22N1A for 4 and 24 hours. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. Supernatants were harvested at the indicated time points and analyzed for VEGF-A induction by ELISA. All cells were treated in triplicate wells and supernatants analyzed as

separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

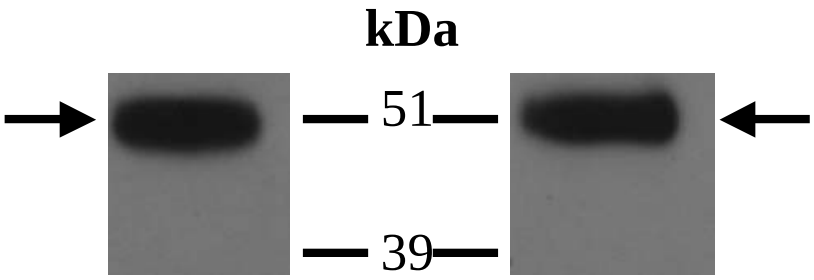
Figure 6: PSG22N1A induces TGF-β_1 and VEGF-A in C166 cells. The C166 murine endothelial cell line was treated for 24 hours with 20-40 μg/ml of PSG22N1A and supernatants were analyzed by ELISA for up-regulation of TGF-β_1 (A) and VEGF-A (B). Statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.05$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 7: PSG22N1A induces TGF-β_1 and VEGF-A in human monocytes and HTR-8/SVneo cells. Human monoctyes were treated with 30 μg/ml, 40 μg/ml or 50 μg/ml PSG22N1A for 24 hours. Up-regulation of TGF-β_1 (A) and VEGF-A (B) was measured in the cell supernatants by ELISA. HTR-8/SVneo cells were treated for 24 hours with 30 μg/ml, 40 μg/ml or 50 μg/ml PSG22N1A and TGF-β_1 (C) and VEGF-A (D) secretion was measured in the cell supernatants by ELISA. For Figure 7a and 7d statistical significance between doses was determined by one-way ANOVA and * indicates $P < 0.05$. For Figure 7b and 7c Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 8: PSG22N1A does not require CD9 for binding or activity in mouse macrophages. Peritoneal macrophages from wild-type and CD9-null mice were treated with 30 μg/ml PSG22N1A for 24 hours and TGF-β_1 up-regulation in the cell supernantants were analyzed by ELISA. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

Figure 1

A



B

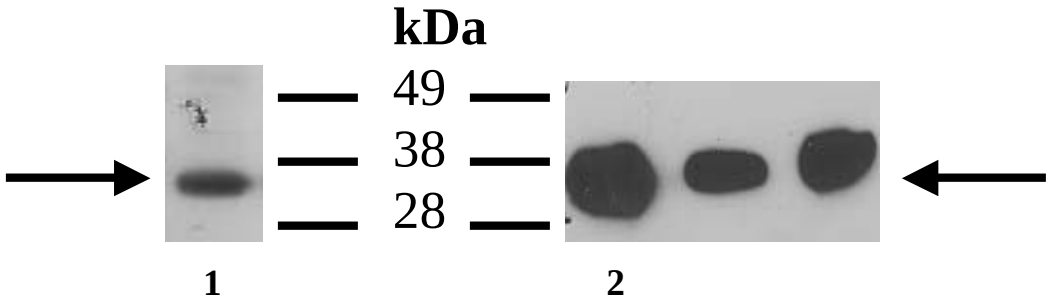
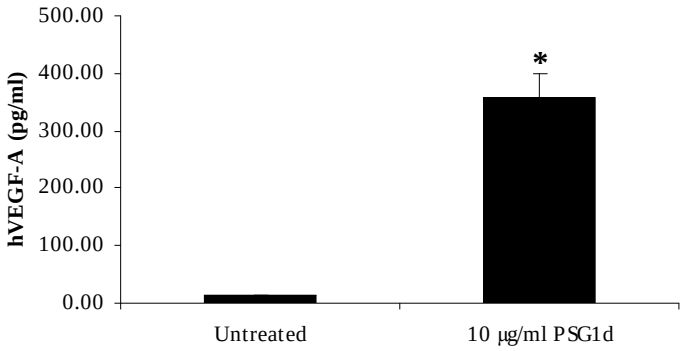


Figure 2

A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200.00
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0.00
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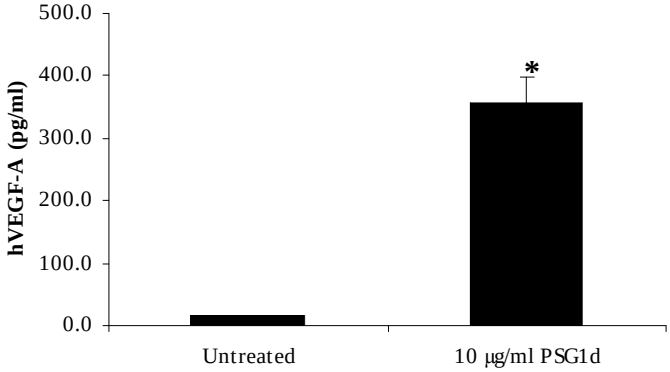
*
Untreated
10 μg/ml PSG1d

B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100.0
0.0
hVEGF-A (pg/ml)
*
Untreated
10 μg/ml PSG1d

Figure 3

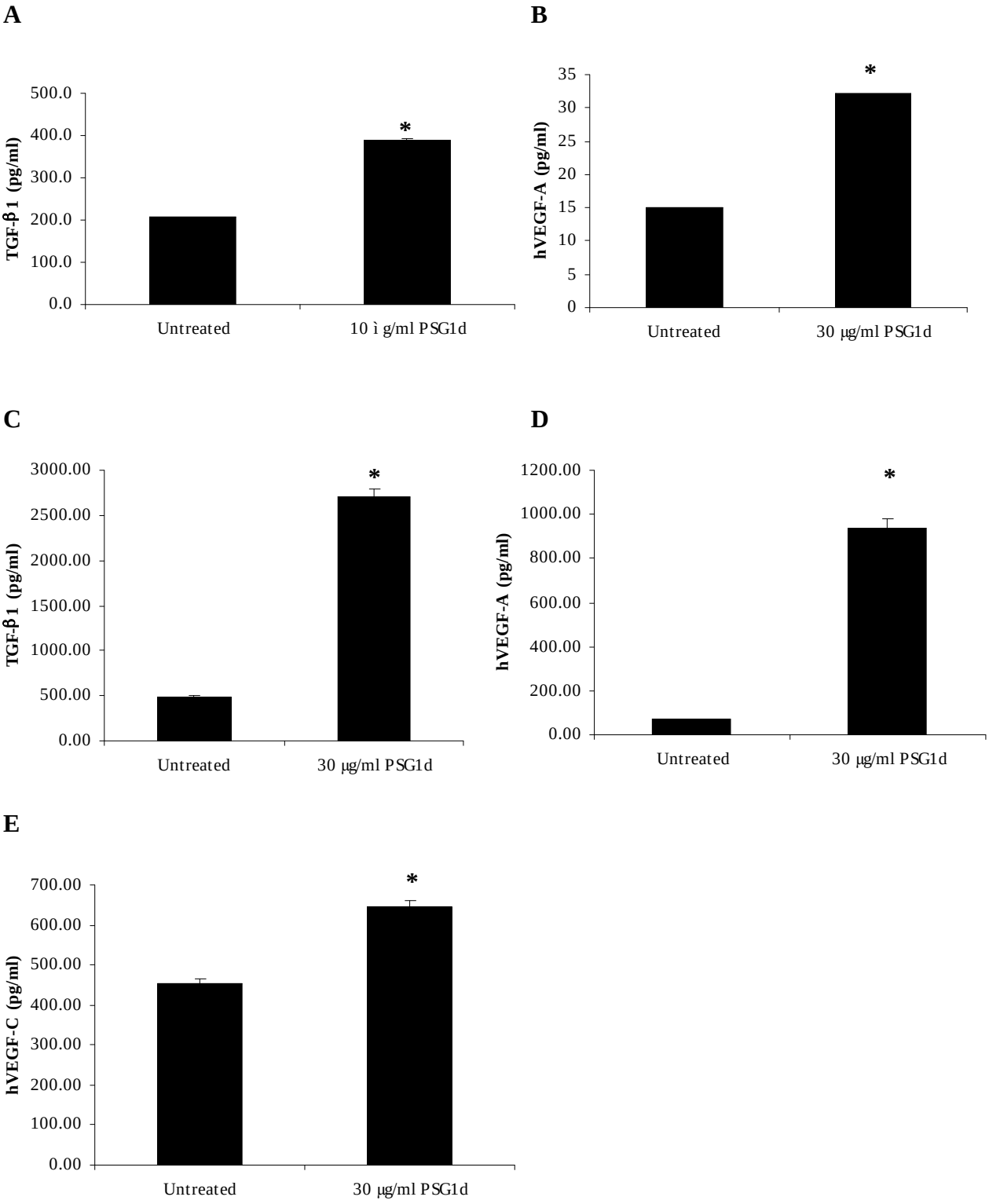
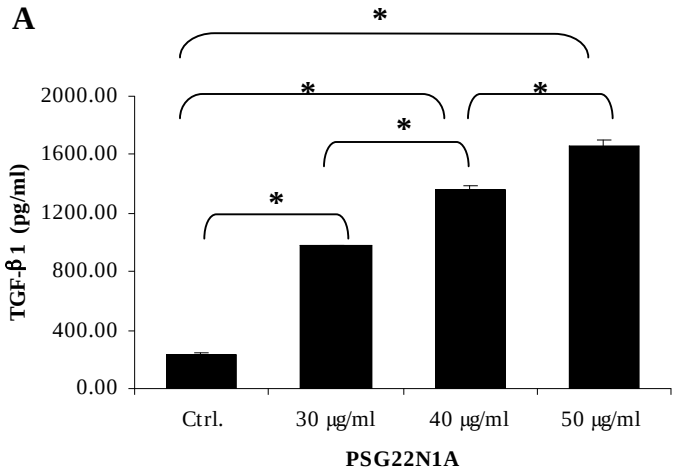

A
TGF-β1 (pg/ml)
500.0
400.0
300.0
200.0
100.0
0.0
*
Untreated
10 ì g/ml PSG1d
B
hVEGF-A (pg/ml)
35
30
25
20
15
10
5
0
*
Untreated
30 µg/ml PSG1d
C
TGF-β1 (pg/ml)
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
*
Untreated
30 µg/ml PSG1d
D
hVEGF-A (pg/ml)
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
*
Untreated
30 µg/ml PSG1d
E
hVEGF-C (pg/ml)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
*
Untreated
30 µg/ml PSG1d

Figure 4

A


*
*
*
*
*
2000.00
1600.00
1200.00
800.00
400.00
0.00
TGF-β1 (pg/ml)
Ctrl.
30 µg/ml
40 µg/ml
50 µg/ml
PSG22N1A

B

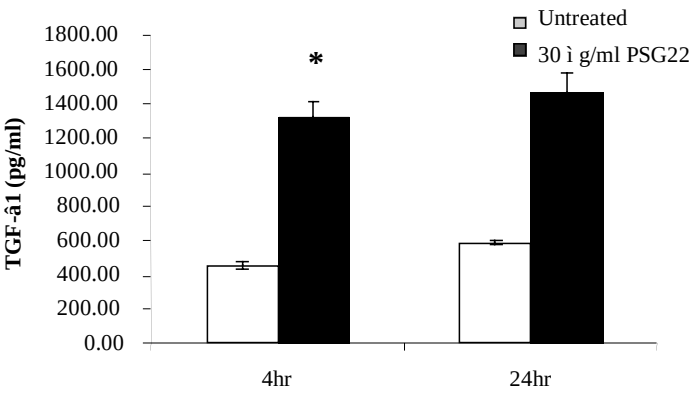
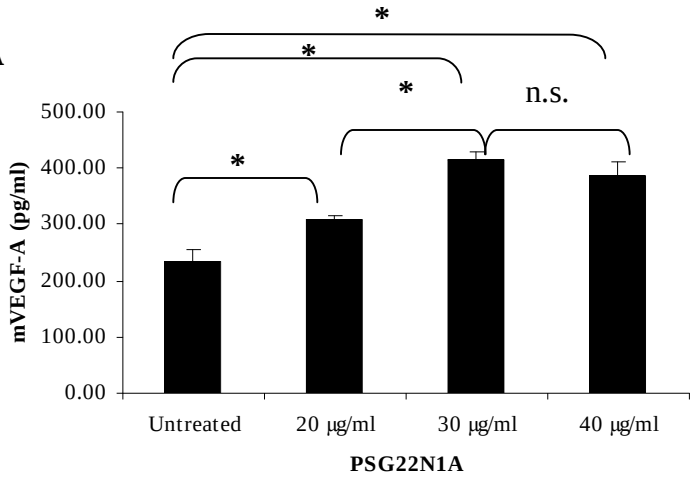

Untreated
30 ì g/ml PSG22
*
1800.00
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
TGF-â1 (pg/ml)
4hr
24hr

Figure 5

A


*
*
*
n.s.
*
500.00
400.00
300.00
200.00
100.00
0.00
mVEGF-A (pg/ml)
Untreated
20 µg/ml
30 µg/ml
40 µg/ml
PSG22N1A

B

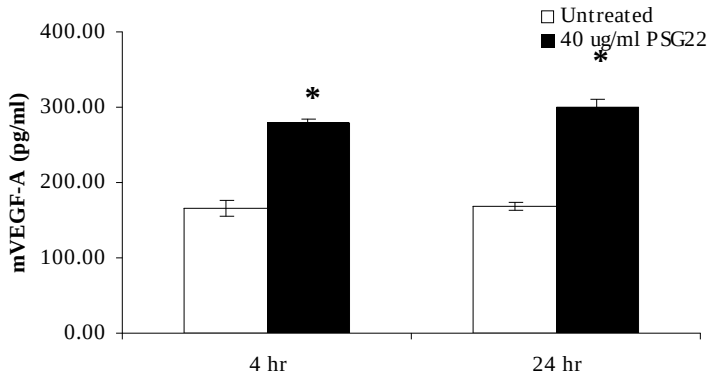

Untreated
40 ug/ml PSG22
400.00
300.00
200.00
100.00
0.00
mVEGF-A (pg/ml)
*
*
4 hr
24 hr

Figure 6

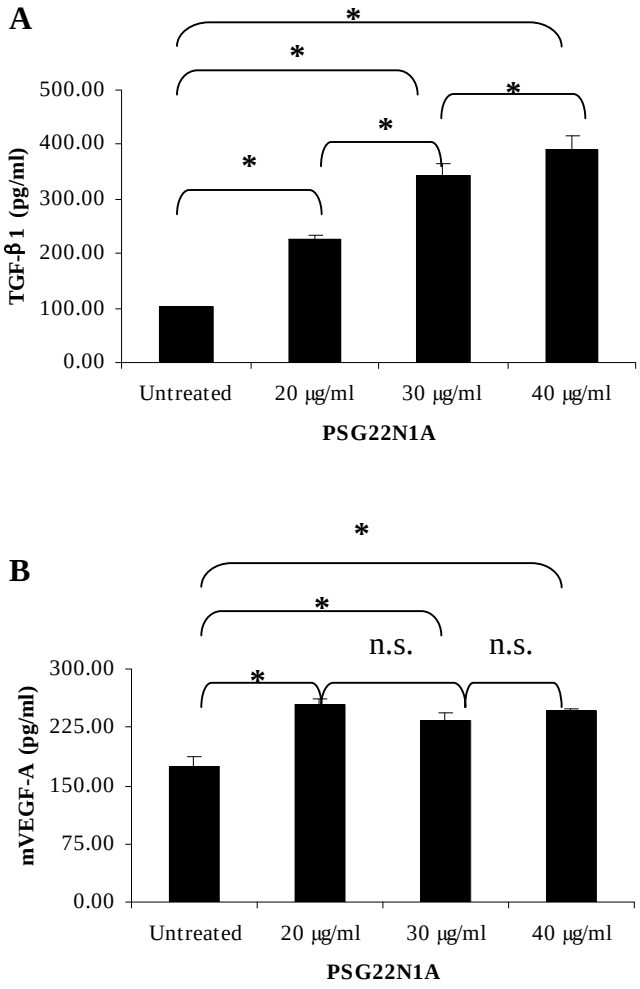

A
*
*
*
*
*
*
500.00
400.00
300.00
200.00
100.00
0.00
TGF-β1 (pg/ml)
Untreated
20 μg/ml
30 μg/ml
40 μg/ml
PSG22N1A
B
*
*
n.s.
n.s.
*
300.00
225.00
150.00
75.00
0.00
mVEGF-A (pg/ml)
Untreated
20 μg/ml
30 μg/ml
40 μg/ml
PSG22N1A

Figure 7

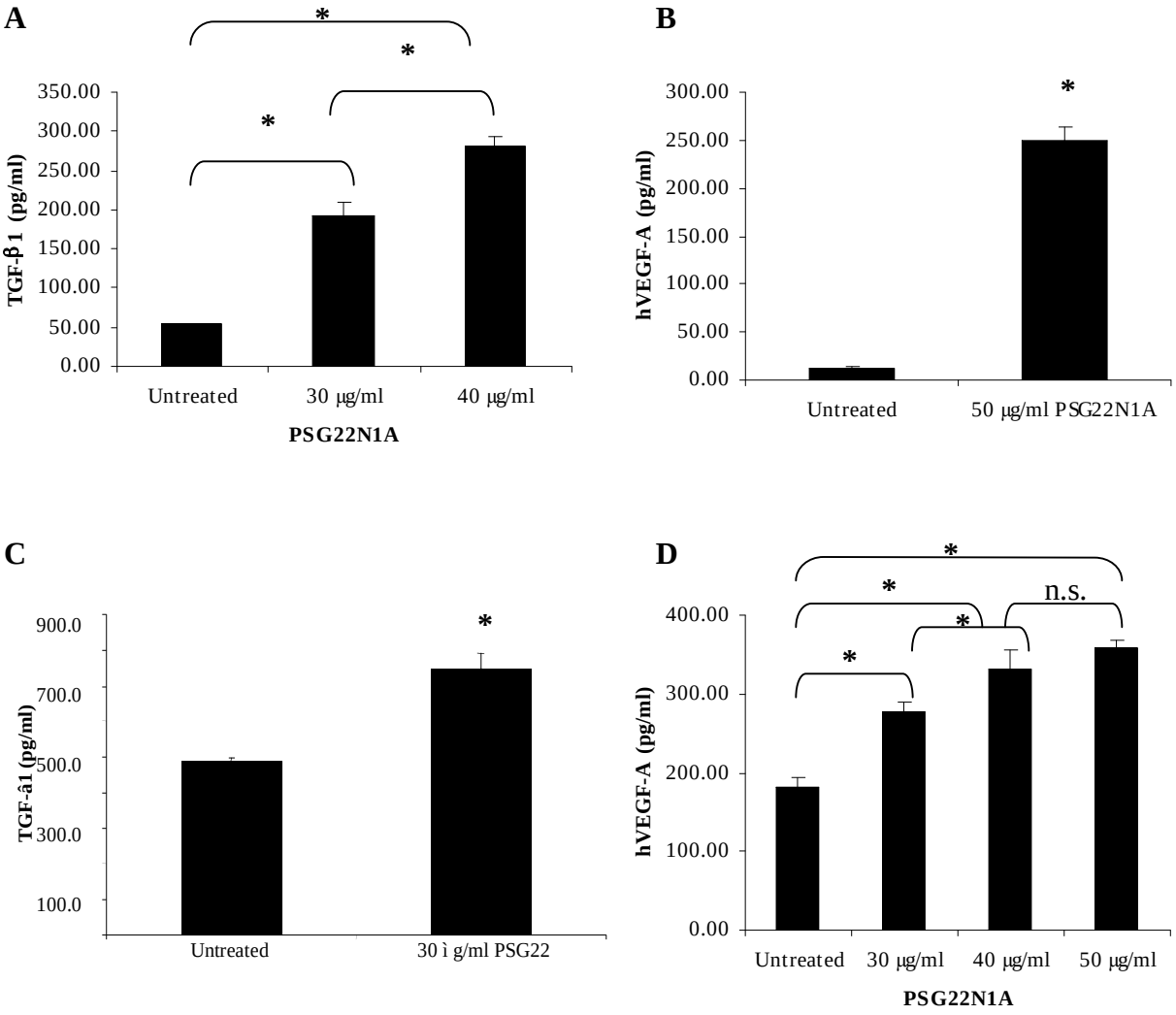
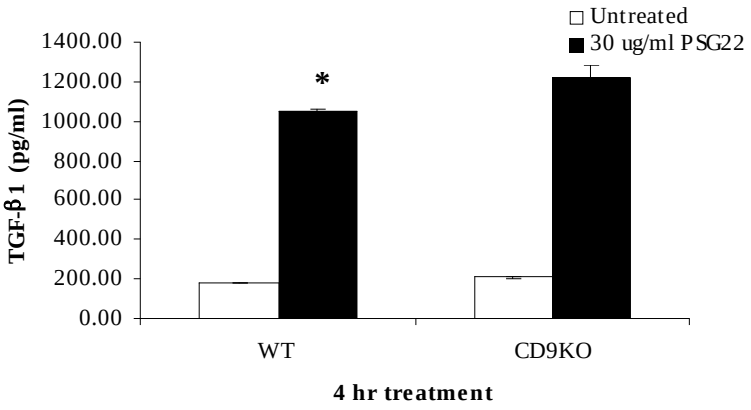

A
*
*
*
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
TGF-β1 (pg/ml)
Untreated
30 µg/ml
40 µg/ml
PSG22N1A
B
*
300.00
250.00
200.00
150.00
100.00
50.00
0.00
hVEGF-A (pg/ml)
Untreated
50 µg/ml PSG22N1A
C
*
900.0
700.0
500.0
300.0
100.0
TGF-â1 (pg/ml)
Untreated
30 ì g/ml PSG22
D
*
*
*
*
n.s.
400.00
300.00
200.00
100.00
0.00
hVEGF-A (pg/ml)
Untreated
30 µg/ml
40 µg/ml
50 µg/ml
PSG22N1A

Figure 8


□ Untreated
■ 30 ug/ml PSG22
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
TGF-β1 (pg/ml)
*
WT
CD9KO
4 hr treatment

PART FOUR:

PROJECT 3

Receptor identification studies

PROJECT 3: Receptor identification studies

Introduction

PSGs are among the most abundant fetally-derived proteins in the maternal circulation [70] and function as inducers of anti-inflammatory cytokines [2-5]. They have been recently identified in the Peyer's Patches outside of pregnancy [6], implying that they play a role in more general immunity. PSGs have also been identified in the tissues associated with the maternal vasculature [7], and we observed PSG induction of pro-angiogenic factors in a number of cell types associated with placentation (Dveskler, unpublished results). Although we are still working to understand this family of proteins, identification of a receptor for additional murine PSGs or any of the human PSGs may provide clues as to all their functions during and outside of pregnancy.

The discovery that PSG17 utilizes the tetraspanin CD9 as its receptor [93] and subsequent identification that PSG19 also uses CD9 as its receptor [79], have wide implications in the field of reproductive immunology. As a member of the tetraspanin superfamily, CD9 is involved in numerous cellular processes, including cell-cell adhesion [103], motility [100] and egg-sperm fusion [12]. A salient feature of tetraspanins is their ability to organize in microdomains, termed a tetraspanin web [104]. CD9 is known to associate with other tetraspanins, including CD151 [102], and a number of other cell surface molecules, including integrins [161]. CD9 has been implicated in helping antigen presentation in DCs [162] and monocytes [17]. It has also been shown that decidual NK cells express CD9, which delineates them from peripheral NK cells that are CD9-negative [163, 164]. Tissues associated with the blastocyst during the intial stages of mouse pregnancy stain positively for

CD9 [165]. As CD9 has pleiotropic functions in a variety of cell types, and PSGs are highly homologous [70] we hypothesized that additional PSGs, if not all, utilize CD9 as their receptor.

This was not the case.

We determined that CD9 was not necessary for human PSG1d and PSG11 action on macrophages based on the observation that macrophages isolated from CD9-null mice respond to PSG1d and PSG11 treatment [89]. We also determined that PSG1d does not bind to the EC2 of CD9 in pull-down experiments and that PSG11 does not bind preferentially to CD9-transfected cells by FACS (Dveksler lab, unpublished results). Human and murine PSGs are 60% homolgous [99] and though they share some similar functions with murine PSGs [5, 22], it was likely that human PSGs used a different receptor from murine PSGs. Employing similar tools that were used to identify the receptor for PSG17 [8, 9], we set out to identify the receptor for human PSGs. A summary of those findings is presented below.

After generating the recombinant PSG23 and determining that PSG23 induces TGF-β_1 in macrophages (Dveksler lab, unpublished results) similar to PSG17N [79, 89] we speculated that PSG23 also binds to CD9. PSG17 and PSG23 have 65% homology in their N1 domains (Accession #NP_031703 and NP_064657, respectively), so it was possible that PSG23 also bound to CD9 to enact its functions on macrophages. However, through binding and functional assays, we discovered that CD9 was not necessary for PSG23 actions on macrophages (Dveksler lab, unpublished results). We did not abandon the idea that PSG23

may use another tetraspanin as its receptor, which stemmed, in part, from our observation that PSG23 does bind to CD9 in a pull-down assay (described below). The idea that PSGs and tetraspanins interact could, therefore, be due to concomitant evolution of these two functionally conserved families.

The data described below summarize ongoing work in our laboratory to identify the receptor for PSG23, and the preliminary work I completed in the search for a receptor for human PSGs.

Part I: PSG23—CD151 interaction

Background

We had established that the target cell type for PSG activity was macrophages [79, 89, 91, 93, 94]; therefore, we began our search for the receptor for PSG23 by examining possible interactions between PSG23 and the tetraspanins expressed in RAW 264.7 cells. The initial ELISA binding experiment was conducted by detecting PSG23 binding to HEK 293T cells transfected with CD81, CD82, CD37, CD53, CD151 or control GFP-encoding plasmids. Our initial results indicated that PSG23 binds to cells overexpressing CD151 compared to control-transfected cells (Figure 1a).

The tetraspanin CD151 has been implicated in cell-adhesion, cell-motility and angiogenesis [106, 166]. CD151 is expressed on numerous cell types, except pre-B cells and resting T cells [25, 26]. One of the strongest interactions is the CD151—$\alpha_3\beta_1$ interaction, which is not dissociated by Triton-X 100 or RIPA buffer and shows a high degree of specificity,

stoichiometry and proximity [166-168]. In addition, CD151 has been shown to form lateral associations with α_6, α_7 and β_4 integrins, which are laminin-binding proteins that are known to interact with the tetraspanin family [100, 103]. Previous studies indicate that CD151 modulates integrin-dependent cell spreading and morphogenesis [29, 30]. Although CD151-null mice undergo normal development and display a primarily normal phenotype [110], inhibition of CD151 by antibodies or silencing can affect cell migration and tube formation, which are processes essential to the formation of new blood vessels [23, 32]. Therefore, the possibility that PSG23 uses CD151 as its receptor would help shed some light on the potential role of PSGs in angiogenesis during pregnancy.

Experimental approach

CD151 is expressed on most cell types [169], therefore, we relied on either overexpression of CD151 in HEK 293T cells or the silencing and subsequent re-expression of CD151 in a human epithelial carcinoma cell line, A431 cells (kind gift from Dr. Christopher Stipp, University of Iowa, Department of Biological Sciences, Iowa City, IA; [170]), in order to perform most of our binding experiments. We were also hampered by the lack of commercially available murine CD151 reagents in our experiments; therefore, we used human CD151 for many of our studies. Human and murine CD151 are 93% homologous at the amino acid level [171], so it is likely that PSG23 binding to human CD151 is similar to PSG23 interaction with murine CD151.

Human CD151 (Accession #CAG46895)
Murine CD151 (Accession #AAC25952)
Red highlighted residues indicate differences between murine and human CD151

```
mgefnekktt cgtvclkyll ftynccfwla glavmavgiw tlalksdyis llasgtylat
mgefnekkat cgtvclkyll ftynccfwla glavmavgiw tlalksdyis llasstylat
```

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ayilvvagtv vmvtgvlgcc atfkerrnll rlyfilllii flleiiagil ayayyqqlnt
ayilvvagvv vmvtgvlgcc atfkerrnll rlyfilllii flleiiagil ayvyyqqlnt

elkenlkdtm tkryhqpghe avtsavdqlq qefhccgsnn sqdwrdsewi rsqeaggrvv
elkenlkdtm vkryhqsghe gvssavdklq qefhccgsnn sqdwqdsewi rsgeadsrvv

pdsccktvva lcgqrdhasn iykveggcit kletfiqehl rvigavgigi acvqvfgmif
pdsccktmva gcgkrdhasn iykveggcit kletfiqehl rvigavgigi acvqvfsmif

tcclyrslkl ehy
tcclyrslkl ehy
```

For overexpression of murine CD151 in HEK 293T cells, we utilized plasmids obtained from Dr. Leonie Ashman (School of Biomedical Sciences, University of Newcastle, Callaghan, NSW, Australia). In the experiments indicated, we used a human CD151-GFP and control GFP plasmid from Dr. Maria Yáñez Mó (Servicio de Inmunología, Hospital Universitario de la Princesa, Diego de León, Madrid, Spain). Transfections were performed using the FuGENE 6 reagent (Roche Diagnostics, Basel, Switzerland), and cells were used in binding experiments 48 hours post-transfection when 80% or greater cells were transfected.

Two additional cell lines were used in our binding experiments: A431 sh3 cells, in which human CD151 is silenced by short hairpin (sh) RNA to the sequence 5'-AGTACCTGCTGTTTACCTACA-3' of CD151 mRNA, and A431 Rx cells, in which A431 sh3 cells were transduced with a retroviral plasmid to re-express human CD151 [170].

Our preliminary findings are based on FACS binding data. Briefly, transfected HEK 293T, A431 sh3 or A431 Rx cells were treated with 10 μg of PSG23 or control protein (Multiple Tag, Genscript Corporation, Piscataway, NJ). Protein binding to the cell surface was determined by subsequent incubation with a biotin-conjugated anti-Flag antibody (Sigma, St. Louis, MO), followed by PE- or FITC-conjugated streptavidin (eBioscience, San Diego,

CA). A431 sh3 and Rx cells were also stained with a live/dead cell discriminating dye (Invitrogen, Carlsbad, CA). Cells were fixed (Cytofix, BD Biosciences, San Jose, CA) and analyzed on the LSR II (BD Biosciences).

To generate the GST-human CD151 extracellular loop (EC) 2 protein, we used the following primers to the EC2, or large extracellular loop (LEL), of full-length human and murine CD151 (cDNA template was a kind gift from Dr. Leonie Ashman): 5'—g(tc)cta (tc)ta(tc)cagcagctgaacac—3' (forward primer) and 5'—cactcgagtcacc(gt)caggtgctc—3' (reverse primer). We subcloned the EC2 of human CD151 into a glutathione S-transferase (GST) fusion vector (pGEX-4T-1, GE Healthcare, Piscataway, NJ). Murine GST-CD151 EC2 was a kind gift from Dr. Peter Monk (Department of Neurology, University of Sheffield, Sheffield, UK). The GST-mouse CD9 EC2 was generated as previously described [34]. Tetraspanin EC2 GST fusion proteins were expressed in BL21 codon-plus *Escherichia coli* cells. Single colony overnight cultures were used to inoculate 4 L of Luria-Bertani (LB) broth, cultures were grown to OD_{600} of 0.5, and induced with 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours. Cells were pelleted and lysed with BugBuster (Novagen, Madison, WI) and GST-fusion proteins were purified on a GSTrap affinity column on the ÄKTAprime chromatography system (GE Healthcare). Protein was quantitated using the Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL).

For the pull-down assays, we used the ProFound Pull-Down GST Protein:Protein Interaction Kit (Pierce). Briefly, 150 μg of GST-tagged tetraspanin proteins (bait) were bound to immobilized glutathione resin, and the coated beads were incubated with 150 μg of BSA

control protein or PSG23 (prey). Bait-prey elutions were run on a SDS-PAGE gel and bait was detected with either an anti-GST antibody (BD Biosciences) or anti-CD151 antibody that cross-reacts with human and murine CD151 (Santa Cruz Biotechnology, Santa Cruz, CA) and the prey was detected with an anti-Flag antibody (Sigma).

Results and discussion

Murine PSGs are 59-70% homologous [98] and are thought to have evolved from a single ancestral gene [98]. Previously, our lab identified the first receptor for two murine PSGs: the tetraspanin CD9 as the receptor for mouse PSG17 [93] and 19 [79]. It is possible that other murine PSGs utilize a member of the tetraspanin family as their receptor. We first investigated a potential PSG23—CD9 interaction. In functional studies, in which we treated macrophages isolated from wild-type and CD9-null mice, we concluded that PSG23 does not require CD9 for its induction of TGF-β_1 (Figure 1b); therefore, we concluded that CD9 does not serve as the receptor for PSG23. However, in pull-down assays, in which PSG23 was incubated with the EC2 of CD9, we observed an interaction between PSG23 and CD9 (Figure 1c), leaving the possibility open that CD9 may act as an accessory to the receptor for PSG23.

Given that tetraspanins form lateral associations with other cell surface molecules and each other [102], we tested possible binding between PSG23 and other tetraspanins on the surface of macrophages and found that CD151 was a candidate. We decided to test our initial findings that PSG23 bind preferentially to CD151 (done by ELISA) by FACS analysis.

First, we tested the binding of PSG23 to HEK 293T cells transfected with empty plasmid versus cells transfected with human or mouse CD151. In some experiments, we used HEK 239T cells transfected with control GFP plasmid or human CD151-GFP. Since HEK 293T cells express human CD151 endogenously, we treated the transfected cells with as much as 25 μg of PSG23 or control (Multiple Tag) protein. We found, however, that 10 μg of protein was sufficient to see PSG23 binding to the CD151-transfected cells over the control-transfected cells (Figure 2).

We next performed binding studies in two cells lines in which human CD151 is knocked-down (A431 sh3 cells) and re-expressed (A431 Rx). The A431 sh3 cells were sorted prior to our use to ensure that human CD151 was knocked-down [170], and the A431 Rx cells were stained with an anti-human CD151 antibody to ensure expression of CD151 in these cells (Figure 3a; [170]). We incubated both cell types with 10 μg of PSG23 or control protein and observed preferential binding of PSG23 to the A431 Rx cells versus the A431 sh3 cells (Figure 3b).

Concomittantly, we discovered that PSG17 binds to the EC2 of CD151 in a pull-down assay (Dveksler lab, unpublished results). Our findings were supported by initial binding studies perfomed using the BIAcore 2000 (BIAcore AB, Öbrink, personal communication) which also demonstrated binding between PSG17 and CD151. Although PSG17 binds with higher affinity to CD9, it is possible that, in the absence of CD9, PSG17 may also utilize CD151 as a lower-affinity receptor. The binding study data is further supported by our observations that in some experiments, peritoneal macrophages from CD9-null mice responded to PSG17

treatment (Dveksler lab, unpublished results). We believed that this response was due to a contaminant in our protein preparations. However, in light of the results of these new binding studies, it is possible that PSG17 was binding to CD151 on the macrophages from CD9-null mice. The promiscuity with which murine PSGs bind to tetraspanins may complicate future receptor binding studies. Ubiquitous expression of tetraspanins on numerous cell types and their ability to compensate for one another [172] also adds another level of complexity to our search for the receptor(s) for additional PSGs.

The tetraspanin CD151 has been implicated in the process of angiogenesis [106] and cDNA microarray analysis has identified CD151 expression in the decidua and placental villi [36]. Since PSG23 induces pro-angiogenic factors in cell types involved in vasculature remodeling during pregnancy possible PSG23—CD151 binding may help to elucidate the role of PSGs in placentation. Futher studies, however, are needed to confirm our initial suspicions that CD151 may be important in PSG23 binding to target cell types. We plan to perform functional studies in macrophages isolated from CD151-null animals [106] to determine if CD151 is required for PSG23-induced TGF-β_1 and VEGF-A. We also want to explore other methods to determine possible binding between PSG23 and CD151. Murine RAW 264.7 cells respond well to PSG23 treatment (Dveksler lab, unpublished results). Silencing CD151 expression in RAW cells may alter their ability to bind to PSG23 in FACS experiments or up-regulate cytokines in functional assays. Additionally, we could test PSG23 binding to Daudi cells, human B cells that do not express CD9 or CD151 [102] to determine if these cells preferentially bind to PSG23. By pull-down we detected binding between PSG23 and

the EC2 of CD9 and CD151. It would be interesting to determine if PSG23 binds to CD9 and CD151, and with what affinity, using the BIAcore 2000.

Part II: Identification of the receptor for human PSGs

Background

We selected human PSG1d, which is believed to be the most abundant human PSG expressed in pregnancy [37], and PSG11, whose lowered expression in the maternal serum has been linked to spontaneous abortion [38], to use in our studies for the human PSG receptor. Human PSG11 contains a RGD sequence [173], which may be involved in binding to its receptor. After numerous binding and functional experiments, we determined that CD9 was not the receptor for human PSG1d or PSG11 (Figure 4a and b). While human and murine PSGs share sequence homology [21] and appear to have similar functions in macrophages [89, 90, 92], we decided to begin our search for the human PSG receptor with an unbiased approach.

Experimental approach

We generated two new constructs for PSG11 and PSG1d in order to perform our receptor identification studies. Our PSG11 construct (Accession #X17610) includes the leader peptide followed by the N1, A1, B2 and C domains of the protein, followed by a 6X His tag and a Flag tag (Figure 5a). The sequence was inserted into the pDW465 plasmid (kind gift from Dr. David Waugh, Macromolecular Crystallography Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, Maryland and described in [174]) in frame with a 17 amino acid biotin acceptor peptide (BAP) and encoding the biotin holoenzyme synthetase

*bir*A gene. The pDW465 plasmid, containing PSG11-6X His-Flag, was used to transform DH10Bac competent cells (Invitrogen), which placed our PSG11 into a baculovirus expression vector. The purified bacoluvirus DNA, containing PSG11, was purified and used to transfect *Spodoptera frugiperda* (Sf-9) cells (Invitrogen) to generate a viral stock. After isolation of the recombinant PSG11 virus from Sf-9 cells, I infected large cultures (typically, 6 L) of Sf-9 cells were infected for protein production. The PSG11-His-Flag-biotin protein was subsequently purified using the HisTrap column (GE Healthcare) and anti-Flag M2 agarose (Sigma) and could be detected by western using a PSG-specific antibody (BAP1, Genovac, Freiburg, Germany), an anti-His antibody (clone sc-803, Santa Cruz Biotechnology, Santa Cruz, CA), an anti-Flag antibody (Sigma) and streptavidin-HRP (Pierce) (Figure 5b). Purified protein was quantitated against BSA standards on a SDS-PAGE gel stained with Coomassie Blue (Pierce).

The human PSG1d used for our binding studies was generated by AmProtein (Camarillo, CA) and contained the full-length human PSG1d gene (Accession #J04539) with a His tag at the N- and C-terminus. The construct was stably transfected into CHO cells and the cells were seeded into a 5 kDa molecular weight cut-off hollow fiber cartrigdge (FiberCell Systems, Frederick, MD). Protein in the supernatant was harvested daily and purified on a HisTrap column (GE Healthcare). Purified HisTrap fractions were concentrated and dialyzed against PBS. Purified protein was quantitated by Western blot using internal standards (Figure 5c).

We screened a human spleen cell cDNA library (Strategene) to identify the receptor for PSG11. Briefly, the spleen library cDNA is inserted directionally into a replicon-deficient retroviral vector (pEF) along with the *gag-pol* and *env* genes into a packaging cell line. The resulting viral stock was used to transform NIH3T3 cells (ATCC, Manassas, VA) and, since we used a retrovirus vector, the human spleen DNA was incorporated into the genomic DNA of the NIH3T3 cells.

For the panning experiments with the spleen cell library-transduced cells, we took advantage of the strong interaction between biotin and streptavidin and coated petri dishes with the streptavidin derivative NeutrAvidin (Pierce), followed by PSG11. As a control, plates were coated with NeutrAvidin only. The spleen library-transduced cells were panned on the PSG11-coated dishes, and the bound cells were recovered, expanded and panned again on PSG11-coated dishes. This process was repeated 6 times and single cell clones were generated from the positive binding cells. The genomic DNA of the single cell clones was isolated (Wizard SV Genomic DNA Purification System, Promega, Madison, WI). The cDNAs from the spleen library were amplified using library-specific primers (Stratagene). The cDNA was subcloned into a sequencing expression vector (TOPO TA Cloning vector, Invitrogen) and sequenced using primers that annealed to the TOPO TA vector. The resulting cDNA sequences were compared to known genes using the BLAST program. Candidate genes were placed into the pEF6 vector (Invitrogen) and used to transfect NIH3T3 cells to determine if expression of the plasmid would confer binding of the cells to PSG11-coated dishes. Positive clones were also tested by FACS or binding to magnetic beads (Dynal, Carlsbad, CA). (Figure 6)

To identify the receptor for PSG1d, we panned a RAW cell cDNA library using the protocol described in Waterhouse et al. with the following exception: to immobilize PSG1d on the Petri dishes, we used the PSG antibody BAP1 (Genovac). Briefly, the RAW cell cDNA library was amplified in the pEAK10CV vector (Edge Biosystems, Gaithersburg, MD), by growing the bacterial stock on LB agar plates and pooling the bacteria with GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8). The plasmid DNA was purified with the Plasmid MaxiPrep kit (Qiagen, Vallencia, CA). We transfected 293 EBNA cells (Invitrogen) with the pooled purified plasmids and selected transformants with 0.5 μg/ml puromycin (BIOMOL International, Plymouth Meeting, PA). Puromycin was increased to 1 μg/ml prior to use in panning experiments.

After 5-6 rounds of panning the RAW library-transfected cells on the PSG1d-coated dishes, single cell clones were isolated. Since the plasmids containing the RAW cell library remained episomal, plasmids were isolated using the Hirt method [175]. Purified plasmids were used them to transform high efficiency XL10 Gold Ultracompetent cells (Stratagene) for sequencing. Sequences were compared to known genes using the BLAST program.

Results and discussion

The genomic DNA of approximately 40 single cells clones was purified after approximately 6 or 7 rounds of panning on the PSG11-coated dishes. Using the library-specific primers, we amplified a number of inserts from each clone. As we expected, more than one library insert resulted per single cell clone as more than one retrovirus virion was incorporated into each

cell (Figure 7a). DNA fragments of 1 kb or larger were selected to subclone into the directional TOPO expression vector suitable for sequencing. Although some fragments lower than 1 kb were sequenced, nothing below 500 bp were cloned, as these fragments would not likely encode for the open reading frame of a gene.

As the methods of purifying and amplifying the library cDNA from the single cell clones, using the library-specific primers, improved we began to see a pattern of gene expression in the positive clones. We saw a band at approximately 1.4 kb, 3 kb and 6 kb. After sequencing these fragments, the 1.4 kb band encoded the receptor for acitivated C kinase 1 (RACK1) and the 3 kb and 6 kb fragments encoded α-aminoadipate semialdehyde dehydrogenase-phosphopantetheinyl transferase (LYS5).

RACK1 was first identified as a binding partner for βII protein kinase C (PKCIIβ) [176] and though it has no known function, RACK1 is thought to act as a shuttling and scaffolding protein (reviewed in [177]). The literature suggests that RACK1 is not on the outer plasma membrane [177], but can associate with the inner leaflet of the cell membrane when it is overexpressed [44]. RACK1 has been reported to associate with β_1 integrins at the cell membrane [178] and is speculated to mediate cross-talk between integrins and growth factor receptors to promote cell migration [179]. Although we did not isolate a tetraspanin during the library screening, we cannot rule out the possibility that PSG11 interacts with a tetraspanin. The primary lateral association between tetraspanins and integrins [104] may explain why we found RACK1 in our clones. Overexpression of RACK1 may have led to RACK1—β_1 integrin association [178]. If the β_1 integrin was also associated with a

tetraspanin, the presence of RACK1 may organize the PSG11 receptor complex and increase its affinity or avidity for PSG11. The possibility remains open as to whether RACK1 acts as an adaptor protein to the true cell surface receptor for PSG11.

LYS5 has been characterized in the lysine synthesis pathway in yeast [45-47] but little is known about its function in humans [48]. Plotting the LYS5 on a hydrophobicity plot indicated that LYS5 may cross the plasma membrane, but we were unsure if this protein was on the outer cell membrane.

To determine whether or not RACK1 and/or LYS5 conferred binding of receptor-negative cells to PSG11, NIH3T3 cells were transiently transfected with plasmids containing RACK1 or LYS5 or both genes and panned on PSG11-coated dishes. The results of these panning experiments were inconclusive. In subsequent binding experiments, HEK 293T cells transfected with RACK1 or LYS5 or both plasmids were used in FACS studies to determine preferential binding to PSG11. However, there did not appear to be a significant difference between the transfected cells and untransfected cells in their ability to bind to PSG11 (Figure 7b). To determine if the single cell clones isolated were truly binding to the PSG11-coated dishes, and not simply artifacts of adherent cells, the single cell clones were panned again. They did not bind preferentially to the PSG11-coated dishes over the NeutrAvidin only-coated dishes.

In reassessing our methods of searching for the receptor for human PSGs, we had to answer a number of basic questions. The first was whether the transduced NIH3T3 cells still had the

spleen cell cDNA library. After purifying the genomic DNA of the cells, and amplifying the cDNA with the library-specific primers, we determined that the cells had retained the spleen library.

We next assessed whether or not PSG11 was the best ligand to use to identify the receptor. Induction of TGF-β_1 in murine RAW 264.7 macrophages after human PSG treatment [89] suggests that there is a receptor for human PSGs on RAW cells (Figure 8a). To characterize the affinity of the RAW cell receptor for human PSGs, we cloned full-length PSG1d and 11 cDNAs into a vector containing an alkaline phosphatase (AP) tag. The AP binding assay was performed as previously described [93]. Briefly, RAW cells were treated with AP-PSG1d or AP-PSG11 fusion proteins or AP only. Cells were lysed and the concentration of AP, AP-PSG1d and AP-PSG11 proteins in the cleared lysates were quantitated by enzymatic assay [180]. Conversion of the binding data to a Scatchard plot revealed that the affinity of PSG1d for its receptor was higher than that of PSG11. The K_D of PSG1d was 3.204×10^{-11} M and the K_D of PSG11 was 1.132×10^{-10} M (Dvkesler lab, unpublished results) (Figure 8b). Therefore, we decided to focus our efforts on identifying the receptor for PSG1d.

Finally, while the human spleen library would have had the receptor for human PSGs, being composed of the genes isolated from cell types targeted by PSG activity, it also contained genes from cells not responsive to PSGs. We, therefore, turned our attention to the RAW cell library previously used to identify the receptor for PSG17 and 19 [79, 93].

After panning 293 EBNA cells, transfected with the RAW cell cDNA library, on the PSG1d-coated dishes for 5 to 6 rounds, approximately 20 single cell clones were isolated. The episomal plasmids, isolated using the Hirt extraction procedure, were used to transform ultracompetent cells. RAW cell cDNA inserts were sequenced; however, upon comparison with known sequences, we determined that only empty plasmids or fragments of human genes were isolated. In order to confirm positive binding of the single cell clones to PSG1d, cells were incubated with magnetic beads coated with PSG1d protein or BSA control protein. After recovering the bound cells and counting the cells with an automated cell counter (Beckman Coulter, Fullerton, CA), the positive clones were determined to be artifacts of the panning procedure (Figure 9).

The difficulty that we have had in cloning the receptor for human PSGs has revealed to us a number of things: (1) the receptor for human PSGs may be a complex of proteins and not just one peptide and identifying multiple receptors will require different experimental methods than we have previously employed; (2) identifying a receptor-negative cell type may be difficult, as in the case of PSG23—CD151 binding where CD151, if it is the receptor for PSG23, is expressed on nearly all cell types [101]; (3) the affinity for human PSGs for their receptors are different and, therefore, identifying the receptor will depend on the cell type we investigate, the protein we use, and the binding conditions (i.e., binding buffers) we employ for our studies. While we are currently exploring the possibility that human PSGs may also use CD151 as its receptor, our experiments have given us mixed results. Further studies exploring possible PSG1d or PSG11 interaction with CD151 are needed.

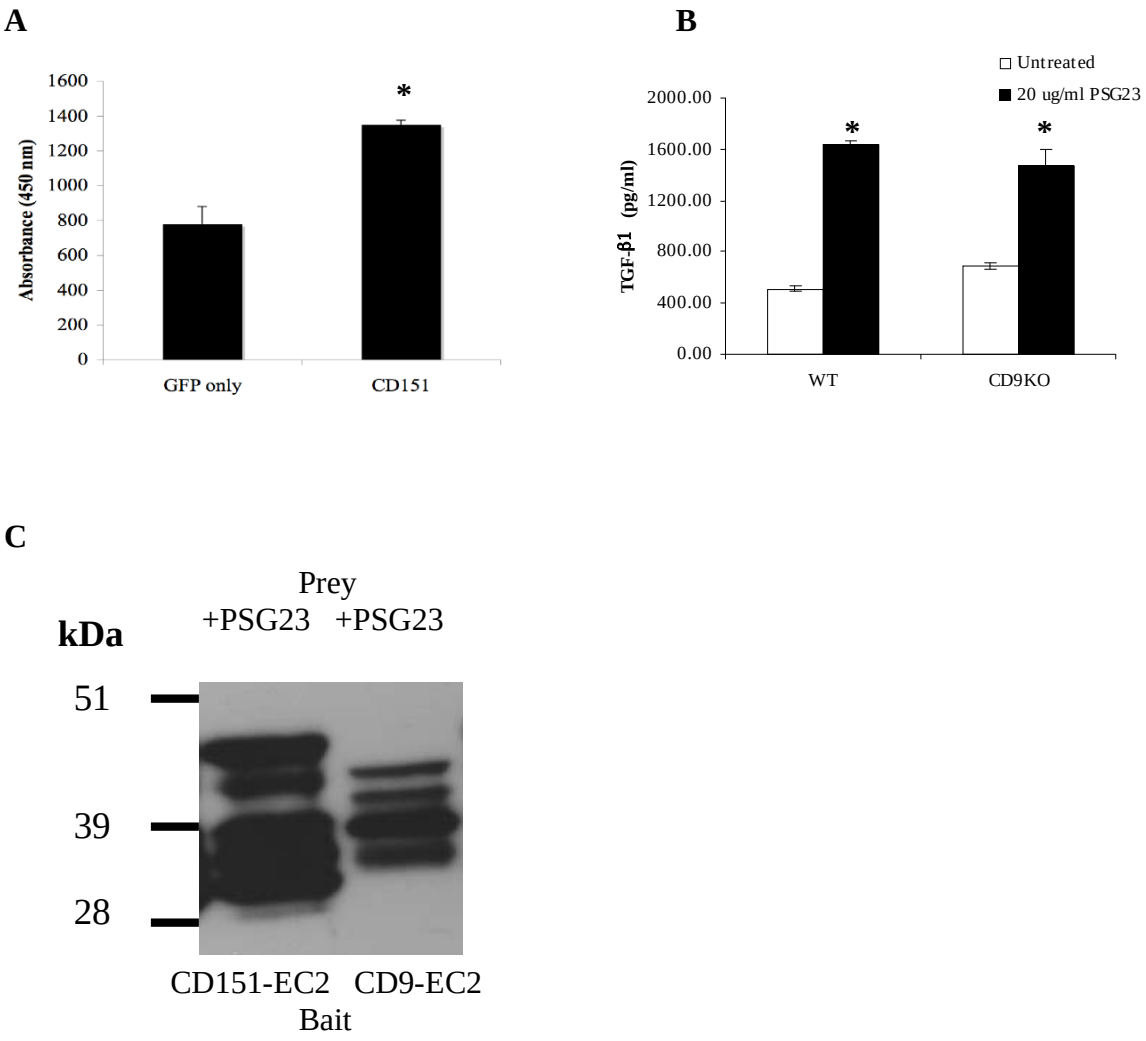


Figure 1: PSG23 may use another tetraspanin as its receptor. (A) PSG23 bound preferentially to cells overexpressing CD151 compared to control-transfected cells. (B) PSG23 does not require CD9 for activity in macrophages, but (C) PSG23 shows low-level binding to CD9 in pull-down assays, as well as binding to CD151. For Figure 1b statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data is representative of at least three separate experiments and error bars represent ± S.E.M.

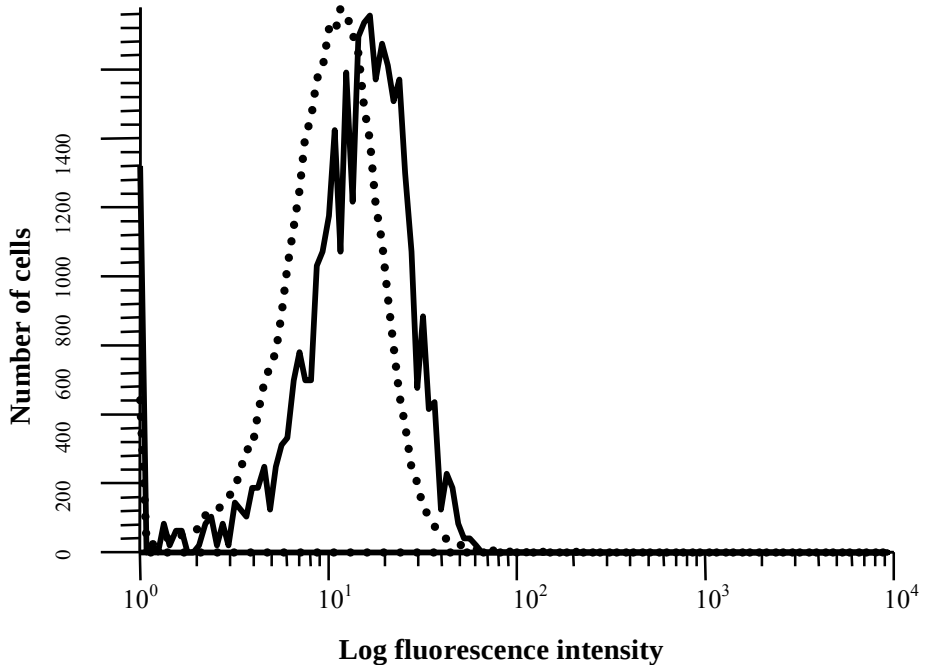


Figure 2: Binding of PSG23 to CD151-transfected HEK 293T cells. HEK293T cells transfected with empty GFP plasmid (dotted line) or CD151-GFP (solid line) and incubated with 10 μg PSG23.

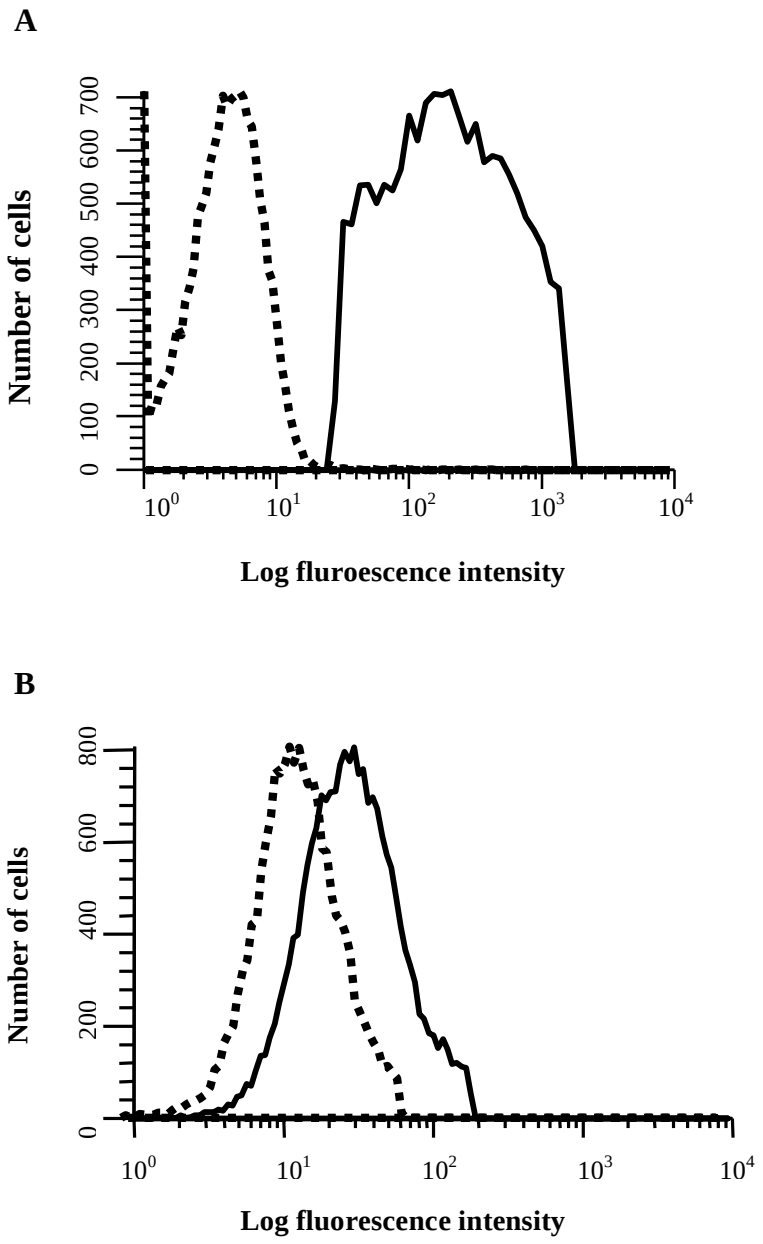
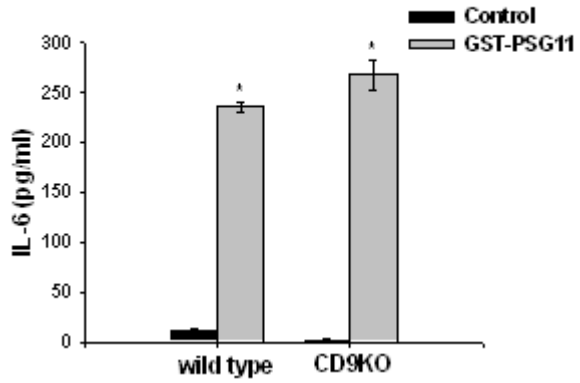


Figure 3: PSG23 binds to CD151. (A) Flow cytometry staining of A431 sh3 cells (dotted line) and A431 Rx cells (solid line) with anti-CD151 antibody. (B) Binding of PSG23 to A431 Rx cells (solid line) over A431 sh3 cells (dotted line).

A



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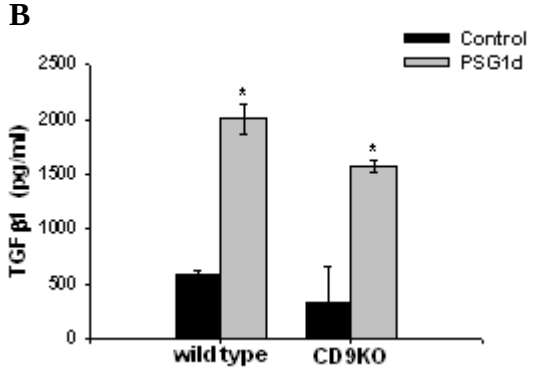
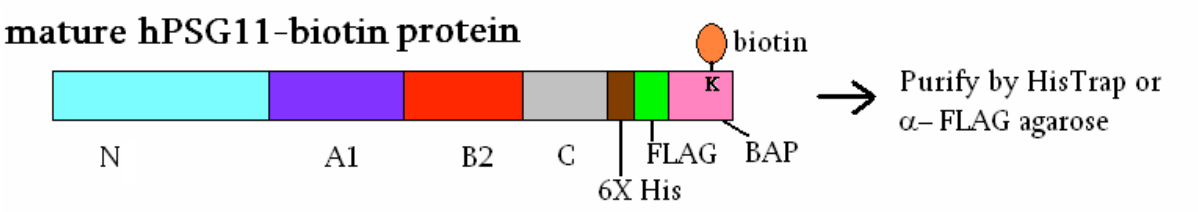


Figure 4: CD9 is not the receptor for human PSGs. (A) IL-6 induction in peritoneal macrophages from wild-type and CD9-null animals after PSG11 treatment. (B) TGF-β_1 induction in peritoneal macrophages from wild-type and CD9-null mice after PSG1d treatment. Statistical significance between protein-treated versus the untreated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.05$. Data is representative of at least three separate experiments and error bars represent ± S.E.M. [2]

A



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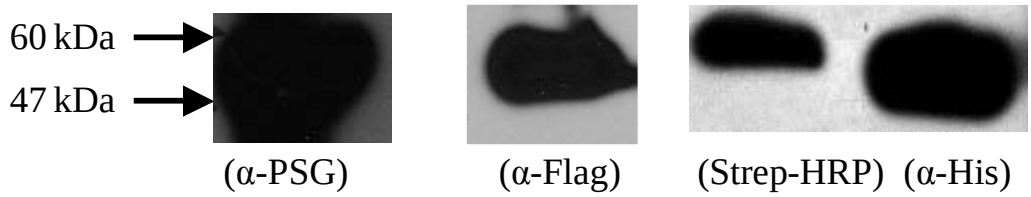

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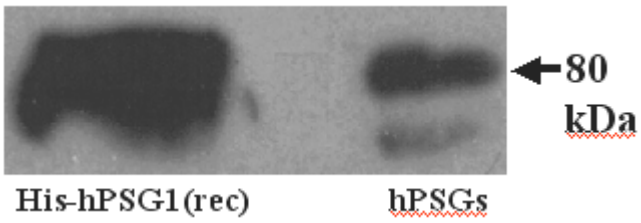


Figure 5: Recombinant PSG11 and PSG1d. (A) Recombinant PSG11 consists of the N, A1, B2 and C domains of PSG11, followed by a 6X His tag, a Flag tag and a biotin acceptor peptide (BAP) that leads to biotinylation of the mature protein. (B) Purified recombinant PSG11-His-Flag-biotin protein detected with anti-human PSG (BAP1), anti-Flag (Sigma), streptavidin-HRP (Pierce) and anti-His (Santa Cruz) antibodies. All antibodies tested detect a protein of approximately 49 kDa. (B) Recombinant His-PSG1d-His purified protein detected with anti-human PSG antibody (BAP1) is approximately 80 kDa and similar in size to human PSGs purified from pregnant serum.

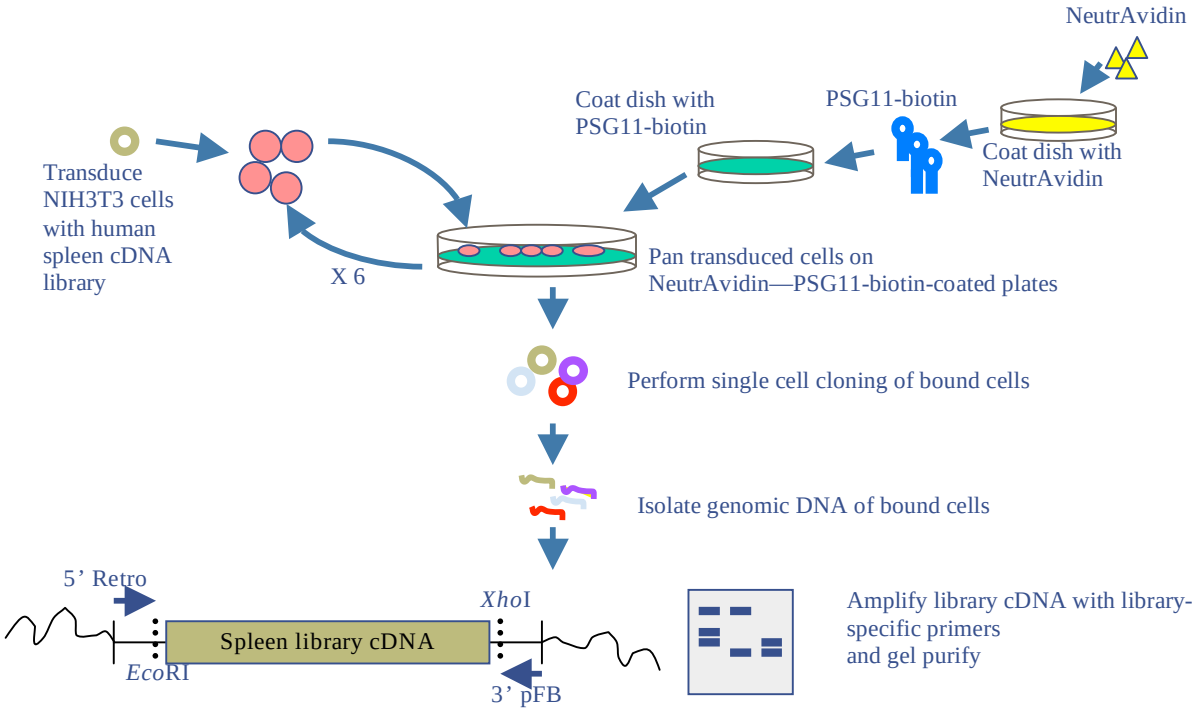
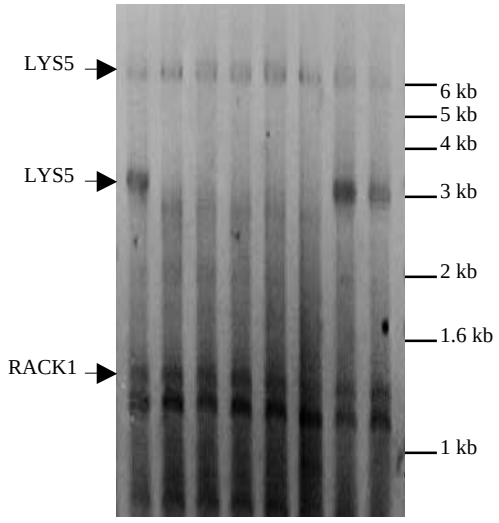


Figure 6: Panning scheme. NIH3T3 cells transduced with a human spleen cDNA library were incubated on Petri dishes coated with PSG11-biotin or control protein (NeutrAvidin). Bound cells were recovered, expanded in culture and panned for five additional rounds of binding (total of 6 rounds of panning). Single cell clones were generated from the bound cells and expanded in culture. Genomic DNA of the single cell clones was amplified using library-specific primers. Bands of 500 bp or larger were purified and sequenced.

A



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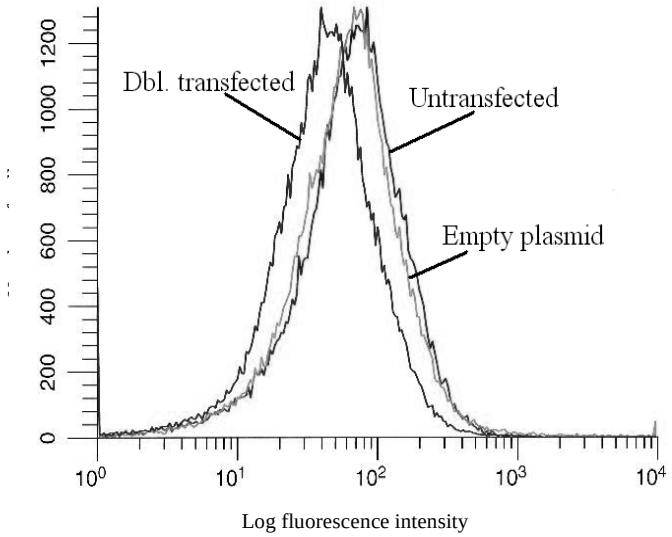
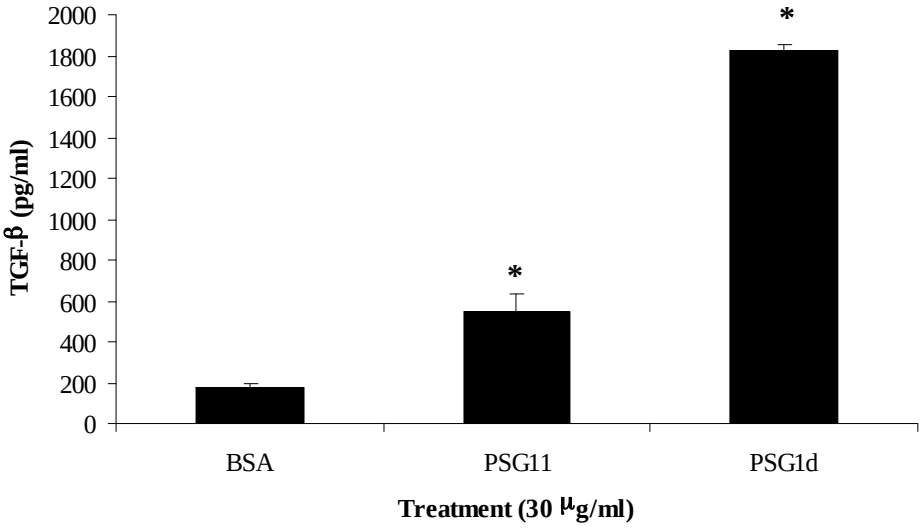


Figure 7: Results of panning the human spleen cDNA library with PSG11. (A) After amplifying the DNA of the single cell clones, DNA bands at 1.4 kb (RACK1), 3 kb and 6 kb (both LYS5) resulted in potential receptors. (B) RACK1-, LYS5- or double-transfected NIH3T3 cells resulted in no shift over control-transfected cells when incubated with PSG11 protein.

A



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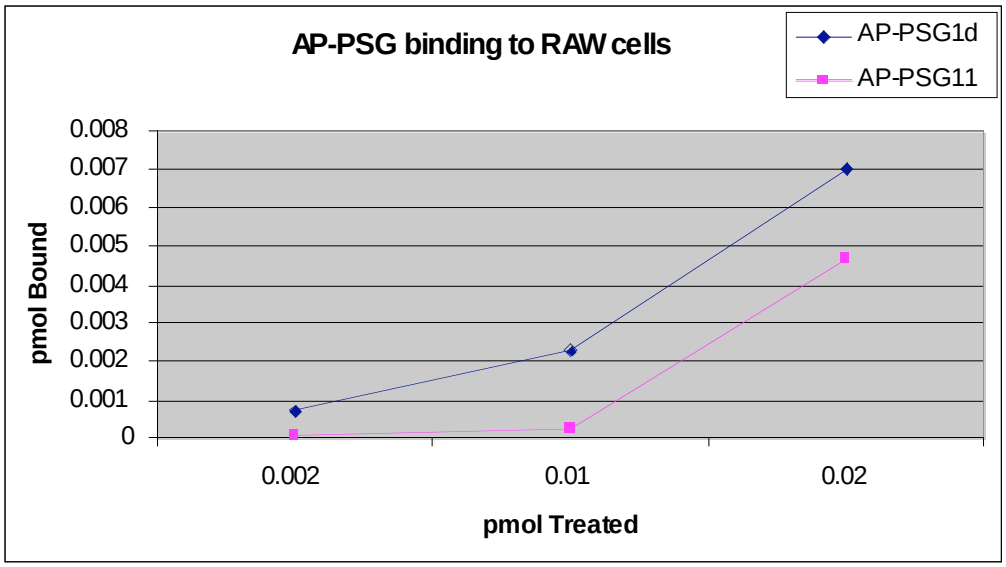

Figure 8: PSG1d may have a higher affinity for its receptor than PSG11. (A) RAW 264.7 cells were treated with 30 μg/ml of BSA (control), PSG11 or PSG1d for 24 hours. TGF-β_1 up-regulation in the cell supernatants was measured by ELISA Statistical significance between protein-treated versus the BSA-treated samples was determined by two-tailed Student's *t*-test and * indicates $P < 0.001$. Data is representative of at least three separate experiments and error bars represent ± S.E.M. (B) RAW 264.7 cells were treated in triplicate with increasing concentrations of AP-PSG1d, AP-PSG11 or AP control. PSG1d and PSG11 binding values were plotted after subtracting levels of non-specific binding by AP from total binding. Similar binding curves were obtained in three independent experiments.

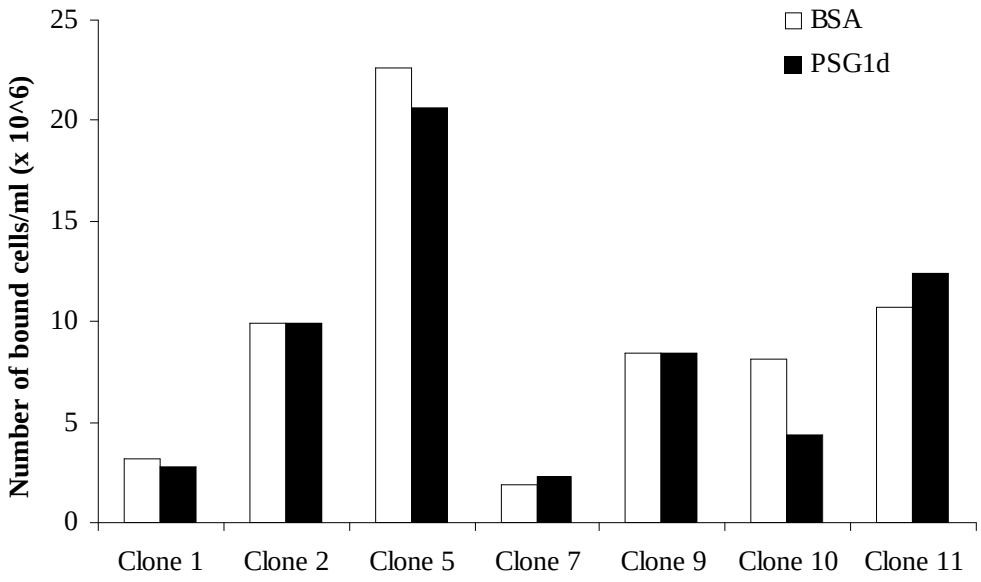

Figure 9: Results of panning the RAW 264.7 cDNA library with PSG1d. The single cell clones were checked for positive binding to PSG1d-coated magnetic beads (Dynal). BSA-coated beads were used as a control. Graph is representative of results for all 20 single cell clones tested.

PART FIVE:

DISCUSSION

DISCUSSION

Summary of the results

This investigation sought to contribute to our current understanding of the biological functions of PSGs in addition to their roles as immunomodulators. The findings indicate that murine PSG22 and PSG23 and human PSG1d induce the pro-angiogenic factors TGF-β_1 and VEGF-A in macrophages. In addition, we identified new target cell types, dendritic cells, endothelial cells and trophoblasts, which secrete TGF-β_1 and VEGF-A in response to these PSGs. We determined that PSG22 and PSG23 activity in macrophages is not dependent on the tetraspanin CD9; however, our initial experiments indicate that another tetraspanin family member, CD151, may be important in the binding of PSG23 to target cell types.

These findings extend our current knowledge of the role that PSGs play in pregnancy. The observation that PSGs induce pro-angiogenic factors may implicate these proteins in the processes of maternal vasculature remodeling, a dynamic process during pregnancy that is vital to the development of the fetus.

PSG induction of pro-angiogenic factors

The issue of incomplete uterine remodeling in pregnancy

In women who develop pre-eclampsia, cytotrophoblast invasion of the decidua remains shallow, leading to restricted uteroplacental blood flow [9]. Although women predisposed to vascular insufficiencies are at higher risk for developing pre-eclampsia [181], the causes of this disorder are still unknown. The only successful treatment of the disease is delivery of the

placenta [9]. It is hypothesized that an imbalance in the circulating angiogenic factors may play a role in the development of pre-eclampsia [23, 182]. The presence of soluble receptors for TGF-β_1 and VEGF, sEng and sFlt-1, respectively, are seen in the serum of women with pre-eclampsia and are believed to sequester and inhibit these pro-angiogenic factors from performing their normal functions during placental development [23, 51].

Recently, pre-eclampsia has been viewed as an immune disorder in which incomplete invasion of the decidua is due to adverse interactions between the invasive fetal trophoblasts and the maternal leukocytes in the uterus [183]. Trophoblast cells express the non-classical major histocompatibility complex (MHC) molecules human leukocyte antigen (HLA)-C, -E and –G [184]. Classical HLA molecules, HLA-A, -B or –D, are the principle stimulators of T cell-mediated immune responses [184]. The unique HLA molecules expressed on EVTs do not present antigen to T cells, but instead interact with killer immunoglobulin-like receptors (KIRs) expressed on uNK cells [184, 185].

Uterine NK cells comprise the majority of leukocytes at the maternofetal interface [149]. During the menstrual cycle, NK cells infiltrate the endometrium and, in the absence of blastocyst implantation, die and shed during menstruation [186]. In the presence of the embryo, NK cells in the endometrium further differentiate into the specialized uNK cells. Uterine NK cells are phenotypically $CD56^{bright}/CD16^{dim}$ or $CD16^{-}$, making them distinct from CD16+ NK cells found in the periphery [57]. Uterine NK cells in the decidua are thought to regulate trophoblast invasion and development of the placenta through their KIR interaction with HLA-C, -E and –G expressed on the surface of EVTs [183]. In normal

pregnancy, the KIR—HLA interaction is thought to prevent uNK cells from becoming cytotoxic against the invading trophoblast cells [184]. Moreover, the specific interaction between HLA-G on trophoblasts and KIR2DL4 on uNK cells stimulates the production of Ang1 and 2, PlGF, VEGF and TGF-β_1 [185, 187, 188]. These observations implicate uNK cells in both the migration of EVTs and the process of placental vasculogenesis. During EVT invasion in pre-eclampsia it has been hypothesized that a mismatch between KIRs on uNK cells and HLA-C on migrating trophoblasts may prevent optimal trophoblast invasion [183].

Currently, it is unknown if PSGs exert effects on uNK cells; however, we can postulate that PSGs will target these leukocytes based on observations that other CEA family members can modulate NK functions in vitro. Studies have shown that the expression of CEA-related cell adhesion molecule 1 (CEACAM1), a member of the CEA family, can inhibit the cytotoxic effects of NK cells [189]. In this study, NK cells were incubated with tumor cells (YTS) transfected with CEACAM1 or untransfected[189]. The amount of NK-mediated killing was reduced in the reactions containing the CEACAM1-transfected tumor cells versus the untransfected cells [189]. Furthermore, when CEACAM1 expression on NK cells is up-regulated [125] it has been shown to bind to CEACAM1 expressed on adjacent cells, resulting in reduced NK function in killing assays [190].

The uNK cells at the maternofetal interface in normal pregnancy do not mount an immune attack against fetal tissue. However, the possible anti-fetal responses of circulating NK cells, which also encounter fetal tissue, must be suppressed. A subpopulation of T cells, known as

regulatory T cells (Tregs), found during pregnancy is thought to promote peripheral immune tolerance by inhibiting the cytotoxic effects of circulating NK cells [191]. Tregs are $CD25+/CD4^-$ and have been reported to produce TGF-β and IL-10 [191]. It is currently unknown if PSGs have any effects on T cells or Tregs, however, other CEA family members have been reported to interact with T cells. CEACAM5, which is expressed on intestinal epithelial cells, has been shown to interact with a unique, but as yet unidentified, receptor on CD8+ T cells and blocks their function through inhibition of phosphatidylinositol-3-kinase (PI3K) activation [72]. CEACAM1 protein has also been demonstrated to elicit IL-10 secretion from Tregs [192]. During pregnancy, CEACAM6 has been shown to interact with placental regulatory CD8+ T cells and inhibit their ability to proliferate, a hallmark of T cell activation, in allogenic mixed lymphocyte reactions [72]. We can speculate that PSGs will also have inhibitory effects on T cell activation.

Human PSG1, 6 and 11 have been found to induce the anti-inflammatory cytokines IL-10 and TGF-β in human monocytes and murine macrophages [90, 94]. Murine PSG17, 18 and 19 have also been shown to induce Th2-type cytokines in monocytes/macrophages [79, 89, 92] which are thought to aid in pregnancy success [65]. We hypothesize that PSGs will have a similar effect on uNK cells at the maternofetal interface and Tregs in the maternal circulation. Our findings indicate that human and murine PSGs also up-regulate pro-angiogenic factors in DCs, endothelial cells, monocytes/macrophages and trophoblasts. Should we discover that PSGs also induce TGF-β_1 and VEGF in uNK cells in particular, this may lend further support for our hypothesis that PSGs play a role in vascular development of the placenta. Based on the role which uNK cells are believed to have in regulating

trophoblast invasion [8, 186], we hypothesize that PSGs may act as signals for uNK cells to allow EVT migration as well as suppress of NK cell cytotoxicity. To tease out this hypothesis, we would first need to treat uNK cells with PSGs and measure possible cytokine up-regulation by ELISA. If we determined that PSGs stimulate uNKs to secrete TGF-β_1 or VEGF, we could then test to see if addition of PSGs to uNK cells in killing assays has any affect.

Human PSG expression is approximately 0.5 μg/L in the circulation of non-pregnant women [193] and appears to increase around week 9 of gestation, coincident with development of the placenta [130]. Wynne et al. demonstrated positive staining for murine PSGs lining the capillaries within the decidua of the implantation site at embryonic (E) day 8-11 of gestation and suggested that PSGs may bind to endothelial cells and contribute to the process of angiogenesis [133]. However, the functional significance of this finding was not determined. Interestingly, other CEA proteins have been implicated in angiogenesis. CEACAM1 has been shown to induce tube formation and up-regulation of VEGF as addition of a neutralizing anti-CEACAM1 antibody block VEGF-induced tube formation in endothelial cells [74, 194]. Studies have also indicated a role for CEACAM1/VEGF synergy in the angiogenesis of neuroblastomas, where CEACAM1 has been isolated on the microvessels of actively growing tumor cells [195]. Moreover, VEGF expression is found on the differentiating cell adjacent to the CEACAM1-positive microvessels [195]. CEACAM1-null mice show reduced arteriole growth and blood flow after femoral artery ligation, whereas mice that overexpress *Ceacam1* show increased revascularization after tissue damage [196]. CEA family members have been isolated in various tissues, including the placenta [73, 131],

and expression of CEA proteins can be linked to the invasive properties of trophoblast cells as anti-CEACAM1 antibodies have been shown to inhibit trophoblast migration [197].

Despite clinical observations that low serum PSG levels are associated with IUGR [198], fetal hypoxia [199] and threatened abortion [200], the methods and reagents used to detect serum PSGs were not uniform, It remains unclear if PSGs directly affect placentation. Although it may be true that women with pregnancy-associated vascular deficiencies have decreased serum PSG levels [201], we cannot extrapolate from the existing data that PSGs directly promote successful fetoplacental circulation. Therefore, this work provides a first step towards understanding the molecular mechanism by which PSGs could contribute to the development of the placenta. (Table 2)

Table 2. Pro-angiogenic factors up-regulated by PSG treatment

PSG family member	Target cell type	Factor induced
Murine PSGs		
22	human monocytes	TGF-β_1, VEGF-A
	murine peritoneal macrophages	TGF-β_1, VEGF-A
	RAW 264.7 murine macrophage cell line	TGF-β_1, VEGF-A
	C166 murine endothelial cell line	TGF-β_1, VEGF-A
	HTR-8/SVneo human EVT-derived cell line	TGF-β_1, VEGF-A
23	human monocytes	TGF-β_1, VEGF-A
	murine peritoneal macrophages	TGF-β_1, VEGF-A
	RAW 264.7 murine macrophage cell line	TGF-β_1, VEGF-A
	bone marrow-derived DCs	TGF-β_1, VEGF-A
	C166 murine endothelial cell line	TGF-β_1, VEGF-A
	murine trophoblast cell line derived from C57Bl/6 x 129	TGF-β_1, VEGF-A
	HTR-8/SVneo human EVT-derived cell line	TGF-β_1, VEGF-A
Human PSG		
1d	human monocytes	TGF-β_1, VEGF-A
	murine peritoneal macrophages	TGF-β_1, VEGF-A
	RAW 264.7 murine macrophage cell line	TGF-β_1, VEGF-A
	C166 murine endothelial cell line	TGF-β_1, VEGF-A
	human UtMVECs	TGF-β_1, VEGF-A
	HTR-8/SVneo human EVT-derived cell line	TGF-β_1, VEGF-A and -C

The importance of TGF-β_1 in pregnancy

TGF-β_1 plays multiple roles in pregnancy and members of the TGF-β family have been implicated in the processes of cell proliferation and differentiation, angiogenesis, immune function and tissue remodeling and repair [202]. TGF-β_1, -β_2 and -β_3, TGF-β receptor I and II, betaglycan and endoglin have been detected at the maternofetal interface [29]. During early pregnancy, expression of TGF-β by uterine tissues is thought to enhance trophoblast attachment to the endometrium [140, 203]. In addition, injection of neutralizing TGF-β antibodies into mouse embryos reduces the rate of implantation [31].

Members of the TGF-β family have a profound influence in the proliferation and differentiation of placental cells. TGF-β production by the decidua is thought to regulate trophoblast invasion [139]. Peak expression of TGF-β mRNA in the human placenta at weeks 17-34 of gestation coincides with the cessation of trophoblast invasion and growth [204]. It has been reported that TGF-β_1 and -β_2 inhibit proliferation of an invasive human trophoblast cell line in vitro [158] as TGF-β has also been shown to enhance trophoblast attachment to the extracellular matrix of the decidua [29]. Shallow trophoblast invasion of the decidua can lead to restricted blood flow to the fetus and result in syndromes such as pre-eclampsia. Conversely, uncontrolled trophoblast migration into the uterine wall is a hallmark of gestational trophoblast diseases such as molar pregnancies and choriocarcinoma [205]. Therefore, TGF-β counterbalances the VEGF-mediated signals promoting trophoblast migration.

During pregnancy, TGF-β acts as a potent immunomodulator by mediating maternal immune tolerance to the developing fetus [206]. Effects of TGF-β in the immune system are mediated through antigen presenting cells (APCs), such as macrophages and DCs [29]. TGF-β has been shown to inhibit Th1 responses, which are thought to be detrimental to successful pregnancy [207]. Importantly, TGF-β modulates the damaging actions of NK cells [208, 209]. In addition, TGF-β has been shown to stimulate Treg generation from $CD4+/CD25^-$ T cells [210]. TGF-β-null mice which survive until birth display numerous immune defects, including expansion of autoreactive T cells [211].

Previous studies from our lab and others have reported on the ability of human PSG1, 6 and 11 and murine PSG17, 18 and to induce TGF-β_1 in monocytes and macrophages [79, 89-94]. These results contributed to the idea that PSGs act as immunomodulators in pregnancy [212], as TGF-β is a potent immunoregulator during gestation [202]. We observed induction of TGF-β_1 in monocytes/macrophages after murine PSG22 and 23 treatment. In addition, we showed that murine PSG23 induced TGF-β_1 in DCs. Macrophages and DCs are important APCs at the maternofetal interface and their ability to secrete TGF-β is linked with pregnancy success [213].

TGF-β is expressed by endothelial cells, leukocytes and trophoblasts during pregnancy [139, 140]. We tested endothelial cell lines from humans and mice and a human invasive trophoblast cell for possible response to PSG treatment. Murine PSG22 and 23 and PSG1d up-regulated TGF-β_1 expression in the cell lines tested. These findings expand our knowledge of the functions of PSGs in pregnancy. Our data suggests that PSGs may play roles outside of

promoting maternal immune tolerance to the fetus. The observation that PSGs target cells, such as endothelial cells and trophoblasts, which are involved in maternal vasculature remodeling [8] indicates that PSGs may also be important in the process of placentation. Normal placenta development is the result of a successful dialogue between migrating EVTs and resident immune cells in the decidua; PSGs may be acting as signaling molecules mediating this interaction.

PSG22 and 23 did not up-regulate TGF-β_1 at the transcriptional level, which was also observed for other PSGs [92]. Regulation of TGF-β_1 at the post-transcriptional level has been observed in other sytems. ELISA data revealed significant up-regulation of TGF-β_1 expression in rat colon tumors compared to normal tissue, however, real-time PCR data revealed no change in the expression of TGF-β_1 mRNA [214]. Additionally, exposure of the A549 lung epithelial cell line to a reactive nitrogen intermediate donor promoted dose- and time-dependent up-regulation of TGF-β_1 [215]. TGF-β_1 up-regulation in A549 cells occurred at the post-transcriptional level because it was not abrogated by addition of a RNA polymerase II inhibitor, but was inhibited by addition of a protein synthesis inhibitor [215]. These data demonstrate that TGF-β expression is regulated at the post-translational level in several systems. Therefore, our observations that PSGs do not affect TGF-β_1 at the level of transcription is valid and was not due to an oversight in the collection times of our samples or data analysis

We do not currently know what post-translational mechanisms are involved in PSG-induced TGF-β_1 up-regulation. Previous work from our laboratory has suggested that PSG17-

mediated TGF-β_1 induction is mediated by a protein kinase C (PKC) pathway [89]. We observed that TGF-β_1 up-regulation in murine peritoneal macrophages and the RAW 264.7 cell line was inhibited by the addition of inhibitors to conventional PKC-α and $-\beta_1$ [89]. In addition, Zhang et al. demonstrated that PKC closely associates with the tetraspanins CD9, CD53, CD81, CD82 and CD151 upon stimulation with activators such as phorbol esters [216]. This work also suggested that PKC links $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrin complexes to tetraspanins [216]. In our system PKC may mediate in the intracellular signaling pathway initiated by PSG17—CD9 binding. Therefore, it is possible that PSG22 and 23 are also regulating TGF-β_1 expression through a PKC-mediated signaling cascade.

A second hypothesis for the post-transcriptional regulation of TGF-β_1 in our system involves MMPs. The work by Yu and Stamenkovic [44] demonstrated that the gelatinase MMP-9 cleaves latent TGF-β in mammary carcinoma cells. Their findings implicate a role for MMPs in post-transcriptional regulation of active TGF-β expression. Gelatinases and collagenases are essential to the breakdown of the extracellular matrix of the decidua prior to trophoblast migration through the uterine wall [217]. The synthesis and activation of MMP-9 by trophoblast cells is thought to be a prerequisite for EVT invasion during pregnancy [218]. Lymphocytes, DCs and monocyte/macrophages produce MMP-9 [219]; however, it is currently unknown if PSGs induce MMP-9 expression. A first step in determining possible involvement of MMPs in our system would be to test for the presence of MMPs in the supernatants of our PSG treated cells by ELISA. If we determine that PSGs induce MMP-9 in target cell types, it is possible that MMP-9 may also cleave latent TGF-β.

Role of the VEGF family in pregnancy

In humans placental vasculogenesis begins about 21 days post-conception [220] and is regulated by the VEGF family [221]. In the 1990s research began to focus on the relationship between VEGF and innate immunity [222]. It had already been reported that macrophages in the villous core of the placenta, known as Hofbauer cells, regulate trophoblast function and express angiogenic factors [223]; however, the paracrine functions of pro-angiogenic factors on immune cells had not been explored. Seminal work showed that dministration of VEGF to myeloid progenitor cells enhanced the growth of mature granulocytes but inhibited the growth of immature cells [224]. Additionally, it was shown that VEGF inhibits DC precursors to develop into mature DCs [225]. VEGF-mediated inhibition of DC maturation occurred through the nuclear factor kappa B (NF-κB) pathway [225, 226]. More recent studies indicate that VEGF inhibits the antigen presenting functions of mature DCs as well [227].

The expression of CEA family members appears to be altered in cancer. CEA expression is known to be down-regulated in colorectal cancer and in endometrial adenocarcinomas in concurrence with an increase in tumor malignancy [197]. Others have reported down-regulation of CEACAM1 expression in malignant breast tumors [228] as well as prostate cancer [229]. Changes in PSG expression have also been associated with cancer. Previous reports indicate that serum PSG is high in women with molar pregnancy, albeit at expression levels lower than normal pregnancy [230]. Expression of PSGs in trophoblastic malignancies may be a predictive factor in establishing disease and tumor type [230]. Expression of PSG9 has been detected in adenomatous polyps and colorectal carcinomas, which may render this

PSG family member a reliable biomarker for certain cancers [231]. PSG expression in human breast cancer has been reported and associated with shorter survival times [232]. Our laboratory has confirmed the expression of PSG family members in breast cancer by RT-PCR (Dveksler lab, unpublished results). Based on findings that PSGs are detected in malignant tumors, it is possible that PSGs may aid in tumor growth through their induction of pro-angiogenic factors.

Previous work by Jeon et al. [41] demonstrated that VEGF expression in macrophages is regulated by TGF-β. We also examined whether PSGs induced VEGF expression in monocytes and macrophages. We observed up-regulation of VEGF-A in human monocytes and murine macrophages (RAW 264.7 cells and peritoneal macrophages) after treatment with murine PSG22 and 23 and human PSG1d. We also observed VEGF-A up-regulation in murine and human endothelial cell lines (C166 cells and UtMVECs, respectively) and a human invasive trophoblast cell line (HTR-8/SVneo) upon PSG treatment. In the mouse RAW 264.7 cells and C166 cells, we determined that up-regulation of VEGF-A was TGF-β_1-dependent as pretreatment with a neutralizing anti-TGF-β antibody amoreliates the induction of VEGF-A by PSG23 treatment in these two cell types. Our findings also revealed that murine PSG22 and PSG23 do not seem to induce other members of the VEGF family important in pregnancy, namely VEGF-C and PlGF. Further investigation is needed to determine if human PSGs induce other pro-angiogenic factors.

Our initial studies indicate that murine PSG22 and 23 do not up-regulate VEGF expression at the level of transcription. VEGF expression is controlled at the level of transcription, mRNA

stability and translation [233]. The VEGF gene encodes for multiple splice variants which are named for the number of amino acids they contain and are predicted to act at various extracellular locations [234]; however, the regulation of VEGF isoform expression remains to be elucidated. VEGF expression is also regulated at the level of mRNA stability, and the actions of Ras and phorbol myristate acetate can increase the stability of mRNA [234]. VEGF translation is also regulated. Previous work showed that $\alpha_6\beta_4$ integrin enhances VEGF translation in breast carcinoma cells through inactivating the translation repressor 4E-binding protein, thereby allowing unregulated translation of VEGF in tumor cells [235].

At the post-translational level, PI3K has been shown to regulate VEGF expression [235]. The PI3K pathway is downstream of PKC activation in tumor cells [235, 236]. Our previous observations that PKC may mediate PSG signal transduction [89]. Although the mechanism of VEGF induction by PSGs is unknown, we observed that VEGF-A up-regulation is TGF-β_1-dependent in a murine endothelial and macrophage cell line. We speculate that TGF-β up-regulation in response to PSGs occurs through a PKC signaling cascade [89]. Previous work suggests that VEGF induction is also regulated by PKC. The PKC-β inhibitor ruboxistaurin was shown to inhibit VEGF expression in glomerular endothelial cells [237]. Therefore, it is possible that TGF-β is regulating VEGF expression post-transcriptionally through a process that is initiated by PKC signaling.

At the level of transcription hypoxia, or low oxygen conditions, is known to induce VEGF expression [238]. Under low oxygen conditions, the transcription factor hypoxia-inducible factor (HIF)-1 is activated and binds to a specific region, the hypoxia response element

(HRE), in the promoter region of the VEGF gene [238]. Hypoxia induction of VEGF mRNA in tumor cells has been linked to malignant outgrowth [239, 240]. We tested whether PSG induction of TGF-β_1 and VEGF-A in macrophages and endothelial cells was enhanced by hypoxia. We treated cells under normoxic and hypoxic conditions. Although we observed up-regulation of both pro-angiogenic factors in cells cultured under low oxygen conditions, PSG treatment did not further increase the up-regulation of TGF-β_1 or VEGF-A in macrophages or endothelial cells (Dveksler lab, unpublished results). These results indicate that PSGs stimulate VEGF up-regulation independently of HIF-1. The VEGF gene promoter also contains a Smad3/4 binding element downstream from the HRE [142]. Jeon et al. suggested that TGF-β_1 regulates VEGF expression in macrophages because Smad3/4 binds to the promoter region of the VEGF gene [41]. Their observations lend support to our results suggesting that PSGs do not induce VEGF expression in a hypoxia-dependent manner.

Based on our findings, we hypothesize that PSGs induce TGF-β_1 release from its latent form, possibly mediated by MMP processing of inactive TGF-β. We can speculate that active TGF-β_1 is secreted and binds back to its receptors on the releasing cell in an autocrine manner or adjacent cells in a paracrine manner. Binding of TGF-β_1 to its receptors leads to Smad3/4 activation and translocation to the nucleus. Smad3/4 binds to the promoter region of the VEGF gene, leading to up-regulation and secretion of VEGF in our target cell types. Although we were not able to detect VEGF mRNA in our samples, this may have been due to collection of incorrect time points in our real-time PCR studies. Expanding our time course studies to include earlier and later time points will be necessary in future experiments. We

could also blocking VEGF transcription by pre-treating cells with transcriptional inhibitors, such as actinomycin D, prior to PSG treatment. The results of these experiments may aid us in determining whether or not PSGs influence VEGF expression at the transcriptional or post-transcriptional level.

Possible PSG23—CD151 interaction

Due to the sequence homology between PSG23 and PSG17 and the overlapping functions both proteins have in terms of inducing TGF-β in monocytes/macrophages [93] (Dveksler lab, unpublished results), we wanted to determine if PSG23 also used the tetraspanin CD9 as its receptor. Through subsequent binding and functional studies, we determined that CD9 was not necessary for PSG23 activity in macrophages. However, it was possible that PSG23 utilized another tetraspanin as its receptor. We then tested a number of tetraspanins expressed in macrophages to determine potential interaction with PSG23, and PSG23 appeared to bind preferentially to CD151.

CD151 is expressed on nearly all cell types [169]. In order to confirm possible binding of PSG23 to CD151, we overexpressed CD151 in HEK 293T cells. In these experiments, we transfected cells with human or mouse CD151 (plasmids were a kind gift from Dr. Leonie Ashman). In some experiments, we transfected cells with mouse CD9 (plasmid described in [93]) as a negative control. We detected binding of PSG23 to the CD151-transfected cells by building a FACS tree in which the transfected cells are incubated with protein, the bound protein is detected with a biotinylated primary antibody (biotinylated anti-Flag, Sigma), and the bound primary antibody is detected with streptavidin conjugated to a fluorescent dye

(FITC or PE labeled streptavidin, eBioscience). The FACS tree amplifies the signal from the protein-receptor interaction, which is useful to us as HEK 293T cells endogenously express CD151 and PSG23 binds well to untransfected or control plasmid-transfected cells. In six independent experiments, we observed positive binding of PSG23 to CD151-transfected cells over control-transfected cells. We did not observe any difference in PSG23 binding to the CD9-transfected cells compared to control-transfected cells.

In subsequent binding experiments, we compared PSG23 binding to A431 sh3 cells, in which CD151 expression is silenced by short hairpin RNA, and A431 Rx cells, in which the A431 sh3 cells were transduced with a retroviral vector to re-express CD151 (both cell lines generously provided by Dr. Christopher Stipp [170]). In five independent experiments, we observed binding of PSG23 to the A431 Rx cells over the A431 sh3 cells; however, we detected binding between PSG23 and the A431 sh3 cells, indicating that CD151, while it may be important to the binding of PSG23 to its target cells, may not be the only receptor for PSG23.

A salient feature of tetraspanins is their ability to form lateral associations with each other and other cell surface molecules [172]. However, the tetraspanin webs formed on the cell surface may differ between cell types [100]. CD151 directly associates with the laminin-binding integrins on endothelial cells [241] but has also been shown to interact with other tetraspanins such as CD81 and CD53 [102]. Previous work by Wright et al. demonstrated by FACS analysis that CD151-null keratinocytes also had significantly reduced expression of CD9 on their cell surface, indicating possible interaction between these two tetraspanins

[110]. In addition, immunoprecipitation of human umbilical vein endothelial cell lysates with an anti-β_1 or anti-α_3 integrin antibodies coprecipitated CD151 and CD9 [241]. We observed that the absence of CD9 did not inhibit PSG23 activity in macrophages. If we hypothesize that CD151 is the receptor for PSG23, and that CD151 is associated with CD9, the absence of CD9 does not appear to affect PSG23 binding to CD151. However, we did observe binding of PSG23 to the EC2 of CD9 in pull-down experiments (Dveksler lab, unpublished results). It is possible that CD9 may act as a lower-affinity co-receptor for PSG23 or may participate in a larger complex of proteins serving as the receptor for PSG23. This finding may, in part, explain why the A431 sh3 cells, which do not express CD151 on their surface [170], still bind to PSG23 over control protein. Previous studies suggest that CD151 may have multiple and distinct binding partners in different cell types. Therefore, PSG23, should it bind to CD151, may not have an effect on every CD151-positive cell type. In addition, PSG23 induction of TGF-β_1 and VEGF-A in macrophages may not occur through the same receptor complexes as are found in endothelial cells.

A secondary observation in our PSG23—CD151 binding studies was that PSG17, previously believed to only bind to CD9, also bound to the EC2 of CD151 (Dveksler lab, unpublished results). Our findings were corroborated by data obtained with the BIAcore 2000 which indicated a PSG17—CD151 interaction, albeit at a lower affinity than the PSG17—CD9 interaction (Öbrink, personal communication). These observations, along with the PSG23—CD9 interaction determined by pull-down, suggest that PSGs may bind to multiple tetraspanins with varying affinities. Due to the lack of commercially available reagents to all 33 mammalian tetraspanins, we cannot currently determine all possible PSG—tetraspanin

interactions. However, utilizing the BIAcore 2000 technology, it would be possible to screen a panel of tetraspanins to determine both binding and affinity to PSGs.

CD151 and angiogenesis

Angiogenesis requires coordinated regulation of endothelial cell migration and tube formation and integrin-type adhesion receptors are involved [242-244]. Among these are the laminin-binding integrin heterodimers, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$, which are thought to halt the proliferative phase of angiogenesis and promote tube stabilization [245]. CD151 is closely associated with the laminin-binding integrins, the CD151—$\alpha_3\beta_1$ interaction being the most well characterized and conserved even under stringent detergent conditions [166]. The CD151—$\alpha_6\beta_1$ complex has been shown to support the formation of cord-like structures by NIH3T3 cells on Matrigel, commonly used to study angiogenesis, suggesting a role for this tetraspanin in vascular development [246]. In addition, CD151 has been shown to regulate endothelial cell migration through its association with integrins [241, 247, 248].

Although CD151-null mice are normal, healthy and fertile [110] they display some selective defects. For example, mice lacking CD151 show longer tail bleeding times, a slight but significant increase in immature myeloid cells, and abnormal wound healing [110]. Endothelial cells from CD151-null mice display impairments in cell spreading, motility and tube formation when grown on Matrigel [106], all processes that characterize vessel formation in culture assays. The association between CD151 and the laminin-binding integrins [241] indicates an important, but as yet undefined, role for CD151 in angiogenesis. Additionally, the laminin-binding proteins can activate a number of signaling pathways,

among them the PI3K-Akt signaling pathway that has been shown to regulate migration, invasion, and capillary formation during angiogenesis [249-251]. Endothelial cells from CD151-null mice displayed reduced laminin-dependent Akt phosphorylation, which may help to explain the observed defects in cell tube formation; however, the reduced Akt phosphorylation did not affect cell survival [106]. At present it is not understood why CD151 is not required for normal vascular development. Speculations about other tetraspanins compensating for the lack of CD151 have been made, and the tetraspanin TSPAN11, which shares some sequence homology with CD151, has been postulated as a candidate molecule [106, 110]. Previous studies have shown that tetraspanins share some overlapping functions, as exemplified in the observation in which microinjection of CD81 into germinal-vesicle stage oocytes reversed the reduced fertility of CD9-null mice [252]. We determined that TSPAN11 was not expressed by A431 sh3 cells by RT-PCR, therefore, the compensatory molecule on the surface of these cells, which accounts for PSG23 binding, remains unknown.

CD151 signaling cascades

CD151 is associated with phosphatidylinositol 4-kinase (PI4K) and the CD151-PI4K- $\alpha_3\beta_1$ interaction is thought to contribute to cell motility [166], with CD151 acting as the linker between the integrin and the intracellular signaling pathway. In addition, it has been reported that CD151 regulates the protein-tyrosine phosphatase PTPμ [253], and inhibits adhesion-dependent activation of Ras [254]. Hong et al. reported that signaling pathways through CD151-$\alpha_3\beta_1/\alpha_6\beta_1$ led to increases in c-Jun-mediated MMP-9 gene expression, thereby enhancing motility and invasiveness of a cancer cell line [247]. It has also been shown that CD151-mediated cell-cell adhesion is modulated by PKCα in A431 cells [255].

PSG23 signaling cascade

Although it remains to be determined if PSG23, or any other PSGs, utilize CD151 as their receptor, if that conclusion is able to be drawn, it will be necessary to examine the downstream signaling consequences of the PSG23-CD151 interaction. CD151 associates with integrins, such as $\alpha_3\beta_{1,}$ via an 11 amino acid motif (residues 195-205) in the extracellular loop 2 (EC2) [167] (Figure 1), and our pull-down assays indicate that PSG23 also interacts with the EC2 of CD151. At present, the residues involved in the PSG23-CD151 interaction are unknown. Based on previous work identifying the residues in the EC2 of CD9, being SFQ (residues 173-175), necessary for PSG17 binding [89], it is possible that CD151 interacts with PSG23 at a similar site in the EC2, which lies outside the integrin-interacting domain. Our observations that PSG23 evokes downstream signaling effects, such as VEGF up-regulation, also seen in cells expressing intact CD151-integrin complexes [256], suggests that PSG23 does not interfere with the interaction of CD151 with integrins. CD151 unbound to integrins is also expressed on the cell surface [110]; therefore, the possibility exists that PSG23 may bind to this free form of CD151. If PSG23 is binding to free CD151 and not integrin-bound CD151, or vice versa, this could limit the cell types that would respond to PSGs.

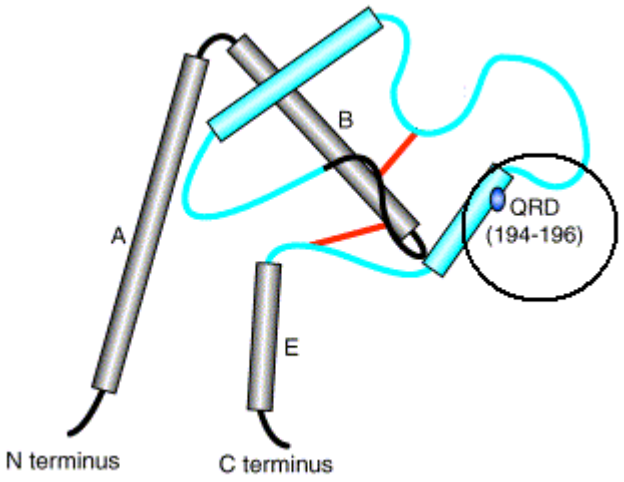

Figure 6: The EC2 of CD151. The ABE helical face (grey) is most likely present in all tetraspanins and the variable region (light blue) is thought to mediate tetraspanin interactions with other proteins. The residues glutamine-arginine-aspartic acid (QRD 194-196) are required for CD151 association with integrins. [101]

Up-regulation of TGF-β_1 and VEGF-A in macrophages as a result of PSG23 treatment may be the result of the activation of the PKC signaling cascade, which may post-translationally regulate secretion of TGF-β_1 and VEGF-A in our system. PKC-mediated regulation of TGF-β_1 and VEGF has been demonstrated in other systems [89, 237, 247, 257], including in our previous work with PSG17—CD9 interaction [89]; therefore, it is possible that PSG23-CD151 signaling activates a similar cascade in macrophages. In addition, the interaction between CD151 and laminin-binding integrins has been shown to activate the PI3K-Akt signaling cascade, which has been shown to regulate VEGF expression at the post-translational level [235]. The induction of TGF-β_1 in PSG23-responsive cell types may also induce signaling events through the PI3K-Akt pathway. TGF-β-mediated PI3K signaling has been shown to induce collagen I expression in glomerular cells [258] and TGF-β-induced growth of airway epithelial cells has been shown to be abrogated by the PI3K inhibitor LY294002 [259]. Additionally, previous studies have shown that CEA and CEACAM6 interact with $\alpha_5\beta_1$ integrin in colon cancer cells and $\alpha v\beta_3$ in pancreatic adenocarcinoma cells upon antibody cross-linking, and this interaction leads to increased expression of phosphorylated Akt in these cell lines [260-262]. Thus, we can speculate that the PSG23-CD151 interaction leads to TGF-β_1 up-regulation and subsequent activation of PI3K-Akt signaling events in target cell types to induce VEGF-A expression.

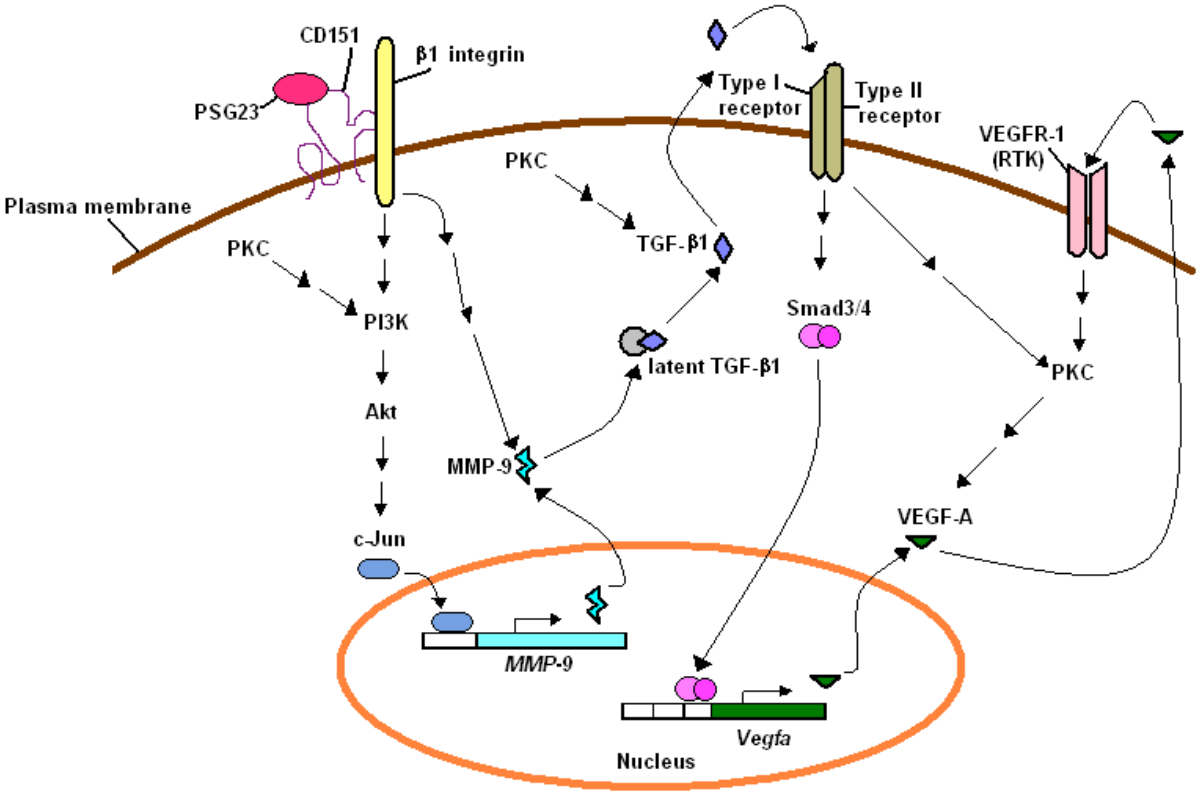

Figure 7: Possible signaling cascades initiated by PSG23—CD151 binding. Upon PSG23 binding to CD151 on the cell surface MMP-9 expression and activation may occur, possibly through a PI3K-Akt-mediated signal transduction pathway. Active MMP-9 cleaves the latent form of TGF-β_1 releasing active TGF-β_1, which can bind to its receptor on the cell surface. TGF-β_1 binding to its cell surface receptor leads to translocation of the transcription factors Smad 3 and 4 to the nucleus. Smad3/4 bind to the response element in the promoter region of the VEGF gene, leading to transcription of VEGF-A. VEGF-A is secreted by the cells and can bind to its cell surface receptor VEGFR-1, leading to activation of PKC. Concomitantly, PKC activation also mediates TGF-β_1 activity and PI3K signaling cascades.

Future Directions

Characterization of the pro-angiogenic properties of human PSG1d

In our initial work with human PSG1d, we observed up-regulation of VEGF-A in human monocytes using a relatively low concentration of recombinant protein (10 μg/ml). However, we did not see induction of VEGF-C in human monocytes. We are currently in the process of determining if PSG1d induces other members of the VEGF family, such as PlGF, in these immune cells. It has been reported that human monocytes up-regulate expression of the Tie-2 receptor for Ang2 during angiogenesis in response to increased Ang2 secretion by neighboring endothelial cells [263-265]. Although the focus of our work has been primarily on pro-angiogenic factors themselves, it will be important for us to explore the up-regulation of their cognate receptors to get a broader sense of the possible actions of PSGs in vessel formation.

We observed in the RAW 264.7 cell line and the C166 cell line that VEGF-A induction was TGF-β-dependent. This is consistent with previous studies that report regulation of VEGF by TGF-β in macrophages [41] and endothelial cells [266]. It has also been observed that TGF-β regulates VEGF in HTR-8/SVneo cells [24], a cell line derived from invasive human trophoblasts [118]. It would be interesting for us to determine if PSG1d induction of VEGF-A and VEGF-C in HTR-8/SVneo cells is also dependent on TGF-β, and, if so, may help us better understand the signaling mechanisms involved when PSGs bind to their cell surface receptors. It would also be of interest to determine if VEGF-A expression in human monocytes or endothelial cells (UtMVECs) is also TGF-β-dependent.

Since PSG1d is the first human PSG we tested to examine the possible induction of pro-angiogenic factors in the cell types involved in maternal vasculature remodeling, we would like to extend our experiments to include other members of the human PSG family. It would be interesting to determine if PSG11, which has also been shown to be important in pregnancy [86], also induces VEGF in monocytes, human endothelial cells or human trophoblast cells. PSG11 contains the proposed integrin-binding RGD sequence in its N-domain [267], and, as integrins, including the fibronectin/vitronectin-binding integrins ($\alpha v\beta_3$, $\alpha v\beta_5$ and $\alpha_5\beta_1$) and the laminin-binding integrins ($\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$), are implicated in the process of angiogenesis [243, 244, 268], it is possible that PSG11 may induce pro-angiogenic factors other than those induced by PSG1d through its potential interaction with integrins on the cell surface.

RGD sequences can act as cell recognition sites for extracellular matrix and platelet adhesion proteins that bind to integrins, including vitronectin, type I collagen, fibrinogen, von Willebrand factor and osteopontin [269]. Indeed, binding of fibronectin to its receptor $\alpha_5\beta_1$ occurs through the RGD sequence on fibronectin [270]. The RGD motif in lymphocyte function-associated antigen (LFA-1/CD11a/CD18) has been shown to contribute to T cell binding to intercellular adhesion molecule (ICAM)-1 on endothelial cells [271]. Membrane activated complex (Mac)-1 (CD11b/CD18) also contains an RGD sequence and its expression on neutrophils is thought to aid in their transendothelial migration [272]. Parallels between the processes of transendothelial cell migration and embryonic implantation have been suggested, and both cellular events utilize some of the same adhesion and migration molecules, including L-selectin, MMP-2 and -9 and vascular cell adhesion molecule

(VCAM)-1 [273]. The presence of RGD sequences on members of the PSG family and the association between laminin-binding integrins and tetraspanins may contribute to PSG binding to their receptors and implicate a role for PSGs at the maternofetal interface in placental development.

The significance of the RGD motif on PSGs remains contentious; not all human PSGs have an RGD motif and none of the murine PSGs contain this sequence. Murine PSGs are believed to have an RGD-like motif within a highly variable β-sheet forming CFG region in their N1 domains [99]. The RGD-like region in the CFG face of murine PSGs is postulated to contribute to cell attachment and aid in tissue remodeling during placentation [99]. Based on the speculation that RGD and RGD-like motifs may play a role in PSG function, we have begun to explore the functional significance of sequence differences between the RGD-like motif of PSG23 and PSG17. We hypothesize that altering the RGD-like region of PSG17 may affect its ability to bind to CD9; therefore, we have generated a mutated PSG17N in which the RGD-like region of PSG17 is mutated to have the same sequence as PSG23 in the same region (Figure 2). Studies are currently underway to determine if the PSG17N mutant protein is still able to bind to CD9 and induce relevant cytokines in macrophages.

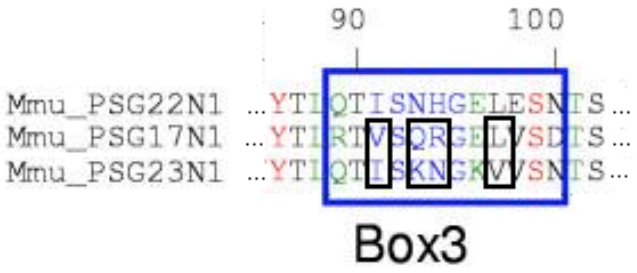

Figure 8: Comparison of the RGD-like sequences of PSG17, 22 and 23. The RGD-like sequence of murine PSGs spans residues 89-100 in the N1 domains. The RGD-like sequence for PSG17 is arginine-glycine-glutamic acid (RGE), for PSG22 is histidine-glycine-glutamic acid (HE) and for PSG23 is asparagines-glycine-lysine (NGK). The residues mutated in PSG17 to the corresponding residues in PSG23 are boxed in black: valine-glutamine-arginine-valine (VQRV) in PSG17 to isoleucine-lysine-asparagine-valine (IKNV) in PSG23. [99]

Pro-angiogenic properties of additional murine PSGs

Our observation that murine PSG23 induces TGF-β_1 and VEGF-A in murine macrophages and human monocytes, murine BMDCs, the C166 cell line and mouse and human trophoblast cell lines lead us to examine whether or not additional murine PSGs share similar functions. We found that PSG22 also induces these pro-angiogenic factors in many of the same cell types, albeit the response requires a higher protein concentration than we observed with PSG23 treatment. Although we do not know the reason for this difference in protein affinity, it is interesting to note that the RGD-like motif in the CFG face of PSG22 and 23 differ (Figure 2), possibly contributing to their binding to target cell types [99]. Our data suggests that other members of the murine PSG family may also play a role in vasculature remodeling. It would be of particular interest to examine VEGF-A induction in macrophages, DCs, endothelial cells and trophoblasts upon PSG17, 18 or 19 treatment, especially as the receptor for PSG17 and 19 has been determined to be the tetraspanin CD9 [79, 93]. Previous reports have implicated CD9 in the process of endothelial cell migration [274] and wound healing [275], processes associated with angiogenesis; therefore, it is likely that PSG17, 18 and 19 share similar abilities with PSG22 and 23 to induce pro-angiogenic factors and further study is needed.

Receptor identification studies

The binding studies that we have performed to determine possible specific interaction between PSG23 and CD151, and the data we have collected thus far, can only provide us with partial information about the receptor for PSG23. Functional studies in CD151-null mice would help us determine whether or not CD151 is biologically significant to the actions

of PSG23 we have observed. However, while it is possible that treating macrophages from CD151-null mice and testing for up-regulation of TGF-β_1 or VEGF-A may give us a negative result, it is also conceivable that we will see cytokine induction in *Cd151*$^{-/-}$ macrophages. From a positive result in these cells, i.e., TGF-β_1 up-regulation in both wild-type and CD151-null macrophages, we could conclude the following:

(1) CD151 is not the receptor for PSG23 and our FACS results are artifacts of non-specific interactions between the glycosylation sites on PSG23 and very adherent cells and blocking non-specific binding, such as pre-incubation with BSA, may reduce background binding of PSG23 to the cells.

(2) CD151 is the receptor for PSG23 but when CD151 is absent other molecules, possibly other tetraspanins or integrins, can compensate for the lack of CD151, and bind with lower affinity to PSG23, which would explain the relatively normal development of CD151-null mice [106, 110]. We observed that PSG23 bound to CD151-silenced, therefore it may be necessary to test PSG23 binding to a cell type that does not express CD151 and CD9, which would provide us with a first step in terms of determining compensatory molecules for CD151.

(3) CD151 is an accessory protein to the receptor for PSG23 and, though PSG23 can bind with some affinity to this unidentified receptor, when CD151 is expressed or overexpressed it modulates the true receptor to bind with higher efficiency to PSG23, which would explain why overexpression of CD151 in cells leads to greater binding to PSG23.

(4) CD151 is the receptor for PSG23 on select cell types, such as endothelial cells or trophoblast cells, but is not the only receptor for PSG23 in macrophages. Our

FACS binding studies have been performed in non-leukocyte populations. Previously, we observed that PSG19 did not bind to CD9 using FACS analysis, however, CD9 was determined to be the receptor for PSG19 [79]. We cannot necessarily translate the FACS binding data to functional data. The expression of CD151 and its binding partners on macrophages may differ from its expression on epithelial cells; therefore, PSG23 may bind to CD151 on epithelial cells and endothelial cells but may not bind to the CD151 expressed on macrophages.

We have come to an impasse in our search for a possible receptor for human PSGs. Although the methods employed to identify the receptor for PSG11 and PSG1d were successful for others in our laboratory to identify the receptors for PSG17 and 19 [79, 93], we were unable to isolate a candidate molecule, possibly due to the following:

(1) The receptor for human PSGs is not a single molecule but a complex of cell surface molecules, and panning, at least with the RAW cell cDNA library, is only effective for single polypeptide receptors.

(2) The receptor for human PSGs is a complex of molecules that were not expressed by the NIH3T3 cells in the proper combination when the cells were transduced with the spleen cDNA library.

(3) The receptor(s) for human PSGs is highly expressed on most cell types and is/are endogenously expressed on NIH3T3 cells and HEK 293 EBNA cells, regardless of the cDNA libraries, and the single cell clones may have expressed the receptor(s) for human PSGs.

(4) We determined that PSG1d had a higher affinity for its receptor(s) on RAW 264.7 cells than PSG11 using an alkaline phosphatase (AP) tag fusion assay (described in [276]); therefore, panning the spleen cDNA library with PSG1d may have been more advantageous than using PSG11 to pan this library.

(5) During the panning procedure human PSG1d was bound to the stable surface (the Petri dish) through its A1 domain by the anti-PSG BAP1 antibody (Genovac) and PSG11 was bound to the Petri dish by NeutrAvidin (Pierce) through its C-terminal biotin group. The antibodies may have anchored the PSGs in a conformation which prohibited the N-domain from binding efficiently to its cell surface receptor(s). In future studies it may be necessary to utilize other antibodies or another stable surface, such as a gel matrix, to immobilize the PSGs.

More sensitive binding methods may be necessary to identify possible receptors for the human PSGs, such as screening a panel of tetraspanins using surface plasmon resonance (BIAcore) to look for candidate receptors. Despite evidence suggesting a common evolutionary ancestor gene for the PSGs [99], they are heterogenic proteins. It has been observed that PSG family members are expressed at different times during pregnancy [98] and studies which report PSG expression outside of pregnancy are specific: only PSG18 has been associated with the Peyer's Patches [132] and PSG9 is specifically detected in colorectal cancer and may be predictive of clinical course [231]. Additionally, initial studies investigating the functions of PSG23 indicate that it does not up-regulate COX-2 or arginase I expression in macrophages (Dveksler, unpublished results), which were reported to be up-regulated upon PSG17 treatment of macrophages [89]. Taken together, these data indicate

that PSGs serve overlapping but divergent functions; therefore, we cannot assume that all human PSGs utilize the same receptor and it will be important to future receptor identification studies to select the proper ligand to perform these studies.

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Despite our knowledge of the existence of PSGs during pregnancy, and their prevalence in the maternal circulation during gestation, their functions are still being elucidated [70]. We observed that murine PSG22 and 23 and human PSG1d induce the pro-angiogenic factor VEGF-A in monocytes/macrophages and discovered that these PSGs target novel cell types, namely endothelial cells and trophoblasts. Previous reports have observed lowered serum PSG levels in conjunction with pregnancy complications [200], among these are fetal hypoxia and pre-eclampsia, which are both due to insufficient trophoblast invasion and incomplete spiral artery remodeling [201]. In addition, it has been observed that tissue biopsies from pre-eclamptic patients have lowered expression of VEGF [23]. We report on a novel link between PSGs and VEGF, opening up the possibility that PSGs may play a role in maternal vasculature remodeling. The findings reported in this dissertation add new insight into the pleiotropic functions of one of the most abundant proteins in pregnancy.

PART SIX:

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REFERENCES

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