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Pregnancy Specific Glycoprotein Function, Conservation and Receptor Investigation

Ronan T. O’Riordan

BSc

NATIONAL UNIVERSITY OF IRELAND, CORK

DEPARTMENT OF BIOCHEMISTRY

Thesis submitted for the degree of

Doctor of Philosophy

December 2013

Supervisor: Dr. Tom Moore

Head of Department/School: Prof. David Sheehan

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List of Abbreviations

AAS  antibiotic antimycotic solution
ACD  acid-citrate-dextrose
APC  antigen presenting cell
BCA  bicinchonic acid
BMDCM  bone marrow derived macrophage
BSA  bovine serum albumin
CD  cluster of differentiation
cDNA  complementary DNA
CDS  coding-domain sequence
CEA  carcinoembryonic antigen
CEACAM  CEA-related cell adhesion molecule
CG  choriogonadotrophin
CTB  cytotrophoblast
DC  dendritic cell
DMEM  Dulbecco’s modified Eagle’s medium
DNA  deoxyribonucleic acid
ECACC  European Collection of Cell Cultures
EDTA  ethylenediaminetetraacetic acid
ELISA  enzyme-linked immunosorbent assay
EMBL  European Molecular Biology Laboratory
EST  expressed sequence tag
List of Abbreviations

EVT  extra-villous trophoblast

FACS Fluorescence Activated Cell Sorting

FBS fetal bovine serum

FLBA Fluorescent Ligand Binding Assay

GI  gastro-intestinal

GPI  glycophosphatidylinositol

HSPG heperan sulphate proteoglycan

iFLBA Indirect Fluorescent Ligand Binding Assay

IFN interferon

Ig  immunoglobulin

IgC  immunoglobulin constant

IgV  immunoglobulin variable

IL interleukin

IMAC immobilised metal affinity chromatography

IP immunoprecipitation

IPTG isopropyl β-D-1-thiogalactopyranoside

ITIM immunoreceptor tyrosine-based signalling motif

IUGR intra-uterine growth restriction

KLF  Kruppel-like factor

LAD  Leukocyte Adhesion Deficiency

LAP Latency Associated Protein

LB  Luria-Bertani
List of Abbreviations

LLC  Large Latency Complex
LPS  lipo-polysaccharide
LTBP  Latent TGF-β1 Binding Proteins
M-CSF  murine-colony stimulating factor
mAb  monoclonal antibody
MHC  major histo-compatibility complex
MWCO  molecular weight cut-off
MYA  million years ago
NO  Nitric Oxide
OGFg  Oregon Green 488 fibrinogen
PBMC  peripheral blood mononuclear cell
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PD-L1  programmed death-ligand 1
PE  phycoerythrin
PFA  paraformaldehyde
PKA  protein kinase A
PRP  platelet rich plasma
PSG  pregnancy-specific glycoprotein
qRT-PCR  quantitative real-time reverse transcriptase-polymerase chain reaction
RARE  Retinoic Acid Response Element
RCSI  Royal College of Surgeons in Ireland
RGD  Arginine-Glycine-Aspartic acid
RNA  ribonucleic acid
SDS PAGE  sodium dodecyl sulphate polacrylamide gel electrophoresis
SEC  Size Exclusion Chromatography
SLC  Small Latency Complex
SMART  Simple Modular Architecture Research Tool
STB  syncytiotrophoblast
T_{h2}  type 2 helper T cell
T_{reg} cell  T regulatory cell
TEMED  tetramethylethylenediamine
TGF-β1  transforming growth factor-β1
TLR  toll-like receptor
TNF-α  tumour necrosis factor-α
TRAP  thrombin receptor activating peptide
vCTB  villous cytotrophoblast
I, Ronan T. O’Riordan, certify that this thesis is my own work and I have not obtained a degree in this university or elsewhere on the basis of the work submitted in this thesis.

Ronan T. O’Riordan
Abstract

Background

Pregnancy-specific glycoproteins (PSGs) are highly glycosylated secreted proteins encoded by multi-gene families in some placental mammals. They are carcinoembryonic antigen (CEA) family and immunoglobulin (Ig) superfamily members. In humans they are composed of one N-terminal immunoglobulin variable (IgV)-type domain (N) and two or three immunoglobulin constant (IgC)-type domains (A1 and/or A2 and B2) with a short C-terminal sequence. PSGs are immunomodulatory, and have been demonstrated to possess anti-platelet and pro-angiogenic properties. Low serum levels of these proteins have been correlated with adverse pregnancy outcomes. Following implantation of the human embryo, PSGs are secreted by the syncytiotrophoblast (STB) and are reported to be detected at 200-400 µg/ml in the maternal serum at term. The N-domains of many of the human PSGs are sufficient to mediate their induction of cytokines as well as their anti-thrombotic function. Motifs representing predicted N-glycosylation sites and a conserved predicted integrin binding Arginine-Glycine-Aspartic acid (RGD)-like tri-peptide have been determined to be unnecessary for cytokine release, anti-platelet or angiogenic functions of PSG1. To date mouse Psgs have been shown to bind differentially to tetraspanin CD9 and both mouse and human PSGs have been shown to have a broad affinity for cell surface heperan sulphate proteoglycan (HSPG).

Objectives

The research within this thesis had goals within three areas:

1) To attempt to replicate previously reported cytokine responses to PSG-treatment of immune cells and subsequently to investigate functionally important amino acids within PSG1. It was further aimed to examine the signalling underpinning the immunomodulatory functions of this hormone through small molecule inhibitors and determination of status of important components of key immune signalling pathways.

2) To determine whether candidate receptor, integrin αVβ3, was a binding partner for PSG1 and to investigate whether PSG1 possessed functionality in a leukocyte-endothelial interaction assay.

3) To determine whether proteins generated from recently identified putative PSG genes in the horse shared...
functional properties with PSGs from other species.

Outcomes

1) Sequential domain deletion of PSG1 showed that multiple domains possess TGF-$\beta_1$ induction activity from a THP-1 monocytic cell line assay. A conserved RGD-like tripeptide was investigated for functional importance in the PSG1N construct and confirmed to not be necessary for TGF-$\beta_1$ release by this domain of PSG1. Deletion of N-domain N-linked glycosylation sites through an asparagine to aspartic acid (N→D) mutation had no effect on TGF-$\beta_1$ release. It was demonstrated that loss of glycosylation sites negatively affected PSG1N expression and secretion with the most dramatic effect caused by deletion of the most N-terminal glycosylation site in PSG1N. Introduction of this site into a novel PSG9N construct rescued protein expression highlighting the contribution glycosylation makes to protein expression. The TGF-$\beta_1$ response was subsequently found to be due to contamination of the purified recombinant proteins with latent TGF-$\beta_1$ and once removed no TGF-$\beta_1$ release could be detected in response to PSG1. The ability of PSGs to attenuate LPS-induced tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin (IL)-6 was found, through use of small molecule inhibitors, to be independent of cAMP-dependent PKA and TGF-$\beta_1$ signalling. 2) Integrin $\alpha_v\beta_3$ was identified as a novel PSG1 receptor mediating an as yet unknown function. Preliminary investigations into a role for PSGs as inhibitors of leukocyte endothelial interactions showed no effect by PSG1. 3) Horse PSG protein, CEACAM49, was shown to be similarly contaminated by latent TGF-$\beta_1$ particle and once removed did not demonstrate TGF-$\beta_1$ release from two previously reported PSG-responsive cell types. Interestingly horse PSG did show anti-platelet properties through inhibition of the platelet-fibrinogen interaction as previously published for mouse and human PSGs.
“I’ve been thinking Hobbes”

“On a weekend?”

“Well, it wasn’t on purpose”

Calvin and Hobbes

- Bill Watterson
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Chapter 1

Introduction

1.1 The Placenta

1.1.1 Structure and Function

The placenta is a complex and dynamic organ. It functions to provide physiological support to the growth of the fetus by facilitating gas and nutrient exchange and mediating the interactions between the mother and the fetus through pregnancy to parturition. Critically, it provides a barrier to prevent direct exposure of the semi-allogenic fetus to the maternal immune system. Interestingly, the placenta, in the relatively short period since it arose in vertebrate evolution (approximately 100 million years ago (MYA), developed significant diversity among placental mammals in terms of structure, cellular differentiation and mechanisms of endocrine function [1]. In humans and rodents, a fully developed placenta is composed of three distinct layers. The outer layer or decidua basalis, is composed of the uterine decidual tissues, including the maternal spiral arteries, which deliver oxygenated maternal blood to the fetus. The middle layer attaches the placenta to the uterus and contains fetoplacental cells derived from the outer cell mass of the developing embryo, called trophoblasts. These cells invade the uterine wall and surrounding blood vessels co-opting them for the survival of the fetus. The final layer contains highly branched villous structures of fetal origin, that are bathed in maternal blood and it is here that the physiological processes that support the fetus are carried out. Broadly, placentas are classified based on the nature of the materno-fetal interface, ie. the number of tissues.
separating the blood supplies of the mother and the fetus. These are haemochorial, endotheliochorial and epitheliochorial.

In haemochorial placentation, as described for the human and rodent, and unlike in the other two placental types, there is direct exposure of the semi-allogenic fetal component of the placenta to the maternal blood supply. While extremely efficient for gas, nutrient and waste exchange it creates physiological complications for maternal haemostasis and also for maintenance of the feto-maternal immunological network during pregnancy [2]. This type of placentation is found in higher order primates and rodents as well as rabbits. In endotheliochorial placentation the fetal epithelium encloses the maternal blood vessels. This type of placentation is common in carnivores but there is evidence of this type of placentation in orders from all four clades of eutherian mammals indicating that they have recently been found to be more widespread and diversified than originally believed [3]. Interestingly, haemochorial placentas of some species of bats and sciurumorph rodents can be seen to go through a transient endotheliochorial stage before establishment of the full haemochorial materno-fetal interface [3]. In epitheliochorial placentation the maternal and fetal epithelia appose each other. This type of placentation is found in ruminants and hooved mammals as well as cetaceans and in the strepsirrhine primates such as the lorisises and the lemurs. A summary table outlining the orders of mammals in 4 superordinal clades with examples and placental type indicated can be found in [4]. All three interhaemal barriers are represented schematically in Figure 1.1

Placentas are further classified based on their shape; discoid, bi-discoid, cotyledonary, diffuse and zonary; and further still based on the nature of the materno-fetal interdigitation which can be: folded, lamellar, trabecular, villous or labyrinthine [4]. Understanding the biology of placentas of different species is becoming more and more relevant. Investigations into the cloning, by somatic cell nuclear transfer, of economically important species such as cattle, give an insight into complications that can arise and potentially only be solved through in-depth investigations of placental biology, which is seen to frequently be central to the onset of pathologies [5]. Interestingly, it was recently demonstrated that gene expression in post-implantation embryos generated by somatic cell nuclear transfer correlated with donor cell type, but only for the embryonic tissue and not the
1. Introduction

1.1 The Placenta

The three main interhaemal barriers found in eutherian mammals are represented diagramatically showing the different types of interaction that occur between fetal and maternal tissues across mammalian placentas.

Figure 1.1: Types of interhaemal barriers present in placentas

The three main interhaemal barriers found in eutherian mammals are represented diagramatically showing the different types of interaction that occur between fetal and maternal tissues across mammalian placentas.
extra embryonic tissues [6]. This study demonstrates that somatic cell nuclear transfer effects on extraembryonic tissue gene expression were not ameliorated based on donor cell choice and reaffirms that the difficulties inherent in this technique are still far from being addressed.

1.1.2 Evolution

With the onset of the genomic era significant advances have been made in the field of phylogenetics that have furthered our understanding of animal evolution. Recent efforts by Song et al. have utilized phylogenetics and a multispecies coalescent model to refine placental mammal phylogeny [7]. The resultant updated evolutionary relationship within mammalian species with specific focus on the eutheria, or true placental mammals, is reproduced in Figure 1.2. It has been claimed that there is considerable adaptive pressure for the development or retention of the haemochorial chorioallantoic structure of placentas [8]. Wildman and colleagues, based on phylogenetic and statistical analysis of molecular and morphologic data, claim that the earliest common therian ancestor may have possessed a haemochorial placental interface with discoid shape and labyrinthine materno-fetal interdigitaion [9]. It has been further argued, again using phylogenetics, that highly invasive haemachorial placentation, as found in humans, is, at least, a very early evolutionary adaptation found in the most ancient ancestors of placental mammals [10]. There is no clear picture of what the placental type of the last common ancestor to therian mammals may have been. Difficulties arising from the lack of agreement with regards to the rooting of mammalian trees, which would inform decisions in this area, affect the determination of this phenotype [11, 12]. While it was held that epitheliochorial placentation was an ancient state based on its presence in what were formerly considered ‘primitive’ animals, consensus appears to have shifted towards it being a derived state that has evolved once in the the common ancestor of pangolins, horses, even-toed ungulates, and whales; as well as once in the primate lineage [11] [12] [13] [14].
1.1.3 The Trophoblast

The process of implantation is a dynamic and complex process that is mediated by a reciprocal sequence of changes in both the embryo and the maternal uterine epithelial surfaces that allow for intial weak interaction through the uterine glycocalyx followed by a subsequent shift to firmer interactions \[15\]. The mechanisms that underpin this are still poorly understood but initial interactions are believed to be dependant on mucins, a family of glyco-proteins in the apical glycocalyx \[16\]. The fundamental importance of these processes is underlined by the fact that errors in them can have adverse effects that perpetuate throughout pregnancy Figure1.3. Trophoderm is the first cell type to be differentiated from the developing embryo and it plays critical roles in implantation and the development of the placenta \[17\]. Once the embryo has been anchored to the cell wall, the development of the extra embryonic cell lineages from the trophoderm begins, an important step that leads to the establishment of the materno-fetal interface \[18\]. The trophoblast is an epithelial-like cell type, derived from the trophoderm, that mediates much of the angiogenic and immune modulations of the maternal physiology that are necessary for the generation of the placenta \[19, 20\]. The outline of early human embryogenesis, illustrating when the trophoblast lineage is determined, as well as days for hatching and implantation, is illustrated in Figure1.4A.

In humans the trophoblast has three distinct types that are generated during placentation. The first layer is the villous cytotrophoblast (vCTB) which is considered the progenitor of the other types. These are located within the villous structures of the placenta. It forms a monolayer of cells separated from the fetal stroma by a basement membrane. The vCTB then undergo differentiation into two lineages represented by the fusion lineage or invasive lineages. The fusion route yields the syncytiotrophoblast (STB). This is a multi-nucleated cell layer that covers the villous structures of the placenta. It is the main site of physiological exchange between the maternal blood supply and the fetal component of the placenta. The invasive lineages forms the extra-villous trophoblast (EVT). These are highly invasive trophoblasts that form the anchoring structures of the anchoring villi, attaching the villi to the maternal decidua. Invasion of the maternal decidua and spiral arteries gives rise to interstitial and endovascular trophoblasts. These initially block the
maternal spiral arteries until weeks 10-12 when bloodflow into the interstitial space begins [22]. Oxygen tension regulates trophoblast proliferation and invasion into the spiral arteries, the literature surrounding this is reviewed in [18]. In contrast to the previous hypothesis that endovascular trophoblasts migrate against flow, it has recently been demonstrated that high shear cause trophoblast migration in the direction of flow [23]. It is argued that the low shear induced by arterial plugging in the early stages of placentation generates a low shear favourable to endovascular migration.

Endovascular and interstitial trophoblasts invade as deep as the maternal myometrium. A recent review of Wnt signalling in trophoblasts highlighted the important role it plays in the formation of the invasive EVT phenotype with significant nuclear accumulation of β-catenin associated with invasive EVT development [24]. Interestingly, it has also been recently demonstrated that TGF-β mediated inhibition of trophoblast invasive capabilities is mechanistically mediated by Snail down-regulation of VE-Cadherin, which is a known interacting partner of β-catenin [25]. In addition, further characterisation of the EVT phenotype has shown that A Disintegrin and Metalloproteinase 12 (ADAM12) is found in anchoring as well as highly invasive decidual EVT and was demonstrated to play a critical role in their invasive phenotype [26]. Figure1.4(B) shows the process of the invasion of the maternal spiral arteries in early placentation.

As a result of obvious ethical constraints, research in this area of human biology is limited to what progress can be made in human embryos and tissue explants in vitro and extrapolations that can be made from model organisms such as the mouse [27, 28]. A comparative systems biology approach investigating protein and mRNA expression data between microdissected vascular exchange regions of both human and mouse term placentas found that of 7000 ortholog genes detected, over 70% were co-expressed in both species [29]. In addition, more than 80% of genes known to cause placental pathologies in the mouse are co-expressed in the human [29]. Thus while there are anatomical and developmental differences between human and mouse placenta, the usefulness of the mouse as a model system for studying early placental development is significant. Progress in this area, while elucidating fundamental areas of biological interest, also has clinical applications. Developments could assist in vitro fertilization technologies as well as the
development of human embryonic and pluripotent stem cells for regenerative therapies.

A key driver which underpins many of the physiological differences observed between placental forms of different species, and even within similar placental forms between very closely related species, such as the rat and the mouse, is the degree and pattern of trophoblast invasion of maternal tissues [30]. Humans, chimpanzees and gorillas possess highly invasive trophoblasts that travel deep into the maternal decidua reaching as far as the myometrium [31, 32, 33]. Failure of this phenotype leads to poor maternal arterial remodelling and results in pre-eclampsia. This disease, which affects almost 4% of pregnancies, was formerly believed to be specific to humans [34]. However, Anthony Carter points out that evidence has been accumulating to suggest that it is a trait shared in most higher primates with this type of invasive placentation [31, 35, 36, 37]. In contrast, murine implantation and trophoblast invasion are far more restricted. The depth of invasion of the trophoblast in humans and apes has been argued to be necessary to accommodate the larger oxygen requirements of the greater brain size found in these species however it has been pointed out that mammals like the dolphin show nearly as large brain development but possess epitheliochorial placentation [38]. It has been suggested that the evolution of the invasive haemachorial phenotype has been driven by maternal-fetal conflict [38, 39].

1.1.4 Maternal-fetal cross-talk and immune modulation in pregnancy

Transcriptomic analysis has revealed that there is expression of reciprocal ligands and receptors between trophectoderm and the endometrium during the implantation window [40]. Following implantation, the trophoblasts are the sole semi-allogenic cell types generating an immune response from the mother as they represent the portion of the fetal component of the placenta in contact with the maternal tissues. It is long known that there is significant leukocyte invasion of the decidua during placentation and it has been demonstrated that there is an enrichment of decidual leukocytes at the trophoblast invasion front [41]. The persistence of the trophoblasts in this context is interesting and points to factors inherent in the trophoblast that suppress or modify the functions of the maternal immune system. Studies on horse trophoblast have contributed greatly to our understanding of the maternal immune response to the trophoblast [42]. Analysis of
lymphocyte subpopulations from peripheral blood and the trophoblastic structures of the
horse, the endometrial cups, show a T regulatory cell (T_{reg} cell) bias in the cup lymphocyte
populations [43]. Furthermore, ectopically transplanted chorionic girdle cells were found
to survive for a time period similar to that of the endometrial cups in the mare [44].

Haemochorial placentation presents a notably complex situation for maternal immunostasis as the trophoblasts are in direct contact with the maternal immune system, even more so in situations of deep trophoblast invasion. A review of the literature surrounding the potential immunological underpinnings of pregnancy disorders in species which evolved deep trophoblast invasion argues that an interesting parallel exists in species with deep trophoblast invasion and the evolution of the major histo-compatability complex (MHC) class I type antigens and their receptors [31]. In species where deep invasion is seen, a polymorphic pattern has evolved in the MHC class I type antigens. Evolution of this polymorphic trait may have been required for deep invasion but has the consequence that certain genotypes for maternal killer immunoglobulin-like receptors (KIRs) and fetal genotype for MHC class I antigens may be incompatible and result in increased risk of pregnancy disorders such as preeclampsia. Genetic studies have indicated that KIR expressed on dNK cells can contribute to preeclampsia [45]. This is mechanistically supported by the fact that interactions between invading trophoblasts and the maternal decidual natural killer cells are a key determinant of the invasive capacity of the trophoblast and alterations in the amount of dNK cells can contribute to preeclampsia [46, 45].

As well as local immune suppression, pregnancy is characterised by a general type 2 helper T cell (T_{h2}) type polarisation of the systemic immune system which is often associated with amelioration of autoimmune conditions such as multiple sclerosis [47]. It has been argued for a modification of the simple Th1/Th2 paradigm to a Th1/Th2/Th17 and regulatory T cell paradigm for pregnancy that is more reflective of the multi-polarity of the T cell responses found in biology [48]. Fetal HLA-C competent T cells are present in the decidua, however, while there is activation of these cells, there is no detrimental effect on pregnancy [49].

Thus, while an adaptive immune response is possible, it is not realised in the decidual environment. The mechanism for this remains elusive but it is believed T_{reg} cells could...
play an important role.

Not only do factors inherent to the trophoblast alter maternal immune function, conversely, decidual factors can also alter trophoblast function. It has been shown that decidual secreted factors alter the composition of invasive trophoblast membrane and secreted proteins \textit{in vitro} \cite{50}. Furthermore, plasma from women with preeclampsia has been shown to inhibit invasion of trophoblast cells \cite{51}. In addition, evidence is accumulating that maternal circulating blood cells make positive contributions to implantation and vascular remodelling during pregnancy in co-operation with the endocrine system \cite{52}. Thus there is broad maternal-fetal cross talk during pregnancy.

\subsection{1.1.5 Placentally Expressed Genes}

One means of trying to elucidate placental evolution is to examine the evolution and function of placenta specific genes. Rawn \textit{et al.} conclude in a lengthy review of the literature concerning the evolution and function of placenta specific genes, that a major limitation of examining placenta-specific gene product function is that many are species-specific, making comparative studies difficult \cite{53}. The presence of convergent evolution in diverse gene families further confounds conclusions on the evolution of these genes. Moreover, subtle differences in promoter sequences can mean while genes are conserved between species, they may only be placentally expressed in a subset of species. The authors finally observe that the number of placenta-specific genes is modest and cannot account for the diversity of placental form and function among mammalian species \cite{53}.

Nonetheless, an interesting observation regarding placentally expressed genes is that some are members of multi-gene families \cite{54,55}. Gene duplication and conversion are known to be critical to the evolution of gene families \cite{56,57}. Moreover, it is known that some of the most rapidly evolving genes have roles in immune responses and reproductive processes \cite{58}. Furthermore, antagonism between maternal and fetal genes, in the placenta, that govern maternal resource allocation and investment in pregnancy is hypothesised to represent an environment of evolutionary conflict and, as such, a selective pressure potentially driving evolution of these genes \cite{39,59,60}. The \textit{CEA} gene family, a sub
group of the Ig superfamily, have roles in both reproduction and immune function. Of particular interest to scientists interested in placental evolution are the PSGs which together with the CEA-related cell adhesion molecules (CEACAMs) make up the CEA family. To date, these are found only in haemachorial placental mammals (rodents, primates and bats) with one recently observed exception being the horse [58]. Understanding the function and evolution of PSGs as well as their role in the haemochorial phenotype and establishment/maintenance of pregnancy may provide insight into the evolution of placentas and also the selective forces that drive this.

1.2 The CEA family: CEACAMS and PSGs

1.2.1 The Carcinoembryonic Antigen (CEA) family

CEA was identified in the 1960s as a tumour associated protein in human colorectal carcinoma [61]. More CEA-like proteins were discovered and the cloning of CEA cDNA sequence allowed the identification of paralogues which led to the designation of a CEA family of proteins [62, 63, 64, 65, 66]. Members of the family are encoded by a specific gene structure which is a determining factor in their assignment to the family [65, 67, 68]. CEA proteins are members of the Ig superfamily. In humans, the CEA cluster of genes is encoded on chromosome 19q13.1-19q13.2 [69, 70, 71]. The first exon encodes the initial residues of a 34/35 amino acid signal peptide that targets the molecules to the secretory pathway. The second exon encodes the remainder of this sequence and also the N-terminal IgV-type domain common to all mature CEA family members. The remaining exons encode different numbers of IgC-type domains. The CEA family is composed of two subfamilies, the widely expressed CEACAMs, and the PSGs which show placenta-specific expression. The nomenclature associated with the CEA family has reviewed [72]; the original CEA is now CEACAM5; nonspecific cross-reacting antigen (NCA) or CD66c is now CEACAM6 and biliary glycoprotein (Bgp), later classified as CD66a is now CEACAM1.

One of the most striking differences between the CEACAMs and the PSGs apart from their expression patterns, is the lack of a C-terminal membrane targeting component in
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1.2 The CEA family: CEACAMS and PSGs

In CEACAMS a hydrophobic transmembrane sequence or glycophosphatidylinositol (GPI) anchoring leads them to attach to the cell surface as opposed to the PSGs which are all secreted. In CEA members, the evolution of GPI anchorage from transmembrane anchored ancestors has been argued to have been a very simple adaptation to give radically different molecular function to Ig superfamily members during evolution \[73\]. This mode of anchorage is found only in primate CEA family members \[58\]. All CEA members are highly glycosylated, a modification that can account for between 30-60% of their mass, with the PSG members showing slightly less glycosylation than the CEACAMs \[74\]. Figure 1.5 shows the domain architecture and predicted glycosylation of the human CEA family subgroups.

Resolution of the crystal structure of murine CEACAM1 IgV and IgC type domains as well as bioinformatic analysis of the CEA family members demonstrated that, structurally, the arrangement of the beta sheets that make up the Ig domains of the proteins is expected to be very conserved based on protein sequence similarity between members of the CEA family \[75\]. This can be seen from an alignment of the N domain protein sequences of the human CEA family members \[75\]. Two interesting characteristics of CEA N domains distinguish them from similar IgV type domains in other immunoglobulins, that is the presence of a unique glycan shielded hydropobic patch and the orientation of the CC' loop of the protein, which, along with the FG loop, are important for homo and heterophilic interactions as well as virus attachment \[76\]. Within CEA family members N/N1 domains, most of the sequence variation occurs on the C'C'CFG faces of these molecules \[75\]. These differences reflect the fact that the C'C'CFG faces of Ig superfamily proteins are frequently used for cell surface recognition. Variations in this face likely confers unique binding capabilities. Furthermore, this face of the CEA family members are frequently subverted as pathogen cell surface receptors reflecting a source of potential selective pressure for alterations to overcome this \[77\] \[78\] \[79\] \[80\] \[81\] \[72\] \[58\]. In addition, the expression of CEACAM and PSG in placental tissues means they could also be subject to selective pressure resulting from maternal-fetal conflict \[58\]. The CEA family has recently been shown to be undergoing positive selection based on their pattern of sequence divergence \[82\].


**Figure 1.2: Phylogenetics of eutherian mammals**

The phylogenetic relationship between the eutherian mammals was further resolved by Song and colleagues and their data is reproduced here [7]. (Copyright National Academy of Sciences)
Figure 1.3: Charting adverse outcomes from errors in processes of early pregnancy
Defective receptivity, implantation, and/or decidualization can lead to infertility. Delayed implantation can lead to incorrect embryo placement and implantation, resulting in placenta previa, ectopic placentation (placenta accreta) or placental insufficiency resulting in IUGR and/or preeclampsia. Implantation beyond the normal window can also give rise to spontaneous abortion, miscarriage and recurrent pregnancy loss, leading to infertility. Premature decidual senescence can lead to preterm birth and fetal death, whereas shallow trophoblast invasion into maternal decidua and/or blood vessels can lead to preeclampsia. Modified from [21].
Figure 1.4: Human embryogenesis and trophoblast development

(A) Day 1 to day 9 in human embryo development showing first stage of differentiation occurring around day 4 giving rise to the trophoblast progenitor cells and inner cell mass. The trophoblast cells subsequently mediate implantation around day 7 before ultimately giving rise to the placenta (http://en.wikipedia.org/wiki/User:Zephyris). (B) Trophoblast differentiation gives rise to syncitiotrophoblast as well as the extravillous trophoblast lineages which invade and modify the maternal spiral arteries through both interstitial and endovascular routes [18].

Pregnancy Specific Glycoprotein Function, Conservation and Receptor Investigation

Ronan T. O’Riordan
Figure 1.5: Domain architecture of human CEA family
Schematics showing domain architecture and predicted N-glycosylation of human CEACAMS (A) and PSGs (B). IgV-type domains are indicated in red and IgC-type domains (A and B subsets) are indicated in blue. Black stick and ball represents predicted N-glycosylation sites. Modified from CEA homepage (http://www.carcinoembryonic-antigen.de)
1.2 The CEA family: CEACAMS and PSGs

1.2.2 CEACAM Expression and Function

CEA-related cell adhesion molecules (CEACAMs) are cell adhesion molecules mediating homo and heterophilic interactions, with expression in several cell types \[83\]. Several CEACAMs contain immunoreceptor tyrosine-based signalling motifs (ITIMs) which are found in an array of inhibitory molecules known to negatively regulate cell activation \[84\]. CEACAM1, the most widely expressed CEACAM is found in epithelial, endothelial and a number of immune cells including B cells, T cells, NK cells, dendritic cells, macrophages and granulocytes \[85\] \[86\] \[83\]. Roles in normal and cancerous tissue development, angiogenesis, immune function and host pathogen interactions have been ascribed to CEACAMS \[87\] \[88\] \[89\] \[83\]. There are 12 human and 15 mouse proteins (http://www.carcinoembryonic-antigen.de). CEACAM expression is restricted to mammals, although evidence of their expression has recently been claimed in fish \[90\] \[58\]. Five human CEACAM genes show orthologs in other therian mammals (CEACAM1, CEACAM16, CEACAM18, CEACAM19, CEACAM20) and are considered the ancient CEACAM gene family present in the last common therian ancestor \[91\] \[58\]. As well as there being 12 human family members, CEACAM1 has 12 splice variants. Two variants, designated L and S, alter the cytoplasmic signalling portion of CEACAM1 by inclusion (L) or exclusion (S) of the 7th exon. This generates CEACAM1 with or without a cytoplasmic ITIM signalling domain. The mechanism underpinning this process in normal tissues, which is deregulated in cancer tissues, was recently found to be dependant on cis acting elements within the flanking introns and exons as well as expression levels of specific rRNPs \[92\] \[93\] \[94\] \[95\].

The relevance of CEACAMs to cancer is reflected in the discovery of the original CEA molecule as a tumour associated protein in 1965 \[61\]. Their potential roles in this heterogeneous disease have been studied extensively since. A recent review covered, in depth, their importance in this field with specific focus on the more widely researched CEACAM1, CEACAM5 and CEACAM6 \[96\]. CEACAMs are used widely as biomarkers with CEACAM5 being utilised to detect colorectal carcinomas and positive lymph node tissues. CEACAM6 expression has been shown to be higher than CEACAM5 in several cancers and it may now represent the most specific marker from the family for a number of more agressive types \[97\] \[98\] \[96\]. CEACAM1, as already outlined, has the widest normal tissue distribution.
of the CEACAMs and deregulation of its splice pattern occurs in transformed tissues \[86,93]. Recent investigations have identified novel splice variants of \textit{CEACAM5} and claim they could improve the prognostic usefulness of this protein marker in gastrointestinal cancers \[99]. Studies on all three of these CEACAMs in A549 lung cancer cells again revealed that forced deregulation of the CEACAM1-L as being functionally important for undifferentiated cell growth and malignant transformation, though the L and S ratios were not observed to be altered endogenously between non-tumour and tumour subpopulations \[100]. It was also demonstrated that alterations in the expression of other CEACAMs may impact on normal CEACAM1 function and furthermore that the secretion of soluble CEACAM5/6 from these cells was also identified, representing a potential source of increased serum levels of these proteins found in lung cancer patients.

More recently, soluble CEACAM5 has been shown to increase proangiogenic endothelial cell behaviour \textit{in vitro} as well as tumour microvascularisation \textit{in vivo} with increased serum levels being correlated with increased tumour microvascularisation in patients \[101]. In addition it has been shown that secreted microvesicles contain CEACAMs and their composition differs between normal and tumour tissue \[102]. These CEACAM1-positive microvesicles were shown to increase CD3 and CD3/CD28-induced T-cell proliferation. CEACAM6 also has functional implications for cancer progression. In myeloma, cancer cell expressed CEACAM6 has been demonstrated recently to neutralise competent cytotoxic T cells \[103]. Moreover it has been demonstrated that silencing of CEACAM6 expression in cancer cells decreases invasiveness and over-expression increases invasiveness in colorectal cancer cells \[98]. Other CEACAMs also have prognostic potential with CEACAM19 recently correlated with poor breast cancer prognosis \[104]. These data highlight the potential for CEACAMs to provide increased prognostic power for stratification of patient cancer risk as well as outlining mechanisms by which acquisition of altered CEACAM expression, both cellular and soluble, can contribute to the development of metastatic disease.

As well as their role in cancer through both disruption of normal tissue homeostasis and immune evasion, CEACAMs play an important role in the normal function of the immune system as well as being exploited by pathogenic bacteria and viruses as cell surface receptors. Pathogenic bacteria exploit human CEACAMs for adhesion to and entry into
their host cells. This generates cell specific targets due to the tissue specific expression of CEACAMs. *Neisseria gonorrhoeae* exploit epithelially expressed CEACAMs; CEACAM1, CEACAM5 and CEACAM6 to colonise mucosal tissues. In contrast, host granulocyte CEACAM3 expression allows for the recognition of *N. gonorrhoeae* by the innate immune system [105, 106]. This binding is through Neisserial colony opacity (Opa) proteins, the type of which can determine whether a patient will develop disseminated disease or not [107, 108]. There are two mechanisms of CEACAM mediated uptake of *N. gonorrhoeae*, lipid raft and CEACAM3 Src dependent [109]. No homologue exists for CEACAM3 in other species and this has led some to argue that it has evolved to allow innate immune control of human specific pathogens [110].

### 1.2.3 Pregnancy Specific Glycoproteins

PSGs are the most abundant fetal proteins in human maternal blood at term in pregnancy [111]. They are produced by cells of the trophoblast lineage; syncytiotrophoblast in higher primates and spongiotrophoblast or giant cells in rodents [112, 113, 68]. The localisation of the expression of PSGs to the placenta, taken with evidence of the correlation of low serum PSG levels with poor pregnancy outcomes, specifically diseases characterised by placental insufficiency, indicates that they may play a fundamental role in the formation, and maintenance, of the materno-fetal unit [112, 114, 115, 116, 117, 118, 119, 120, 121, 122].

While PSGs were believed to be found solely in haemochorial placental mammals of the primate and rodent lineages, there is bioinformatic and experimental evidence of candidate PSG-like genes in the bat and the horse [58]. Two related expressed sequence tags (ESTs) from equine trophoblast complementary DNA (cDNA) libraries corresponding to secreted equine CEACAMs lend further evidence to the existence of horse PSG-like genes [58]. PSGs, like many placental hormones, are found in multi-gene families in all species in which they are found [54, 58].

The domain architecture of the PSGs are very similar to the CEACAMS being composed of one N-terminal IgV type domain (N) followed by a number of IgC type domains (A and B). However there is a clear pattern of divergence between primate (Fig. 1.6A) and rodent (Fig. 1.6B) PSG domain architecture with expansion of the IgV type domains within the rodents.
Rodent PSGs are composed of a series of three or more N domains and a single C-terminal A domain. There is however a high degree of similarity in the amino acid composition of the human N and rodent N1 domains. This conservation of general structure (N terminal IgV type domain) and sequence composition as well the conserved sites of expression imply a conserved function between primate and rodent PSGs. In addition, the conservation of RGD and RGD-like motifs on the solvent exposed F-G loop in the human and rodent PSGs supports a role for these motifs in PSG function. Conversely, the expansion and diversification of the rodent family may also imply novel functions for rodent Psgs.
1. Introduction

1.2 The CEA family: CEACAMS and PSGs

A  Human PSG

B  Mouse PSG

Figure 1.6: Human and mouse PSG domain architecture
(A) Human PSG domain architecture showing conservation of general structure among family members. (B) Mouse domain architecture showing expansion and diversification of N domains within the family.
1.2.4 PSG Expression and Regulation

In contrast to the widely expressed CEACAMS, PSG expression is normally restricted to the placenta. There is limited evidence of expression of PSG outside the placenta. In the mouse Psg16 has been reported in the brain [124]. One study examined the expression of PSG in breast cancer and correlated it with poor prognosis [125]. There is one report of expression of PSG in haematopoetic cells [126]. Evidence of aberrant expression of PSG9 in colorectal cancers has also been found [127]. Psg18 has been found expressed in the murine intestine, demonstrated by in-situ hybridization and with anti-Psg18 polyclonal antibody against a 16 amino acid carboxy-terminal sequence from Psg18 [128]. The expression was localised to follicular associated epithelium overlaying Peyers patches where there are high levels of antigen presenting cells present. Furthermore, recent work has demonstrated that murine Psg can be detected throughout the gastro-intestinal (GI) tract (Williams, J., PhD thesis). Moreover, unpublished work from our group has shown that PSG staining can be detected in the human oesophagus and lower GI tract, sites where mucosal immune regulation is critical for tissue homeostasis. The presence of PSG at sites of immune contact in the placenta and the mucosal tissue of the GI tract indicate that PSGs may have immunoregulatory functions that are common to both these sites in normal biology.

Human PSGs are tightly linked on the long arm of chromosome 19 and their expression is co-ordinately regulated [129]. PSG can be detected secreted from cultured human embryos as early as 3-4 days after fertilisation [130]. The expression of PSG is upregulated upon cytotrophoblast (CTB) syncytialisation, as can be seen from the detection of PSG1, PSG3, PSG5, PSG7 and PSG9 transcripts in differentiated Jeg-3 human choriocarcinoma cells and CTBs but not from undifferentiated precursors [131]. In agreement with the believed co-ordinated regulation of the human PSGs, the promoter sequences show strong homology between family members [132]. However it has been argued that subtle differences between promoter sequences could allow for differential expression [133]. Some of the factors that have been functionally implicated in the regulation of the expression of human and mouse PSG are summarised in Table 1.1. This clearly indicates that most of the work concerning PSG regulation has been carried out on human PSG, most notably, PSG5.
Human PSGs do not possess conventional promoters, but their minimal promoter regions have been defined as spanning -172 to -34 bp \cite{132, 133, 134}. An SP1 binding site mapped within this region and SP1 is known to co-express with PSG5 in the placental villous, notably in the STB layer \cite{135}. All human PSGs contain a putative Retinoic Acid Response Element (RARE) and Lopez-Diaz et al. confirmed the functionality of this in response to 9-cis retinoic acid treatment as well as by mutation of PSG5 reporter constructs and analysis of RXRα binding \cite{136}. Two Kruppel-like factors (KLFs) have been implicated in PSG regulation; KLF4 and SP1 act synergistically to increase PSG5 expression \cite{137}; KLF6 was also implicated in the regulation of PSG3 and PSG5 \cite{138}. The demonstration of the response of PSG gene expression to KLFs is interesting in the context of the recent reports of PSG expression in the GI tract both in normal and disease settings as KLFs play a critical role in development, regulation and carcinogenesis in these tissues \cite{139}. There is also evidence to suggest that PSG genes are regulated at the higher order chromatin structure level \cite{140}.

<table>
<thead>
<tr>
<th>Regulator</th>
<th>PSG</th>
<th>Responsive Cell Lines</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPBP</td>
<td>PSG5</td>
<td>Jeg3</td>
<td>\cite{141}</td>
</tr>
<tr>
<td>KLF4</td>
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<tr>
<td>KLF6</td>
<td>PSG3, PSG5</td>
<td>Jeg3</td>
<td>\cite{142, 138}</td>
</tr>
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<td>SP1</td>
<td>PSG5</td>
<td>Jeg3, HP-A1</td>
<td>\cite{133, 135, 137}</td>
</tr>
<tr>
<td>RXR</td>
<td>PSG5</td>
<td>Jeg3</td>
<td>\cite{136}</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XBPI, IRE1a</td>
<td>Psg18, Psg28</td>
<td>SM-10, MEF</td>
<td>\cite{143}</td>
</tr>
</tbody>
</table>

### 1.2.5 PSG Function

The importance of PSGs to successful pregnancy is underscored in the already outlined correlation between low PSG and diseases of placental insufficiency. In addition, it is recognised that dysfunction of the syncytiotrophoblast, the site where PSGs are synthesised, and changes in the soluble factors generated there, such as soluble endoglin (sEng), as well as the anti-fibrinolytic factor plasminogen activator inhibitor-1 (PAI-1), contributes to preclampsia \cite{144}. Furthermore, it has been observed that the application of anti-PSG antibodies or vaccination with PSG induces abortion in mice and monkeys, respectively, and reduces the fertility of non-pregnant monkeys \cite{145, 146}. However, these studies are
relatively old and there are uncertainties surrounding the quality/specificity of antibodies utilised. While the exact function of PSGs remain uncertain, a body of literature has accumulated over the last number of decades examining immunomodulatory, angiogenic and anti-platelet functions of these hormones.

Thirteen years of published work has shown that PSGs are immunomodulatory and pro-angiogenic hormones that can directly induce various cytokines from several cell types in a cross-species reactive manner [147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157]. Table 1.2 outlines the published cytokine responses reported for individual PSGs and the responsive cell types. The identification of these cytokines as induced by PSGs and the particular cell types responsive to PSG treatment represent critical steps in the characterisation of the fundamental role that PSGs play in the establishment of feto-maternal unit and its maintenance throughout pregnancy. Many of these cytokines have well documented roles in the promotion of an immune environment that is accommodating of the semi-allogenic fetus as well as angiogenic functions.

**Table 1.2:** Published cytokine responses for PSGs

<table>
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<th>Cytokines</th>
<th>Responsive Cell Types</th>
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</thead>
<tbody>
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<td>monocytes/macrophages, dendritic, endothelial, trophoblastic</td>
</tr>
<tr>
<td>6</td>
<td>IL-6 [149], IL-10 [149], TGF-β,</td>
<td>monocytes/macrophages</td>
</tr>
<tr>
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<td>IL-10 [148]</td>
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</tr>
<tr>
<td>19</td>
<td>TGF-β,</td>
<td>macrophages</td>
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<td>dendritic, natural killer</td>
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<tr>
<td>23</td>
<td>TGF-β, VEGF-A</td>
<td>monocytes/macrophages, dendritic, endothelial, trophoblastic</td>
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</tbody>
</table>

IL-10 expression is up-regulated in the first and second trimesters of pregnancy and lower in the third concurrent with a gestational age based function for this cytokine, with high levels required during early to mid gestation and decreasing levels potentially contributing to parturition [159]. There is also an increase in IL-10 release from uterine lymphocytes and NK cells compared to peripheral blood mononuclear cells (PBMCs) in response to LPS [160, 161]. Furthermore IL-10 is lower in placental tissue of women with spontaneous abortion compared to elective controls [162]. IL-10’s role as a vascular protective agent and the contribution this could make to pregnancy and prevention of preeclamptic symptoms...
is also recently coming to light as reviewed by [163]. Thus, IL-10 has multifaceted roles in pregnancy [164].

As can be seen from Table 1.2, TGF-β1 is the most widely documented PSG induced cytokine. It has well documented roles in the regulation of inflammation and angiogenesis [165]. TGF-β1 is an interesting cytokine as it is quite extensively post-transcriptionally regulated. It is a member of the Transforming Growth Factor β1 superfamily. TGFβ1 is synthesised as a homodimeric proprotein. This is processed intracellularly cleaving the propetide from the cytokine, however they remain associated and the propeptide and cytokine are secreted together as a complex as is the case for all TGFβ proteins [166]. This Small Latency Complex (SLC) containing the cytokine and propeptide or Latency Associated Protein (LAP) requires activation by proteolytic cleavage before the cytokine can interact with its receptors [167]. An additional layer of regulation is found in the association between the SLC and Latent TGF-β1 Binding Proteins (LTBP) which regulate the availability of the SLC [168]. This triprotein complex is referred to as the Large Latency Complex (LLC). Almost all publications examining PSG1 induction of TGF-β1 examined total secreted TGF-β1 by ELISA following acid activation of samples which cleaves the cytokine from the LAP.

Further work using transient in-vivo expression of PSGs as well as co-culture systems examined the immunomodulatory effect of PSG1 further. It was demonstrated that untagged PSG1 produced from a transient vaccinia based expression sytem in HeLa or J774 cells could up-regulate arginase expression in primary human monocytes and human and mouse monocyte cell lines as well as suppressing LPS induced Nitric Oxide (NO) production [147]. This study also demonstrated that PSG1 altered accessor cell dependant activity of T-cells both in vivo and in vitro. The observations were further characterised by Motran et al. where the authors demonstrated that ovalbumin challenged splenic monocytes from vaccinia PSG1 mice showed a T_h2 polarised pattern of cytokine response (high IL-4, IL-5 and IL-10, and low interferon (IFN)γ and IL-2 production) compared to splenic monocytes from control mice [152]. It was demonstrated that this response could be mediated by IL-10, as IL-10 blocking antibodies rescued the induction of IL-2 and IFN-γ in some situations of stimulation. IL-10 block also rescued the suppression of splenic monocyte proliferation observed in monocytes isolated from vacPSG1 mice. Furthermore,
this study confirmed the alternative activation state of monocytes and macrophages in vacPSG1 mice by demonstrating a bias towards arginine metabolism in LPS challenged monocytes and macrophages as well as increased levels of TGF-β release from monocytes and macrophages from vacPSG1 mice. An additive effect on IL-10 release in LPS-stimulated monocytes and macrophages from vacPSG1 mice, versus controls. It was shown that macrophages from vacPSG1 mice strongly inhibited naive T-cell proliferation compared to macrophages from control mice as well as inhibiting their ability to secrete IL-2 and IFN-γ in response to Concanavalin A.

The Th2 polarising effect of PSG1 was further investigated by examining the effect of PSG1 on dendritic cells (DCs) which are recognised as highly specialised antigen presenting cells (APCs) playing a crucial rule in the regulation of innate and adaptive immunity. [157] demonstrated that PSG1 treated immature DCs showed lower cell surface expression of co-stimulatory molecules, such as cluster of differentiation (CD)40, CD80, and MHC class II, compared to control DCs when stimulated with toll-like receptor (TLR)-4 and -9 ligands and CD40 ligation. In addition, it was found that PSG1 treatment had an additive effect on CpG- and CD40-stimulated upregulation of programmed death-ligand 1 (PD-L1). The authors also showed that PSG1-treated DCs showed a stimulus-dependent capability to modulate the profile of secreted cytokines, thus showing that PSG1 treatment appears to generate a unique phenotype in DCs. The effect of this Th2 polarising of DCs on the adaptive immune response was examined in an elegant in vivo experiment where mice that had received labelled ovalbumin-specific KJ1-26+ CD4+ T cells were injected intraperitoneally with untreated, PSG1 treated, or LPS treated DCs. Five days later, freshly isolated small mononuclear cells were cultured with ovalbumin peptide for 72 hrs and cytokine release evaluated by ELISA. IL-4,-5,-10 and -17 release was increased in small mononuclear cells isolated from mice that were immunised with PSG1 treated DCs. Having demonstrated the ability of PSGs to modulate the adaptive immune response the authors then demonstrated that this does not compromise the ability of the immune system to defend against pathogens using Listeria monocytogenes infection with and without PSG1. The authors also demonstrated the ability of PSG1 to increase the population of T<sub>reg</sub> cells in this environment. This paper provides clear evidence of the ability of PSG1 to modulate the
innate as well as the adaptive immune systems generating a unique immune context, that is replicated in pregnancy, without compromising anti-microbial defence or tissue repair. These functions in the context of pregnancy were reviewed in [169].

As well as their immunological functions PSGs are also reported to possess angiogenic properties. The induction of TGF-β1 and VEGF-A in response to both human and murine PSGs implies angiogenic functions. It has been further demonstrated that PSG1 induces tube formation by endothelial cells and thus it has been claimed that PSGs have a role in placental vascular morphogenesis [158,170,155]. Moreover, Psg22 was also shown to induce edothelial tube formation in the presence or absence of VEGF-A [156].

To date a number of receptors/binding partners have been identified for PSGs. The tetraspanin, CD9, was shown to bind PSG17 [150]. PSG17 binding was subsequently located to the EC-2 extracellular loop of CD9, a site important for sperm-egg fusion [151]. The interaction between CD9 and a truncated PSG17N1 domain protein was shown to be necessary for PSG17N1 induction of anti-inflammatory cytokines from macrophages [153]. It was also demonstrated for the the first time that human and mouse PSGs could have different receptors, as absence of CD9 had no effect on PSG1 cytokine induction [153]. [154] further showed that a PSG19N1 protein bound to CD9 and that bacterially produced PSG17 could not bind to CD9, implying a fundamental role for protein glycosylation in this interaction. In a critical finding, [171] showed that PSG23N did not bind CD9. In addition, it was shown that the amino acid sequence of PSG17 required for CD9 binding is located at the region of highest divergence between murine PSGs. Furthermore it was shown that murine PSGs bound to cell surface proteoglycans. Importantly, this demonstrated that murine PSGs could have evolved different receptors potentially mediating novel divergent functions. It further showed that at least one PSG could bind more than one molecule at the cell surface.

Glycans, oligo and polysaccharides are found almost everywhere in the body and possess a structural diversity and regulatory capacity beyond that of any other bio-molecule [172]. Because they play a role in cellular interactions they are believed to have evolved these diverse properties in response to evolutionary conflict [172]. Interestingly, a theory called The Glyco-Evasion Hypothesis has been proposed to explain how microbial products can manipulate host glycosylation to the benefit of the pathogen, which could represent a
1. INTRODUCTION

1.2 The CEA family: CEACAMS and PSGs

selective force for rapid evolution of cell surface glycans [173]. Proteoglycans consist of a protein core and covalently attached glycosaminoglycan side chains [174].

The binding of PSGs to cell surface proteoglycans was first demonstrated for PSG1 when glycosaminoglycan side chains were found to be necessary for PSG1-induced endothelial tubulogenesis [170]. It was also demonstrated that transfection with syndecans1-4 or glypican-1 rescued PSG1 binding to cells lacking heparan and chondroitin sulphate. This binding to cell surface proteoglycans was subsequently demonstrated for PSG17N1, PSG23N1A and PSG22N1A [171, 156]. Importantly, proteoglycans often act as co-receptors or scaffold proteins for organising clustering of receptors at the cell surface with roles in adhesion, cell migration, morphogenesis and differentiation [175]. Cell surface proteoglycans could also act like chaperones for PSG mediating cell membrane binding and bio-availability, much as has been described for other ligand interactions with HSPGs such as VEGF [176]. Notably, syndecans and glypicans are known to interact with, organise and regulate integrin function [177, 178]. Integrins have been hypothesized to be targets of PSG function for more than a decade [179].

More recently it was demonstrated that PSGs could bind platelet integrin αIIbβ3 mediating a novel anti-platelet function, abrogating the interaction between platelets and fibrinogen [180]. This interaction was not dependent of the KGD tri-peptide that PSG1 shares with snake venom disintegrin barbourin. Furthermore, it was demonstrated to be mediated by multiple domains of the protein and to occur with PSG9 and PSG23, indicating a conserved function. Shanley et al. hypothesised that PSGs evolved to counteract high levels of fibrinogen (>2 mg/ml) and prevent thrombosis at the placental surface and in the maternal circulation during pregnancy in haemachorial placental mammals [180]. It has been further argued this could represent an alternative explanation for the high levels of PSG in the maternal serum during pregnancy, as opposed to the Maternal-fetal-Conflict theory [39, 59, 180].

The evidence presented here of both cell surface proteoglycan that interact with and regulate integrins, and a β3 integrin both binding human and mouse PSGs provides justification for investigating whether other integrins with roles in immune function, reproduction and angiogenesis could represent novel binding partners for PSGs. Moreover,
1.3 Integrins

The ability of cells to interact with each other, the extracellular matrix and the endothelial surfaces is mediated by membrane proteins called adhesion molecules. These are composed of selectins, cadherins, mucins, the Ig superfamily and finally the integrins. Integrins are heterodimeric cell adhesion molecules that affect a number of cellular functions [181]. They are known to have roles in normal development, metastasis, angiogenesis and immune function as well as serving as binding partners for pathogens such as *H. pylori* and Kaposi’s sarcoma-associated herpes virus [182, 183, 184, 185, 186]. The 24 known integrin heterodimers are generated by the association of 18 α and 8 β subunits which have so far have been identified in mammals [181]. The expression of integrins, while wide, is tissue specific, reflecting the specificity of the heterodimers for particular subsets of extracellular matrix proteins and cell surface ligands. This is important for normal tissue function and homeostasis. Ligand/integrin interactions activates intercellular signalling that, depending on the cell type, can be pro-survival, pro-proliferation, pro-migratory and pro-differentiation. Conversely, detachment of an integrin from its cognate ligand can induce pro-apoptotic signals [187]. Integrin signals occur in two directions, both from the cytoplasm out, 'inside-out' and vice-versa 'outside-in'. One example is the platelet integrin α<sub>IIb</sub>β<sub>3</sub> which is seen to undergo a confirmational change to an active 'open' state for fibrinogen binding upon stimulation of platelets with agonists such as thrombin receptor activating peptide (TRAP) and adenosine diphosphate[188, 189]. 'Outside-in' signals are generally a result of binding a specific ligand and generate signals instructing the cell
about the external environment, directing cellular response to the same [190]. Moreover, integrins are regulated by extracellular factors such as lipid and/or divalent cation presence [191]. Divalent cations such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ are essential for integrin binding and different cations can differentially affect integrin binding [192].

Integrins are not enzymatic molecules, however they have the ability to recruit a wide array of intercellular signalling molecules and modulatory enzymes at sites of adhesion, generating what has been referred to as the adhesome [193]. Integrin adhesions, while stable, are dynamic structures and their composition can rapidly change altering adhesive capacity and internal cell signalling [194]. The molecular mechanisms underlying the crosstalk between the microenvironment and the adhesome are poorly understood [194]. Interestingly, compelling evidence that $\beta_2$ and $\beta_3$ integrin subunits in various leukocyte cell types utilise the Immunoreceptor-Tyrosine based Activation Motif (ITAM) adaptors has recently been generated [195].

### 1.3.1 Integrins in Immunology and Haemostasis

A fundamental function of leukocytes is their ability to home to an inflammatory site through attachment to endothelium, extravasation and migration along a chemokine gradient to the site of inflammation where they carry out their immune functions [196]. The process of leukocyte recruitment is recognised as an intrinsic part of the immune response and drugs that target mediators of this represent potential therapeutic compounds. Snake venom disintegrins have been examined extensively in their ability to modulate leukocyte activity as reviewed by [197]. RGD disintegrins, Echistatin, Flavoridin and Kirstrin, have been shown to bind to and modulate T-cell function [198]. Moreover, another disintegrin, Jararhagin-C was demonstrated to increase rolling of leukocytes in vivo in a mouse model [199].

Circulating leukocytes generally keep their integrins in an inactive or ‘bent’ conformation which represents a low affinity state for their cognate ligands [190]. Chemokine or cytokine signals activate inside-out signalling on leukocytes leading to rapid activation of leukocyte integrins to a high affinity state. There are currently believed to be five steps involved in
leukocyte recruitment from the vasculature: capturing and rolling, adhesion, postadhesion strengthening, crawling and transmigration and these are reviewed in [184]. Briefly, endothelial luminal selectins mediate the capturing of leukocytes from the circulating blood. This initial interaction causes activation of leukocyte integrins through inside out signalling, thereby slowing the rolling of the attached leukocyte [200, 201]. Integrins roles in this are less well studied. In vitro studies suggest integrin α4β1 as β2 integrin blockade has no effect on monocyte rolling on IL-4 activated HUVEC cells [202]. Integrin α4β1 also appears to be important to lymphocyte rolling [203]. The physiological relevance of rolling to leukocyte function remains unclear. Adhesion and post adhesion strengthening in lymphocytes appears to be mediated by integrins α4β1 and αLβ2 whereas in monocytes it is α4β1 and broadly β2 integrin mediated. Inside out signalling activates these integrins to bind substrates such as ICAM-1 and VCAM-1 [204]. Intravascular crawling is when attached monocytes start to migrate to points of cell junctions on the endothelium. These monocytes have been labelled as ‘patrolling’ [205]. αLβ2 blockade decreased monocyte crawling in vivo however it has been suggested that the crawling of monocytes is dependant on the cell surface molecule expression with a more αMβ2 dependent crawling found on TNF-α stimulated endothelial cells [205, 206]. Recently endoglin, an RGD containing endothelial protein, was shown to be important in integrin α5β1 mediated adhesion and extravasation of leukocytes [207]. The importance of integrins to this process is evidenced in the symptoms of patients with [Leukocyte Adhesion Deficiency (LAD)] disorders. Patients suffering from LAD-I have mutations in β2-integrin that cause errors in leukocyte/endothelial interactions. Patients with this type of disorder suffer from recurrent infections due to their compromised immune response [208, 209]. Patients with LAD-III suffer an additional complication of bleeding disorders as well as recurrent infections. The reason for this is that these patients have mutations in an intercellular activator common to both β2 and β3 integrins, kindlin-3. Platelets from these patients have dramatically lower activation levels [208, 209]. As well as talin and kindlin, paxillin phosphorylation is known to regulate LFA-1 or integrin β2 activity and lymphocyte migration [210].

Integrins are known to interact with and regulate latent [TGF-β] [211, 212]. In addition,
1. Introduction

1.3 Integrins

knockout mice for integrins $\alpha V\beta 6$ and $\alpha V\beta 8$ show similar deformities to TGF-$\beta 1$ null mice confirming the functional role of these integrins in TGF-$\beta 1$ regulation [213]. Furthermore, this interaction between TGF-$\beta 1$ and integrins is believed to contribute to tumour growth and metastasis [214]. $\alpha V$ containing integrins appear to share the ability to activate latent TGF-$\beta 1$ [215, 216].

1.3.2 Integrins in Reproduction and Placentation

Integrins have widely acknowledged roles in fertilisation, implantation and placentation. Given that physiological processes such as cell-cell contact, invasion and adhesion are critical to normal biology in these processes, this is not surprising. Fertilization is a complex process that involves the fusion of the gametes of two individuals. Sperm-egg interaction has multiple players such as ADAMs, CRISPs, SLLP1, SAMP14, SAMP32; immunoglobulins such as Izumo; integrins; GPI-APs; and tetraspanins [217, 218]. Only Izumo and the tetraspanin CD9 have been shown to be essential for fertilization [219, 220, 221, 222]. While multiple integrins have been demonstrated on the murine oocyte surface, knock out mice for these integrins are fertile leading to the conclusion that "none of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm-egg binding and fusion" [223]. However data exists demonstrating a contribution to fertilisation by sperm and oocyte integrins, including $\beta 1$, $\alpha 9$ and $\alpha 6\beta 1$ [224, 225, 226]. It has been demonstrated that RGD tri-peptides can inhibit fertilisation in IVF assays [227]. In addition, FEE tri-peptides which are recognised by integrin $\alpha 6\beta 1$ potentiate the affect of RGD tri-peptides [228]. This has led to the hypothesis that there is a cooperation between RGD-sensitive and RGD-insensitive integrins during human fertilization [229]. Furthermore, integrin $\alpha 5\beta 3$ is present in both human gametes where it is believed to play an important role in fusion events between the two via its ligand vitronectin [230]. This role of sperm $\alpha 5\beta 3$ has also recently been outlined in the mouse [231]. Moreover, it is seen that post fertilisation entry into normal cell cycle appears to require integrin signalling and phosphorylation of Focal Adhesion Kinase (FAK) [232].

After fertilisation the embryo develops into a blastocyst which once hatched attaches to and invades the endometrial surface in a process that has parallels with leukocyte...
extravasation [233]. Cross talk between the endometrium and the blastocyst established prior to implantation mediates the activity of cell surface receptors, including integrins [234]. The uterine epithelium expresses selectins and mucins as well as αV integrins as reviewed in [235]. Blocking antibodies against integrins αvβ3 and α4β1 impair implantation in the mouse [236, 237]. Conversely, administration of calcitonin increases implantation through up-regulation of integrin β3 expression on endometrial epithelium [238]. Several RGD-containing ligands of integrins have been implicated in the implantation process including osteopontin, fibronectin and thrombospondin [239, 240, 241].

During placental development, trophoblasts require integrins to carry out their normal barrier and migratory functions. Integrin α6β4 is expressed at the basal surface of both CTB and STB and is important for barrier functions of the trophoblast [242]. However, during placental development, and the acquisition of the more invasive phenotype by the trophoblast lineages, there is a shift to an altered integrin expression pattern compared to that of the CTB and STB which is characterised by increased expression of integrins α5β1, α4β1, αvβ1 and αvβ3 and decreased integrin α6β4 as reviewed in [51]. The importance of the β3 integrins to the peri-implantation process as has been demonstrated in the mouse where it was shown that a switch to integrins αvβ3 and αIIbβ3 from α5β1 occurs during the invasion of the blastocyst after attachment [243]. This important adhesive role in early pregnancy for the integrin αIIbβ3 has been demonstrated in trophoblasts isolated from early human pregnancy also [244]. Together these findings illustrate an important regulatory role for the platelet integrin αIIbβ3 as part of the orchestrated changes in trophoblast integrin expression patterns during trophoblast differentiation.

Interestingly, the activity of other adhesion molecules and hormones can modify integrin activity. It was recently shown that MUC-1 is over expressed in patients suffering from preeclampsia and that forced over expression of this molecule in HTR8/SVneo EVT cells inhibited invasion and β1 integrin activity [245]. In addition, estradiol was recently shown to modify integrin expression in baboon pregnancy where artificially raised estradiol levels negatively impacted on EVT invasion and maternal spiral artery remodelling with integrin α1β1 and integrin α5β1 expression reduced 2-fold and by 40% respectively [246]. These accumulated findings confirm the importance of the pattern of orchestrated changes to
integrin expression, but also demonstrated that maternal signals could play important roles in the correct execution of these expression changes. Furthermore, it has been found that atypical expression of integrins is associated with diseases of pregnancy such as preeclampsia and ectopic pregnancy [247,248].
Chapter 2

Materials and Methods

2.1 Molecular cloning

All blunt ligations were performed on end-converted gel extracted PCR fragments into PSTBlue1 and transformed into NovaBlue Singles ultra-competent *E. Coli* (Novagen). Insert-positive colonies were selected by growth on 100 µg/ml ampicillin plates with [isopropyl β-D-1-thiogalactopyranoside (IPTG)] and X-gal to allow for white/blue colony screening. Positive clones were grown overnight shaking examined for insert by quickscreen or isolation of DNA and diagnostic restriction enzyme digest. Insert was ligated into vector cut with the same restriction enzymes at room temperature for two hours with T4 DNA ligase (New England Biolabs). Ligations were transformed into NEB Turbo ultra-competent *E.coli* cells (New England Biolabs) and grown on 100 µg/ml ampicillin [Luria-Bertani (LB)] agar plates. Clones were grown overnight in LB broth with 100 µg/ml ampicillin shaking at 250 rpm at 37°C. Bacterial cells were centrifuged at 2500 rpm for 5 min, plasmid DNA isolated using QIAprep spin mini-prep kit (QIAGEN, UK) and sent for sequencing (GATC Biotech, Germany) with the following primers: GATC-pTT3up-611629 5’-CTCTCAAAAGCGGGCATTA-3’ and GATC-pTT3Reversesequencingprimer-612690 5’-GAGGGATCTCGACCAAATGA-3’.
2.2 Generation of pTT3hCEACAM1Δ430-465 construct

An expression construct encoding a c-terminal V5 6xHis tagged truncated form of the human CEACAM1 gene minus the c-terminal membrane targeting component designated pTT3CEACAM1Δ430-465, was generated by polymerase chain reaction (PCR) amplifying the CEACAM1Δ430-465 sequence incorporating 5’ EcoRI and 3’ HindIII restriction sites from pcDNA3.1 hCC1-4S which encodes the human CEACAM1-4S isoform (Genbank accession number DQ975209) (a kind gift from Dr. Bernhard Singer, Institute for Anatomy, Clinical University of Essen) using the following primers: hCC1For 5’ aGAATTCaccatggggcacctctcagcc 3’ and hCC1Rev430-465del 5’aAACCTTggccccaggtgagaggccattttat3’. The PCR amplified band was blunt cloned as described in Section 2.1. The insert was digested out of pSTBlue1 with EcoRI and HindIII and ligated into a digested, modified pTT3 construct that had been mutated to incorporate EcoRI and HindIII RE sites before the pBluescript polylinker and V5 6xHis tag (pTT3-B). pTT3 is a modified version of the pTT vector with an extended multiple cloning site. See Appendix Fig.7.1 (A) and (B) for vector maps of pTT3 and pTT3-B PSG1.

2.3 Mutation of pTT3 PSG constructs by PCR

pTT3PSG1, pTT3PSG1ΔN, pTT3PSG1ΔA1A2, pTT3PSG1ΔB2term, pTT3PSG1 KGD→AAA, pTT3PSG1N, pTT3PSG1N KGD→AAA, pTT3PSG9 and pTT3PSG23 were generated as previously described[180]. PCR mutagenesis of pTT3PSG1 and pTT3PSG9 constructs for this study was performed with Phusion II Taq Polymerase (Finnzymes) as outlined in Table 2.1. This was followed by ligation of PCR product with T4 DNA ligase (New England Biolabs) and transformation into NEB Turbo ultra-competent E. coli (New England Biolabs). pTT3PSG4 was generated by Mrs Melanie S. Ball, by amplifying the PSG4 coding-domain sequence (CDS) from a commercially available clone (Source Bioscience, UK) incorporating 5’ EcoRI and 3’ HindIII restriction sites. This was blunt cloned into PSTBlue1 and subcloned into pTT3-B using EcoRI and HindIII restriction enzymes as described in section 2.1.
Table 2.1: Templates, primers and conditions for pTT3 PSG mutagenesis

<table>
<thead>
<tr>
<th>Template</th>
<th>Primers</th>
<th>Conditions</th>
<th>Construct</th>
</tr>
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<tbody>
<tr>
<td>pTT3PSG1ΔN</td>
<td>F’ aagcttaggcctggtaagcctaccca 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td>pTT3PSG1A1</td>
</tr>
<tr>
<td></td>
<td>R’ aatcagggtgactgggtcactgc 3’</td>
<td>[98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td></td>
</tr>
<tr>
<td>pTT3PSG1ΔB2</td>
<td>F’ cccaagccctacatcaccatc 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td>pTT3PSG1A2</td>
</tr>
<tr>
<td></td>
<td>R’ ggcagtggtgggcaggtt 3’</td>
<td>[98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td></td>
</tr>
<tr>
<td>pTT3PSG1ΔA1A2</td>
<td>F’ tagttccagactcctacca 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td>pTT3PSG1B2</td>
</tr>
<tr>
<td></td>
<td>R’ ggcagtggtgggcaggtt 3’</td>
<td>[98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td></td>
</tr>
<tr>
<td>pTT3PSG1ΔN</td>
<td>F’ cctggtaagcctatccctaaccctct 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td>pTT3PSG1A1A2</td>
</tr>
<tr>
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<td>R’ cctaacttgaggacattcagg 3’</td>
<td>[98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td></td>
</tr>
<tr>
<td>pTT3PSG1N</td>
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<td></td>
</tr>
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<td>F’ gatcttaccggctacatctggtacaaag 3’</td>
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<td></td>
</tr>
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<td>pTT3PSG1N</td>
<td>F’ gcatattccgatgcatccctgct 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
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<td>R’ tgtttctcgtccactatatgcag 3’</td>
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<td></td>
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<tr>
<td>pTT3PSG1N</td>
<td>F’ gatcttaccggctacatctggtacaaag 3’</td>
<td>98°C 3 min, [98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
<td>pTT3PSG1N111D</td>
</tr>
<tr>
<td></td>
<td>R’ ctggggcaaattgtggacaagtagaaga 3’</td>
<td>[98°C 10 sec, 65°C 30 sec, 72°C 3 min] x 25, 72°C 10 min</td>
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2.4 Generation of pTT3CEACAM49 construct

The pTT3CEACAM49 construct was generated by **PCR amplifying the CEACAM49 CDS** from pRcCMV-ecaCEACAM49 (a kind gift from Dr. Robert Kammerer, Institute of Immunology, Friedrich-Loeffler-Institute, Teubingen, Germany) incorporating 5’ EcoRi and 3’ HindIII restriction sites by Mrs Melanie Ball using the following primers; ecaCEACAM49For 5’gGAATTCaccatgcaatcaccctca3’ and ecaCEACAM49Rev 5’cAAGCTtggctctgtaactgggg3’. The **PCR** amplified band was blunt cloned into PSTBlue1 and sub-cloned into pTT3-B using EcoRI and HindIII restriction enzymes as described in Section 2.1.
2.5 Generation of Recombinant Proteins

2.5.1 Endotoxin Free plasmid DNA Preparation

*E. coli* containing the PSG construct to be expressed were grown in 300 ml of 25 g/L Luria Broth (Sigma) with 100 µg/ml ampicillin overnight, shaking at 250 rpm at 37°C. Cells were isolated by centrifugation at 6,000 rpm in a Beckman Avanti™ J-25 centrifuge. Endotoxin free plasmid DNA was isolated using the Nucleobond Xtra Maxi EF plasmid DNA extraction kit (Machery-Nagel, Germany) as per the manufacturer's instructions. DNA reconstituted in endotoxin free water was quantified by measuring absorbance at λ280 and aliquoted in endotoxin free eppendorf tubes and stored frozen at -20°C.

2.5.2 Transient transfection of Freestyle™ 293 cells

250 µg of plasmid DNA and 250 µl of Freestyle™ MAX Reagent (Invitrogen Life Technologies) were each diluted independently in 5 ml of OptiPRO™ serum free media (Gibco® Life Technologies) and subsequently mixed and incubated at room temperature for 12 minutes. This 10 ml transfection mix was added slowly to 250 ml of antibiotic antimycotic solution (AAS)-free Freestyle™293 cells at 1.0 x 10⁶ cells/ml. The transfected cells were incubated for a further 72 hrs. The PSG containing media was separated from cells and particulate matter by centrifugation at 1,500 rpm and stored at -80°C.

2.5.3 Nickel Agarose Affinity Chromatography

250 ml PSG media was thawed and Imidazole added to a 10 mM final concentration. 2.5 ml of HisPur Ni-NTA resin in 20% ethanol (Fisher-Thermo Scientific) was washed in wash buffer (500 mM NaCl, 20 mM NaH₂PO₄, pH 6.0) and added to the imidazole supplemented PSG media. This was incubated rotating at 8 rpm overnight at 4°C. All subsequent steps were carried out at 4°C. The beads were settled out in a disposable polypropylene column and washed with 60 ml of wash buffer. The last 4 x 1.5 ml of wash was collected to check for absence of contaminating protein. PSG was eluted by increasing concentrations of imidazole (50 - 500 mM) in wash buffer and collected in 1.5 ml fractions (4 x 50 mM, 5 x 200
mM, 3 x 300 mM, 3 x 500 mM). 22.5 µl of fractions were separated by SDS-PAGE and stained with Gelcode® Blue to examine purity and elution pattern of PSG. Gels were de-stained further for 1 hr in distilled water and scanned on the Odyssey infrared imaging system (Li-Cor Biosciences, UK) using protein gel system settings at 700 nm.

**2.5.4 Concentration**

Positive fractions free from contaminating protein were pooled and added to a washed Centrifugal Filter Unit with a 30 KDa molecular weight cut-off (MWCO) (Millipore, Ireland). This was centrifuged at 3,800 rpm at 4°C until the PSG solution was concentrated greater than 100-fold.

**2.5.5 Dialysis**

The concentrated PSG solution was injected into a pre-hydrated Slide-A-Lyzer® Dialysis cassette (Millipore) with a 10 KDa cut-off and 0.5 - 3 ml capacity. The solution was dialyzed in 500 times its own volume in sterile-A PBS in an LPS free container for 2 x 3 hrs and overnight at 4°C.

**2.5.6 Quantification**

The PSG protein, now in PBS, was removed from the cassette and quantified by measuring the absorbance of 5 µl of the protein in 95 µl water at λ280. This figure was then divided by a protein specific figure for predicted absorbance at λ280 at 0.1% (=1g/L), determined by entering the protein sequence minus the 35 amino acid leader sequence into the Expasy ProtParam online tool. The predicted absorbance at at λ280 at 0.1% (Abs 0.1%) for each protein is indicated in Table 2.2. Protein purity was assessed by SDS-PAGE and Gelcode® Blue staining as in section 2.5.3.
Table 2.2: Protein Abs @ 0.1% from Expasy ProtParam tool

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2.5.7 Size Exclusion Chromatography

1-4 mg of purified recombinant protein was applied to a HiLoad 16/60 Superdex S200 prep grade column in a 1 ml volume with a flow rate of 1 ml/min using the Akta explorer system in PBS. Determination of 1 ml fractions to pool was based on chromatogram peaks and is indicated on graphs. Resultant protein solution was concentrated and visualised as previously described to confirm removal of non-specific contaminating bands. The presence/absence of contaminating TGF-β1 was determined by enzyme-linked immunosorbent assay (ELISA).

2.6 Cell Culture

THP-1 cells were purchased live from the European Collection of Cell Cultures (ECACC). Cells were maintained between 0.3 - 1.0 x 10^6 cells/ml in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin antibiotic and 20 mM L-glutamine. HUVEC-2 endothelial cells were purchased frozen from BD Biosciences. Cells were resuscitated and maintained in Enhanced Growth Media (EGM-2) (Lonza). Cells were seeded at a 2,500 cells/cm^2 through each passage and not allowed grow to confluency. THP-1 cells and HUVEC-2 cells were grown at 37°C in a 5 % CO2 atmosphere. Freestyle™ 293 cells (Invitrogen-Life Technologies) were maintained in Freestyle™ expression medium (Gibco®-Life Technologies) supplemented with AAS between 0.3 - 1.0 x 10^6 cells/ml shaking at 100 rpm, at 37°C in an 8 % CO2 atmosphere. For transfection Freestyle™ 293 cells were twice passaged free of AAS and grown to 1.0 x...
2. MATERIALS AND METHODS

2.7 Treatment of cells with PSG

10^6 cells/ml. Mock and integrin α_IIbβ_3 dual over-expressing CHO-K1 cells were grown in DMEM-F12 media supplemented with 10 % FBS, 100 U/ml penicillin-streptomycin antibiotic, 20 mM L-glutamine, G418 and Zeocin. RAW 264.7 and integrin α_Vβ_3 dual over-expressing CHO-K1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS, 100 U/ml penicillin-streptomycin antibiotic and 20 mM L-glutamine. Integrin β_3 sub-unit over-expressing CHO-K1 cells were grown in DMEM supplemented with 10 % FBS, 100 U/ml penicillin-streptomycin antibiotic, 20 mM L-glutamine and G418.

2.7 Treatment of cells with PSG

THP-1 cells were treated at 3 x 10^5 cells/ml in 1 ml volumes in 24 well plate. HUVEC-2 endothelial cells were treated in 1 ml in 24 well plate with 3 x 10^5 cells/well. All treatments were carried out in the same volume in phosphate buffered saline (PBS). PBS alone was included as a vehicle control. Where indicated, human IgG and human CEACAM1-Fc along with CEACAM1ΔTM were used as non-specific protein controls. Cells were treated for the indicated times, at the indicated concentrations and media or nucleic acids were harvested for analysis.

2.8 Western Immunoblotting

2.8.1 Preparation of Whole Cell Lysates

Cells were lysed in NP-40 lysis buffer (20mM Tris, pH 8.0, 50mM NaCl, 50mM NaF; 1% NP40). Lysis buffers were supplemented with inhibitors (1 mM Sodium Orthovanadate, 2 μg/ml Aprotinin, 1 mM pefabloc, and 1 mM pepstatin-A). All samples were lysed on ice for 20mins, centrifuged at 14,000rpm at 4°C for 20 mins and supernatents removed to fresh tubes and maintained at -80°C. Large lysates were aliquoted to avoid multiple freeze/thaw cycles.
2.8.2 Protein Concentration Determination

The bicinchonic acid (BCA) Protein Assay Kit (Novagen, CA, USA) was used to determine protein concentration of all samples. Serial dilutions of bovine serum albumin (BSA) were used to generate a standard curve, from which sample protein concentrations were estimated. Absorbance values at 562 nm were obtained using a SpectraMAX340 spectrophotometer and SOFTMax Pro software (Molecular Devices Corp.).

2.8.3 SDS PAGE and Immunoblotting

Proteins were resolved on 10 or 12% acrylamide gels (1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, dH$_2$O and tetramethylethylenediamine (TEMED)), depending on the molecular weight of the protein of interest. Equal amounts of proteins were loaded into a 5% acrylamide stacking gel (1M Tris (pH6.8), 10% SDS, dH$_2$O and TEMED) using Laemmli buffer (62.5mM Tris-HCL (pH 6.8), 2% w/v SDS, 15% v/v glycerol, 3% v/v β-mercaptoethanol). Proteins were denatured by boiling at 100°C for 5 mins before being resolved at 120-150V using standard electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Proteins were then transferred electrophoretically to 0.2 µm pore nitrocellulose membranes (Millipore, ) using transfer buffer (48mM Tris, 39mM glycine and 20% ethanol) at 20 V for 60 mins at room temperature with a semi dry transfer apparatus. Membranes were incubated with a blocking buffer containing PBS, 0.1% Tween-20 (PBS-T) and either 5% w/v non-fat desiccated milk (Marvel, Chivers Ltd, Dublin, Ireland) or 5% BSA for 1hr at room temperature on a rocking platform at approximately 35rpm. Immunoblots were then incubated overnight at 4°C with primary antibody solutions diluted in 5% non-fat milk or 5% BSA on a rocking platform. Membranes were washed 3 x 5mins and 2 x 10mins in PBS-T and incubated for 1hr with Alexa Fluor 680 and 800-coupled anti-mouse secondary antibodies (LI-COR Biosciences, UK) diluted in PBS-T (1:10,000) containing 5% non-fat milk or 5% BSA for 1hr at room temperature on a rocking platform. Immunoblots were then washed in PBS-T as above. Immunoreactive proteins were visualized on the Odyssey Infrared imaging system using manufacturers settings (LI-COR Biosciences, UK). An exception was rabbit anti IκBα primary antibody which was detected with a horse radish peroxidase conjugated
2. MATERIALS AND METHODS

2.9 Fibrinogen Binding Assay

2.9.1 Preparation of Human Platelets

Platelets were obtained from healthy volunteers that were free of medication under Royal College of Surgeons in Ireland (RCSI) Ethical Review Protocol. Platelets were collected into 0.15 vol/vol acid-citrate dextrose (ACD, 75 mM trisodium citrate, 124 mM dextrose and 38 mM citric acid) anticoagulant and washed using a modification of a previously described method (ref). Briefly, blood was centrifuged at 150 x g for 10 min at room temperature. In order to avoid any contamination from the buffy coat, 0.5 ml of the platelet-rich suspension above the buffy coat layer was left behind in the centrifugation tube. Platelet rich plasma (PRP) was then acidified to pH 6.5 with acid-citrate-dextrose (ACD) and Prostaglandin E1 (PGE1, 1 mM) was added. The platelets were pelleted by centrifugation at 750 x g for 10 min at room temperature. The supernatant was removed and the platelet pellet was gently resuspended in JNL buffer (130 mM NaCl, 3 mM KCl, 10 mM trisodium citrate, 9 mM NaHCO3, 6 mM dextrose, 0.9 mM MgCl2, 0.81 mM KH2PO4, 10 mM Tris pH 7.4). Platelet count was adjusted to 3 x 10^8/ml using a Sysmex XE K-1000 counter (Toa Medical Electronics Co. Ltd, Kobe, Japan). Platelets were allowed to stand at room temperature for 45 min to let PGE1 dissipate. Calcium chloride (CaCl2, 1.8 mM) was added to platelets immediately before use.

2.9.2 Fibrinogen Binding Assay

10 µl of 2.5 mg/ml Oregon Green 488 fibrinogen (OGFg)-conjugate labeled Fibrinogen (Invitrogen, UK) was added to 20 µl aliquots of washed platelet suspension along with the indicated concentrations of wildtype PSG1 and CEACAM49 in successive tubes. All
2. MATERIALS AND METHODS

2.10 Primary Bone Marrow-Derived Macrophage Isolation and Culture

Experiments were run in duplicate at least three times. The platelet suspension was vortexed and allowed to stand at room temperature for 10 min before the addition of 3.4 µM TRAP (Bachem, UK), a dose known to produce a 50% maximal response as measured by aggregometry in pilot studies. Assay tubes were incubated at room temperature for a further 10 min. The reaction was stopped by addition of 1 ml ice-cold buffer. The association of OGFg with platelets was detected using the BD FACS Calibur system. Data acquisition and analysis were performed with the Cell Quest program. Platelet populations were gated, and histograms of mean fluorescence were generated for each sample. Statistical analysis was performed on the geometric scale.

2.10 Primary Bone Marrow-Derived Macrophage Isolation and Culture

6-8 week old CH3/HEN mice were sacrificed by rapid cervical dislocation. Femur and tibial bones were harvested and cleaned. Marrow was flushed with 5 ml of 10% FBS DMEM with Pen/Strep and L-glutamine. Cells were centrifuged at 1500 rpm for 7 minutes, supernatant discarded and resuspended in BMM media (20% DMEM with Pen/Strep, L-glutamine supplemented with 10 ng/ml murine-colony stimulating factor (M-CSF)). Cells were seeded and allowed to differentiate over 7 days. Cells were supplemented with fresh media on day 3, washed on day 5 with PBS and fresh media applied. On day 7 cells were washed in PBS and harvested by incubation with non-enzymatice cell dissociation solution (PBS with 5 mM ethylenediaminetetraacetic acid (EDTA) 4 mg/ml lidocaine, 0.22 µm filtered) for 20 minutes and scraped. Cells were centrifuged for 7 minutes at 1000 rpm and resuspended in 10% DMEM counted and seeded at required density. Cells were stained for F4/80 and scanned on a BD FACS Calibur system to verify macrophage phenotype.

2.11 PSG1 pre-treatment and LPS challenge of BMDM

Cells seeded at 2 X10^4 cells/well in a 96 well plate overnight were pre-treated with 5 µM (250 µg/ml) PSG1 for 30 minutes. Cells were subsequently challenged with 10 ng/ml, 100...
ng/ml or 1 µg/ml LPS for 6 and 18 hrs. Media was harvested, centrifuged at 5000 rpm to remove cellular debris and stored at -20°C. Supernatants were applied to IL-6, IL-10 and TNF-α ELISAs (e-biosciences, Germany) as per the manufacturers instructions.

2.12 Quantitative Real-time PCR

Total ribonucleic acid (RNA) was isolated using TRI-Reagent® (Sigma-Aldrich, UK). cDNA was generated using High Capacity cDNA Reverse Transcription kit (Applied Biosciences) with 1 µg of RNA per 20 µl reaction. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was carried out using the Roche Universal Probe Library and the Lightcycler® 480 Probes Master on the LC480 real time PCR system with 10 µl PCR reactions in triplicate composed of 7.5 µl LC480 PCR mix and 2.5 µl of 1:2 dilution of cDNA. Primer and fluorescent probe pairs used are shown in Table 2.3.

Table 2.3: Primer-Probe pairs for Quantitative Real-time PCR

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2.13 *In-vitro Integrin Binding Assay*

To bind integrins $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ to Protein G agarose beads, 2 $\mu$g of purified integrin $\alpha_{IIb}\beta_3$ from human platelets (Calbiochem, UK) and 2 $\mu$g of purified integrin $\alpha_V\beta_3$ from human placenta (Millipore, US) were added to a tube containing 700 $\mu$l of immunoprecipitation (IP) buffer (100 mM NaCl, 0.1% NP-40, 1 mM CaCl$_2$, 20 mM TrisHCl pH 7.4 plus the tyrosine phosphatase inhibitor Na$_3$VO$_4$ (1 mM) and the protease inhibitors PMSF (1 mM), pepstatin (1 $\mu$M) and aprotinin (1.5 $\mu$g/ml)). 3 $\mu$g of the anti- integrin $\alpha_{IIb}\beta_3$ monoclonal antibody (mAb) (clone SZ22, Beckman Coulter, Ireland) and 5 $\mu$l of anti integrin $\alpha_V$ mAb (clone LM142, Millipore, Ireland) were added to the respective tubes overnight at 4°C with gentle rocking. The next day Immune complexes were obtained by adding 40 $\mu$l of Protein G agarose beads (Thermo-Fisher, UK) for 3 h at 4°C followed by three washes with ice-cold IP buffer. For the *in vitro* binding assay, the last wash was removed from each of the samples and controls and the Protein G agarose beads were incubated with 2 $\mu$g of PSG1. All samples were incubated on a rotator for 1 h at room temperature. The beads were recovered by centrifugation at 3,000 rpm for 3 min followed by extensive washing to remove unbound PSG1. Protein was removed from the beads by boiling for 5 min in 20 $\mu$l of 2X SDS-PAGE sample buffer before electrophoresis and western immunoblot analysis as previously described. For immunoblotting all primary and secondary antibodies were diluted in 5% milk. Mouse anti- integrin $\alpha_{IIb}\beta_3$ mAb (clone SZ22, Beckman Coulter, Ireland) was used at 1 in 800 dilution, mouse anti-CD51 (BD Biosciences) was used at 1 in 500 dilution, mouse anti-PSG mAb11 ascites was used at 1 in 200 dilution.

2.14 Fluorescence Activated Cell Sorting

*(Assisted by Ken Martin, Centre for Research in Vascular Biology, UCC)*

Integrin $\alpha_{IIb}\beta_3$ dual transfected CHO-K1 cells were enriched for highly expressing population by Fluorescence Activated Cell Sorting (FACS) using the FACS Aria II system (BD Biosciences). Briefly, mock and integrin $\alpha_{IIb}\beta_3$ dual transfected CHO-K1 cells were non-
enzymatically detached (0.2% EDTA) and washed in cell sorting buffer (1% FBS, 1 mM EDTA, 25 mM HEPES in PBS). 1 x 10^6 cells in 100 µl of cell sorting buffer were incubated with 20 µl of phycoerythrin (PE) conjugated mouse anti-CD41a (BD Pharmingen) for 30 mins on ice in the dark. Cells were washed twice in 5 ml of cell sorting buffer, resuspended in 1 ml and sorted as indicated using a BD FACS Aria-II. Enriched cells were propagated and used in ligand binding experiments. The protocol for integrin β3 CHO cells and mock controls was the same but with APC-labelled anti-CD51 (BD biosciences). FACS on integrin α5β1 cells and mock controls was performed with anti-α5β1 antibody (Beckman Coulter) and FITC-labelled secondary antibody (Jackson immunoResearch).

2.15 Fluorescent Labelling of PSG1

1 mg of recombinant PSG1 was labelled with an N-hydroxysuccinimide (NHS) ester-activated Dylight®-800 or -488 fluorophore (Thermo Scientific), and excess fluorophore removed, as per the manufacturer’s instructions. Labelled protein was aliquoted and stored at -20°C.

2.16 Multi-channel FACScan analysis of fluorescent PSG1 binding to primary leukocytes

(Assisted by Andreia Ribeiro, ReMedI, NUIG)

Leukocyte samples were obtained using Ficoll-Hypaque separation of whole blood isolated from healthy volunteers in EDTA Vacutainers (BD, UK) under NUIG Ethical Review Protocol. Buffy coat was harvested and washed in FACS buffer (PBS pH 7.4, 3 % BSA, 0.05 % NaN₃). Cells were counted on a BD Accuri®C6 flow cytometry system and adjusted to the same concentration in FACS buffer. Fluorescent PSG1 was added along with mouse anti-CD14-APC (e-biosciences, Germany) for identification of monocyte sub-population alone or mouse anti-CD14-APC in conjunction with mouse anti-CD45-PerCP Cy5.5 (e-biosciences, Germany) for separation of monocyte and lymphocyte sub-populations. PSG1^{488} was added at 50 µg/ml unless stated otherwise for 30 mins at RT in the dark.
2. MATERIALS AND METHODS

2.17 FACScan analysis of fluorescent PSG1 binding to cell lines

Treatments were in 100 µl volumes. Blocking antibodies were used at 20 µg/ml and added as a cotreatment. Post acquisition compensation and analysis was carried out using the Infinicyt™ software package.

2.17 FACScan analysis of fluorescent PSG1 binding to cell lines

THP-1 cells were washed and re-suspended in FACS buffer at a concentration of 1 x 10^7 cells/ml. Fluorescent PSG1^488 was added at 50 µg/ml to 100 µl of cell suspension and incubated for 1 hr on ice with before washing and fixing at 37°C for 10 mins in 4% paraformaldehyde (PFA) PBS pH 7.4. Cells were washed in PBS and re-suspended in 1 ml of PBS before being scanned on a BD FACSCalibur system. Data were analysed using the BD Cell Quest Pro software.

2.18 Fluorescent Ligand Binding Assay

Stable empty vector transfected (Mock) and integrin α_IIbβ_3 over-expressing cells were seeded in quadruplicate in 96-well plates in selective media at 60,000 cells per well and allowed to adhere overnight. The next day the wells were washed in PBS and 100 µl of selective media with 50 µg/ml of PSG1^800 was applied to the cells for 30 mins at 37°C. Wells that had been blocked with media overnight were used as controls for background binding of PSG1^800. Media was removed and wells were washed extensively and fixed in fresh 4% PFA PBS pH 7.4 for 20 min at room temperature or overnight at 4°C. The cells were washed once in PBS and a 1:10,000 dilution of the SYTO®60 red fluorescent nucleic acid stain (Molecular Probes) in dH_2O was applied was applied for 1 hr at room temperature. Wells were washed three times with dH_2O and plate allowed to dry. The plate was scanned on a LI-COR Odyssey® Infrared Imaging System using the manufacturer's microplate settings. The mean fluorescence intensity for each well in the red and green channels was determined using the supplied software. Data from the green channel were normalized to the red channel.
2.19  **Indirect Fluorescent Ligand Binding Assay**

Mock and over-expressing cells were seeded as previously described in section 2.18. The next day the wells were washed in PBS and 100 µl of selective media with 50 µg/ml of PSG1 or a 1 in 4 dilution in culture media of a pool of six third trimester plasma samples from pregnant women were applied to the cells for 30 mins at 37°C. The cells were washed in PBS and fixed in 4% PFA-PBS pH 7.4 overnight at 4°C. Subsequently 100 µl of mouse anti-PSG mAb5 hybridoma supernatant was applied to the indicated wells for 1 hr at room temperature. The wells were washed three times and 75 µl of a 1 in 75 dilution of goat anti-mouse IRDye680 was applied to the wells for 1 hr at room temperature. The wells were washed and scanned on a LI-COR Odyssey®Infrared Imaging System using the manufacturer’s microplate settings. A 1:10,000 dilution of the SYTO®60 red fluorescent nucleic acid stain (Molecular Probes) in dH₂O was applied was applied for 1 hr at room temperature to the unstained wells to control for cell number. The plate was washed and scanned again at a lower intensity. The mean fluorescent intensity for the background was subtracted from the sample mean fluorescent intensity which was subsequently normalised to the SYTO®60 staining.

2.20  **Endothelial-monocytic interaction assay**

HUVEC-2 endothelial cells were seeded in a 24 well plate at a density of 2.5 x 10^5 cells/well and allowed to adhere before being treated for 12 hr with 10 ng/ml TNF-α. Monocytic THP-1 cells were subsequently labelled with Cell Tracker™Green CMFDA (Molecular Probes®) according to the manufacturers instructions. Labelled cells were pre-incubated with treatments indicated for 30 min before application of 1 x 10^6 cells/well to the washed monolayer and allowed to adhere for the indicated times before being washed in PBS and fixed overnight in 4% PFA-PBS pH 7.4 overnight at 4°C. Wells were washed gently in PBS allowed to dry and imaged on a Leica DMI3000 B microscope attached to a Leica DFC420 C CCD camera.
2.21 Bioinformatics

All human and rodent PSG sequences were taken from publicly accessible genome browsers; National Centre of Biotechnology Institute (NCBI) (http://www.ncbi.nlm.nih.gov), University College of Santa Cruz (UCSC) (http://genome.ucsc.edu) and the Ensembl Genome Browser (http://www.ensembl.org/index.html) as represented in Table 2.4 compiled by (Williams, J. 2013). Horse, Ape and Baboon sequences were taken from Kammerer and Zimmermann 2010 and supplemented with extra Horse sequence data provided by Robert Kammerer in a personal communication [58].

2.21.1 Alignments

Sequence alignments were performed using the online ClustalW alignment software (http://www.ebi.ac.uk/tools/clustalw2/index.html)

2.21.2 Generation of Phylogenetic Trees

Utilising ClustalW aligned PSG N domain CDSs lacking the leader sequence, the MEGA5 Molecular Evolutionary Genetics Analysis software was employed to construct phylogenetic trees (Neighbour-joined pairwise comparison). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (citation). The bootstrap consensus inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (citation). The percentage of replicate trees in which the taxa clustered together in the bootstrap test are shown next to the branches. All major branches yielded values of 95-100%.

2.22 Statistical Analysis

All statistical analyses were carried out using Graph Pad Prism 4 statistical analysis software package (GraphPad software Inc, La Jolla, CA, USA). Analyses used and sample numbers are
Table 2.4: Mouse, Rat and Human accession numbers

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Pregnancy Specific Glycoprotein Function, Conservation and Receptor Investigation

Ronan T. O'Riordan
indicated under figures. In all figures * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.001$. 
Chapter 3

Mutation of PSG1 and analysis of its immunomodulatory function

3.1 Introduction

As described earlier, thirteen years of published work has shown PSGs to be immunomodulatory, and pro-angiogenic hormones that can directly induce various cytokines from several cell types with roles in pregnancy and placentation, in a cross-species reactive manner [147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157]. More recently it was demonstrated that they possess novel anti-platelet properties, disrupting the interaction between platelet integrin αIIbβ3 and fibrinogen, a function that it is argued evolved in answer to the haemostatic complications associated with haemochorial placentation [180]. Many of the studies listed have investigated functional elements within PSGs through the expression of truncated constructs and the mutation of residues with proposed functional importance. Constructs studied for cytokine induction include: PSG6N [149], PSG17N1 [150, 151, 153, 158, 171], PSG18N1 [148], PSG19N1 [154, 171], PSG22N1A [156], PSG23N1A [158, 171], and PSG23N1 [171]. It must also be noted that two of the recent papers published on PSG1, make use of a truncated form of the protein lacking the A2 domain [155, 170]. This list shows that most studies on the immune functions of PSGs have examined truncations of the murine proteins and have almost entirely focused on the IgV
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3.1 Introduction

McLellan et al. proposed that a conserved RGD-like tri-peptide in primate N and rodent N1 domains may be of functional importance. Ha et al. examined this in a mutant PSG1 construct lacking the A2 domain where the Glycine-Aspartic acid-Aspartic acid (GDD) on the solvent exposed F-G loop was mutated to Serine-Aspartic acid-Leucine (SDL), the sequence that is found in this location in CEACAM1. The authors concluded these residues were unimportant for PSG1-induced TGF-β release. In the same paper, an asparagine (N) important for the formation of a salt bridge in conjunction with two conserved glycosylation sites in the PSG1 N-domain were also investigated in a single mutant and collectively were proposed to ablate PSG1 function. Mutations in glycosylation site number in proteins are known to affect protein function, as is the case in ocuculocutaneous albinism, a pigment disorder caused by mutations associated with a single N-linked glycoylation site within the Tyrosinase enzyme, a critical component of the melanin biosynthesis pathway. Conversely, mutation of the glycosylation sites in horse radish peroxidase changing the asparagine to aspartic acids have been shown to increase its heat stability and enzymatic activity, indicating that alterations in glycosylation site number can also lead to increased function or stability.

The nature of glycosylation modification of proteins, as distinct from variation in glycosylation site number, is extremely diverse and widely reported to affect protein function, through contributions to protein folding and quality control as well as roles in many biological recognition events. This understanding that glycosylation can impart an additional layer of information content to polypeptide sequences has led to the rise of the fields of glycobiology and glycomics. The role of glycosylation as an extra layer of information is widely investigated in the field of immunoglobulin research where glycoform variation is known to have an effect on the functionality of monoclonal antibody therapeutics. Moreover glycosylation patterns of the endothelium are believed to represent specific homing patterns that identify different locations in the vasculature. Recently, variation in glycosylation modification has been correlated with disease pathogenesis and as such its potential as a biomarker for a given physiological state of disease is of increasing interest. The predictive power of hyper-glycosylated...
human choriogonadotrophin (CG) was also recently examined \cite{257}. Consequently, there have been calls for the development of glycoproteomics and systems glycobiology, though these are currently limited by technology \cite{256, 258}. At present no data exists on the glycoforms of PSGs present endogenously, due to the inability to sufficiently purify PSGs, collectively or individually, from biological samples for characterisation.

However, the effects of broad glycoform variation on PSG function were investigated by Ha et al. using different recombinant protein expression systems \cite{154}. The authors utilised mammalian and bacterial expression systems, as well as enzymatic removal of glycosylation to investigate the effect it has on the ability of murine PSG17 to bind its receptor, tetraspanin CD9. It was shown that bacterially produced PSG17 did not bind CD9. It was also shown that mammalian PSG possessed terminal sialic acid modification, a modification insect cell expression systems cannot produce. Interestingly, however, production of PSG17 in insect cells did not affect its ability to bind CD9. Nor did it affect the reported ability of PSG17N to induce a TGF-\beta response. However, a mammalian expression system is required to ensure as near to what is understood to be correct glycosylation of the proteins as is possible. While it is known that there exist differing glycosylation patterns between mammalian, yeast and insect cells, it has only recently started to come to light that there may be biologically significant differences between commercially utilised cell lines such as HEK and CHO cells \cite{259}. The choice of mammalian expression system has also been proposed to be of relevance to the functionality of the resultant recombinant PSG by altering its affinity for its receptor/s at the cell surface. Sulkowski et al. demonstrated that PSG17N and PSG23N from CHO-K1 cells and HEK293T cells have different affinity for L929 cells which the author proposes to be as a result of differing glycosylation patterns between the two cell types used for production of the proteins \cite{171}.

Thus, it is concluded that glycosylation modifications of PSGs could play a role in modifying their affinity for their receptors/the cell surface. Furthermore, much evidence has been gathered implying an important functional role for the N/N1 domains, and some specific residues within these domains, of PSGs in their ability to induce a cytokine response from the listed responsive cell types. However, little work has been done to elucidate a role, if any, for the IgC-type domains of the PSGs in this important aspect of their biology. Shanley
et al. attempted to approach this question of functionality of PSG IgC-type domains in a platelet-fibrinogen interaction assay when investigating the anti-platelet properties of PSGs \[180\]. It was shown that a mutant PSG1 protein lacking the N-domain still retained full functionality in this assay. This implied that multiple domains of PSGs specifically PSG1, possess biological activity in this assay. In light of this finding the KGD tri-peptide, perhaps concluded pre-maturely to be unimportant for PSG1 function in earlier cytokine release studies, requires re-investigation in a PSG1 N-domain only construct to rule out the activity of other domains as confounding factors. Furthermore, the functionality of the remaining domains is also in need of investigation.

In addition to the requirement for the investigation of the functional elements within PSG1, the signalling underpinning the immunomodulatory functions of the hormone are in need of greater elucidation. Ha et al. demonstrated that PSG17N induction of IL-6 and IL-10 was PKA dependent \[153\]. Beyond this there has been no investigation of the signalling following PSG1 treatment of immune cells.

### 3.2 Objectives and Outcomes

This chapter had two broad areas of investigation; the first was the study of the functional importance of domains and motifs within PSG1 for the hormone's expression, cytokine induction and regulation of LPS-induced cytokines. There is a dearth of knowledge on the functionality of the various domains of PSGs in humans. As such, a previously published expression system for recombinant PSG generation was examined for suitability for study in cytokine induction assays. In addition, a mutational approach was undertaken that involved the deletion of the domains individually as well as attempts at their production independently of one another. It was found that PSG1 induced TGF-β1 in a multi-domain manner, that was not KGD or glycosylation dependant for the N-domain. Furthermore, the deletion of the N-domain from PSG1 had no effect on PSG1 attenuation of LPS-induced chemokine Cxcl2 from a mouse macrophage cell line. The induction of TGFβ1 mRNA was examined and only found in monocytic THP-1 cells indicating that the major source of TGF-β1 is latent particle release from cellular stores. It was subsequently found that
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TGF-β1 contaminates preparations of PSGs from mammalian cells [260]. Size Exclusion Chromatography (SEC) was utilized to remove the contaminating TGF-β1 from our protein and PSG1 was shown to, in fact, not induce TGF-β1 release in the monocytic cells utilised in this study. Sequential ablation of glycosylation sites within PSG1N had a negative effect on PSG1N expression that was additive. Moreover, it was found that deletion of the N61 site had the most significant impact of protein expression. Furthermore, forced introduction of this site into a novel PSG9N protein rescued the expression of the protein.

The second area of investigation was the immunomodulatory role of PSG1. This was examined through cytokine expression and release studies in monocytic and macrophage cells. The only investigation into the signalling underpinning PSG1 function was by Ha et al. who found that PSG1N induced IL-6 and IL-10 was PKA dependent [153]. In contrast, here it was found that PSG1 pre-treatment attenuated LPS-induced TNF-α and IL-6 in mouse and human monocytic and macrophage cells and that this was not PKA dependent. In addition, and in contrast to Blois et al., it was found also that inhibition of TGF-β1 signalling did not affect this [260]. Moreover IL-6 and IL-10 induction in response to PSG1 treatment could not be detected. The status of the key NFκB regulator, IκBα was also investigated in PSG1 pre-treated LPS-challenged cells to see whether an alteration in NFκB regulation could be detected. No effect could be observed.

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3.3.1 Validation of protein production, and function in TGF-β1 induction assay

The transient Freestyle™293 mammalian protein expression system was chosen to study PSG functionality as it was amenable to the generation of a series of mutants without the requirement for the establishment of stably over-expressing cell lines. It was first investigated whether it was possible to generate pure PSG in sufficient quantities for functional studies.

Figure 3.1(A) shows the chromatogram from a nickel agarose affinity chromatography column of beads that had been batch-bound overnight in PSG-containing medium
supplemented with 10 mM Imidazole and eluted with indicated washes of increasing imidazole concentrations. This indicates that large quantities of protein remains attached to the Ni-NTA beads through the extensive volumes of washes. Note, also, that the elution of protein from the beads gives a peak in absorbance in the 200 mM fractions. The increasing imidazole concentration starts to reach background column eluate absorbance in the 300 mM range. Fractions were separated by SDS PAGE and the elution pattern of the recombinant PSG1 visualized by coomassie staining (Fig. 3.1B). Most of the recombinant PSG1 elutes in the 50 and 200 mM fractions. The indicated fractions were pooled and processed further as indicated and described in the materials and methods chapter. 2 µg of the resultant protein was separated by SDS PAGE and visualized by coomassie staining to examine purity. Figure 3.1(C) demonstrates that the PSG1 generated for this study is > 95% pure as determined by densitometric analysis of the coomassie stained gel as outlined in the materials and methods.

A further requirement for this study is that the protein production process be endotoxin free. To that end a series of batches were tested for LPS contamination and demonstrated to be endotoxin free by LAL assay (Lonza biosciences, UK) confirming that our reagents, disposable plasticware and production system generated endotoxin free product. It was also necessary to determine fully the processing that happened to the primary translated protein sequence and whether the initial Signal-P v4.1 predicted 34 amino acid signalling peptide was indeed cleaved [261]. A sample of frozen aliquoted PSG1 was sent to be N-terminally sequenced by Edman degredation (Alta-biosciences, UK). This yielded a 5 amino acid sequence corresponding to the N-terminus of the secreted purified PSG1. The amino acid sequence 'VTIEA' reveals that the secretion process in fact cleaves a 35 amino acid peptide from the N-terminus of PSG1 (Fig. 3.2). This was also confirmed to be the case with protein made from two endogenous splice variants of mouse PSG22 (Williams, J. unpublished).

The protein generated through this system was previously demonstrated to have biological function in a platelet-fibrinogen interaction assay [180]. This study aimed to investigate the function of these proteins in cytokine induction assays. As mentioned earlier, several publications provide evidence that PSGs induce the release of cytokines from various cell
types. As a further control for the effects of non-specific contaminants of the purification process a protein control was required that could be used as a negative control for functional analysis of PSG1. The closely related membrane bound CEACAM1 was mutated by PCR to remove the hydrophobic membrane targeting sequence and subcloned into pTT3 as described in the materials and methods, incorporating a C-terminal V5-His tag. This construct was sequenced and verified to contain the CEACAM1 protein coding sequence in frame incorporating the tag sequence. The protein, designated CEACAM1\(\Delta\)TM, was expressed as described and purified in a similar fashion to PSG1. Figure 3.3 (A) shows the chromatogram of 1.5 ml imidazole fractions from the CEACAM1\(\Delta\)TM bound nickelagarose beads. The fractions were resolved by SDS PAGE and the resultant protein is indicated (Fig. 3.3 (B) and (C)). Interestingly this protein was smaller than previously published reports examining soluble CEACAM1 proteins. Critically, the protein was larger than PSG1 and showed significantly more glycoform variation. The size difference between PSG1 and soluble CEACAM1 is due to the greater amount of predicted glycosylation sites found in CEACAM1. We hypothesize that the predicted glycosylation sites were not all modified in our expression system yielding a lower molecular weight CEACAM1\(\Delta\)TM protein. The role of this recombinant protein as a negative control for the purification process can still be carried out.
Figure 3.1: Purification of recombinant PSG1 protein

(A) Chromatogram of 1.5 ml washes and imidazole elutions of PSG1 from nickel agarose affinity column. (B) Coomassie stain of 22.5 µl of highlighted fractions separated by 10 % SDS PAGE. (C) Coomassie stain of 2 µg of PSG1 resulting from processing of fractions.
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Figure 3.2: N-terminal protein sequencing of PSG1 confirms 35 amino acid signal peptide cleavage

PSG1 protein sequence was applied to the SMART at EMBL which utilizes the Signal-P signal peptide prediction algorithm to determine signal peptide cleavage \[262, 263\]. This predicted a 34 amino acid signal peptide in PSG1. N-terminal sequencing revealed the first five residues of the processed and secreted protein showing that 35 amino acids had been cleaved.
3. Mutation of PSG1 and Analysis of its Immunomodulatory Function

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Figure 3.3: Purification of recombinant CEACAM1ΔTM protein
(A) Chromatogram of 1.5 ml washes and imidazole elutions of CEACAM1ΔTM from nickel agarose affinity column. (B) Coomassie stain of 22.5 µl of highlighted fractions separated by 10 % SDS-PAGE. (C) Coomassie stain of 2 µg of CEACAM1ΔTM resulting from processing of fractions.
In response to 24 hr treatment with increasing concentrations of PSG1 protein, monocytic THP-1 cells release TGF-β1 over CEACAM1ΔTM control treated cells (Fig. 3.4 (A)). Other previously reported responsive cell types, HUVEC-2 human endothelial cells and murine macrophage cell line, RAW264.7, also demonstrated PSG1 induced TGF-β1 release (Fig. 3.4 (B) and (C)).

The method by which PSG1 regulates TGF-β1 was examined further by looking at the mode of induction of TGF-β1 that PSG1 elicits from several cell types by qRT-PCR and ELISA. I found that PSG1 produces significant induction of TGFB1 mRNA in monocytic THP-1 cells but, interestingly, not in the PMA-differentiated macrophages derived from these cells or the endothelial HUVEC-2 cell line (Fig. 3.5 (A)). The release of TGF-β1 could be detected from as early as 1 hr in the media of all cells tested, indicating that PSG1 is causing the release of latent TGF-β1 from cellular stores as the main means of inducing the cytokine (Fig. 3.5 (B)). The induction of TGFB1 mRNA observed in monocytes, taken with the lack of induction in the other cell types tested indicates that this result is most likely a cell or cell line specific and would need further investigation.

I also investigated whether IL-10, a previously reported PSG-induced cytokine, could be detected following PSG1 treatment of monocytic THP-1 cells. IL-10 release could not be detected in the supernatant of cells treated with PSG1 at all time points tested from 6-24 hrs as determined by ELISA. We further examined IL10 gene expression by qRT-PCR however there is no significant upregulation (Fig. 3.5 (C)). Interestingly, LPS also did not induce il-10 transcription or protein release in the systems tested indicating that perhaps the cells are LPS insensitive or the cell system was not competent for studying IL-10 release and regulation by PSG1. Having established the baseline regulation of TGF-β1 and being unable to replicate previously reported IL-10 induction, I determined to use TGF-β1 induction as a measure of PSG1 activity for subsequent functional tests on mutant PSG1 constructs.
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Figure 3.4: PSG1 induces TGF-\(\beta_1\) in a dose dependant manner from previously reported responsive cell lines

(A) TGF-\(\beta_1\) release from 24 hr PSG1 treated THP-1 cells was measured by ELISA and showed a dose-dependant response to PSG1 treatment over CEACAM1\(\Delta TM\) protein control. 50 \(\mu\)g/ml, equivalent to approximately 1 \(\mu\)M, PSG1 treatment of HUVEC-2 endothelial cell line (B) and RAW264.7 murine macrophage cell line (C) show that PSG1 induces TGF-\(\beta_1\) from other previously reported responsive cell types. n=3, Two-way ANOVA with Bonferroni post-test.
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Figure 3.5: PSG1 regulation of TGF-β1 and IL-10

(A) qRT-PCR analysis of TGFβ1 expression in 50 µg/ml PSG1 or 100 ng/ml LPS treated monocytic THP-1, PMA-differentiated THP-1 and HUVEC-2 endothelial cells shows gene expression is only significantly upregulated in monocytic THP-1 cell line. (B) Analysis of release of TGF-β1 into the media in the same samples by ELISA shows that it is induced to significant levels from as early as 1 hr in all cells tested. (C) Analysis of IL10 expression in monocytic THP-1 cells shows that no significant expression could be detected in response to PSG1 treatment. IL-10 could not be detected by ELISA in any samples tested. n=3, Two-way ANOVA with Bonferroni post test.
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3.3.2 Multiple domains of PSG1 induce TGF-β1 release

As the IgV-type N/N1 domains of human and murine PSGs have been so heavily studied for their ability to induce cytokine release, it was decided to employ a previously generated PSG1 mutant lacking the N domain [180], PSG1ΔN, to the TGF-β1 release assay to determine if the PSG1 IgC-type domains also induce its release. Figure 3.6 demonstrates that PSG1ΔN does still induce TGF-β1 release. In addition, this lack of a requirement for the PSG1 N-domain was observed in the newly identified ability of PSG1 to attenuate the LPS-induced expression of the chemokine Cxcl2 in the RAW264.7 murine macrophage cell line (Fig. 3.7). Thus, it is concluded that the N-domain, though sufficient, is not in fact necessary for PSG1 induction of TGF-β1 release or for attenuation of LPS-induced chemokines by PSG1. This raises questions about much PSG1 research where point mutations of individual residues of PSG1 N-domains, in the context of the full length protein, may have led to premature conclusions on the functional importance of these residues. It may be required that mutations in the N-domain be studied in an N-domain-only context to draw firm conclusions as to their importance. Moreover it identifies the functional importance of the remaining the domains of the PSG1 molecule.

Subsequently a further domain was deleted from PSG1ΔN generating a novel construct encoding both the PSG1 A-domains, PSG1A1A2, and protein was successfully produced (Fig. 3.8(A) and (B)). When tested in the TGF-β1 assay in equimolar treatments along with PSG1 it was found that PSG1A1A2 induces significant levels of TGF-β1 release (Fig. 3.8(C)). Thus, it is evident that there is TGF-β1-releasing activity in the A domains of PSG1.

To further refine our understanding of the remaining domains of the PSG1 molecule it was decided to generate constructs designed to express individual A1, A2 and B2 domains of PSG1. Figure 3.9(A) shows anti 6xHis western immunoblot of cell lysates and supernatants from transient transfection of Freestyle™293 cells with pTT3 constructs encoding the individual domains of PSG1. These indicate that PSG1N, as previously reported, is successfully expressed and secreted, as are PSG1A1 and PSG1A2. PSG1A1 and PSG1A2, however, both show signs of degradation indicating that they may be unstable proteins. PSG1B2, while showing expression at low levels, is not however secreted at detectable levels.
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Figure 3.6: PSG1 N-domain is not necessary for TGF-β1 induction
(A) Schematic showing domain structure and relative amino acid count of PSG1 and PSG1ΔN proteins. (B) Comassie stained 10% polyacrylamide gel showing 2 µg of PSG1 and PSG1ΔN. (C) Monocytic THP-1 cells were treated for 24 hrs with 1 µM PSG. TGF-β1 release was determined by ELISA n=6, One-way ANOVA with Tukey post-test.
Figure 3.7: PSG1 attenuation of LPS-induced *cxcl2* is not N-domain dependant

RAW264.7 macrophage cell line was pre-treated for 10 mins with either PSG1 or PSG1ΔN and subsequently challenged for 3 hrs with LPS. *cxcl2* expression was analysed by qRT-PCR. n = 6, One-way ANOVA with Tukey post-test.
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Figure 3.8: PSG1A1A2 induces TGF-β1
(A) Schematic of PSG1 proteins used. (B) Coomassie stained polyacrylamide gel showing 2 µg of PSG1 and PSG1A1A2. (C) PSG induction of TGF-β1 release from monocytic THP-1 cells showing PSG1A1A2 induces significant levels of the cytokine. n=3, Two-way ANOVA with Tukey post-test. Samples were compared to PBS control.
PSG1A1 and A2 were transfected into 100 mls of Freestyle™293 cells. The PSG containing media was processed as normal. Fractions 50.4 - 300.1 were pooled, concentrated and dialyzed but only minimal amounts of protein were recovered. To confirm protein was not being lost, the fractions, the concentrated sample, and the centrifugal force spin column flow-through were separated by 12 % SDS PAGE transferred to a membrane and probed with an anti-6xHis antibody. Figure 3.9(B) indicates that small amounts of protein are present in 50 mM fractions but not in sufficient quantities to make a significant difference to overall protein recovery as they could only be detected by Western blotting. There also appears to be two distinct species in the PSG1A1 sample, indicating that most likely proteins are not stably expressed and are extremely unlikely to be recovered in sufficient quantities to perform functional experiments.

As multiple individual domains of the protein cannot be independently produced to study their function in inducing TGF-β1 release, it was decided to focus on the N-domain of the protein since it is relatively better characterized and more mutations of this domain have been published, which need re-examining in the N-domain only context.
Figure 3.9: PSG1 individual A1, A2 and B2 domains cannot be purified

(A) Time course of transfections of Freestyle™293 cells with pTT3PSG1N, pTT3PSG1A1, pTT3PSG1A2 and pTT3PSG1B2. (B) 22.5 µl of fractions and purified protein, as well as flow through from centrifugal force spin column were probed for presence of recombinant protein with anti 6xHis antibody.
3.3.3 Mutation of a conserved RGD-like tri-peptide does not affect PSG1 N-domain function

Primate N- and rodent N1-domains contain a conserved RGD-like tri-peptide that has been proposed to have a role in their function [81]. It was observed that PSG1 differs slightly in that it has a GDD tri-peptide in this position but one position back, the presence of a lysine (K) gives PSG1 a KGD tri-peptide sequence on the solvent exposed loop of its N domain. This motif is found in snake venom disintegrins like barbourin. Work from our group demonstrated that mutation of the KGD tri-peptide sequence from PSG1N to AAA does not affect the function of the domain in a series of platelet integrin-fibrinogen interaction assays. Ha et al. demonstrated that a PSG1 GDD→SDL mutation did not affect PSG1 induced TGF-β release a result confirmed with our PSG1 KGD→AAA mutant (Fig. 3.10[A] and [B]) [155]. This, however, must be revisited in the context of the earlier findings of this chapter that demonstrate the multi-domain functionality of the PSG1 molecule, with a focus on the N-domain specifically. Figure 3.10[C] and [D] demonstrates that mutation of the KGD tri-peptide from the PSG1 N-domain also does not affect the ability of this domain to induce TGF-β release from THP-1 cells confirming that the KGD tri-peptide is not essential for this function of PSG1.
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Figure 3.10: Mutation of KGD tri-peptide does not affect PSG1 or PSG1N induced TGF-β1 release

(A) Coomassie stained polyacrylamide gel showing 2 µg of PSG1 and PSG1 KGD→AAA. (B) Monocytic THP-1 cells were treated for 24 hrs with 1 µM PSG. TGF-β1 release was determined by ELISA. (C) Coomassie stained polyacrylamide gel showing 2 µg of PSG1N and PSG1N KGD→AAA. (D) Monocytic THP-1 cells were treated for 24 hrs with 1 µM PSG. TGF-β1 release was determined by ELISA. n=3 both mutants, One-way ANOVA, with Tukey post-test.
3.3.4 Glycosylation site number alteration negatively affects PSG secretion

An interesting feature of the human, and other, PSG gene families is that there is variation in predicted glycosylation site number between different family members. I investigated if glycosylation site number variation could have functional consequences. First it was necessary to examine, as reported for other proteins, whether glycosylation site number variation could affect secretion of the PSG. To that end, the PSG1N construct was mutated to remove all the glycosylation sites individually and in pairs and to examine the expression and secretion of these mutants in Freestyle™293 cells by western immunoblot. The choice of the PSG1N protein as a model had the double benefit of reducing the number of mutants it was necessary to generate, while also making it more likely that the negative affects of the mutations would be more easily observed due to the mutant nature of PSG1N. Alteration of the asparagine (N) in the N-Xaa-S/T glycosylation site consensus sequence to an aspartic acid (D) removes the substrate for the attachment of the glycosidic bond while inserting an amino acid with a similar charge to the glycosylated asparagine, recapitulating the deamination reaction that occurs through PNGaseF.

Figure 3.11 shows that deletion of an individual site negatively affects secretion and that this effect is additive. Interestingly, constructs lacking the N61 glycosylation site seem to be the most negatively affected indicating that this site might more fundamentally affect protein stability and expression than other sites. This is in keeping with observations in other proteins that the more N-terminal a glycosylation site is, the more it can affect stable expression and secretion of recombinant proteins, as was shown for von-Willebrand factor. Thus PSG1N expression responds negatively to alterations in its glycosylation pattern. As an additional experiment a construct encoding PSG9N, which was generated but abandoned due to the inability to express the protein successfully, was utilised to examine whether introduction of the N61 glycosylation site consensus sequence, which is endogenously lacking in PSG9 as well as PSG3, PSG4, PSG5, and PSG11, could rescue the expression and secretion of the protein. A PSG9NP63T mutant which has the N61 glycosylation consensus sequence was generated and examined for expression and secretion in Freestyle® 293 cells. Figure 3.12 demonstrates that introduction of the glycosylation consensus sequence into PSG9N rescued protein secretion, indicating that
this site is indeed important for secretion of the PSG N domain. It is unlikely that this site is fundamental to PSG9 secretion as that protein expressed and was purified successfully in Shanley et al. and later in this chapter, however lower yields were obtained compared to PSG1 [180].
Figure 3.11: Mutation of predicted glycosylation sites negatively effects PSG1N expression and secretion

Anti-6xHis and β-actin immunoblots of Freestyle™293 cells transfected with glycosylation mutant constructs of PSG1 N-domain. 60 µg lysate and 45 µl of supernatant were loaded into the wells for each sample.
Figure 3.12: Restoration of a glycosylation consensus site in PSG9N rescues protein expression
Anti-6xHis and β-actin immunoblots of lysates and cell culture supernatants from Freestyle™293 cells transfected with pTT3PSG9N and pTT3PSG9N P63T constructs show P63T mutation rescues secretion of PSG9N. 50 µg lysate and 22.5 µl of supernatant were added for each sample.
3.3.5 Glycosylation site number alteration does not affect PSG induced TGF-β₁ release

PSG4 and PSG9 proteins were generated as described in the materials and Methods (Fig. 3.13(A)). While all PSG family members tested induced TGF-β₁, an observation was made that members of the gene family with reduced predicted glycosylation site number demonstrated significantly increased induction of TGF-β₁ release (Fig. 3.13(B)). As the PSG4 and PSG9 preparations were of lesser purity to PSG1, further investigations were required to implicate glycosylation site number variation in PSG-TGF-β₁ induction.

To that end it was undertaken to mutate the variable (N61) and one of the conserved (N111) glycosylation sites of the human PSG N-domains in the context of the full length PSG1 protein. As previously outlined the mutation was N→D abolishing the site for the attachment of the glycosidic bond. Fig. 3.14(A) shows the resultant proteins. PSG1 N61D shows a marginally lower molecular weight on the gel in comparison to the other proteins. However when proteins were examined for ability to induce TGF-β₁ release in it was found that no significant difference could be observed between the mutants and the wildtype. This would appear to rule out any major functional role for glycosylation site number variation within the PSG N domains in induction of TGF-β₁ release. However to conclusively prove it was again necessary to ask the question in an N-domain only context where any differences are free from potential confounding impacts of the remaining domains and functional effects could likely be more easily observed. Thus the previously generated mutants of the PSG1N protein, PSG1N N61D and PSG1N N111D were utilised to generate proteins for functional studies.

Both PSG1N N61D and PSG1N N111D could be produced in sufficient quantities for experimentation, though lower yields were obtained for PSG1N N61D (data not shown). When examined for purity by 12 % SDS PAGE and coomassie staining it was found that both glyco mutants had a lower molecular weight than the PSG1N and PSG1N N111D had a tighter band compared to PSG1N indicating reduced glycoform variation, as would be expected (Fig. 3.15(A)). Interestingly, PSG1N N61D had a larger amount of glycoform heterogeneity, however this is not conclusive as the pattern on the gel could equally be as
a result of degradation due to reduced protein stability. An attempt was made to further purify the proteins by Size Exclusion Chromatography (SEC) on a Superdex 75 column. The resultant proteins showed significantly less contaminating high molecular weight bands, though some were still present (Fig. 3.15(B)). These proteins were tested in the TGF-β\(_1\) induction assay and Fig. 3.15(C) shows that glycosylation site mutation does not affect PSG1N domain-induced TGF-β\(_1\) release.
Figure 3.13: PSGs 4 and 9 induce significantly more TGF-β₁ release compared to PSG1

(A) Coomassie stained polyacrylamide gel showing 2 µg of PSG1, PSG4 and PSG9. (B) PSG induction of TGF-β₁ release from monocytic THP-1 cells showing PSG4 and PSG9 induce significantly more compared to PSG1. n=3, One-way ANOVA with Bonferroni post test.
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Figure 3.14: Deletion of conserved or variable glycosylation sites does not affect PSG1 TGF-β1 induction

(A) Coomassie stained polyacrylymide gel showing 2 µg of PSG1, PSG1 N61D and PSG1 N111D. (B) Treatment of HUVEC-2 endothelial cells with 50 µg/ml PSG shows no significant difference between mutants in their ability to induce TGF-β1 release. n=3
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Figure 3.15: Glycosylation site number does not affect PSG1 N-domain TGF-β1 induction (A) 3 µg of indicated PSG1N proteins. (B) Same proteins after further purification by SEC using an S75 prep grade size exclusion column. (C) 50 µg/ml treatment of THP-1 cells with PSG1N, 1N N61D and 1N N111D for 24 hrs shows all induced significant levels of TGF-β1 release with no significant difference between mutants observed. n=3, One way ANOVA
3.3.6 Reported PSG1-induced TGF-β1 release as a result of contamination from mammalian culture expression system?

Thus far, all mutants examined appeared to be incapable of ablating PSG1-induced TGF-β1 release, negating fundamental roles for the N domain or its constituent conserved KGD/RGD tri-peptide or its conserved/variable glycosylation sites in this reported function of PSGs. However, a number of interesting observations were made at this point of the research into PSG function. Firstly, the level of variation in TGF-β1 induction between batches of proteins used throughout the course of this work was considered to be large in comparison to the published literature \[153, 154, 158, 155, 170, 171, 156\]. Secondly, the variation in TGF-β1 induction between members of the same family (PSGs 1, 4 and 9) was larger than anticipated given their sequence similarity and was observed for PSG23 as well (data not shown). Taken in the context of an inability to knockout the function under investigation I became concerned that, in spite of the wide body of literature in support of the finding, the examined release of TGF-β1 in response to PSG treatment may be artefactual.

A subsequent personal communication from Prof. Gabriela Dveksler indicated that there may be evidence for an interaction between PSG and TGF-β1 in that TGF-β1 (latent and active) appears to contaminate the PSG produced in mammalian cell expression systems utilised in previous studies \[153, 154, 158, 155, 170, 171, 156\]. A publication followed which demonstrated the contamination and furthermore outlined a role for PSG in activating latent TGF-β1 \[260\]. This represented a major confounding factor for much of the functional work preceding this point in the chapter.

Finally, a publication from the end of 2012 showed that the widely used immobilised metal affinity chromatography (IMAC) which is employed in this study and several of the PSG-related publications with TGF-β1 contaminated protein, co-purifies TGF-β1 from HEK293 cells based on a specific interaction between the immobilized metal ions and TGF-β1 \[265\]. As this would represent a further confounding factor, PSG1 utilised in this chapter was investigated for TGF-β1 contamination. Following on from this it was decided to investigate if, as in Kaur \emph{et al.}, SEC could be utilised to remove the contaminating TGF-β1 \[265\].
Figure 3.16 (A) shows that TGF-β1 does contaminate PSG1 purified from Freestyle™293 cells utilising nickel agarose beads, however, the levels of TGF-β1 vary dramatically and are entirely composed of the latent particle. Active TGF-β1 is probably removed during the concentration step in the purification protocol which utilises 30 kDa MWCO ultra-centrifugal force filters. This is important as the lack of active TGF-β1 precludes any signalling events being induced by this potent signalling molecule, which could confound any functional work on the immunological or angiogenic roles of these hormones. However the presence of latent particle in such quantities in some preparations represents an obvious potential confounding factor and highlights the need for a second round of purification. The authors of the aforementioned study identified high salt gel filtration as an effective means of removing TGF-β1 from the purified recombinant protein utilised in their study. As such, gel filtration chromatography was employed to determine if the TGF-β1 contaminant could be removed. Figure 3.16 (B) shows the chromatogram of PSG1 applied to a HiLoad 16/60 Superdex 200 prep grade column using an Akta explorer system. The peak fractions pooled are indicated in Figure 3.16 (C) and the resultant proteins were seperated by 10% SDS PAGE followed by coomassie staining (Fig. 3.16 (D)). The staining pattern indicated that the gel filtration removed high molecular weight contaminants. TGF-β1 ELISA analysis of the proteins demonstrated the the system was effective at removing the TGF-β1 contamination from the PSG1 protein (Fig. 3.16 (E)). Interestingly, high salt buffer was not required in contrast to Kaur et al. [265]. Finally, SEC-purified protein was applied to HUVEC-2 endothelial cells, reported in the literature and earlier in this chapter, to release TGF-β1 in response to PSG1 treatment. Figure 3.16 (F) shows that PSG1 does not, in fact, induce release of TGF-β1 as previously reported.
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Figure 3.16: PSG1 does not induce TGF-β1 release
(A) Large batch to batch variation detected in amount of TGF-β1 contaminant in PSG1. Chromatogram from PSG1 applied to a Superdex 200 prep grade size exclusion column with red box indicating area of interest (B) from which fractions representing two peaks were seperated and pooled (C). (D) Coomassie stained 10% polyacrylamide gel with 8 µg of indicated protein per lane. (E) TGF-β1 ELISA indicating that SEC removed latent TGF-β1 contaminant. (F) SEC purified PSG1 does not induce TGF-β1 release from HUVEC-2 endothelial cells, n=3. SM, Starting Material.
Thus having ruled out TGF-β₁ as a reliable readout of PSG1 function, it was decided to focus on its immunomodulatory properties by examining whether PSG1 could affect the expression of pro-inflammatory cytokines induced by LPS stimulation of leukocytes. As outlined earlier, a novel finding was that PSG1 could attenuate LPS-induced Cxcl2 from the RAW264.7 macrophage cell line and that this was not N domain dependent (Fig. 3.7). It was aimed to determine whether other pro-inflammatory cytokines could be attenuated in this way and whether the signalling mediating this could be elucidated. TGF-β₁ is an obvious signalling candidate for many reasons. The earlier described contaminant though composed almost entirely of the latent particle does not preclude the activity of pg amount of active TGF-β₁ present. In addition, the proposed novel function ascribed by Blois and colleagues, whereby PSG1 binds and activates TGF-β₁ requires that it is investigated here [260]. In the same paper, though in lymphocytes, the effects being examined here of attenuation of pro-inflammatory cytokines, were shown to be TGF-β₁ dependant by application of a TGF-β₁ neutralising antibody. Furthermore, TGF-β₁ has recently been demonstrated to have a role in the regulation of genes necessary for the full polarization of macrophages to an M2 phenotype [266].

3.3.7 PSG1 attenuates LPS-induced pro-inflammatory cytokine expression and secretion independant of TGF-β₁ and PKA signalling

Early on in our studies of PSG function it was found that IL-10, previously reported to be induced by PSG1, could not be found to be induced in response to our protein in the cell systems tested while a small induction was observed at the transcriptional level (Fig. 3.5). It was, however, noted that it was not possible to detect il-10 in the LPS positive control either. Due to the widely acknowledged ability of cell-line systems to become LPS insensitive, a new stock of THP-1 cells were purchased to re-examine the immunomodulatory capacity of PSG1. It was found that co-treatment with 1 μM PSG1 (50 μg/ml) and low dose LPS (1 ng/ml) had opposing effects on LPS-induced il-10 and il-6 expression in PMA-differentiated THP-1 cells. A very small additive effect on il-10 expression was observed associated with an attenuating effect on LPS induced il-6 and tnf-α (Fig. 3.17). No induction of il-6 from PSG1 treatment alone could be detected contrary to previous reports for PSGs.
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[149, 153, 157]. However this could be a cell line-specific effect. Taken together these data indicate a pattern of differential gene regulation consistent with an M2 polarising effect of PSG on monocytes/macrophages where immunosuppressive cytokines such as IL10 are upregulated in inflammatory situations and pro-inflammatory cytokines are attenuated.

It was next aimed to examine this effect on LPS induced IL-6, TNF-α and IL-10 at the protein level in primary bone marrow derived macrophages (BMDMs) using higher doses of LPS, and PSG1 at endogenous levels of approximately 250 µg/ml, or 5 µM. It was observed that while there is a trend of attenuation for all samples, it is significant only for IL-6 when compared to the PBS control in the 10 and 100 ng/ml LPS samples at 6 hrs Fig. 3.18. IL-10 could not be detected in any samples tested indicating that this cell system is likely not suitable for studies of IL-10 induction.

Subsequently, the signalling mediating the observed attenuation was investigated by using small molecule inhibitors against candidate signalling pathways that could be implicated in this function of PSG1. As already outlined TGF-β1 represents a good candidate for mediating the pro-inflammatory cytokine attenuation of PSG1. A second candidate of interest was cAMP-dependant PKA. PKA has been implicated in PSG17 induced up-regulation of IL-6 and IL-10 [153]. While neither of these cytokines could be induced by PSG1 in this study, it does not necessarily preclude PKA signalling as a candidate signalling pathway mediating PSG1’s effects. cAMP has long been known to have an attenuating effect on pro-inflammatory signalling in monocytes and macrophages [267]. More recently cAMP, through PKA, has been shown to be able to illicit differential effects on genes controlled through the same transcription factor, namely NFκB, by means of association with PKA anchoring proteins [268]. Similarly, PKA mediated cross-talk with other TLRs has been shown to be responsible for attenuating their pro-inflammatory signals through NFκB [269]. Therefore, given the pattern of differential regulation of LPS-induced NFκB regulated genes that we are observing in response to PSG1 treatment, PKA is a good candidate signalling pathway to be mediating this.

Two inhibitors were used for studying both candidate signalling pathways due to recent publications outlining shortcomings and potential off target effects of the historically more widely utilised H-89 and LY364947 [270, 271]. SB504124 is a more highly selective inhibitor...
than other TGFβRI inhibitors [271, 272]. PKI(6-22) is a palmitoylated peptide analogue specific for the cAMP binding pocket of PKA.

Critically no significant attenuation of TNFA or IL6 could be observed (Fig. 3.19). While there was a trend of attenuation of IL6 which was not affected by either H89 or LY34, it is not possible to draw solid conclusions from this. The SB50 inhibitor appeared to affect the LPS-treated sample and as such it is believed further optimisation of the dose of this inhibitor may be needed. No TNFA attenuation could not be detected. This is in keeping with the earlier findings in BMDMs Fig. 3.18. In addition, IL10 expression showed no induction in response to PSG1 however a large induction of IL10 was observed in the LPS-treated samples indicating it is likely PSG1 does not directly induce IL10.

The convergence of multiple inflammatory receptor pathways on the NFkB pathway confirms its importance to the regulation of the response to inflammatory stimuli [273]. It was finally aimed to investigate whether PSG1 could attenuate the pro-inflammatory signal by affecting the degradation of IκBα. IκBα degradation induced by 1 µg/ml of LPS is conserved in PSG1 treated THP-1 cells (Fig. 3.20).
Figure 3.17: PSG1 attenuates \textit{IL6} and \textit{TNFA} expression but has additive effect on \textit{IL10}.

PMA-differentiated THP-1 cells were treated with PSG1 at 50 \(\mu\)g/ml. qRT-PCR analysis shows that a small additive effect on \textit{il-10} expression was observed associated with an attenuating effect on \textit{LPS} induced \textit{il-6} and \textit{tnf-\alpha}. \(n=3\), One-way ANOVA with Tukey post-test.
Figure 3.18: PSG1 attenuates IL-6 and TNF-α release in mouse primary macrophages
BMDMs were pre-treated with 5 µM PSG1 for 30 minutes and subsequently challenged with indicated dose of LPS for 6 hrs and 18 hrs. IL-6 and TNF-α release were determined by ELISA. n = 6, Two-way ANOVA with Bonferroni post-test. Error bars are Mean and Standard Deviation.
Figure 3.19: TGF-β1 and PKA signalling can not be ruled out as mediating the observed PSG1 mediated pro-inflammatory cytokine attenuation

Monocytic THP-1 cells were pre-incubated for 1 hr with PKA inhibitors, H89 [5 µM] and PKI(6-22) [1 µM] as well as the TGFβRI inhibitors LY364947 [10 µM] and SB505124 [0.5 µM] followed by 30 min 5 µM PSG1 treatment and subsequent 100 ng/ml LPS challenge for 3 hrs before nucleic acids were harvested for qRT-PCR analysis of IL6, IL10 and TNFA expression. No significant attenuation was observed, n=3, Two-way ANOVA
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Figure 3.20: PSG1 pre-treatment does not inhibit $I\kappa B\alpha$ degradation
THP-1 cells treated for 12 hours with PSG1 or PBS control were challenged with LPS for the indicated times and lyzed to evaluate $I\kappa B\alpha$ degradation by western immunoblot.
3.4 Discussion

Several novel mutants were generated during the course of this research to study the functionality of PSG1. It was aimed to investigate cytokines induced by PSG1 as well as examining the expression and release of LPS-induced cytokines in PSG1-treated cells. It was concluded that IL-6 and IL-10 could not be induced in response to PSG1, contrary to previous reports for PSG1, PSG6, PSG11, PSG17N and PSG18 [149, 157, 153, 148]. This could be a cell line specific effect or even due to the choice of cell line to produce the proteins for this study. It has been reported that the choice of cell line in which PSGs are produced can affect their function [171]. It was found that PSG1 induced TGF-β1 release and in a screen of LPS-induced cytokines, suppressed Cxcl2 expression in a mouse macrophage cell line. Both of these immunomodulatory functions were found to not require the N domain of PSG1 through application of a PSG1ΔN mutant. To further study the functionality of domains and residues in PSG1, it was decided to focus on PSG1-induced TGF-β1 release through testing of the mutants in this assay, as this is a more widely reported PSG response.

However, after much of the work reported in this chapter had been completed, a report demonstrating TGF-β1 contaminates proteins produced in HEK293 cells purified by immobilised metal affinity chromatography (IMAC) was published [265]. This report showed an interaction between the immobilised metal ions and the latent TGF-β1 particle. Both the cell type examined as well as the purification process are applicable to work in this chapter. Further to this, [260] demonstrated that PSG from different mammalian expression systems and also endogenous PSG associate with TGF-β1 during different forms of affinity chromatography. They also demonstrated that PSG1 activates TGF-β1 in a cell free manner. We investigated this in our protein and it was subsequently found that latent TGF-β1 contaminates the proteins used in this study and that after removal by SEC no TGF-β1 induction could be found in response to PSG1 treatment. These publications and our data raise serious questions about a number of earlier publications on PSGs as the immunomodulatory and angiogenic functions ascribed to PSGs are possibly confounded by the presence of TGF-β1. Equally, it is no longer certain whether PSG1 itself induces TGF-β1 or if it is solely an artefact of contamination. It was reported, however that the lymphocytes isolated from PSG1 treated mice in [260] had higher cell surface levels of latent TGF-β1 as
such it is still possible that PSG1 induces TGF-β1. Moreover, insect cell induced PSG1 will be free from TGF-β1 and this has been reported to induce TGF-β1 [149].

While much of the functional data studying domains and motifs in the PSG1 protein in this chapter are confounded by the discovery of a TGF-β1 contaminant at the end of the study, much can still be inferred from the expression studies carried out on the individual domains and the panels of glycomutants. Firstly, the inability to generate individual domains was circumvented by generating the PSG1A1A2 domain protein. This is still a valuable tool that can be used to study whether important functionality exists in the PSG1 N or B2 domains, if utilised with the PSG1 and PSG1ΔN proteins, as was attempted in this chapter.

During the study of the glycoylation sites of the PSG1 protein it was decided to focus again on the more widely studied PSG1N protein. The effect that differences in glycosylation site number can have on the expression and secretion of the PSG1N protein was examined as it was a novel area yet to be examined in PSG research. Sequential mutation of glycosylation sites has an additive negative impact on the secretion of the PSG1N protein. Interestingly however, mutation of the N61 glycosylation site of PSG1N was shown to have a larger, negative impact on secretion of the protein compared to the other single glycosylation mutants. The was also true comparing the N61, 111D to the N104, 111D mutant. Thus, it was concluded that the N61 glycosylation site makes a larger contribution to protein stability and secretion than the other tested sites. The determination that mutation of most N-terminal N-glycosylation site most negatively impacts protein expression and secretion has been found for other proteins also [264]. As this site is absent in fully half of all human and primate PSGs through alterations in the third amino acid of the consensus sequence, it was decided to carry out the reciprocal experiment and introduce the glycoylation consensus sequence into a novel PSG9N mutant through a P63T mutation. This introduced the PSG1-like ‘NLT’ amino acid sequence, and thus glycosylation at this site, in PSG9N. This mutation rescued PSG9N secretion confirming the contribution that the most N-terminal glycosylation site makes to PSG secretion. It must be noted, however, that the PSG9, and PSG4, proteins can be successfully expressed and secreted without this mutation, though lower yields of protein are observed. This raises interesting questions about why this site is then absent in half of human and primate PSGs. It is possible that the detrimental effects on
secretion are balanced by an increase in activity as a result of the structural changes arising from the incorporation of a proline or alanine at this site in the PSG N domain of those proteins which do not possess the glycosylation consensus sequence. This glycosylation site is located on the B-C loop of the protein, an area that shows significant variation in the CEA family \[\text{75}\]. Importantly, this site is also absent in human CEACAM1 implying either loss of this site from CEACAM1 subsequent to PSG radiation from the CEACAM gene family or acquisition in the PSG family after this event. Moreover, it is also absent in mouse Psgs N1 domains except for Psg29 and PSG31 while the N104 and N111 sites are conserved. Further research examining the evolution of this sequence could shed interesting light on PSG function but also on the pressure driving evolution of both the PSG and CEACAM families.

In the second section of this chapter examining the immunomodulatory roles of PSG1, it was demonstrated that the previously reported PSG-induction of IL-6 and IL-10 could not be replicated at the protein level or transcriptional level. In fact, PSG1 attenuated LPS-induction of IL-6 and TNF-\(\alpha\) from monocyctic and differentiated THP-1 cells, as well as primary BMDMs at the transcriptional and protein levels. However there was not significant attenuation in some experiments with results appearing to show variation based on cell choice.

The abortogenic properties of TNF-\(\alpha\) are widely acknowledged with high circulating levels of TNF-\(\alpha\) found in patients with miscarriages \[\text{274}\]. It has, however, been found that while circulating levels of TNF-\(\alpha\) are increased in the serum of preeclampsia patients, that placental levels of the hormone show no difference between normal and preclamptic patients, indicating a different source for the elevated levels of the cytokine \[\text{275}\]. As already mentioned, low levels of PSG correlate with increased preeclampsia incidence as well as higher levels of spontaneous abortion. The very high level of PSG in the serum could potentially be to prevent the overexpression of TNF-\(\alpha\) seen in preeclamptic patients that could result from an over-active maternal immune response to pregnancy.

Increased circulating levels of IL-6 were also found in miscarriage patients \[\text{274}\]. A recent review of the literature surrounding IL-6 in pregnancy outlines that elevated IL-6 has been found in unexplained infertility, recurrent miscarriage, preeclampsia and preterm delivery \[\text{276}\]. Moreover, IL-6 has been mechanistically implicated in the systemic activation of...
endothelium that is a hallmark of pre-eclampsia \[277\]. As already mentioned, these are all disorders correlated with low levels of PSG. The same review highlights evidence indicating "...altered systemic IL6 trans-signalling in women prone to recurrent miscarriage, with excessive IL-6 bioavailability potentially inhibiting generation of CD4+ T regulatory cells required for pregnancy tolerance". Thus PSG could very well contribute to the generation of an environment that is more conducive to a successful pregnancy outcome by attenuating systemic IL-6 and TNF-\[\alpha\] with one of those effects being the increase of the generation of T\(_{\text{reg}}\) cells. Though not studied here, a number of groups have demonstrated a functional role for PSG in this, in different immunological and physiological settings \[157, 260\]. Further research is necessary to elucidate what mechanism is responsible for this.

The discovery of the presence of TGF-\(\beta_1\) as already outlined, represents a confounding factor for much published PSG research directly for work investigating the reported PSG-induced TGF-\(\beta_1\) release. Importantly, given TGF-\(\beta_1\)'s potent angiogenic and immunomodulatory properties, it further represents a confounding factor for the previously reported roles for PSG in these areas also. With regards to the study of immunomodulatory functions attributed to PSGs in this work, it is relevant that active TGF-\(\beta_1\) is largely absent from the proteins utilised in this study. This is likely as a result of the size cut off during the concentration step of purification. However, the high doses of PSGs required make micro-contaminants a concern. Moreover, it could not be ruled out that the large amounts of latent TGF-\(\beta_1\) in some preparations of PSG1 were not being activated in experiments. \[260\] also demonstrate the ability of PSG1 to attenuate IL-6 and TNF-\(\alpha\) as well as IFN-\(\gamma\) measured in lymphocytes isolated from a DSS-induced mouse colitis model. Interestingly, they demonstrate this to be TGF-\(\beta_1\) dependant. To address this TGF-\(\beta_1\)-RI inhibitors were included in the experiments examining PSG1 attenuation of LPS-induced pro-inflammatory cytokines in this chapter. While there was a slight trend for attenuation of IL-6 in the THP-1 cells used there was no significant attenuation of pro-inflammatory cytokines. Thus it was not possible to draw conclusions as to the roles of signalling pathways in these effects.

It could not be demonstrated that TGF-\(\beta_1\) signalling is not having an affect on the attenuation of pro-inflammatory cytokines by PSG1 in monocytic THP-1 cells. Moreover,
the hypothesised role of PKA signalling could not be ruled out as contributing to this. It is possible that differences exist in mechanisms of PSG1 action on myeloid and lymphocytic cells. Further tests in more suitable cell lines are required.

In addition, to further examine the mechanism by which PSG regulated the induction of LPS-induced cytokines, the levels and degradation pattern of a key regulator of NF$\kappa$B activity and localisation, I$\kappa$B$\alpha$, were assessed in untreated and PSG1 treated THP-1 cells. It was found that LPS-induced degradation of I$\kappa$B$\alpha$ were unaffected by PSG1 treatment, implying that PSG1 must be regulating the expression of NF$\kappa$B-regulated genes in a different way. either through inhibition of this pathway downstream of I$\kappa$B$\alpha$ or through the inhibition of NF$\kappa$B sites in these genes.
Chapter 4

PSG1 is an integrin $\alpha_V\beta_3$ ligand

4.1 Introduction

The exact function of PSGs in normal pregnancy remains elusive but, as already outlined, they have angiogenic, immunomodulatory and anti-platelet properties. The cell surface interactions that mediate much of the functions of PSGs are incompletely understood. It is known that both human and mouse PSGs have affinity for cell surface proteoglycan side chains, specifically for syndecan family members and glypican-1 \[158, 170, 156]\. Mouse Psgs have been shown to differentially bind tetraspanin CD9 while PSG1 has been shown to not bind this receptor \[150, 154, 158, 171]\. The interaction of PSG17 with CD9 has been demonstrated to mediate the cytokine induction from this PSG \[153]\. In addition, it has been shown that the angiogenic properties of PSG1 require cell surface proteoglycans \[170]\. In addition protein N-glycosylation has been demonstrated to affect the affinity of recombinant PSGs for the cell surface \[154, 171]\.

Integrins have been hypothesised by many to represent potential binding partners for PSGs \[179, 278, 81]\. This has been largely due to the presence of a conserved proposed integrin binding RGD-like tri-peptide motif in primate and rodent N and N1 domains \[81]\. Moreover, the accumulating evidence for heperan sulphate proteoglycan (HSPG) interactions with PSGs supports this, as these cell surface proteins, especially syndecans, are known organisers of integrin activity and clustering at the cell surface, as is the case for
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Integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ in carcinoma and endothelial cells \[279\]. This is reviewed further in \[175, 178, 280\]. Syndecans not only function as regulators of adhesion and migration through modulation of integrin activity but also as docking receptors for a multitude of extracellular ligand receptor interactions \[280\]. Syndecans can regulate extracellular events without signalling to the cytoplasm through their protein core by binding and modifying the bio-availability of ligands at the cell surface through their glycan sidechains \[281, 177\]. This activity is seen in ligands such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), and transforming growth factor b1 (TGFb1) which have been shown to interact with and in may cases require syndecan glycan sidechains as well as their cognate receptors \[282, 283, 284, 285, 286, 287, 288\]. PSG interaction with syndecans has not been demonstrated to induce cell signalling events, thus it is possible that the binding of PSG to the glycan sidechains of HSPGs serves a docking function.

PSG interaction with an integrin was demonstrated for the first time by \[180\] where it was found that the interaction of PSG with the platelet integrin $\alpha IIb\beta 3$ inhibited the interaction between platelets and fibrinogen thus identifying a novel anti-platelet property for PSGs. It is argued that this function could have evolved to "antagonise abundant (3 mg/ml) fibrinogen in the maternal circulation, which may be necessary to prevent platelet aggregation and thrombosis in the prothrombotic maternal environment of pregnancy" \[180\]. Interestingly this interaction was found to reside in multiple domains of PSG1 and to occur independent of the solvent exposed N-domain KGD tri-peptide that PSG1 has in common with some snake venom disintegrins \[289, 180\].

Even though the interaction of PSG1 with integrin $\alpha IIb\beta 3$ was KGD independent, nevertheless, a strong case exists for investigating other RGD-recognising integrins as potential receptors for PSGs. Many RGD-recognising integrins are expressed on cells types responsive to PSG or are to be found exposed to PSG contact in the vasculature or the placenta as outlined in the introduction. These include integrins $\alpha M\beta 2$, $\alpha 5\beta 1$ and $\alpha V\beta 3$. Moreover there is evidence of interactions of RGD containing compounds with RGD-recognising integrins in an RGD-independent manner, as is the case for tumistatin and the integrin $\alpha V\beta 3$ \[290\]. In addition, it has been demonstrated that blocking of integrin $\alpha V\beta 3$ by
cyclic RGD inhibits the additive effect ligation of this integrin has on LPS-induced and TNF-\(\alpha\)-induced pro-inflammatory cytokine expression in macrophages\[291\]. This correlates with PSG1s pro-inflammatory cytokine attenuation as demonstrated earlier in this thesis. 

Integrins \(\alpha V\beta 3\) and \(\alpha IIb\beta 3\) have been implicated in interactions between platelets and HUVEC cells \[292\]. Furthermore, as outlined earlier, leukocyte adhesion and transmigration through the endothelium are critical steps in the inflammatory process in which integrins play a fundamental role. In addition it has been demonstrated that integrin \(\alpha V\)-specific RGD mimetics attenuate mononuclear cell invasion into an allogenic renal graft model \[293\]. Given PSG1s inhibitory function on platelet integrin, as well as evidence of the association of PSG with the maternal vasculature in pregnancy \[294\] it is hypothesized that PSG1 could also have a similar effect on this aspect of normal leukocyte biology by inhibiting the interaction of leukocytes with endothelial cells.

4.2 Objectives and Outcomes

In generating my evidence supporting the existence of an interaction between the platelet integrin \(\alpha IIb\beta 3\) and PSG1, a cell based Fluorescent Ligand Binding Assay was developed to demonstrate affinity for integrin \(\alpha IIb\beta 3\) using stable empty vector transfected (Mock) control cells and integrin over-expressing cells and fluorescently labelled PSG1. One objective of this work was to determine if this assay could be modified to allow for the detection of endogenous PSG binding using anti-PSG mAbs. It was shown that endogenous PSG from pooled term pregnant plasma behaved similarly to recombinant PSG1 in this assay. In addition, other RGD-recognising integrins were selected as candidate receptors for PSG1. Integrin \(\beta 3\), \(\alpha V\beta 3\) and \(\alpha 5\beta 1\) over-expressing CHO cells were sourced and the over-expression of integrins on these cells was characterised to determine their suitability for application to the Fluorescent Ligand Binding Assay. Only integrin \(\beta 3\) CHO cells showed sufficient over-expression of the respective integrin \(\alpha V\beta 3\) heterodimer to justify application to the assay from which a trend for increased binding of PSG1 to integrin over-expressing cells was observed. This assay was further supported by \textit{in vitro} binding data demonstrating PSG1 interacting with integrin \(\alpha V\beta 3\) purified from placenta. In addition, the hypothesis that PSG1
4. PSG1 is an integrin $\alpha_V\beta_3$ ligand

could affect monocyte-endothelial interactions was tested in a static fluorescent monocyte-endothelial interaction assay but found to not have any affect.

4.3 Results

4.3.1 Development of a cell-based Fluorescent Ligand Binding Assay (FLBA)

During the investigations into the interaction between PSG and integrin $\alpha_{IIb}\beta_3$, a plate based Fluorescent Ligand Binding Assay (FLBA) was developed to provide further evidence in support of the interaction. Integrin $\alpha_{IIb}\beta_3$ over-expressing CHO-K1 cells (a kind gift from Prof. Niamh Moran, Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland) were enriched by FACS to select for highly over-expressing cells relative to the empty vector transfected mock control cells. The cell sorted integrin over-expressing cells showed an approximate 100 fold enrichment of the integrin $\alpha_{IIb}\beta_3$ relative to the mock control cells (Fig. 4.1(A)). Though these cells were over-expressing the integrin, this over-expression dropped when cells were routinely maintained in culture, requiring periodic enriching of the cells by FACS. These cells were seeded and fluorescently labelled PSG1 was incubated with the cells and the plate processed as outlined in the materials and methods. Figure 4.1(B) shows fluorescent PSG1 binding to enriched integrin $\alpha_{IIb}\beta_3$ over-expressing cells and mock controls, as well as SYTO60® nucleic acid staining acquired on an Odyssey infrared imaging system. Densitometric analysis of this staining, when normalised to nucleic acid stain, shows a greater than 50% increase in binding of fluorescent PSG1 to integrin $\alpha_{IIb}\beta_3$ over-expressing cells relative to the mock control cells confirming the in vitro binding data (Fig. 4.1(C)) [180].

With a goal of adapting the FLBA for the examination of endogenous PSG binding, the assay was modified to optimise detection of the binding of PSG1 by monoclonal antibodies against PSG, which we are characterising, namely mouse anti-PSG1 mAb5 (a kind gift from Stepan Jonjic, Croatia). The mAb binding was detected using a fluorescently labelled secondary antibody. Cell density was again controlled for using the SYTO60® nucleic acid stain. Figure 4.2(A) shows a representative Indirect Fluorescent Ligand Binding Assay.
4. PSG1 is an integrin αVβ3 ligand

4.3 Results

(iFLBA) image. When images were quantified and normalised to nucleic acid stain the iFLBA detects a greater than 50% increase in PSG1 binding to the integrin αIIbβ3 over-expressing cells (Fig. 4.2(B)). Thus a cell-based assay system for PSG-binding has been developed which can be applied to see if a similar increased binding to integrin αIIbβ3 over-expressing cells by PSG from plasma samples of pregnant women can be detected.

Plasma samples from 6 pregnant women at term were mixed and applied to the assay in quadruplicate. Figure 4.3(A) shows a representative iFLBA. A greater than two-fold increase in PSG binding to integrin over-expressing cells versus mock controls was detected using the plasma samples (Fig. 4.3(A)). This indicates that endogenous PSG appears to have a similar affinity for platelet integrin αIIbβ3 supporting published work [180]. Non-pregnant sample controls proved toxic to cells and so were not investigated further.
4. PSG1 is an integrin α\textsubscript{V}β\textsubscript{3} ligand

4.3 Results

Figure 4.1: Fluorescently labelled PSG1\textsuperscript{800} shows increased binding to integrin α\textsubscript{IIb}β\textsubscript{3} dual overexpressing CHO-K1 cells in Fluorescent-Ligand Binding Assay (FLBA)

(A) Empty vector transfected mock control cells and α\textsubscript{IIb}β\textsubscript{3} dual over-expressing CHO-K1 cells were incubated with fluorescently labelled anti-CD41 antibody and highly expressing cells were selected by FACS for propagation and subsequent experimentation.  
(B) Representative image of PFA-fixed CHO-K1 cells incubated with PSG1\textsuperscript{800} and stained with SYTO60® nucleic acid stain and (C) quantification of PSG1\textsuperscript{800} binding normalized to nucleic acid stain. n=5, students t-test.
4. PSG1 is an integrin αvβ3 ligand

4.3 Results

Figure 4.2: PSG specific mouse monoclonal detects increased PSG1 binding in Indirect FLBA

(A) Representative image showing increased binding of PSG1 to αIIbβ3 dual over-expressing CHO-K1 cells over empty vector transfected Mock control with SYTO60® nucleic acid stain to control for cell number. (B) Graph of mean fluorescent intensity showing this difference is significant, p < 0.05, students t-test, n=3.

Pregnancy Specific Glycoprotein Function, Conservation and Receptor Investigation

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4. PSG1 IS AN INTEGRIN $\alpha_v\beta_3$ LIGAND

4.3 Results

Figure 4.3: Endogenous PSG shows increased binding to $\alpha_{IIb}\beta_3$ dual over-expressing CHO-K1 cells in anti-PSG mAb LBA

(A) Representative image showing increased binding of edogenous PSG from maternal plasma to $\alpha_{IIb}\beta_3$ dual over-expressing CHO-K1 cells over empty vector transfected Mock control cells with SYTO60® nucleic acid stain to control for cell number. (B) Graph of mean fluorescent intensity showing this difference is significant, $p<0.05$, students t-test, $n=3$. 

$\begin{array}{c|c|c}
\text{gt-$\alpha$-ms}^{680} & \text{Mock} & \alpha_{IIb}\beta_3 \\
\hline
\alpha$-PSG & + & ++ \\
Plasma & + & + \\
\hline
\alpha$-PSG mAb5 & + & + \\
Syto60 & - & + \\
\end{array}$
4.3.2 PSG1 binds integrin $\alpha_V\beta_3$

As part of the research into whether RGD-recognising integrins could serve as receptors for PSG1, a number of functional blocking experiments were carried out to examine whether integrin $\alpha_V\beta_3$ and $\alpha_M\beta_2$ blockade of the THP-1 monocytic cell line could affect PSG1 induction of TGF-$\beta_1$. However, given the subsequent finding that latent TGF-$\beta_1$ contaminates recombinant PSG1 generated for this study and that SEC purified PSG1 did not induce TGF-$\beta_1$ release, these data have not been included. Investigations into candidate integrin $\alpha_5\beta_1$ were carried out on over-expressing cells (a kind gift from Prof. Errki Ruoslahti, Sandford Burnham Medical Research Institute, La Jolla, CA, USA). Empty vector transfected Mock control and over-expressing cells were screened for over-expression to ascertain suitability for application to the iFLBA. However these cells proved negative for integrin over-expression as determined by FACS meaning no population of enriched integrin over-expressing cells could be isolated for further study Figure 4.4.

The identification of a $\beta_3$ integrin containing binding partner for PSG1 led us to hypothesize that other $\beta_3$ integrins might represent binding partners for PSG, mediating known immunomodulatory properties or, given the anti-thrombotic role underpinned by the PSG-platelet integrin interaction, novel anti-adhesive or anti-migratory functions for PSGs. To that end integrin $\beta_3$ over-expressing and integrin $\alpha_V\beta_3$ dual over-expressing cells as well as mock controls were obtained (a kind gift from Prof. Yoshikazu Takada, Department of Dermatology, UCDavis, California, USA) and assayed by FACSscan for over-expression of the $\beta_3$ subunit and $\alpha_V\beta_3$ heterodimer respectively, using $\beta_3$ specific and $\alpha_V\beta_3$ heterodimer specific antibodies. Figure 4.5(A) shows that the $\beta_3$ integrin over-expressing cells were positive for the $\beta_3$ subunit and that this subunit was contained in similar levels of $\alpha_V\beta_3$ heterodimer. The $\alpha_V\beta_3$ dual over-expressing cells did not stain positively. The $\beta_3$ CHO cells were applied to the iFLBA. The integrin over-expressing cells were enriched for FACS using an APC-labelled anti CD51 antibody and applied to the iFLBA. Figure 4.5(B) shows a representative iFLBA image. Figure 4.5(C) demonstrates that PSG1 shows a trend of increased binding to the $\beta_3$ CHO cells over mock controls.

The next step was to confirm the interaction between these two proteins biochemically.
Integrin αVβ3 purified from human placenta was purchased to carry out in vitro binding assays with PSG1 as has been demonstrated for integrin αIIbβ3. Briefly, purified integrin was immunoprecipitated as outlined in the Materials and Methods chapter section 2.13. Protein G bound integrin was then re-suspended in buffer with 2 µg of PSG1 for 1 hr at room temperature. Protein bound beads were recovered boiled and analysed by SDS-PAGE and western immunoblot for integrin and PSG1. Figure 4.6 shows that, as previously identified, PSG1 interacts with integrin αIIbβ3 and the integrin αVβ3, confirming the earlier iFLBA result with the β3 CHO cells. Following the confirmation of this interaction by iFLBA and by the in vitro binding assay, it was aimed to investigate whether blocking the integrin would alter the affinity of PSG1 for circulating blood cells.

To identify whether blocking of integrins negatively affected PSG1 affinity to monocytes. A preparation of peripheral blood mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation were incubated with blocking antibodies against integrins αVβ3 and another important integrin on monocytes and macrophages, αMβ2. PBMCs were subsequently incubated with fluorescently labelled PSG1488 and anti-CD14 antibody. After washing, multi-channel FACS analysis was used to examine whether integrin blockade had any affect on PSG1488 affinity for CD14+ gated monocytes. Figure 4.7(A) shows the staining of CD14+ monocytes with fluorescent PSG1. From the mean fluorescent intensity graph it can be seen that blockade of integrins αVβ3 or αMβ2 had no affect on PSG1488 binding to cells.

A large body of evidence has been gathered implicating a role for HSPGs as mediators of attachment of PSGs to the surface of cells. The affinity of two murine PSGs for several HSPGs was investigated by Sulkowski and colleagues. The authors demonstrated that PSGs 17 and 23 shared an interaction with HSPGs but that only PSG17 bound to the tetraspanin CD9. Lisboa and colleagues demonstrated that the presence of glycosaminoglycans on the cell surface is required for PSG1 attachment and further demonstrated that transfection of cells deficient in condroitin and herparan sulphate, with Syndecans 1-4 and glypican-1, increased binding of PSG1 the authors also demonstrated that PSG1-induced tubuleogenesis is dependant on the interaction of PSG1 with cell surface proteoglycans. Subsequently Blois and colleagues demonstrated PSG22 binding to heparan sulphate chains of syndecans.
Consequently, the effect of heparin and heperan sulphate on fluorescent PSG1 binding to a monocytic cell line was investigated. Figure 4.8 shows that both heparin and heperan sulphate inhibit the interaction between fluorescent PSG1 and the THP-1 monocytic cell line. This confirms the previous findings that cell surface proteoglycans are significant mediators of the attachment of PSG1 to the cell surface. As such it is unlikely that integrin blockade would affect the affinity of PSG1 for the cell surface.

**Figure 4.4: Integrin $\alpha_5\beta_1$ cells are negative for the integrin**
Empty vector Mock control cells and integrin $\alpha_5\beta_1$ over-expressing cells were stained with anti-integrin $\alpha_5\beta_1$ antibody and FITC-labelled secondary before being analyzed by FACS. The $\alpha_5\beta_1$ cells appeared to have lost over-expression of the $\alpha_5\beta_1$ integrin.
4. PSG1 is an integrin $\alpha_v\beta_3$ ligand

4.3 Results

$\beta_3$ mAb  $\alpha_v\beta_3$ mAb

Mock 2°  $\beta_3$ mAb  $\alpha_v\beta_3$ mAb

Mock mAb  $\beta_3$ mAb  $\alpha_v\beta_3$ 2°

Mock β3

700
600
500
400
300
200
100
0
Fluorescent Intensity

Figure 4.5: PSG1 binding to integrin $\alpha_v\beta_3$ cannot be detected by indirect FLBA

(A) Integrin $\alpha_v\beta_3$ and $\beta_3$ over-expressing CHO cell lines as well as mock controls were examined for expression of $\beta_3$ and $\alpha_v\beta_3$ by FACS with antibodies that distinguish the individual $\beta_3$ and $\alpha_v\beta_3$ heterodimer molecules. $\beta_3$ CHO cells stain positive with both antibodies but $\alpha_v\beta_3$ CHO cells showed no over-expression compared to controls. (B) Application of mock and $\beta_3$ CHO cells to indirect FLBA could not detect significantly increased PSG1 binding to $\beta_3$ over-expressing CHO cells versus control though a trend of increased binding was present.
4. PSG1 is an integrin $\alpha\_V\beta\_3$ ligand

4.3 Results

Figure 4.6: PSG1 interacts with purified integrins $\alpha_{IIb}\beta\_3$ and $\alpha\_V\beta\_3$ in vitro in a protein pull down assay

Immunoblots of immunoprecipitations showing PSG1 pulls down with integrins $\alpha_{IIb}\beta\_3$ and $\alpha\_V\beta\_3$ in solution. Work of Pat Kiely, University of Limerick, Ireland.

| Integrin $\alpha_{IIb}\beta\_3$ | + | - | - |
| Integrin $\alpha\_V\beta\_3$ | - | + | - |
| PSG1 | + | + | + |
| Anti-integrin IP antibodies | + | + | - |
4. PSG1 IS AN INTEGRIN $\alpha_v\beta_3$ LIGAND

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Blocking antibodies do not affect PSG1-488 binding

Figure 4.7: Anti integrin $\alpha_v\beta_3$ and $\alpha_M\beta_2$ blocking antibodies do not affect PSG1$^{488}$ binding to primary monocytes

(A) Representative FACS staining of CD14+ gated cells for PSG1$^{488}$ binding in presence of integrin blocking antibodies. (B) Graphical representation of MFI of n = 3 from 3 separate donors showing integrin blocking antibodies have no effect on PSG1$^{488}$ binding, n=3.
4. PSG1 IS AN INTEGRIN α\textsubscript{V}β\textsubscript{3} LIGAND

4.3 Results

Figure 4.8: Heparin and heperan sulphate block PSG1\textsuperscript{488} binding to THP-1 monocytic cell line

(A) Representative FACS staining of THP-1 cells for PSG1\textsuperscript{488} binding in presence of indicated concentrations of heperin and heperan sulphate.  (B) Graphical representation of MFI of PSG1\textsuperscript{488} binding. One-way ANOVA, n=3.
4.3 Results

4.3.3 PSG1 and endothelial interactions

The final stage of this body of work was to determine if a novel functional role for the interaction between PSG1 and integrins could be determined. Given the nature of integrins as adhesion molecules and furthermore given the published function of PSG as an inhibitor of integrin \( \alpha_{IIb}\beta_3 \), taken with the notably high levels of PSG in circulation, it was decided to investigate whether PSG1 affected the attachment of monocytes to TNF-\( \alpha \)-activated endothelium. This physiological process represents a critical step in the process of inflammation and as targeting this could significantly attenuate the immune response it is considered a good candidate for investigating in the context of potential PSG functions in pregnancy. Figure 4.9(A) shows representative false coloured ImageJ analysed immunofluorescence images of wells with fluorescently labelled monocytes adhered to HUVEC-2 endothelial cells for the indicated times and with the indicated treatments. Densitometric analysis reveals that PSG1 has no significant effect on monocyte adhesion Fig. 4.9(B).
Figure 4.9: PSG1 attenuates monocyte attachment to activated endothelium

(A) Representative Image-J output, false-coloured images from analysis of one replicate of endothelial attachment assay. (B) Graphical representation of measurement of pixel area of fluorescent THP-1 attachment to endothelial cells, n=3.
4.4 Discussion

The identification of the intergrin $\alpha_{\text{IIb}}\beta_3$ as a binding partner for PSG demonstrated a novel anti-platelet function for these hormones [180]. In this chapter the published assay demonstrating increased binding of fluorescent PSG1 to integrin over-expressing cells was modified allowing for the confirmation of this interaction with endogenous PSG as detected by anti-PSG monoclonal antibody. To further extend this assay format, several stable integrin over-expressing cells were sourced and screened for over-expression of the respective integrins. When tested, integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$ over-expressing cells were negative for integrin over-expression. Integrin $\beta_3$ CHO cells tested positive for $\alpha_v\beta_3$ heterodimer over-expression and were enriched and applied to the modified iFLBA. This identified a second potential integrin binding partner for PSG1 as the assay detected a trend for increased PSG1 binding to the $\beta_3$ CHO cells versus mock controls. While the assay did not give a statistically significant result, it is believed that this is likely due to the low level of integrin over-expression in comparison to the integrin $\alpha_{\text{IIb}}\beta_3$ CHO cells. These cells showed an almost 100-fold increase in expression while the $\beta_3$ CHO cells showed $<10$ fold over-expression. A major difficulty found in utilising stably over-expressing cell lines is the cumulative loss of expression from these cells over time, leading to a need for continual enrichment through FACS. Moreover, significant differences in the quality of the cells used makes accurate comparisons difficult. It is felt that a switch to a transient dual over-expression system might yield better results for the type of assays utilised here.

However, to verify the integrin $\alpha_v\beta_3$ assay biochemically, purified integrin $\alpha_v\beta_3$ from placenta was incubated with PSG1 in an in vitro binding assay. This confirmed the earlier identified interaction with platlet integrin $\alpha_{\text{IIb}}\beta_3$ and further confirmed the iFLBA result with the $\beta_3$ CHO cells. Thus a novel integrin binding partner for PSG1 has been identified in the integrin $\alpha_v\beta_3$, mediating an as yet unknown function. As already outlined functional blocking experiments were carried out with anti-$\alpha_v\beta_3$ blocking antibody LM609 on THP-1 cells to investigate whether this antibody could block PSG1 induced cytokine release. The data proved negative, however no conclusions could be drawn from the data due to the earlier described contaminating latent TGF-β1. This contamination has been published and it was shown that TGF-β1 free PSG1 immunomodulatory effects were still TGF-β1 dependant.
4. PSG1 is an integrin αVβ3 ligand

4.4 Discussion

based on a novel cell-free ability of PSG1 to activate latent TGF-β1 [260], though earlier work in this thesis appears to contradict this. While not investigated in this body of work, RGD tri-peptides have been shown to attenuate the ability of integrin αVβ3 to potentiate the inflammatory response of macrophages to LPS and TNF-α [291]. It is possible that PSG1 treatment has a similar effect on αVβ3 integrin and inflammation. This evidence is in agreement with the earlier pro-inflammatory cytokine attenuation observed in this thesis. The application of a PSG1N KGD→AAA mutant to the in vitro binding assay would elucidate whether, as is the case with integrin αIIbβ3, integrin αVβ3 binds PSG1 in an KGD-independent manner. This requires further investigation. Moreover, the possibility that PSG1 binds other RGD recognising integrins such as α5β1 and αMβ2 cannot be ruled out. In addition, if PSG1 is found to bind multiple integrins, it is possible that some integrin interactions may prove to be KGD-dependant.

Investigations into the affinity of fluorescently labelled PSG1 for CD14+ primary monocytes showed that blocking antibodies had no effect on PSG1 cell surface binding. However, as outlined, several publications have shown HSPGs such as Syndecans and Glypican-1 to be important mediators of the binding of PSGs to the cell surface. In agreement we this we demonstrated that soluble heparin and heparan sulphate blocked PSG1 binding to monocytic THP-1 cells.

To investigate the hypothesis that, based on the identified inhibitory effect of PSG1 on platelet integrin αIIbβ3, PSG1 could play a role in attenuating inflammation through affecting the activities of integrins involved in the process of recruitment of leukocytes to sites of inflammation, we performed a static fluorescent leukocyte-endothelial adhesion assay. The data demonstrate that PSG1 did not negatively impact on the attachment of monocytic THP-1 cells to TNF-α activated HUVEC-2 cells. While the preliminary investigations into this area have proved negative, more refined approaches may yet yield a result. Moreover, potential roles for PSG in altering vascular permeability and leukocyte transmigration were not investigated due to time constraints and represent interesting areas for continued research. Importantly RGDS peptides have been demonstrated to not exhibit anti-adhesive effects on HUVEC cells seeded on collagen IV, however the did attenuate FGF-2 mediated chemotaxis [295]. Moreover, a role for integrin αVβ3 ligand vitronectin in
vascular permeability has recently been identified lending further support to the hypothesis that PSG1, an integrin $\alpha_v\beta_3$ ligand, requires investigation in these types of assays [296].
Chapter 5

Conservation of Function in Novel Horse PSG-like CEACAM49

5.1 Introduction

As highlighted in the introduction, PSGs are found in higher primates and rodents and their proposed immunological, angiogenic and haemostatic functions imply fundamental roles in the haemochorial placental phenotype common to these two orders of mammals. PSG genes are, however, also found in bats and horses. In bats there is evidence of invasive haemochorial placentation in many species, therefore presence of PSG genes in this order correlates with the invasive haemochorial phenotype. However, in horses this correlation breaks down somewhat as they do not possess a haemochorial placental phenotype, rather, an epitheliochorial one. Interestingly, the horse placenta does possess unique highly invasive, immunoreactive, trophoblastic structures that exist for a limited period during gestation, the endometrial cups. These are small, 1-10 cm long structures composed of invasive trophoblasts that form in the early stages of equine placentaion. Following the arrest of the equine blastocyst in the uterus due to its dramatically increasing size and a concomitant increase in uterine tone, the girdle of proliferative trophoblast cells on the conceptus are directly apposed to the maternal epithelium. These are the source of the endometrial cup cells. They digest through the maternal basement membrane and
5. CONSERVATION OF FUNCTION IN NOVEL HORSE
PSG-LIKE CEACAM49

5.1 Introduction

establish themselves in the maternal tissue, progressively invading maternal uterine glands. They are the source of equine CG \(^{299, 300}\). The cells of the endometrial cups induce a strong innate and cell mediated immune response from the mother, eventually leading to destruction of the cups 2-3 months into gestation, which normally lasts approximately 360 days. A correlation has been observed between the stage of the developing trophoblast cup cells and the maternal immune response \(^{301}\). There is an initial strong response that is reduced between days 45 and 60 which subsequently increases again following this stage, where the leukocytes are seen to invade from the peripherary and base of the cups, where they have been accumulating, and begin actively destroying the necrotic cup tissue. The other changes involved in the early stages of horse placentation that ultimately lead to the extensive non-invasive microcotyledonary endotheliocorial placenta, but which are not directly related to the biology of the trophoblast, are reviewed extensively in \(^{302}\).

Adams and colleagues utilised a skin allo/auto-graft experiment in mares followed by mating with the stallion allograft donors to the, now, immunologically primed mares, to demonstrate the assymetric immune response observed between the endometrial cups and the circulating immune system during equine pregnancy \(^{303}\). The development of strong humoral antibody responses, in first and second pregnancies in primed mares, or in second pregnancies in un-primed mares, was not concurrent with a strong cellular immune response in the endometrial cups. Early invasive chorionic girdle cells in the horse, as distinct from other species, express high levels of paternal MHC antigens \(^{304, 305}\). These are down-regulated during differentiation into the endometrial cup cell lineage \(^{306}\). However, the short-term expression of MHC is sufficient to induce an early and stronger maternal anti-MHC response in horses than in any other species examined \(^{307}\). Thus factors inherent to the endometrial cups are attenuating the maternal immune response to the cup tissue for the duration of the cups in pregnancy. Flaminino and colleagues demonstrated that isolated invasive trophoblastic cup cells can reduce lymphocyte proliferation and alter expression of cytokine mRNA such as IFNgamma \(^{308}\). Analysis of the lymphocyte sub-populations in equine pregnancy confirmed this split immunological tolerance between the peripheral blood lymphocytes and those at the maternal fetal interface with higher levels of IFN-\(\gamma^+\) and FOXP3+ lymphocytes found in the populations.
from the endometrial cups [43]. In a further extension of this concept, ectopically transplanted chorionic girdle cells were found to survive for a time period similar to that of the endometrial cups in the mare while reproducing physiological changes associated with pregnancy such as estrus suppression [309] [44]. This confirms the importance of innate immunoregulatory functions of equine trophoblasts to their prolonged survival.

Therefore, as pointed out by Kammerer and Zimmerman, the presence of PSGs in the horse, while not adhering to the correlation between PSG and haemochorial placentation in species, does still correlate with a local invasive trophoblastic phenotype [58]. Furthermore, the immunological suppression associated with the cup environment, where the equine PSG-like CEAM expression is seen, is similar to the functions proposed to be carried out by the PSGs in their sites of expression in other species. We hypothesised that horse PSGs might function similarly to human and mouse PSGs in immunomodulatory and anti-platelet assays. We therefor tested putative horse PSG CEAM49 in assays examining these functions.

### 5.2 Objectives and Outcomes

The putative equine PSG genes identified by Kammerer and Zimmerman were supplemented with further genes identified by the author subsequent to publication [58]. Phylogenetic analysis of the horse CEA family confirmed the relationship between equine CEAM and the putative horse PSG genes. Furthermore, given the unique nature of placentation in the horse and the distant nature of the relationship between it and other mammals with PSGs, I looked for othalogous relationships between the putative horse PSGs and the PSGs of other animals. No orthologous relationships were found. An alignment of protein sequences of the horse PSG1 leader sequence and N-domains with human PSG1 leader sequence and N-domain was generated to illustrate sites of conserved amino acids. To establish whether the putative equine PSGs are indeed functionally equivalent to a member of this family, CEAM49, was cloned into a mammalian expression system and protein was made to test in various assays where PSG function has been explored earlier in this thesis. It was found that horse PSGs do not induce TGF-β_1.
however, and in contravention to the expected hypothesis given the epitheliochorial nature of the equine interhaemal barrier, CEACAM49 did disrupt the human platelet-fibrinogen interaction.

5.3 Results

Determining the relatedness of the horse putative PSGs to the horse CEACAM family supports their identification as CEACAM1-related genes, as alignment and phylogentic tree analysis follows the pattern outlined in [58] whereby the PSGs cannot be significantly discriminated (50% cut-off) from a broad CEACAM1-like cluster (Fig. 5.1(B)). Within the CEACAM1-like cluster the putative PSG genes do not cluster together.

In order to determine whether any putative horse PSGs showed orthology to the PSGs in other species, a pairwise alignment of the N-domains of available PSG sequences excluding the leader sequence, in baboon, chimpanzee, human, mouse, rat and horse was carried out. A phylogenetic tree generated using maximum parsimony was utilized to infer the closeness of relatedness of the genes (Fig. 5.2). The tree reveals the previously reported orthologous relationship between some rodent PSGs (Williams, J., PhD thesis). The horse PSGs cluster together indicating the lack of any orthologous relationships supporting the hypothesis that these genes were likely an independent adaptation in the horse from a CEACAM1-like ancestor common to all species examined.

Next, the horse putative PSGs were examined by aligning the protein sequences encompassing the leader sequences and N domains against the leader sequence and N domain of PSG1 (Fig. 5.3(A)). This allowed for the identification of conserved amino acid sequences that may be critical to the structure and function of the horse proteins. The sequences were analyzed by the Signal-P database to predict the cleavage site of the leader peptide. The cleavage site for PSG1 was earlier identified by N-terminal protein sequencing (Fig. 3.2). For most of the horse PSGs the software predicts cleavage after amino acid 35, except in instances of a substitution at this site of a lysine (K) for a glutamine (Q). Here the software predicts a more N-terminal cleavage site. There is considerable variation in amino acid conservation around this site. Generation of the proteins and
confirmation of the cleavage site by protein sequencing would be required to confirm these data. Furthermore, horse PSGs show considerable variation in the solvent exposed F-G loop where the conserved RGD-like tri-peptide is located in primate and rodent N- and N1-domains respectively. Horse PSGs do possess an RGD-like tri-peptide in the C-C' loop. However, based on homology modelling of human PSG1, this loop is not believed to be as solvent exposed as the F-G loop in PSGs. The lysine (K34) of this loop is predicted to be surrounded by residues of the C' and E strands reducing its bioavailability (personal communication, Dr. Rob Meijers, EMBL, Hamburg) (Fig. 5.3(B)). In human and mouse PSGs these residues would be located in a difficult to access region of the proteins N- or N1-domains that would require complex folding of the remaining PSG domains for access. However, as the horse PSGs are only composed of N-domains access to these residues is not restricted by other domains. There is also significant variation in the NetN glyc database predicted glycosylation pattern of the horse PSGs. CEACAM49 has five predicted glycosylation sites, while the remaining horse PSGs have between one and three sites.

To assay the functionality of the horse putative PSGs, CEACAM49 was subcloned from pRcCMV-ecaCEACAM49 into the pTT3 expression vector incorporating an C-terminal V5-6xHis tag as outlined in the materials and methods section. This allowed for the generation of horse PSG-like CEACAM49 protein in the Freestyle™293 expression system. Figure 5.4(A) shows the chromatogram of the imidazole elutions from the CEACAM49 bound beads. The elution pattern was further observed by coomassie staining of the indicated fractions which had been separated by 12% SDS PAGE (Fig. 5.4(B)). The indicated fractions were pooled, concentrated and dialyzed and 2 µg of the resultant protein was separated by 12% SDS PAGE and stained by coomassie. This showed that there were contaminating high molecular weight bands. To remove these the protein was further purified by SEC. Figure 5.5(A) shows the chromatogram from application of the affinity purified CEACAM49 to an Superdex 200 prep grade column in PBS. The indicated fractions were pooled and concentrated. The resultant protein along with the pre-SEC starting material was separated by 12% SDS PAGE and stained with Gel Code Blue stain reagent (Fig. 5.5(B)). This showed that the SEC effectively removed the contaminating proteins. Furthermore, as TGF-β1 was identified as a contaminant of PSG preparations, the starting material and SEC
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Purified CEACAM49 were tested for TGF-β1 contamination. Figure 5.5(C) shows that SEC efficiently removed the contaminating TGF-β1 from CEACAM49. It was then investigated whether, as published for PSG from other species, CEACAM49 could induce TGF-β1 release from two previously reported PSG-responsive cell types. Figure 5.5(D) and (E) show that 24 hr treatment of human monocytic THP-1 cells and HUVEC-2 endothelial cell lines with CEACAM49 did not induce TGF-β1 release relative to untreated, PBS and CEACAM1 controls. This is in keeping with earlier findings that PSG1 does not induce the release of this cytokine from these cell lines.

To test the hypothesis that due to the physiology of the horse placenta and the possibility that the endometrial cups contact the maternal blood before their destruction three months into gestation, that the horse PSGs might exhibit the ability to inhibit the platelet-fibrinogen interaction, the CEACAM49 protein was applied to a fluorescent fibrinogen-platelet interaction assay as described in the materials and methods and in [180]. The result showed that CEACAM49 inhibited the interaction in a dose dependant manner similar to PSG1. Figure 5.6(A) shows the FACS graph for the 200 µg/ml treatment with PSG1 and CEACAM49. Figure 5.6(B) is a graphical representation of the fluorescent fibrinogen binding as a percentage of the activated platelet sample showing the dose response.
Figure 5.1: Horse PSG-like CEACAMs cluster with CEACAM1-like horse CEACAMs
(A) Bootstrapped minimum parsimony tree of equine CEACAM N-domain sequences from [58] shows horse PSGs (in red) do not cluster together on an individual branch. (B) Allowing for a 50% confidence cut-off shows that horse PSG-like CEACAMs cluster with other CEACAM1-like horse CEACAMs supporting their identification as CEACAM1-like genes.
5.3 Results

Figure 5.2: Horse PSG-like CEACAMs do not have orthology with rodent or primate PSGs. Individual species PSGs or CEACAMs cluster together with high confidence with primates on one arm and rodents and equine on the other. An orthologous relationship is highlighted as previously reported between mouse (Mmus) and rat (Rn). Chimp (Pt) is separated from human (Hs) and baboon (Ph) with high confidence. The branching of human with baboon is not significant.
5.3 Results

A

Figure 5.3: Sequence alignment of putative horse PSGs with human PSG1N domain
(A) Invariant residues are coloured orange, while physico-chemically conserved residues (with no more than one exception) are coloured blue. (B) Homology model of PSG1 N-domain generated by Rob Meijers (EMBL, Hamburg) from CEACAM1 crystal structure with position of F-G loop and C-C' loop residues highlighted to indicate predicted availability of residues on PSG N-domains.
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Figure 5.4: Purification of recombinant CEACAM49
(A) Chromatogram of 1.5 ml washes and imidazole elutions of CEACAM49 from nickel agarose affinity column. (B) Coomassie stain of 22.5 µl of highlighted fractions separated by 12 % SDS-PAGE. (C) Coomassie stain of 2 µg of CEACAM49 resulting from processing of fractions.
Figure 5.5: CC49 does not induce TGF-β₁ release from human cell lines

(A) Chromatogram from CC49 applied to a Superdex 200 prep grade size exclusion column with red box indicating area of interest from which fractions were pooled and concentrated.

(B) Coomassie stained 12% polyacrylamide gel with 8 µg of indicated protein per lane.

(C) TGF-β₁ ELISA indicating that SEC removed latent TGF-β₁ contaminant. SEC purified CC49 does not induce TGFβ₁ release from HUVEC-2 endothelial cells, n = 3 (D) or THP-1 cells, n = 3 (E). SM, Starting Material.
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Figure 5.6: Equine PSG-like CEACAM49 causes dose dependent inhibition of platelet-fibrinogen interaction

(A) FACS showing fluorescent Oregon Green Fibrinogen binding to resting, TRAP-activated and 200 µg/ml PSG1 and CEACAM49 pre-treated and TRAP activated human platelets. (B) Graphical representation of dose dependent inhibition of OgFg binding to activated human platelets, relative to untreated control, by PSG1 and CEACAM49. n = 3, One way ANOVA.
5.4 Discussion

No orthologous relationship could be identified for the horse PSG-like CEACAMs confirming their likely independent evolution from a horse CEACAM1-like ancestor rather than a common PSG-like ancestor. The horse PSG-like CEACAMs are very different from PSGs in other species being composed solely of a single IgV-like N-domain. Furthermore, there is significant variation in predicted glycosylation site number between the family members, much more than is evident in the N- or N1-domains of primate and rodent PSGs respectively. This variation could reflect a diversification in function among horse PSG-family members.

Consistent with the earlier findings for PSG1, there is no TGF-β1 induction from the cell types tested following 24 hr CEACAM49 treatment. There were detectable levels of TGF-β1 present in the preparations of the protein pre-SEC. This is relevant due to the recently proposed association of PSG1 with TGF-β1 [260]. However as the Ni-NTA beads have also been demonstrated to bind the latent TGF-β1 particle, it is not possible to determine if a specific association of TGF-β1 with CEACAM49 occurs [265, 260]. Furthermore, the presence of high molecular weight contaminants which could be carriers of TGF-β1 similarly confounds this. It is also possible that the horse protein may not be able to react in a cross species manner in this assay due to differences between species receptors. Critically, without access to a horse cell type of relevance and a horse TGF-β1 ELISA, it is not possible to conclusively rule out the potential for TGF-β1 induction from horse PSG-like CEACAMs functions in immune regulation, especially in the regulation of T-cell activity, make it an excellent candidate for further investigations of the function of horse PSG-like CEACAMs.

While not examined further in this work, it is most likely that the horse PSGs have important roles in T-cell regulation, given the large cell mediated response from the maternal immune system but the delayed destruction of the endometrial cup structures in horse pregnancies. It is clear that factors inherent to the invasive equine trophoblast alter the maternal cell mediated immune response. Evidence that in vitro co-culture model of invasive horse trophoblast cells and peripheral blood lymphocytes has an inhibitory effect on the...
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proliferative capacity and cytokine expression pattern of the lymphocytes [308], as well as the earlier cited studies demonstrating the asymmetric immunological response to ectopically transplanted invasive trophobласт and the endometrial cups, versus the systemic immune system, clearly illustrate this. Evidence for the role of PSGs in T-cell function has been increasing since it was identified in [170] that they bind CD4+ T-cells in a heperan sulphate proteoglycan (HSPG) mediated fashion and since [157] demonstrated a role for PSG1 in generating a T<sub>reg</sub> cell phenotype in vivo mediated by PSG1 treated dendritic cells. This was further extended by evidence from [260] that showed PSG1 treated mice have increased CD4+Foxp3+Latency Associated Particle-TGF-β1+ T-cells isolated from the lamina propria of the gut which is TGF-β1 dependant.

The main functional evidence generated in this chapter was that CEACAM49, similar to human and mouse PSG, exhibits the ability to inhibit the interaction between activated platelets and fibrinogen. No investigations have yet looked for horse putative PSG expression in the serum of pregnant mares due to technical constraints and the relatively recent discovery of these genes. While no evidence of contact between the maternal blood and the fetal tissue could be found in the literature, it is highly likely that horse PSGs will still be present in the maternal blood, as one of the major secretions of endometrial cups is choriogonadotrophin, which is found at very high levels in the maternal blood of the pregnant mare while the cups persist. PSGs have been shown to bind other integrins, besides platelet integrin and it is possible, though not investigated here, that this binding also exists in horse PSGs. Significantly this work represents the first functional evidence ascribing PSG-like properties to this family of proteins in the horse. Moreover, it shows that potentially the anti-platlet properties of the PSGs are not strictly tied to the haemochorial phenotype, an intriguing observation that could potentially widen the list of species in which PSGs could potentially be found.
Chapter 6

Discussion and Future Directions

The work contained in this thesis aimed to extend our understanding of PSG expression, secretion, function and evolution by examining domains and motifs in PSG1 through mutagenesis and functional experimentation, as well as investigating novel binding partners and conservation of function in novel putative PSG genes in the horse. During the course of the work a major confounding factor for much of the functional experiments with the PSG1 mutants was discovered and confirmed. This raises questions not only about some of the earlier work in this study but also much of the published work examining proposed functions of PSGs as well as functional elements in PSG1. In spite of this, several novel findings were successfully generated.

Firstly, a significant insight gained from the mutagenesis of N-glycoylation sites in PSG1N was that the N61 site appears to make a greater contribution to the stability of this domain than the other mutants. Moreover, introduction of this site into the PSG9N construct rescued expression of this protein. The significance of this site to full length PSG function was not confirmed due to the contamination by TGF-β, however, its absence in half of human PSGs, due to differences in the amino acid at position 63, thus ablating the glycosylation consensus sequence, is relevant. In addition, all human CEACAMs (bar CEACAM16) as well as most mouse PSGs do not possess this consensus sequence which would seem to imply that it is not essential for function. Production of PSG4 and PSG9 for this thesis, which both lack this sequence, gave lower yields when purified in a similar
manner to PSG1. As such, an important future experiment would be to compare yield of purified protein for PSG9 and a PSG9 P63T mutant. Importantly, PSG1 is the most highly transcribed PSG at term in human pregnancy [180]. While no evidence yet exists quantifying individual PSG levels in the blood during pregnancy, this might indicate PSG1 is highly expressed. Until the relative levels of PSGs in the blood during pregnancy are determined it is not possible to make an estimation as to the importance to pregnancy of PSGs containing the N61 glycosylation site compared to the remaining PSGs. In terms of the retention/loss of this site in human PSGs over evolutionary time, it is possible that selective disadvantage arising from decreases in the ability to secrete PSG proteins may be counteracted by increased activity of these proteins resulting from conformational changes due to the presence of a proline (P) or alanine (A) at position 63 in human PSG N domains. This would make the absence of glycosylation at the N61 site neutral and could explain why half of human PSGs do not possess this. This was not examined here. Further bioinformatic and structural analysis of this site could yield interesting answers and provide intriguing insights into the evolution of the human PSG gene family.

The immunomodulatory properties of PSG1 were examined in a screen of LPS-induced cytokines. It was found that PSG1 attenuated LPS-induced Cxcl2 in a mouse macrophage cell line. This effect was shown to not require the N domain. The panel of LPS-responsive genes affected by PSG1 was expanded to include IL6 and TNFA, which were also confirmed at the protein level in mouse primary BMDMs. IL-6 and IL-10 had been reported as being induced by PSGs [149, 153, 157, 148], a result that could not be replicated here. Importantly, these findings were potentially confounded by the earlier mentioned TGF-β1 contaminant. It was noted however that active TGF-β1 was almost entirely absent in all tested preparations of proteins used in this study. Nonetheless, to examine this an extra experiment examining PSG1 attenuation of pro-inflammatory cytokines was performed that included small molecule inhibitors against TGF-βRI. The result could not identify any contribution by TGF-β1 signalling to PSG1s attenuation of pro-inflammatory cytokine signalling in monocytic THP-1 cells. This was in contrast to the findings of [260] who showed that PSG1 attenuation of IL-6, TNF-α and IFN-γ were TGF-β1 dependent in primary lymphocytes isolated from the lamina propria of mice with DSS-induced colitis. This contradiction requires further
investigation. The means by which PSG attenuates NFκB-regulated gene expression is still not understood, though we have ruled out alterations in the pattern of IκBα degradation as being responsible. In addition, inhibition of PKA signalling was also ruled out as a mechanism underpinning PSG1s attenuation of LPS-induced pro-inflammatory cytokine expression.

Though not examined here, it has been proposed that PSGs have an important role to play in T-cell biology and the polarization of the immune system during pregnancy along the recently called for, more sophisticated Th1, Th2, Th17 and T regulatory cell spectrum [48, 169]. More refined immunological experimentation such as that utilised in [157] and [260] will yield interesting data on the functions of PSGs in this area.

Taken together this work shows that much work of the published work into PSG function and the elucidation of functionally important elements in PSG1 requires further analysis. Future efforts in this area will require more stringent protocols for the generation of recombinant PSG, incorporating multiple different forms of chromatography such as tandem affinity and size exclusion chromatography, as was shown here. Many of the mutants generated for this study were novel and as such of general value to the field. In addition, the importance of the pattern of glycosylation modifications on the recombinant proteins, though not investigated here, also needs to be addressed to allow for appropriate studies of PSG function. Critically, the absence of a knock out mouse model for Psgs is one of the main hurdles to furthering our understanding of the exact functional roles of these hormones which still remains unknown.

The second main aim of this thesis was the investigation of PSG1 receptor identity. It was known, and confirmed here, that HSPGs are the major mediators of PSG binding at the cell surface. This is a pattern that is found in many growth factor cell surface interactions. However, data showing that PSGs bind integrin αIIbβ3 mediating a novel inhibitory function provided increasing evidence that PSG interaction with a different integrin could be mediating the other reported functions of the protein. PSGs have a conserved RGD-like tri-peptide on a solvent exposed loop in their N and N1 domains in human and mouse [81]. Thus RGD-recognising αMβ2, α5β1 and αvβ3 were selected as good candidates for investigation as potential PSG1 receptors. Moreover these integrins are found
on cell types that PSG are regularly in contact with such as endothelial, trophoblastic, and leukocytic [243, 207, 51, 310, 311]. Functional experiments with blocking antibodies against integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$ were negative and also confounded by the TGF-$\beta_1$ contaminant and so not included here. In addition, plans at examining integrin $\alpha_5\beta_1$ binding to PSG1 by means of the iFLBA could not be attempted as the over-expressing cell line proved negative. However, data was generated showing increased binding of PSG1 to $\beta_3$ CHO cells which was subsequently supported by in vitro binding data. Thus a novel integrin binding partner had been identified mediating an as yet unknown function. Given the important roles that integrins play in the recruitment of leukocytes to sites of inflammation [184], I investigated whether PSG1 could affect this process in a static fluorescent leukocyte-endothelial adhesion assay. It was found that PSG1 had no effect on the adhesion of leukocytes in this assay. This finding represents a preliminary investigation into a complex and developing area of immune research. Further experimentation would be required to rule out a role for PSG in this process. It was aimed to examine leukocyte transmigration through endothelial layers in a transwell experiment but time constraints prevented this. There remains interesting observations to be made in this area. Moreover, an inhibitory function of PSG on leukocyte invasion makes sense in the environment in which they are found during pregnancy.

Finally, a recently identified group of CEACAM genes in the horse showed an expression pattern that indicated they may in fact represent PSG genes [58]. We investigated the relationship of these genes within the horse CEA family as well as their relationship with PSG genes from primates and rodents. We confirmed the identification of the genes as CEACAM1 related and moreover showed that no orthologous relationship exists between the horse putative PSGs and those of other primate and rodent species examined. This showed that the genes were evolved from a CEACAM1 like ancestor as well as the likelihood that they arose independently in the horse lineage. The presence of PSGs in the horse does not correlate with haemochorial placentation which is seen in all species in which PSGs have been found to date. It does however correlate with an invasive trophoblast phenotype that is unique to the horse among epitheliocorial mammals [58]. As such, I investigated whether horse CEACAM49 possessed immunomodulatory properties or anti-
platelet functions found in PSGs from human and mouse. Horse CEACAM49 was found to not induce TGF-β1 from human moncytic or endothelial cell lines. The horse PSG was however found to possess anti-platelet properties, a fascinating finding given the epitheliocorial nature of horse pregnancy. This would seem to imply that CEACAM 49 and possibly other horse PSGs may have a role in haemostatic regulation during the early stages of horse pregnancy while the endometrial cups persist. Though not investigated here it is likely that horse putative PSGs play an important role in regulating T-cell function at the materno-fetal interface as it has been demonstrated that there is a distinct population of Treg like cells located at the maternal-fetal interface in the horse [43].

In summary, the findings of this thesis show that glycosylation plays an important role in the expression of PSGs in the human and that the N61 glycosylation site in particular, being absent in half of human PSGs, represents an intriguing site for investigating the forces driving the evolution of the human PSG gene family. Furthermore, complications associated with the identification of TGF-β1 as a contaminant of PSG from mammalian expression systems requires that much PSG research into functional elements within PSG1 is revisited. Further research is needed to elucidate the recently identified associated between PSG1 and latent TGF-β1 [260]. A novel integrin receptor for PSG1 has been identified mediating an unknown function for these hormones. Investigations into whether PSG plays a role in leukocyte adhesion and invasion during inflammation is expected to reveal interesting findings in the future. Finally, horse PSG-like CEACAMs have been demonstrated to possess PSG-like properties lending support to them being PSGs opening upon chances for the investigation of PSG biology in a species with a radically different type of placenta.
Chapter 7

Appendix
Figure 7.1: pTT3 and pTT3-B PSG1 vector maps

(A) Vector map of pTT3, a modified version of the pTT construct with an extended Multiple Cloning Site (MCS). PSG1 was originally cloned into this vector as outlined in Shanley et al. 2013 [180] (B) pTT3 PSG1 was modified to introduce a HindIII site before the pBluescript V5His sequence and to remove the EcoRI site 3’ of the insert sequence yielding pTT3-B PSG1 (Mrs Melanie Ball). This allowed cloning of other sequences into pTT3-B incorporating the pBluescript V5His tag sequence.
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