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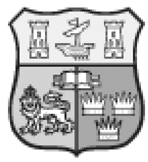

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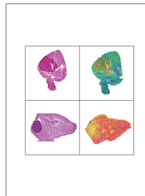

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Pregnancy-specific glycoprotein expression in normal gastrointestinal tract and in tumors detected with novel monoclonal antibodies

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ABSTRACT

Pregnancy-specific glycoproteins (PSGs) are immunoglobulin superfamily members related to the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family and are encoded by ten genes in the human. They are secreted at high levels by placental syncytiotrophoblast into maternal blood during pregnancy, and are implicated in immunoregulation, thromboregulation, and angiogenesis. To determine whether PSGs are

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expressed in tumors, we characterized 16 novel monoclonal antibodies to human PSG1 and used two that do not cross-react with CEACAMs to study PSG expression in tumors and in the gastrointestinal (GI) tract using tissue arrays and immunohistochemistry. Staining was frequently observed in primary squamous cell carcinomas and colonic adenocarcinomas and was correlated with the degree of tumor differentiation, being largely absent from metastatic samples. Staining was also observed in normal oesophageal and colonic epithelium. PSG expression in the human and mouse GI tract was confirmed using quantitative RT-PCR. However, mRNA expression was several orders of magnitude lower in the GI tract compared to placenta. Our results identify a non-placental site of PSG expression in the gut and associated tumors, with implications for determining whether PSGs have a role in tumor progression, and utility as tumor biomarkers.

KEYWORDS

Oesophagus, squamous epithelium, squamous cell carcinoma, colon, colonic adenocarcinoma, pregnancy-specific glycoprotein, PSG1, carcinoembryonic antigen, CEACAM, placenta, trophoblast, monoclonal antibody

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ABBREVIATIONS AND ACRONYMS

CEA Carcinoembryonic antigen

CEACAM Carcinoembryonic antigen-related cell adhesion molecule

FACS Fluorescence activated cell sorting

GI Gastrointestinal

hCG Human chorionic gonadothropin

IHC Immunohistochemistry

mAb Monoclonal antibody

PSG Pregnancy-specific glycoprotein

qRT-PCR Quantitative reverse transcription-polymerase chain reaction

SCC Squamous cell carcinoma

WB Western blotting

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INTRODUCTION

Pregnancy-specific glycoproteins (PSGs) are abundant fetal proteins in the human maternal bloodstream during pregnancy.[1,2] The 10 *PSG* genes in the human, and 17 in the mouse,[3-5] are expressed predominantly in specialised secretory tissues of the placenta: human syncytiotrophoblast and rodent spongiotrophoblast and trophoblast giant cells.[6,7] *PSG*s are members of the immunoglobulin gene superfamily and are closely related to another multigene family, the carcinoembryonic antigen-related cell adhesion molecule genes (*CEACAM*).[3,8] CEACAMs are widely expressed in embryonic and adult tissues, and several members of the CEACAM family are expressed in tumors and used clinically as serum biomarkers and as targets for therapeutic development.[9-13] For example, CEA (CEACAM5) is widely used as a serum biomarker in colorectal carcinoma therapy and may be useful in monitoring pleural effusions with inconsistent cytologic results.[14,15]

There is extensive evidence of PSG mRNA expression in non-trophoblastic tumors.[16-21] For example, in one study, PSG9 mRNA upregulation was detected in 78% (14/18) of FAP adenomas and 75% (45/60) of sporadic colorectal cancer cases tested.[21] We speculated that PSGs may be similar to other placental hormones, such as human chorionic gonadotrophin (hCG), that are expressed in many tumors and may contribute to tumor progression.[22] Indeed, the proposed anti-inflammatory and pro-angiogenic functions of PSGs suggest possible pro-tumorigenic functions in cancer.[23-25] Moreover, the highly restricted pattern of PSG expression in the embryo,[26,27] the evident absence of PSG protein expression in normal adult tissues, and the fact that PSGs are secreted molecules that accumulate in the bloodstream makes them attractive candidates as potential tumor biomarkers.

Due to the high similarity of PSG and CEACAM peptide sequences and the widespread expression of CEACAMs in normal tissues and in tumors, the specific detection of PSG protein expression in tumors is challenging and requires the development of well-validated

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PSG-specific antibodies. However, previous studies reporting PSG protein expression in tumors have not provided extensive validation of antibody specificity. BAP-3, a well-validated PSG-specific monoclonal antibody (mAb) has been developed,[28] but tumor studies using it have not been reported.

Here, we describe the production and characterization of novel anti-PSG1 mAbs, and their use to describe PSG protein expression in tumors and in normal tissues.

RESULTS

Characterization of anti-PSG1 monoclonal antibodies

To obtain antibodies that distinguish between PSGs and CEACAMs, sixteen mAbs PSG.01 - PSG.16 were raised against recombinant immunoglobulin Fc-tagged PSG1 (PSG1-Fc) and were tested for positive staining using immunohistochemistry (IHC) and Western blotting (WB), and for crossreactivity to CEACAMs using fluorescence-activated cell sorting (FACS). All mAbs were of the IgG1 isotype (data not shown). In preliminary experiments, six mAbs (mAbs PSG.01, PSG.03, PSG.04, PSG.05, PSG.06, PSG.11) detected a band of ~60 kD on WB of serum from pregnant women, but not from non-pregnant women or men, consistent with detection of a pregnancy-specific protein (data not shown). mAbs PSG.05 and PSG.11 were chosen for further characterization because they gave the most robust staining on WB (Fig. 1A, B). These mAbs also produced robust staining on IHC of human placental syncytiotrophoblast, which is consistent with detection of endogenous PSGs as shown with mAb PSG.11 (Fig. 1C). Similar staining was observed with mAb PSG.05 (data not shown).

To determine whether mAbs PSG.05 and PSG.11 cross-react with CEACAMs, we tested them in FACS experiments against a panel of CEACAM-expressing Hela cell lines as previously described.[29] Although no cross-reactivity was detected (Fig. 2A), we did not have a suitable positive control for sensitivity of mAbs PSG.05 and PSG.11 in these experiments because PSG1 is a secreted protein unlike CEACAMs, which are anchored to the cell surface.

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We therefore probed western blots containing a subset of Hela-CEACAM cell lines and recombinant PSG1 as a positive control with mAbs PSG.05 and PSG.11 and observed strong staining of PSG1 with no cross-reactivity to CEACAM1 and CEACAM5 (Fig. 2B).

Epitope mapping of mAbs PSG.05 and PSG.11 was carried out by probing western blots of supernatant from Freestyle 293 cells transiently transfected with V5/His-tagged PSG1 wildtype protein and variants possessing single domain deletions. mAb PSG.05 staining was abolished by deletion of PSG1 N domain and mAb PSG.11 staining was abolished by deletion of PSG1 A1 domain (Fig. 2C). We attempted to refine further the mAb PSG.05 and mAb PSG.11 epitopes using binding of mAbs to an array of overlapping PSG1 18-mer peptides. However, high background staining was observed, which may reflect non-specific cross-reactivity in this experimental platform, or that relevant epitopes include glycans or other post-translational modifications that are not represented on the peptide array (data not shown). As PSG proteins are highly similar, we screened the entire human PSG family (PSG1 - PSG9, PSG11) for cross-reactivity with mAbs PSG.05 and PSG.11 by western immunoblot of supernatants from Freestyle 293 cells transiently over-expressing the human PSGs, PSG1 - 9, and PSG11. mAb PSG.05 detected PSG1 and the closely related PSG7 and PSG8, and mAb PSG.11 detected PSG1 and PSG8 (Fig. 2D, E).

Detection of PSG expression in human oesophageal and colonic epithelium and in tumors using mAbs PSG.05 and PSG.11

To determine whether PSG proteins are expressed in tumors, we conducted a preliminary screen of commercial tumor arrays with mAb PSG.11. Using a semi-quantitative scoring scheme, localised patchy staining was observed in 75 (17%) of 448 tumor samples (data not shown). Ninety-five percent of positive samples were from the GI tract and 73% were described by the supplier as squamous cell carcinomas (SCC). Sixty-six percent of the positively stained squamous cell carcinomas were oesophageal in origin.

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Based on these findings indicating that PSGs are most likely expressed by tumors of oesophageal or GI tract origin, PSG expression was analysed by IHC using mAb PSG.11 in surgically resected human oesophageal squamous cell carcinomas (n=10) and human normal colon (n=6) and colonic adenocarcinomas of varying Dukes' stage (n=36). IHC using mAb PSG.05 was used on a subset of samples to confirm mAb PSG.11 staining. Matched normal and metastatic paraffin-embedded specimens were available for six of the 10 human oesophageal squamous cell carcinomas. Examination of the stained tissue specimens revealed that PSGs are strongly expressed at the crypt surface of the normal colon (Fig. 3A) and in the squamous epithelium of the normal oesophagus (Fig. 3F) in all specimens examined. Expression of PSGs decreased with increasing colon tumor stage (Figs. 3B-E). PSG immunostaining in more than 50% of the tumor cells was only seen in two of the five Dukes' A colon tumors (Fig. 3B), whereas eight of the 10 Dukes' C tumors and five of the eight Duke's D tumors had lost PSG expression (Fig. 3D, E; Table 1).

Progression from oesophageal squamous cell carcinoma to metastatic oesophageal carcinoma (Fig. 3G, H) was also associated with a reduction in PSG expression. There was an absence of staining in five of the six matched metastatic oesophageal carcinomas examined, and for the one sample that did show positivity, the matched tumor oesophageal squamous cell carcinoma showed strong PSG positivity in 50-75% of the tumor cells. Furthermore, all three poorly differentiated (grade 3) tumors were negative for PSG expression, while four of the six moderately differentiated (grade 2) tumors were either negative or exhibited PSG immunostaining in less than 25% of the tumor cells (Table 2).

Detection of PSG expression in human and mouse gastrointestinal tract using qRT-PCR

We used qRT-PCR to determine the level of human PSG expression in the oesophagus and colon compared to whole brain and term placenta using a pair of redundant PCR primers

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spanning *PSG* intron 1 that amplify all *PSG* family members. Consistent with our IHC studies, qRT-PCR analysis revealed that PSG was expressed in normal colon and oesophagus. Expression in the oesophagus was almost two orders of magnitude higher than in the brain and between four and five orders of magnitude lower than in term placenta. Similarly, expression in the ascending colon was four orders of magnitude higher than in brain and between two and three orders of magnitude lower than in term placenta (Fig. 4A). For comparative purposes, we carried out a similar study in GI tract tissues from one male and one female C57Bl/6j mouse. Similar to the human, qRT-PCR analysis of mouse *Psg* expression using a set of redundant PCR primers showed that GI tract (oesophagus and ascending colon) expression is higher than brain, but approximately four orders of magnitude lower than placenta (Fig. 4B).

DISCUSSION

We developed novel mAbs raised against human PSG1, and we extensively characterized two (mAbs PSG.05 and PSG.11) that specifically detect PSG expression and do not cross-react with CEACAMs. These mAbs were used to screen tumor arrays because of numerous reports of PSG mRNA expression in tumors.[16-21] The restriction of PSG expression to placental trophoblast in normal individuals suggested that PSGs would be useful tumor biomarkers, particularly as they are secreted proteins and potentially detectable in the blood.

Our initial observation of PSG staining in squamous cell carcinomas (SCC) of GI tract origin on tumor arrays prompted us to examine GI tract tissues and tumors in detail. Our IHC analysis of well-characterized normal oesophageal and colonic tissues, and oesophageal SCC and colonic adenocarcinomas at progressive stages of malignancy, indicated that PSG staining is strongest in normal tissues, with staining reduced or absent in metastatic oesophageal samples and in advanced colonic adenocarcinomas (Dukes C & D stages). We cannot determine from our study whether loss of PSG expression is incidental or contributory

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to tumor progression. One possibility is that as epithelial cells lose their differentiated state, or are overgrown by metaplastic non-expressing cells, PSG expression becomes incidentally lost apart from in residual cells that retain some features of normal epithelial cells. Alternatively, there is evidence that senescing cells express PSGs,[30] and we speculate that there may be de novo expression of PSGs in senescing tumor cells. Loss of PSG expression in tumors may not support the use of PSGs as tumor biomarkers detectable in blood because expression in normal GI tract tissues might mask expression arising from tumors. However, observed loss of PSG expression in biopsied tumor material might be indicative of the stage of tumor progression and a prognostic indicator. Moreover, the secretion of tumor-specific isoforms of PSGs might provide clinical biomarkers detectable in the blood, particularly if expression arises de novo due to tumor cell senescence.

The pattern of PSG expression in the GI tract detected by mAb PSG.05 and PSG.11 is similar to the expression of the CEACAMs, which are widely expressed in epithelial and myeloid cell lineages. CEACAM expression is particularly high in the GI tract, including the oesophageal squamous epithelium and colonic epithelia.[31,32] CEACAMs are also expressed in multiple tumor types and CEACAM5 (formerly CEA) is used extensively as a biomarker in clinical oncology.[13,33]

The function of PSG expression in the GI tract is unknown, but there is increasing evidence that PSGs may have multiple functions in immunoregulation, thromboregulation, and angiogenesis.[34] Consistent with their co-expression, PSGs and CEACAMs both exhibit immunoregulatory functions.[5] An immunoregulatory function for PSGs in the GI tract is also supported by the expression of mouse *Psg18* in the follicle-associated epithelium overlaying Peyer's patches in the small intestine,[35] possibly contributing to a tolerogenic response to commensal bacteria and food antigens, or regulating immune responses to pathogens.[36,37] The recent finding that PSGs induce and activate TGFβ1 and prevent colitis in a mouse

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model,[38,39] suggest a possible anti-inflammatory role in the colon. Manipulation of PSG expression in animal models will be required to test these possibilities. Human studies will be facilitated by novel anti-PSG specific mAbs reported here.

Because of the high degree of sequence identity between the 10 predicted human PSG proteins, it is likely that mAb PSG.05 and PSG.11 each detect multiple PSG family members. This was confirmed by western immunoblot against the supernatants containing the human PSG family members, where mAb PSG.05 detected PSG1, PSG7 and PSG8, and mAb PSG.11 detected PSG1 and PSG8. BAP-3, a mAb raised against PSG purified from human retroplacental blood serum, recognises an epitope in the B2 domain and cross-reacts with all six PSG proteins, and none of seven CEA proteins, expressed in cell lines.[28] While less fully characterized and apparently less specific than the mAbs described in this study, BAP-3 may nevertheless be useful as a complement to our mAbs described herein.

The levels of PSG mRNA are approximately four orders of magnitude lower in mouse and human GI tract compared to their respective placentas, which is unsurprising given the extremely high levels of PSG expression in the placenta.[40] Notwithstanding the comparison with placenta expression, human and mouse GI tract PSG expression is two orders of magnitude higher than in brain, suggesting that GI tract expression is significantly higher than background. Combined with previous studies, our findings suggest that PSG protein expression occurs extensively in the GI tract, with implications for understanding immune, epithelial and tumor cell regulation in these tissues.

MATERIALS AND METHODS

BIOINFORMATICS

Phylogenetic tree of human PSG Amino Acid sequences. Phylogenetic trees (Neighbour-joined pairwise comparison phylogenetic trees) were constructed using the MEGA4.0 software programme (http://www.megasoftware.net/). Data were bootstrapped 1000 times

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and all major branches yielded values of 95 - 100%. The scale bars represent 0.5 nucleotide substitutions per site.[41-43]

PRODUCTION OF RECOMBINANT PSG1 PROTEINS

PSG1 wildtype and N-domain deletion mutant (PSG1ΔN) proteins were made as previously described by transient transfection of pTT3-based expression vectors in HEK293 cells.[44] PSG1 protein with a deletion of the A1 domain (PSG1ΔA1) was made as previously described.[44] Briefly, the mutated ORF was created using site-directed mutagenesis of the PSG1pTT3 plasmid using primers: PSG1ΔA1 F: 5'CCGAAGCTGCCCAAGCCCTAC, R: 5'GTGTAAGGTGAAGGTGAAACGTCCA.

TISSUE COLLECTION

First, second and third trimester maternal blood samples from uncomplicated pregnancies were collected at Cork Unified Maternity Services as approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Samples (1- 5 ml) were collected by venipuncture into Greiner Bio-one EDTA K3 bottles (Cruinn Diagnostics Ltd, Dublin, Ireland). After low speed centrifugation, serum was stored at -80°C. First trimester placentas (8 - 9.5 weeks post-conception) were collected at elective termination at St. Mary's Hospital, Manchester, under approval of the Central Manchester Local Ethics Committee. Normal colonic tissue was collected during surgical resection of colon cancer at the Mercy University Hospital Cork under a protocol approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Tumor arrays were obtained commercially from Super Bio Chips, Seoul, South Korea (catalogue numbers: MC4, BB6, BC7, MB3, CDA2, BA4) and Biomax, Insight Biotechnology Ltd, Wembley, UK (catalogue number LY802). Arrays contained a total of 448 tumor sections representing a variety of cancers and clinical grades and were 1.5 mm (Biomax) and 2 mm (Super Bio Chips) in diameter and 5 μm thick. Tumors were graded by

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the supplier according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (6th Edition). Human colonic adenocarcinoma (n=36) and oesophageal squamous cell carcinoma (n=10) samples were obtained from the archives of the Mercy Hospital, Cork, under a protocol approved by the Cork Teaching Hospitals Clinical Research Ethics Committee. Histological sections of normal human oesophagus were obtained from AMS Biotechnology Ltd; Abingdon, U.K. Mouse gastrointestinal tissues were dissected from adult male and female C57Bl/6j obtained from the Biological Services Unit, University College Cork.

ANTIBODIES

MAbs against PSG1 were produced in BALB/c mice immunized at two-week intervals with 40 µg of recombinant PSG1 protein fused to human IgG1 Fc (PSG1-Ig), produced by Prof. Mandelboim (data not shown), in complete or incomplete Freund's adjuvant (Sigma-Aldrich). Several dilutions of sera were screened by ELISA on 96-well plates coated with the PSG-Ig protein diluted (1 ug/ml) in coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6). Three days preceding the fusion, the animal showing the best titre was boosted by intraperitoneal injection of 40 µg PSG-Ig protein diluted in PBS. Splenocytes from the boosted mouse were fused with Sp2/0 murine myeloma cells. The hybridomas were selected by growing in RPMI 1640 medium (GIBCO-BRL) supplemented with HAT (GIBCO-BRL) and 10% FCS. Supernatants of hybridomas were collected and screened against the PSG-Ig protein by ELISA using the same procedure as for the sera. Positive hybridomas were subcloned to clone density, and supernatants were collected from mAbs PSG.01 - PSG.16. mAbs were purified from supernatants by protein G affinity chromatography using an ÄKTAprime plus system (GE Healthcare).

Commercially sourced primary antibodies were mouse anti-actin mAb and rabbit anti-His-tag polyclonal (pAb) (Abcam; Cambridge, U.K.), and rabbit anti-CEA pAb

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(DakoCytomation; Eching, Germany). Secondary antibodies were horseradish peroxidise (HRP)-conjugated donkey anti-mouse IgG, biotinylated horse anti-mouse IgG (Vector Laboratories; Peterborough, U.K.), HRP-conjugated goat anti-rabbit IgG (Abcam) and FITC-conjugated goat anti-mouse F(ab´)$_2$ (Jackson ImmunoResearch; Suffolk, U.K.). Detection of secondary Abs was achieved using Vector ABC, Vector VIP peroxidase substrate, HRP-conjugated avidin and DAB substrate from the Vectastain ABC detection kit (Vector Laboratories).

WESTERN BLOTTING

Cellular protein extracts were prepared by washing cells with PBS and lysing in lysis buffer consisting of Tris HCl, pH 7.4, 150 mM NaCl, 1% NP40 plus the tyrosine phosphatase inhibitor Na_3VO_4 (1 mM), and the protease inhibitors PMSF (1 mM), pepstatin (1 µM) and aprotinin (1.5 µg/ml). After incubation at 4°C for 20 min nuclear and cellular debris were removed by microcentrifugation at 14,000 rpm for 15 min at 4°C. Total protein was quantified using BCA Protein Assay Kit (Calbiochem; San Diego, California) according to manufacturer's instructions and lysate stored at minus 80°C until further use. Samples were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk (Marvel) in PBS containing 0.1% Tween (PBS-T) for 1 hr at RT. Membranes were probed with either anti-PSG1 mAb PSG.05 or mAb PSG.11 diluted 1:200 in 1% milk, or anti-actin mAb diluted 1:1000 in 1% milk, or anti-CEA pAb diluted 1:2000 in 1% milk. Membranes were then probed with either HRP-conjugated donkey anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG secondary antibodies diluted 1:100,000 in PBS-T for 1 hr at room temperature. Immunoreactive bands were visualized using Immobilon™ Western HRP-substrate chemiluminescence detection kit (Millipore) according to manufacturer's instructions.

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Serum and recombinant proteins samples were analysed as described above for cell lysates except that anti-His-Tag pAb diluted1:1000 in 1% milk was used to demonstrate equal gel loading of recombinant proteins.

FLOW CYTOMETRY

Stably transfected Hela cell lines: Hela-CEACAM1 (CC1), Hela-CEACAM3 (CC3), Hela-CEACAM5 (CC5), Hela-CEACAM6 (CC6), Hela-CEACAM7 (CC7) and Hela-CEACAM8 (CC8) were stained with 20 µg/ml CEACAM-specific rabbit anti-CEA pAb (DakoCytomation) as described,[29] or 20 µg/ml mAb PSG.05 or PSG.11 diluted in 3% fetal bovine serum (FBS) in PBS for 1 hr on ice, washed with ice-cold PBS, and incubated with FITC-conjugated goat anti-mouse $F(ab')_2$ (Jackson ImmunoResearch). Background fluorescence was determined using isotype-matched Ig. Subsequently, the stained cell samples were examined in a FACS Calibur flow cytometer (BD Biosciences; San Diego, CA) and the data were analyzed utilizing CellQuest software. Dead cells, identified by propidium iodide staining, were excluded from the determination.

IMMUNOHISTOCHEMISTRY

Tissue sections and tumor arrays were de-paraffinized in xylene and rehydrated prior to analysis. Antigen retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Endogenous peroxidase was quenched with 3% hydrogen peroxide in distilled water for 10 min. For IHC localisation of PSGs in tumor arrays, sections were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Triton-X 100 (TBS-Tx) for 1 hr at RT and then incubated with mAb PSG.11 diluted 1:200 in 3% BSA/TBS-Tx overnight at 4°C. Sections were then washed three times for 10 min in TBS-Tx before incubating with biotinylated horse anti-mouse pAb IgG secondary antibody diluted 1:200 in TBS-Tx, washed again three times for 10 min in TBS-Tx and detected using the ABC system and Vector VIP peroxidase substrate kit (Vector Laboratories). Alternatively,

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for IHC localisation of PSG protein in tissue sections, non-specific binding was blocked with 5% normal goat serum (NGS) in TBS containing 0.001% saponin (TBS-SAP). Sections were incubated overnight at 4°C with mAb PSG.05 or PSG.11 diluted 1:2000 in 1% NGS/TBS-SAP. Antibody binding was localized using a biotinylated secondary antibody (Santa Cruz Biotechnology), and HRP-conjugated avidin and DAB substrate using the Vectastain ABC detection kit (Vector Laboratories). Following IHC, sections were counterstained with Mayers' haematoxylin (BDH chemicals, Poole, U.K.), differentiated, dehydrated, and mounted with DePeX (BDH) permanent mounting medium. Parallel negative controls were performed using normal goat serum instead of primary antibody. Sections were viewed using an Olympus Provis microscope with a 10x eyepiece and either a 20x or 40x objective lens. Samples displaying immunoreactivity in more than 5% of tumor cells were regarded as positive. Qualitative analysis of the extent of PSG staining was performed for each tumor using the following 4-point scale: 0 = negative; + = 1 to 25% of cells stained; ++ = 26 to 50% of cells stained; +++ = 51 to 75%; ++++ > 75% of cells stained. For each tumor, 10 random high power fields were scored.[45]

QUANTITATIVE RT-PCR

Human normal brain cDNA (pool of 5 males, aged 21 to 66) was purchased from Biochain (AMS Biotech, UK). The Normal Human Digestive System MTC Panel was purchased from Clontech (oesophagus: pool of 39 male/female Caucasians aged 17 to 72). Tissue from pooled normal colon (n = 3) and pooled normal term placentas (n=10) was homogenised in 1 ml TRI Reagent (Sigma, UK) and total RNA was isolated. First strand cDNA was synthesised using 1 μg total RNA in a 20 μl reaction using random hexamer priming and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK).

Quantitative RT-PCR (qRT-PCR) primers were designed to give unbiased amplification of all PSG transcripts: PSG-all F: 5'GACCATGGGAACCCTCTCAGC; PSG-

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all R: 5'GAACATCCTTCCCCTCGGAAAC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalise mRNA input and cDNA synthesis efficiency using the primers: GAPDH F: 5'AGCCTCCCGCTTCGCTCTCT; GAPDH R: 5'CCAGGCGCCCAATACGACCA. qRT-PCR was carried out in duplicate 10 μl reactions using SYBR Green PCR Master Mix (Applied Biosystems, UK), 1 μl cDNA and primers at 600 mM using the ABI PRISM 7900HT instrument. PCR cycle was: initial denaturation (95°C for 10 min), amplification and quantification repeated for 40 cycles (95°C for 45 sec, 61°C for 45 sec and 72°C for 60 sec with a single continuous fluorescence measurement), followed by a melting curve program (60 - 95°C, with a heating rate of 1°C per 30 sec and a continuous fluorescence measurement). Human term placental cDNA was used to produce the standard curve. PCR products were identified by generating a melt curve and results were expressed as mean PSG expression relative to mean GAPDH expression.

qRT-PCR of mouse tissues was carried out essentially as described previously using Psg-all2F/R and HprtF/R primer sets.[46] PCR cycle was: initial denaturation (95°C for 10 min), amplification and quantification repeated for 40 cycles (95°C for 1 min, 60°C for 1 min and 72°C for 60 sec with a single continuous fluorescence measurement), followed by a melting curve program (60 - 95°C, with a heating rate of 1°C per 30 sec and a continuous fluorescence measurement).

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Table 1 PSG staining in colonic adenocarcinomas[a]

Grade	Dukes A	Dukes B	Dukes C	Dukes D
Negative	0	6	8	5
+	1	3	1	3
++	2	4	1	0
+++	2	0	0	0

[a]n=36

0, negative;

+, 1 to 25% of tumor cells stained positively;

++, 26 to 50% of tumor cells stained positively;

+++, 51 to 75% of tumor cells stained positively;

++++ > 75% of cells stained positively.

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Table 2 PSG staining in oesophageal squamous carcinomas[a]

Grade	1	2	3
Negative	0	1	3
+	1	3	0
++	0	1	0
+++	0	1	0

[a]n=10

0, negative;

+, 1 to 25% of tumor cells stained positively;

++, 26 to 50% of tumor cells stained positively;

+++, 51 to 75% of tumor cells stained positively;

++++ > 75% of cells stained positively.

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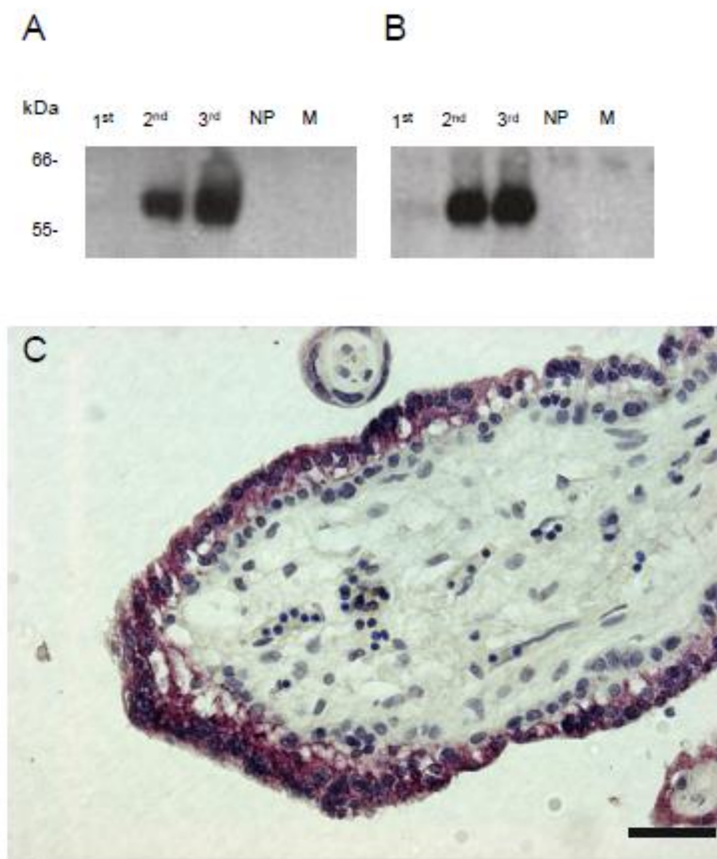

Figure 1. Western blot of 1st, 2nd and 3rd trimester maternal pregnant sera, non-pregnant female serum (NP), and male serum (M), probed with mAb PSG.05 (A) and mAb PSG.11 (B). Immunohistochemical staining of 1st trimester chorionic villus syncytiotrophoblast with mAb PSG.11 (C). Similar staining was observed with mAb PSG.05 (data not shown). Scale bar = 50 µm.

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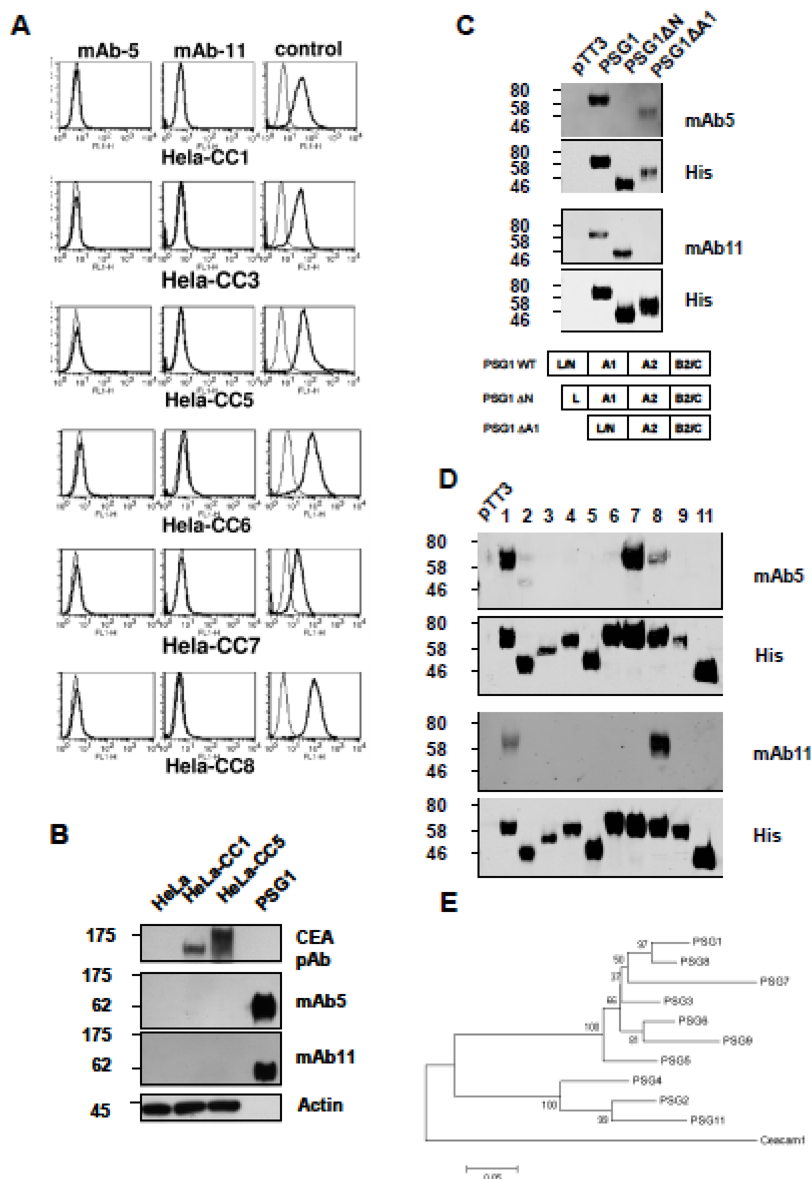

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Figure 2. FACS analysis using mAb PSG.05, mAb PSG.11 and rabbit anti-CEA pAb positive control, of Hela cell lines stably expressing CEACAMs (CC1, CC3, CC5, CC6, CC7, CC8) (A). Western blots of Hela, Hela-CC1 and Hela-CC5 cell lysates, and purified recombinant PSG1, probed with rabbit anti-CEA polyclonal, mAb PSG.05, mAb PSG.11, and anti-Actin mAb as loading control for cell lysates (B). Western blots of supernatants from Freestyle293 cells 72 hrs post-transfection with pTT3-PSG1 expression vectors lacking either the N or A1 domain and probed with mAb PSG.05, mAb PSG.11, and an anti-His-Tag polyclonal as positive control. A schematic of human PSG1 wildtype and deletion mutant protein domain

structures are shown (C). Western blots of supernatants from Freestyle293 cells 72 hrs post-transfection with pTT3-PSG constructs (PSG1-9, 11) probed with mAb PSG.05, mAb PSG.11, and an anti-His-Tag pAb as positive control (D). Phylogenetic tree of human PSG amino acid sequences. Phylogenetic trees (Neighbour-joined pairwise comparison phylogenetic trees) were constructed using the MEGA4.0 software programme (http://www.megasoftware.net/). Data were bootstrapped 1000 times and all major branches yielded values of 95–100%. The scale bars represent 0.5 nucleotide substitutions per site (E).

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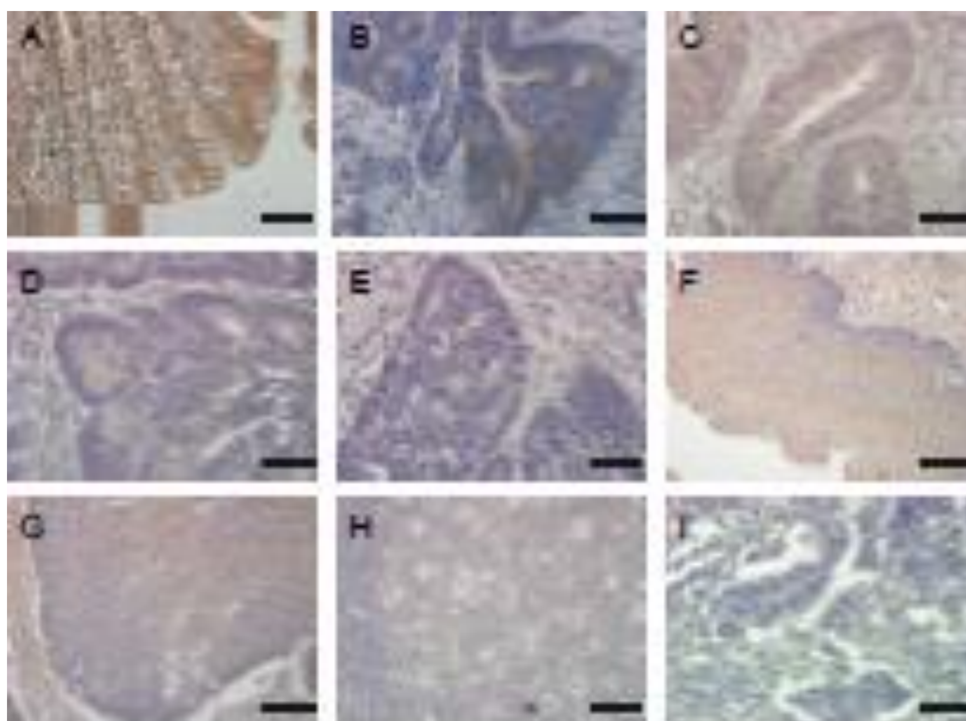

Figure 3. Immunostaining of PSG (brown) using mAb PSG.11 in normal colon (A), colon adenocarcinoma of increasing stage (B–E), normal oesophagus (F), oesophageal squamous cell carcinoma (G) and metastatic oesophageal carcinoma (H). PSG staining decreases with increasing Dukes' stage (B, Dukes' A; C, Dukes' B; D, Dukes' C; and E, Dukes' D) and on progression from oesophageal squamous cell carcinoma (G) to metastatic oesophageal carcinoma (H). No staining observed in goat serum control (I). All sections were counterstained with haematoxylin (blue). Scale bars = 200 µm (F) and 100 µm (A-E, G-I).

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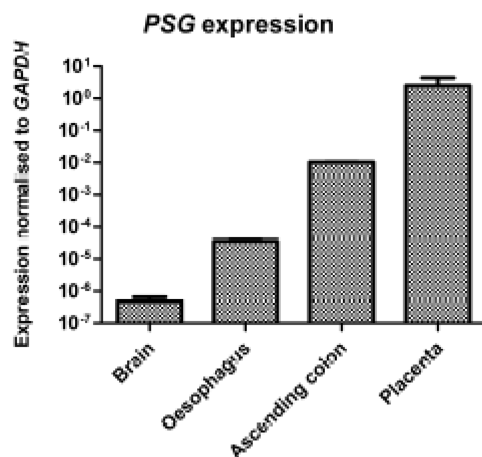
A



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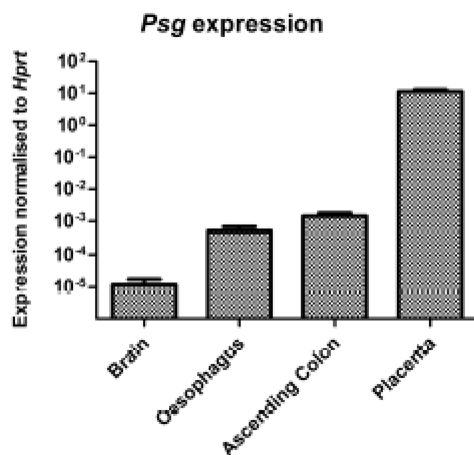

Figure 4. qRT-PCR analysis of PSG expression in selected human (A) and mouse (B) tissues. Pooled samples of human brain (n=5), oesophagus (n=39), colon (3) and placenta (n=10) were analysed in duplicate and the experiment was repeated once. For each tissue the four data points were combined, normalised to *GAPDH*, and expressed ± S.E.M. (A). Similarly, tissues from two C57BL/6 mice were analysed in duplicate and the experiment was repeated once. Data were combined, normalised to *Hprt*, and expressed ± S.E.M. (B). Note logarithmic scale in A & B.

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