Mouse pregnancy-specific glycoproteins: tissue-specific expression and evidence of association with maternal vasculature

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Abstract

The pregnancy-specific glycoproteins (Psg) are secreted hormones encoded by multiple genes in rodents and primates, and are thought to act as immune modulators. The only Psg receptor identified is CD9, through which Psg17 induces cytokine production from macrophages cultured *in vitro*. We examined temporal and spatial aspects of Psg and CD9 expression during mouse pregnancy to determine whether their expression patterns support a role in immune modulation. Using *in situ* hybridisation, immunohistochemistry and RT-PCR we found Psg expression in trophoblast giant cells and in the spongiotrophoblast. Psg22 is the predominant Psg family member expressed in giant cells. Detectable Psg is associated predominantly with endothelial cells lining vascular channels in the decidua, rather than with maternal immune cell markers. CD9 expression exhibited partial overlap with Psg, but without exclusive co-localisation. CD9 was observed in decidual cells surrounding early implantation sites, and in the endometrium. However, embryo transfer of wild-type embryos to CD9-deficient females indicates that maternal CD9 is not essential for successful pregnancy.

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Introduction

The human pregnancy-specific glycoproteins (PSG) are the most abundant fetal proteins in the maternal bloodstream in late pregnancy (Lin *et al.* 1974). They are synthesised in the syncytiotrophoblast of the human placenta and in the giant cells and spongiotrophoblast of the rodent placenta (Lei *et al.* 1992, Rebstock *et al.* 1993, Kromer *et al.* 1996, Zhou *et al.* 1997). The PSG family belongs to the carcinoembryonic antigen (CEA) family, which also includes the CEA-related adhesion molecules (CEACAMs), and is part of the immunoglobulin (Ig) superfamily (Brummendorf & Rathjen 1994). There are eleven members of the PSG family in humans that are encoded by genes clustered on chromosome 19q13.2, and seventeen mouse genes (Psg16–32) located on chromosome 7 (Thompson *et al.* 1990, Teglund *et al.* 1994, McLellan *et al.* 2005a). PSG proteins have a similar domain structure to the CEACAMs, but the majority lack a membrane anchor and are secreted (Teglund *et al.* 1994). Membrane-anchored CEACAMs are widely expressed during embryonic development and in adult tissues, and are implicated in multiple aspects of cell

signalling, tissue homeostasis and disease, including carcinogenesis and regulation of immune and metabolic functions (Han *et al.* 2001, Zebhauser *et al.* 2005). PSGs and some CEACAMs are expressed almost exclusively in trophoblasts of the haemochorial placenta of rodents and primates, suggesting potential functional convergence between these otherwise divergent gene families (Rebstock *et al.* 1993, Zhou & Hammarstrom 2001).

The biochemical properties and physiological functions of the members of the PSG family remain to be fully elucidated. Currently, multiple lines of evidence suggest an immunomodulatory function to prevent rejection of the allotypic fetus (Majumdar *et al.* 1982, Harris *et al.* 1984). Specifically, low PSG levels in the human maternal circulation are associated with threatened abortions, intrauterine growth retardation and fetal hypoxia, and the application of anti-PSG antibodies or vaccination with PSG induces abortion in mice and monkeys, and reduces the fertility of non-pregnant monkeys (Bohn & Weinmann 1976, Hau *et al.* 1985). In addition, PSG-mediated suppression of T cells is correlated with increased maternal morbidity in purulent septic complications of abortion

(Repina *et al.* 1989), and elevated circulating PSG levels are correlated with improved symptoms of rheumatoid arthritis (Fialova *et al.* 1991). Human and mouse PSGs induce secretion of anti-inflammatory cytokines from monocytes and macrophages *in vitro* (Wessells *et al.* 2000, Snyder *et al.* 2001), consistent with the observation of PSG-mediated switching of the immune system from a predominantly TH1 response to a predominantly TH2 response, which is considered more compatible with successful pregnancy (Motran *et al.* 2003).

The only PSG receptor identified to date is the integrin-associated cluster of differentiation 9 antigen (CD9) receptor. In macrophages it was found to bind the N1 domain of both Psg17 and Psg19 (Waterhouse *et al.* 2002). The interaction of Psg17 and CD9 was found to be necessary for the induction of secretion of anti-inflammatory cytokines (Ha *et al.* 2005). Psg17 has also been shown to prevent sperm-egg fusion by interrupting the binding of CD9 to a ligand on the egg surface (Ellerman *et al.* 2003). No receptor for human PSG has been identified and, unlike mouse Psg17, human PSG do not require CD9 to induce cytokine production from mouse macrophages (Ha *et al.* 2005).

CD9 is a tetraspanin, which is an integral membrane protein with four transmembrane domains and two extracellular domains. Tetraspanin family members have been implicated in a variety of cellular and physiological processes, such as cell motility, aggregation, signalling, and fusion (Boucheix & Rubenstein 2001). Tetraspanins are believed to act as 'molecular facilitators', grouping together cell-surface proteins and thus increasing the formation and stability of functional protein complexes (Maecker *et al.* 1997). CD9 associates with a great variety of membrane proteins, such as membrane anchored growth factors, integrins, members of the immunoglobulin superfamily and other tetraspanins (Boucheix & Rubenstein 2001). Although little is known about the role of CD9 in reproduction, human CD9 may function in extravillous trophoblast invasion of maternal tissues (Hirano *et al.* 1999). CD9 expression has not been analysed in mouse pregnancy, and it is not known whether PSG and CD9 expression patterns overlap *in vivo*.

In this study we examined the expression of Psg and CD9 during mouse placental development, and we investigated whether CD9 is necessary for successful pregnancy. Our results provide evidence of tissue-specific regulation of mouse *Psg* genes, and a possible association of secreted Psg protein with vascular endothelium.

Materials and Methods

Mice

Mouse tissues were obtained from the Biological Services Unit, University College Cork. Mouse strains used were CD1, C57Bl6/J, B6CBF₁, and the CD9 null mutant on the C57Bl6/J background (hereafter CD9), which was obtained from Dr Claude Boucheix, Institut National de la

Santé et de la Recherche Médicale (INSERM), unite 268, Hopital Paul-Brousse, 94800 Villejuif, France. Embryonic (E) stage refers to the gestational age of the embryo. The morning on which the vaginal plug was found is counted as day one (E1) of gestation.

Production of recombinant baculovirus mouse Psg21-V5/His and human PSG1-V5/His

Recombinant Psg21 and PSG1 proteins were produced using the baculovirus protein expression system (Invitrogen Life Technologies).

Subcloning in pBlueBac4.5V5/His

Psg21wt was cloned previously into pcDNA3.1 (Ball *et al.* 2004). The Psg21wt open reading frame (ORF) was reamplified by PCR, using primers incorporating restriction enzyme sites at each end (XhoI at 5' and EcoRI at 3') to allow ligation into pBlueBac4.5V5/His (Invitrogen Life Technologies) in frame with the V5/His tag. A vector with a carboxyl (C) terminal V5/His tag was used to prevent interference of the tag with putative Psg functional domains at the N terminus. The human PSG1a ORF was amplified by PCR from an existing cDNA clone (Zimmermann *et al.* 1989).

Production of viral DNA

Recombinant plasmid and Bac-N-Blue DNA (Invitrogen Life Technologies) were co-transfected into adherent *Spo-doptera frugiperda*-9 (Sf9) cells in Grace's Insect media (Invitrogen Life Technologies). Cultures were closely observed for signs of viral infection and serial dilutions of the infected culture media were then used for plaque assay. Blue plaques representing sites of recombinant viral infection were picked and used to infect Sf9s in adherent culture. The cultures were observed for signs of recombinant only viral infection (seen as absence of occlusion bodies (OCC-)). The cell suspensions from OCC- wells were split into two fractions, one for PCR analysis to confirm absence of wild-type baculovirus and the other to be kept as the initial (P1) viral stock. The P1 viral stock of a recombinant-only culture was then used to generate a small-scale high titre (P2) viral stock, which was then used to generate a large-scale, high titre stock (P3). Plaque assay was then carried out to determine the titre of the P3 viral stock.

Protein production and purification

Small-scale protein expression was performed to establish the optimal multiplicity of infection (MOI) and time course for expression of the recombinant Psg21 and PSG1 protein in Sf9 in suspension in serum-free medium (SF900 II, Invitrogen Life Technologies). Recombinant protein production was determined by Western blot with antibody targeted against the V5 epitope tag. Large-scale expression was carried out in one-litre spinner flasks using the MOI and time course determined previously, in this case, an MOI of

3 phage particles per Sf9 cell and time course of 5 days in SF900 II. After 5 days culture the cells were spun out and the Xpress Protein Purification system (Invitrogen Life Technologies) was used to purify the recombinant protein from the medium. The medium was batch bound to the ProBond nickel chelating resin (Invitrogen Life Technologies), the resin was allowed to settle and the supernatant drained off. The resin was then washed, and bound protein was eluted using increasing concentrations of imidazole. The eluate was collected in 1 ml fractions and analysed for recombinant protein by spectrophotometer and Western blot. Fractions with contaminating proteins were pooled and subjected to a second round of purification on the ProBond resin. All clean fractions of recombinant protein were pooled and dialysed against 50 mM Tris pH 7.5, and then further purified by anion exchange chromatography using an increasing step gradient of NaCl in 50 mM Tris pH 7.5. The clean fractions were then pooled, dialysed against phosphate buffered saline pH 7.5 (PBS) and concentrated using centrifuge concentrators (Millipore, Ireland BV, Cork, Ireland). Concentrated protein was used for polyclonal antibody production.

Production of polyclonal antisera to Psg21 and PSG1

Antiserum production

Preimmune bleeds were taken prior to inoculation of rabbits with recombinant Psg21 and PSG1 proteins. An initial injection of 500 µg recombinant protein was administered with Freund's Complete Adjuvant, followed by four booster doses of 250 µg protein with Freund's Incomplete Adjuvant at 3-week intervals. Test bleeds were taken 10 days after each injection and tested on Western blot against recombinant baculovirus Psg21 and PSG1. A final bleed from each rabbit was taken by exsanguination.

Antiserum validation

Polyclonal antisera were tested against recombinant baculovirus Psg21 and PSG1, placental tissue homogenates from pregnant and non-pregnant mice, or tissue homogenates from human term placentas or maternal pregnant serum, by Western blotting.

Immunohistochemistry and immunofluorescence

Tissue preparation

Tissues were collected from non-pregnant and pregnant mice at varying stages of pregnancy, washed in cold (4 °C) PBS, embedded in OCT cryopreservation compound (BDH Laboratory Supplies, Poole, UK) and frozen in an iso-pentane bath in liquid nitrogen or at -80 °C. Frozen tissue blocks were stored at -80 °C until use. Sections (5 µm thick) were cut and mounted on Superfrost Plus microscope slides (BDH Laboratory Supplies), air dried for 15–30 min, fixed in cold (4 °C), fresh 4% paraformaldehyde in PBS for 10 min and then rinsed in cold PBS. The slides

were then equilibrated in Tris-buffered saline at room temperature (TBS, pH 7.4) for 5 min. Sections for immunohistochemistry were treated for endogenous peroxidase activity by incubating in 2% hydrogen peroxide in TBS for 30 min.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using the Vectastain Elite ABC system (Vector Laboratories Ltd, Peterborough, UK). For immunohistochemical localisation of Psg, sections were blocked with 5% non-fat milk in TBS containing 0.1% Triton-X 100 (TBS-Tx) for 1 h at room temperature (RT) and then incubated with primary antiserum or preimmune serum (Psg21 1:500, preimmune serum 1:500) in blocking buffer overnight at 4 °C. Sections were then washed three times for 10 min in TBS-Tx before incubating with biotinylated anti-rabbit secondary antibody (Vector Laboratories, 1:200), washed again three times for 10 min in TBS-Tx and detected using the ABC system and Vector VIP peroxidase substrate kit (Vector Laboratories), following the manufacturer's instructions. Optimisation of different blocking buffers showed that non-fat milk gave the best results; negative controls were therefore needed in all experiments to ensure endogenous biotin in the milk did not interfere with the ABC system. To avoid possible variation in biotin levels from the non-fat milk, TBS-Tx non-fat milk was prepared in bulk, aliquoted and frozen at -20 °C and used for all procedures. Following IHC, sections were counterstained with Harris' haematoxylin (BDH), differentiated, dehydrated, and mounted with DePeX (BDH) permanent mounting medium.

Immunohistochemical staining with a rat anti-mouse CD9 antibody (RDI-MCD9-C8, Research Diagnostics Inc., Concord, MA, USA) was carried out using the Vector Mouse on Mouse (MOM) kit (Vector Laboratories) following the manufacturer's instructions, to minimise cross reactivity between the rat secondary antibody and mouse tissues. Briefly, sections were blocked overnight with MOM IgG blocking solution in TBS-Tx at 4 °C, washed twice for 5 min with TBS-Tx, incubated with MOM solution in TBS-Tx for 15 min, incubated with anti-CD9 antibody at a dilution of 1:1000 for 1 h at RT, washed three times for 10 min in TBS-Tx, incubated with secondary biotinylated anti-rat (Vector Laboratories) at 1:1000 for 1 h then washed, detected and mounted as for Psg. An anti-Ceacam antibody, AgB10 (Kuprina *et al.* 1990), was used at a dilution of 1:50 in conjunction with the MOM kit as for CD9.

Immunofluorescence

Single antibody immunofluorescence was carried out using the same basic procedure as for immunohistochemistry except fluorescent secondary antibodies were used instead of the ABC system. Donkey anti-rabbit rhodamine (Abcam Plc, Cambridge, UK) for Psg was used at a concentration of 1:100 and sheep anti-rat FITC (Abcam) for CD9 at a concentration of 1:100. After incubation with secondary

antibody, sections were washed three times for 10 min with TBS-Tx, once for 5 min in PBS, post fixed with cold 4% paraformaldehyde in PBS for 10 min, washed twice for 5 min in PBS and then mounted with Vectashield Mounting medium with DAPI (Vector Laboratories).

Double immunofluorescence was carried out sequentially due to the incompatibility of the rabbit (for Psg) antibodies with the MOM kit required for the rat antibodies (for CD9 and CD31). Psg staining was carried out first, followed by CD9 or CD31. The anti-CD31 antibody (Abcam) was used at 1:20, with blocking and washing steps as for the anti-CD9 antibody.

In situ hybridisation

In situ hybridisation was carried out using digoxigenin-labelled RNA probes. A 252 bp fragment was amplified from the *Psg21* A domain incorporating EcoRI restriction sites at the 5' and 3' ends (forward (F) 5'GCGGAATTCTGTTC AAGTCAACATCTACAAGC; reverse (R) 5'CGCGAATTCGGGTTGAAGGCCTCACATT). The resulting PCR fragment was ligated into the pSPT18 multiple cloning site at the EcoRI site. Two constructs were created with the insert in opposite orientations for generating sense and antisense probes. RNA probes were synthesised in the presence of digoxigenin-labelled UTP using T7 polymerase. *In situ* hybridisations were carried out on 5 µm thick cryosections mounted on Superfrost Plus microscope slides (BDH). All solutions were prepared using diethylpyrocarbonate-treated water or RNase-free molecular biology grade water (Sigma); all glassware, bench tops and equipment were treated with RNase Away (Invitrogen Life Sciences) to remove RNase contamination prior to each experiment. Sections were treated as follows: air dried for 30 min, fixed in cold (4%) paraformaldehyde in PBS for 10 min, rinsed three times for 5 min in TBS, 200 mM HCl for 10 min, 0.5% acetic anhydride in 100 mM Tris for 10 min, rinsed in TBS for 5 min, baked on a hotplate at 95 °C for 4 min, rinsed in TBS and then incubated at 55 °C with hybridisation buffer for 1 h. Sections were then incubated with 0.1 ng/µl probe in hybridisation buffer at 55 °C in a humid chamber for 2 h. Unbound probe was removed by washing twice for 5 min in 4 × SSC at RT, three times for 5 min in 60% deionised formamide/0.2 × SSC at 55 °C, twice for 5 min in 2 × SSC at 37 °C, two times for 10 min in 0.1 × SSC at 37 °C. Sections were then equilibrated in TBS and blocked overnight at 4 °C with TBS-Tx containing 2% normal sheep serum (NSS). For immunological detection of bound digoxigenin (DIG) probe, anti-DIG antibody (Roche) was used at a dilution of 1:700 in TBS-Tx NSS for two hours at room temperature. Sections were washed twice for 10 min in TBS and then either detected using Vector Red alkaline phosphatase substrate kit (Vector Laboratories) following the manufacturer's instructions and counterstained with haematoxylin, or detected using NBT-BCIP and the DIG Nucleic Acid detection kit (Roche) following the manufacturer's instructions.

CD9 expression analysis of maternal tissues

Decidual tissue was dissected from implantation sites of pregnant CD1 mice at E8, E9, E10 and E11 along with the corresponding uterine tissues. Lung, liver and brain from an E10 pregnant mouse were also included. Tissues were homogenised in lysis buffer (0.1 M Tris-HCl pH8, 0.1% Triton-X 100) containing protease inhibitors (phenylmethylsulphonyl fluoride 100 µg/ml, leupeptin 0.5 µg/ml, aprotinin 0.5 µg/ml, pepstatin A 1 µg/ml), insoluble debris was removed and the supernatants quantified and used for SDS-PAGE and Western blotting analysis. SDS-PAGE (for Western blotting) for CD9 was carried out under non-reducing conditions, with 15 µg protein of each sample being heat denatured at 95 °C for 10 min in a non-reducing loading buffer and resolved on a 5% stacking/15% resolving gel. A duplicate gel was set up, as for CD9, for the β-actin loading control but run under reducing conditions. The gels were then electrotransferred to nitrocellulose membrane and immunodetection was carried out. Briefly, the blots were blocked with 5% non-fat milk in phosphate buffered saline 0.1% Tween 20 (PBS-T) for 1 h at RT, incubated with primary antibody overnight at 4 °C, washed three times for 10 min in PBS-T, incubated for 1 h with secondary horseradish peroxidase (HRP)-conjugated antibody in PBS-T, washed three times in PBS-T for 10 min and then detected using Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc, Rockford, IL, USA) and exposed to Kodak X-OMAT AR film (Sigma). Unless stated otherwise incubations and washes were carried out at room temperature and on a rocker. The primary antibodies, CD9 (used previously) and β-actin (Sigma), were used at a dilution of 1:1000 in blocking buffer. The secondary antibodies, anti-rat-HRP (Sigma) for CD9 and anti-mouse-HRP (Sigma) for β-actin, were used at a dilution of 1:20 000 in PBS-T.

Psg gene expression analysis in trophoblast giant cells

Placentas from CD1 mice were collected at E8, E9, E10, E11, and primary trophoblast giant cells were dissected from the surrounding tissue. For each developmental stage, cDNA was analysed from two litters (cDNA was pooled from approximately six placentas per litter). Extracted tissue was homogenised in 1 ml TRI-reagent (Sigma-Aldrich) and total RNA was isolated. First-strand cDNA was synthesised using 1 µg total RNA as template in a 20 µl reaction using random hexamer priming and Moloney-murine leukaemia virus reverse transcriptase (Invitrogen).

Quantitative RT-PCR

Primers were designed that amplify all known mouse *Psg* gene sequences: PsgF: 5'-TYCAYCCDKTGGHTCTTCAAYA; PsgR: 5'-CACAYYGRTAMTYTCCASCATC. Normalisation of expression level to the housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), was used to avoid discrepancies caused by variations in input RNA or in reverse transcription efficiencies. The following primer

sequences were used: *Hprt* forward (*HprtF*): 5'-CTCATGG-
ACTGATTATGGACAGGAC; *Hprt* reverse (*HprtR*): 5'-GCA-
GGTCAGCAAAGAACTTATAGCC. Quantitative PCR was
performed using the ABI PRISM 7900 sequence detection
system (SDS) and the SYBR GREEN qPCR kit (Applied Bio-
systems, Foster City, CA, USA). The SYBR GREEN PCR mas-
ter mix consists of Amplitaq Gold DNA polymerase, opti-
mised PCR buffer, 25 mM MgCl₂, dNTP mix and AmpErase
UNG. PCR amplifications were performed in a total
volume of 15 µl in duplicate wells.

The following PCR protocol was used: denaturation pro-
gram (95°C for 10 min), amplification and quantification
program repeated for 40 cycles (95°C for 15 s, 55°C for
30 s, 72°C for 45 s with a single fluorescence measurement),
melting curve program (60°C – 95°C with a heating rate of
1°C per 30 s and a continuous fluorescence measurement).
Thereafter, PCR products were identified by generating a
melting curve, which was also used to assess the occurrence
of putative PCR artefacts (primer-dimers) or non-specific
PCR products. The sizes of the RT-PCR products were con-
firmed by gel electrophoresis on a standard 1.5% agarose
gel stained with ethidium bromide and visualised by
exposure to ultraviolet light. Results were described as
mean *Psg* expression relative to mean *Hprt* expression.

Identification of *Psg* gene transcripts in trophoblast giant cells

To identify *Psg* genes expressed in giant cells, *PsgF* and
PsgR primers previously described were used to amplify
Psg cDNA transcripts present (*Psg22* and *Psg25* are of
identical sequence in the amplicon generated by these
primers). PCR was performed in 50 µl using 0.4 U Accu-
zyme (Bioline, London, UK) according to the manufac-
turer's instructions. RT-PCR products were purified using
the Qiaquick PCR purification kit (Qiagen, Crawley, West
Sussex, UK). Purified amplicons were subcloned into
pSTblue-1 vector and transformed into NovaBlue Singles
competent cells (Novagen, EMD Bioscience, Madison, MI,
USA). Positive clones were identified by a diagnostic PCR
screen of bacterial colonies. For each developmental stage
tested eight clones per litter were bi-directionally
sequenced (Macrogen Inc, Gasan-Dong, Gaumcheon-gu,
Korea). In order to distinguish between *Psg22* and *Psg25*
and to ensure that there was no preferential amplification
of any particular *Psg*, the above experiment was repeated
using the primer set *Psg-all2*, which has the following
sequences: *Psg-all2F*: 5'GTGTTGACAATCTGCCAGAGAA-
TCTT; *Psg-all2R*: 5'CTCCTGGGTGACATTTGGATC. PCR
was performed in 50 µl using 0.4 U Accuzyme according
to the manufacturer's instructions (Bioline). The following
amplification protocol was used: denaturation at 95°C for
5 min, amplification repeated for 40 cycles at 95°C for
30 s, 55°C for 30 s, 72°C for 45 s and elongation cycle at
72°C for 10 min. An amplicon of 176 bp was generated
which was confirmed by gel electrophoresis on a standard
1.5% agarose gel as described above.

Embryo transfers to *CD9* $-/-$ and *CD9* $+/+$ mice

Matings between *CD9* heterozygous ($+/-$) males and
females were used to generate homozygous null ($-/-$)
females as test subjects and wild-type ($+/+$) females as lit-
termate controls on the same genetic background.
CD9 $+/+$ (B6CBF₂) embryos were produced, collected
and transferred at the late morula or early blastocyst stage
to the uterine horns of *CD9* $+/+$ or *CD9* $-/-$ pseudo-
pregnant females as described (Nagy *et al.* 2003), with
eight embryos being transferred to each uterine horn.
Recipients were monitored daily for recovery, signs of
pregnancy and pregnancy loss. Mice were killed at either
E10 or E16, the uterus was removed and the number of
successful and failed implantation sites was recorded.

Results

Specificity of anti-*Psg21* and anti-*PSG1* antisera

To determine the location of *Psg* protein during mouse pla-
centation, a polyclonal antiserum was generated against
recombinant *Psg21*. In parallel, human *PSG1* protein and
antiserum were also produced. On Western blot, recombi-
nant *PSG1* and *Psg21* proteins had molecular weights of
approximately 55 kDa and 62 kDa respectively, close to
the predicted size for the glycosylated isoforms observed *in*
vivo (Sorensen 1984, our unpublished observations). Using
these recombinant proteins, anti-*Psg21* and anti-*PSG1*
polyclonal antisera were produced in rabbits.

Using immunohistochemistry, *Psg21* antiserum pro-
duced a pattern of staining on tissue sections of mouse
placenta that was broadly consistent with expectations (see
below). However, although it detected recombinant *Psg21*-
V5/His on Western blots, it did not detect *Psg* in mouse pla-
cental lysates or in maternal serum of pregnant mice. It also
failed to detect recombinant *Psg21* that was over-expressed
in HeLa cells from a transfected pcDNA3.1 (Invitrogen Life
Technologies) expression vector (data not shown). In con-
trast, the anti-human *PSG1* antiserum worked effectively
on Western blots, detecting bands consistent with endogen-
ous *PSG* in human pregnant sera and placental lysates, and
recombinant *PSG1* and *PSG9* over-expressed in HeLa cells
from transfected pcDNA3.1 expression vectors (data not
shown). These results were identical to those obtained
using AB653, a commercially sourced anti-human *PSG*
antibody raised against *PSG* purified from the urine of preg-
nant women. When tested against mouse placental tissues
or transgenically over-expressed recombinant mouse *Psg21*
in HeLa cell lysates, *PSG1* antiserum did not detect mouse
Psg on Western blots.

To exclude the possibility of cross-reaction of the *Psg21*
antiserum to the widely expressed Ceacam proteins,
which are closely related to *Psg*, the antiserum was tested
by immunohistochemistry on mouse tissues that express
Ceacam, but not *Psg* proteins including liver, colon, small
intestine, testis, ovary, brain and non-pregnant uterus.

The anti-Ceacam antibody, AgB10 (Kuprina *et al.* 1990), was used as a positive control (Fig. 1b). The Psg21 antiserum did not cross-react with Ceacam in bile canaliculi of liver (Fig. 1a), nor in any of the other tissues tested (data not shown). Pre-immune serum for the Psg21 antiserum was used as a negative control (Fig. 1c).

Psg staining is associated with trophoblast and maternal vascular endothelium

The expression of Psg in the developing mouse placenta was investigated by *in situ* hybridisation to mRNA using a digoxigenin-labelled Psg21 RNA probe, and by immunofluorescence and immunohistochemistry using Psg21 antiserum. Unexpectedly, *in situ* hybridisation did not detect Psg mRNA in giant cells between E8 and E11, which may reflect lack of sensitivity of our technology combined with relatively low levels of Psg mRNA in giant cells (see below). However, strong staining of spongiotrophoblast was evident at E14 to E16 (Fig. 2e). Psg protein was detected abundantly in giant cells from E8 to E11. Psg was distributed in vesicular structures, reminiscent of secretory granules, throughout the cytoplasm (Fig. 2a). However, at E15 there was only faint, diffuse Psg immunoreactivity in the cytoplasm of giant cells (Fig. 2b). Unexpectedly, the Psg21 antiserum did not detect Psg protein in the spongiotrophoblast of E14 and later stage placentas (Fig. 2c), in spite of the high levels of mRNA detected by *in situ* hybridisation.

Psg staining was observed lining the capillaries within the decidua of the implantation site from E8 to E11 using the anti-Psg21 antiserum. This staining was located predominantly on the antimesometrial aspect of the implantation site but also extended laterally towards the mesometrial pole. An antibody to CD31 (DeLisser *et al.* 1997, Newman 1997), an endothelial cell marker, was used to confirm this localisation (Fig. 3a-f). Staining for Psg was much less obvious at the mesometrial pole of the implantation site from E8 to E11 and was not seen in earlier implantation sites on E6 and E7.

CD9 is expressed in maternal decidual tissues

From the earliest post-implantation stages examined, CD9 completely surrounds the embryo within the implantation site, as determined by immunohistochemistry and

immunofluorescence (Fig. 4A, a-c). CD9 expression is located on the cell surface and in the cytoplasm of maternal decidual tissue cells immediately surrounding the embryonic tissue. As pregnancy progresses, from E6 onwards, the intensity of CD9 staining increases and extends throughout the rest of the decidua, with a distinctly polarised pattern of expression (Fig. 4A, c, e-j). Mesometrially, CD9 expression is strongest lining the endothelial cells of capillaries and vascular spaces within the decidua (Fig. 4A, c, e, f), whereas antimesometrially, CD9 staining is observed throughout the tissue, with both the cytoplasm and surface of decidual stromal cells staining strongly (Fig. 4A, c, g, i, j). Subsequent to E12, there is reduced CD9 staining of the implantation site as the decidual tissue is reduced on the antimesometrial and lateral aspects of the implantation site; however, the endothelium of the maternal sinuses in the remaining mesometrial decidual tissue still exhibits strong CD9 staining (Fig. 4A, d). Staining is also observed in the uterine luminal epithelium, uterine glandular epithelium, myometrium and in blood vessels cross-sectioned within the myometrium. To determine whether CD9 expression levels in decidual tissues are similar to those found in other tissues, Western blotting was carried out using decidual tissues from E8 to E11, and a range of adult tissues. There was no observable difference in expression levels between decidual tissues and other tissues (Fig. 4B).

Co-localisation of CD9 and Psg in the placenta

CD9 staining is present in several trophoblast cell types. From E8 onwards, CD9 staining is found both on the cell surface of primary trophoblast giant cells and also as an intense punctate distribution within the cytoplasm (Fig. 4A, l). Cell surface staining of secondary trophoblast giant cells is found from E10 onwards (Fig. 4A, h, k). The labyrinthine layer of the placenta also shows extensive CD9 staining, whereas staining is less intense in the spongiotrophoblast and even further reduced in glycogen cells (Fig. 4A, d).

Double immunofluorescence staining experiments with the anti-CD9 and anti-Psg21 antisera revealed that both proteins are found contemporaneously at several locations in fetal and maternal tissues. Psg and CD9 are found on the cell surface of primary and secondary trophoblast giant cells (Fig. 4A, h, k, l).

Figure 1 Immunohistochemistry of adult liver tissue sections, which express Ceacams, using (a) anti-Psg23 antiserum, (b) the anti-Ceacam antibody, AgB10, and (c) preimmune serum control for anti-Psg23 antiserum. Scale bar, 100 μ m.

Figure 2 Immunofluorescent detection of Psg with anti-Psg23 antiserum in (a) E9 giant cells, (b) E15 giant cells, (c) E15 spongiotrophoblast (no staining observed). *In situ* hybridisation using digoxigenin-labelled Psg23 RNA probes in (d) E15 giant cells and (e) E15 spongiotrophoblast is shown. GC, giant cells; SP, spongiotrophoblast layer. Scale bar, 100 µm.

In the decidua, from the earliest observed expression of Psg (E8), there is evidence of co-localisation of Psg and CD9 in the endothelium of capillaries and vascular spaces within the decidua, which is evident until E11 (Fig. 4A, e-g, i, j). CD9 expression in the decidua starts at a much earlier stage (E6) and is both intense and ubiquitous.

Psg expression levels exhibit tissue and developmental stage specificity

Quantitative RT-PCR was used to determine the level of *Psg* gene transcripts in the trophoblast giant cells and in

the spongiotrophoblast. PCR primers were designed to amplify a region in the N domain of all known mouse *Psg* genes. Primer concentration was optimised to determine the lowest threshold cycle (Ct) while minimising non-specific amplification. This concentration was found to be 300 pmol for the PsgF and PsgR primer set, and dissociation curves for the PCR product demonstrated a single specific peak indicating absence of non-specific amplification.

In trophoblast giant cells there is increased *Psg* expression between E8 and E11 (Fig. 5). *Psg* transcript levels double from E8 to E9 and from E9 to E10. In

Figure 3 Double immunofluorescence detection of Psg (red) and CD31 endothelial cell marker (green) in decidual tissue surrounding the implantation site on E8 (a-c) and E10 (d-f). (a and d) Anti-Psg23 antiserum, (b and e) anti-CD31 antibody, (c and f) merged anti-Psg23 and anti-CD31. EN, endothelium; GC, giant cell layer. Scale bar, 100 µm.

Figure 4 (A) (a-d) Immunohistochemical localisation of CD9 in E6 implantation site at low magnification: (a) scale bar 200 μ m and (b) scale bar 100 μ m. Low power image of CD9 (green) and Psg (red) immunofluorescence on E10 (c), and CD9 (green) only on E15 (d); scale bars, 100 μ m. (e-l) High magnification double immunofluorescence staining of Psg and CD9: (e and f) E8 lateral to mesometrial, (g) E8 antimesometrial, (h) E10 mesometrial, (i) E10 lateral, (j) E10 antimesometrial, and (k) E15 secondary giant cell. (l) Punctate Psg and CD9 staining in primary giant cell. Scale bars in (e-l), 50 μ m. GL, glycogen cells; GC, giant cell layer; DC, decidua; SP, spongiotrophoblast layer; LB, labyrinthine layer; EN, endothelium; M, mesometrial aspect; AM, antimesometrial aspect. (B) Relative CD9 expression levels in mouse tissues. Lanes 1-4: E8, E9, E10 and E11 decidual tissues; lanes 5-8: E8, E9, E10 and E11 uterine tissues; lane 9: E10 ectoplacental cones; lane 10: E10 giant cells; lane 11: adult brain; lane 12: adult lung; lane 13: adult liver. β -Actin staining indicates approximately equal loading of lanes. In this instance, E10 decidual tissues (lane 3) exhibit low levels of CD9 expression. However, in other experiments, E10 CD9 expression was similar to other stages.

Figure 5 Quantification of *Psg* gene transcription relative to *Hprt* in giant cells on E8 to E11, ectoplacental cones on E9 to E11, and entire placentas on E12, E15, E18. Data are means $\pm$ standard deviations, $n = 2$ litters (cDNA was made from pooled mRNA from approximately six placentas per litter).

ectoplacental cone (EPC), there is a fivefold increase in *Psg* transcript levels between E9 and E11 (Fig. 5). However, absolute levels in the EPC are low, with E10 giant cells having approximately sixfold higher levels than E10 EPC. In dissected whole placenta samples (of which only the spongiotrophoblast compartment supports *Psg* gene transcription), *Psg* transcript levels increased fourfold from E12 to E15 (Fig. 5), and thereafter declined by approximately fifty per cent to E18.

Psg22 is the most abundant Psg gene transcript in trophoblast giant cells

Previous studies have shown that *Psg21* and *Psg23* gene transcripts together constitute the bulk of *Psg* gene expression in the spongiotrophoblast (Ball *et al.* 2004, McLellan *et al.* 2005a). To determine whether specific *Psg* gene transcripts similarly dominate in giant cells, *Psg* transcripts were analysed in giant cells at four stages in development: E8, E9, E10 and E11. cDNA samples were amplified with degenerate primers designed to amplify all murine *Psg* gene transcripts (*Psg16* – *Psg32*). *Psg22* and *Psg25* sequences are identical in the amplicon generated. Amplicons were subcloned into pSTBlue vector and eight of the resulting clones from each litter were sequenced in both directions for each developmental stage. As detailed in Table 1, the clones were overwhelmingly derived from either *Psg22* or *Psg25*. Other *Psg* gene transcripts were represented at lower levels (10% or less), and included *Psg19*, *Psg24* and *Psg29* at E8, *Psg19* and *Psg29* at E9, *Psg29* at E10 and *Psg29* at E11. The experiment was repeated using alternative degenerate PCR primers (*Psg-all2*) that allow *Psg22* and *Psg25* to be distinguished. Of the 10 clones derived from E10 giant cells, all were *Psg22*.

Table 1 *Psg* gene transcripts in CD1 trophoblast giant cells from E8-E11

Developmental stage	PCR primers	No. clones sequenced	<i>Psg</i> transcripts present (<i>n</i>)
E8	<i>PsgF&R</i>	18	<i>Psg22/25</i> (11) <i>Psg19</i> (13) <i>Psg24</i> (1) <i>Psg29</i> (1) <i>Ceacam9</i> (2)
E9	<i>PsgF&R</i>	16	<i>Psg22/25</i> (12) <i>Psg19</i> (1) <i>Psg29</i> (1) <i>Loc384557</i> (2)
E10	<i>PsgF&R</i>	16	<i>Psg22/25</i> (15) <i>Psg29</i> (1)
E11	<i>PsgF&R</i>	17	<i>Psg22/25</i> (14) <i>Psg29</i> (3)
E10	<i>Psg-all2</i>	10	<i>Psg22</i> (10)

Maternal CD9 is not essential for successful pregnancy

To determine whether maternally expressed CD9 is required for embryo implantation and pregnancy, wild-type embryos of the B6CBF₂ strain were transferred to wild-type (CD9 +/+) and null (CD9 –/–) pseudopregnant females. To control for genetic background effects on fertility, both wild-type and null recipient females were derived from offspring of the CD9 +/– $\times$ CD9 +/– intercross on the C57BL/6 genetic background.

CD9 was not required for a successful pregnancy up to E16, the latest stage at which embryo recipients were examined (Table 2). No difference was observed between CD9 +/+ and CD9 –/– recipients with respect to number of implantations, number of resorptions, developmental stage or gross morphology of embryos or placentas.

Discussion

We analysed the expression of the mouse *Psg* and CD9 proteins during pregnancy to determine whether their expression patterns are consistent with mutual interactions *in vivo*. We also sought to determine whether specific *Psg* genes are expressed at different developmental stages, and to attempt to assess the likely importance of *Psg*/CD9 interactions for successful pregnancy.

Psg expression and localisation were analysed using a combination of *in situ* hybridisation to mRNA and immunohistochemistry and immunofluorescence. At the time of this study, a specific anti-mouse *Psg* antibody was not

Table 2 Embryo transfer to CD9 +/+ (wildtype) and CD9 –/– (null) mice.

	CD9 +/+	CD9 –/–
Total recipient females	14	17
Recipients pregnant at E10 or E16	10	12
Mean implantation sites/pregnancy	7.33	7.16

available, and stocks of commercially available anti-human PSG (AB653) were low. We therefore produced antisera to recombinant mouse *Psg*21 and human PSG1. Our anti-PSG1 antiserum behaved predictably on Western blots of human tissues, comparable to AB653. However, the anti-*Psg*21 antiserum did not detect endogenous mouse *Psg* on Western blot, but was successfully used for immunohistochemistry of trophoblast giant cells. Unexpectedly, however, the *Psg*21 antibody did not detect *Psg* in spongiotrophoblast, the major site of *Psg* gene transcription. Potential explanations for this anomaly are that the *Psg*21 antiserum recognises a restricted set of epitopes that may only be present in the limited set of *Psg* genes expressed in giant cells (see below), or there may be masking of relevant epitopes (e.g. by glycosylation) in spongiotrophoblast. Alternatively, in spite of high levels of *Psg* mRNA, there may be low steady state levels of *Psg* protein due to translational regulation or rapid turnover. An alternative explanation, given our failure to validate our anti-*Psg*21 antiserum to the same standard as our anti-PSG1 antiserum, is that staining of giant cells is artefactual. While we cannot formally exclude this possibility subject to the generation of further anti-mouse *Psg* antibodies, we consider it unlikely because staining is confined to giant cells, which express *Psg* mRNA, and to maternal vasculature expressing the *Psg* receptor CD9 (see below), which would be directly exposed to secreted *Psg*.

Subject to the aforementioned caveat, *Psg* staining was observed on the endothelial lining of vascular spaces in the decidual tissue surrounding the implantation site from E8 to E11. Since there was no evidence from this or previous studies of *Psg* gene expression in endothelium of decidual tissues, we conclude that this staining may represent secreted *Psg* from fetal tissues, which becomes associated with maternal vascular endothelium. There is extensive angiogenesis and vascular remodelling associated with pregnancy, and trophoblast giant cells produce a complex array of angiogenic and anti-angiogenic and vasoactive compounds (Cross *et al.* 2002). Currently, *Psg* are thought to regulate the maternal immune system during pregnancy; however, the evidence in support of this hypothesis has largely been generated *in vitro* using immune cell models (Wessells *et al.* 2000, Snyder *et al.* 2001, Motran *et al.* 2003), and there remains the possibility that different *Psg* proteins encode different functions. One interpretation of our finding that *Psg* may associate with maternal vasculature is that some *Psg* may exhibit vasoactivity or angiogenesis-related functions.

Using immunohistochemistry and immunofluorescence, we found that CD9 is widely expressed in the fetal compartments of the placenta and in maternal decidual and other uterine tissues throughout pregnancy. CD9 is evident both intracellularly and on the cell surface of giant cells from E8 to E11. Rodent trophoblast giant cells are analogous to extravillous cytotrophoblast cells of the human placenta; both are polyploid and invasive, and have similar patterns of trophoblast cell subtype-specific gene

expression (Hemberger & Cross 2001). In the human placenta, the intensity of CD9 expression in the extravillous trophoblast cells differs between early pregnancy and term: expression in the extravillous trophoblast (EVT) invading the endometrium in early pregnancy is weak, but in the placental bed and chorionic laevae of the term placenta (where EVTs have ceased invasion into the endometrium) expression is intense (Hirano *et al.* 1999). This suggests that human CD9 might have a role in inhibiting cell invasion; however, from our data, we cannot confidently propose an analogous function for mouse CD9. From E13 onwards, CD9 staining is found in both the spongiotrophoblast and the labyrinth; in the latter case staining is predominantly associated with the cells lining the vascular channels.

Staining of decidual tissues was particularly intense, with evidence for both cytoplasmic and cell surface staining from E6. The significance of the different distributions of CD9 staining of decidual cells on the mesometrial and antimesometrial aspects of the implanted embryo at later stages is unclear: the intense staining of decidual cells on the antimesometrial aspect is particularly striking, whereas on the mesometrial aspect, staining was mostly confined to the surface of endothelial cells lining vascular spaces. The strong cytoplasmic staining of CD9 in decidual cells is somewhat unusual; however the overall magnitude of CD9 staining in decidual cells is similar to other tissues, as judged by Western blot. CD9 is generally found on the cell surface; however, occasionally, it is located intracellularly e.g. in eosinophils and platelets, where it is stored pending transfer to the cell surface upon activation (Fernvik *et al.* 1995, Brisson *et al.* 1997).

In summary, our findings show that there is considerable scope for secreted *Psg* to interact with maternal CD9. However, our results suggest that the bulk of CD9 expression in pregnancy is not associated with immune cells but, rather, with maternal decidual and vascular tissues. Our related finding of an association of *Psg* and vascular endothelium supports a scenario whereby *Psg* secreted from fetal tissues interacts with CD9 on the maternal vasculature. However, whether this putative interaction would result in the activation of signalling pathways relevant to endothelial cell function is unclear. An alternative scenario, based on the concept of maternal-fetal conflict (Moore & Haig 1991, Haig 1993), is that maternal CD9 expressed on vascular endothelium acts as a 'sink' or decoy receptor for *Psg*, thereby reducing the amount of *Psg* available for interacting with maternal immune cells. In addition, we add the caveat that, because of the widespread expression of CD9 in maternal tissues, the observed co-localisation with *Psg* could be coincidental. Confirmation of putative functional interactions between these proteins at overlapping sites in maternal tissues will therefore require further analysis.

The independent expansion of primate and rodent *PSG* gene families in evolution suggests convergence of function (McLellan *et al.* 2005b). However, a previous

semi-quantitative study of *Psg* gene expression in mouse pregnancy indicated that different family members exhibit different expression levels between E11 and E18, suggesting the possibility of divergent functions, at least within the mouse *Psg* family (McLellan *et al.* 2005a). To investigate this possibility further, we analysed the expression profile of *Psg* genes in the giant cells and spongiotrophoblast using quantitative methods. The earliest that *Psg* transcripts have been detected in the murine placenta is E6.5 in trophoblast giant cells (Finkenzeller *et al.* 2003). It is difficult to isolate cDNA from specific embryonic tissues at this stage, so our analysis began at E8. First, we analysed levels of total *Psg* gene expression using PCR primers designed to amplify transcripts from all *Psg* genes. Total levels of *Psg* transcripts in giant cells increased between E8 and E11, but remained considerably lower (approximately tenfold) than in whole placenta from mid to late gestation. This is consistent with our failure to detect *Psg* transcripts in giant cells using *in situ* hybridisation. Very low levels of *Psg* transcription were also detected in dissected ectoplacental cones; this may represent contamination from adherent giant cells or, alternatively, the earliest manifestation of differentiating spongiotrophoblast from late E10 and E11. *Psg22* was overwhelmingly the most abundant *Psg* transcript in giant cells between E8 and E11, consistent with a previous study that did not detect *Psg22* expression from E11 onwards, when the giant cells form a progressively reduced proportion of placental tissues (McLellan *et al.* 2005a). *Psg22* may be expressed virtually exclusively in giant cells and may encode a specific or additional function not associated with other *Psg* proteins. In this context, it would be interesting to determine whether *Psg22*, like *Psg17* and *Psg19*, binds CD9.

In spite of the widespread expression of CD9 in the pregnant uterus, the transfer of wild-type embryos to CD9-deficient females resulted in comparable pregnancy rates to embryos transferred to wild-type females, suggesting that maternal CD9 expression is not essential for successful implantation or maintenance of pregnancy. If CD9 represented the sole receptor for secreted fetal *Psg*, the CD9 null female would represent a surrogate *Psg* null mutant, with important implications for understanding the significance of *Psg* in pregnancy. However, it is currently unclear whether all mouse *Psg* bind CD9, or whether those that do, do so exclusively. In this context, it is noteworthy that human *PSG* do not bind CD9, but nevertheless induce expression of a similar range of cytokines to mouse *Psg* from monocytes (Ha *et al.* 2005). The elucidation of *Psg* function in mouse pregnancy will therefore require further developmental and biochemical studies using techniques such as gene targeting to assess the importance of specific *Psg* genes, and proteomics to collate the full spectrum of putative *Psg*/receptor interactions on trophoblast cells, maternal vascular endothelium and maternal immune cells.

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