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Early Expression of Pregnancy-Specific Glycoprotein 22 (PSG22) by Trophoblast Cells Modulates Angiogenesis in Mice[1]

Sandra M. Blois,[3,4,5] Irene Tirado-González,[4,5] Julie Wu,[6] Gabriela Barrientos,[5] Briana Johnson,[6] James Warren,[6] Nancy Freitag,[5] Burghard F. Klapp,[5] Ster Irmak,[7] Suleyman Ergun,[8] and Gabriela S. Dveskler[2,6]

[5]*Charité Centrum 12 für Innere Medizin und Dermatologie, Reproductive Medicine Research Group, University Medicine of Berlin, Berlin, Germany*
[6]*Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland*
[7]*Institute of Anatomy, University of Duisburg-Essen, University Hospital Essen, Essen, Germany*
[8]*Institute of Anatomy and Cell Biology, Julius-Maximilians-Universität Würzburg, Würzburg, Germany*

ABSTRACT

Mouse and human pregnancy-specific glycoproteins (PSG) are known to exert immunomodulatory functions during pregnancy by inducing maternal leukocytes to secrete anti-inflammatory cytokines that promote a tolerogenic decidual microenvironment. Many such anti-inflammatory mediators also function as proangiogenic factors, which, along with the reported association of murine PSG with the uterine vasculature, suggest that PSG may contribute to the vascular adaptations necessary for successful implantation and placental development. We observed that PSG22 is strongly expressed around the embryonic crypt on Gestation Day 5.5, indicating that trophoblast giant cells are the main source of PSG22 during the early stages of pregnancy. PSG22 treatment up-regulated the secretion of transforming growth factor beta 1 and vascular endothelial growth factor A (VEGFA) in murine macrophages, uterine dendritic cells, and natural killer cells. A possible role of PSGs in uteroplacental angiogenesis is further supported by the finding that incubation of endothelial cells with PSG22 resulted in the formation of tubes in the presence and absence of VEGFA. We determined that PSG22, like human PSG1 and murine PSG17 and PSG23, binds to the heparan sulfate chains in syndecans. Therefore, our findings indicate that despite the independent evolution and expansion of human and rodent PSG, members in both families have conserved functions that include their ability to induce anti-inflammatory cytokines and proangiogenic factors as well as to induce the formation of capillary structures by endothelial cells. In summary, our results indicate that PSG22, the most abundant PSG expressed during mouse early pregnancy, is likely a major contributor to the establishment of a successful pregnancy.

angiogenesis, implantation, pregnancy, pregnancy-specific glycoprotein 22, trophoblast

INTRODUCTION

Pregnancy-specific glycoproteins (PSG) are members of the carcinoembryonic antigen family of immunoglobulin-like genes [1]. They are highly homologous proteins secreted by the placenta and represent the most abundant fetal proteins in the maternal blood at term of pregnancy [2]. The PSG family comprises 11 genes in humans and 17 in mice, which are localized on chromosome 19q13.2 and chromosome 7, respectively [3, 4]. Mouse PSG expression is detected exclusively in trophoblast giant cells (GC) and spongiotrophoblast of the placenta [5, 6], where it has been associated with regulatory mechanisms promoting pregnancy maintenance [7]. Treatment with anti-PSG antibodies or immunization with PSG have, accordingly, been found to induce fetal loss in mice and monkeys [8, 9]. Low systemic human PSG levels are often linked to different complications of pregnancy, including fetal hypoxia, intrauterine growth retardation, preeclampsia, and spontaneous abortion [10–12].

The pregnancy protective mechanisms induced by PSG appear to rely on the immunomodulatory functions of these proteins, particularly through the induction of anti-inflammatory cytokines produced by immune cells [13, 14]. For example, the ability of human PSG1, PSG6, and PSG11 and murine PSG17 and PSG18 [7, 14] to stimulate interleukin 10 (IL10) secretion by macrophages and monocytes has been demonstrated [13, 15], often showing cross-species reactivity. In addition, an important anti-inflammatory mediator induced by human and mouse PSG is TGFB [13]. As further suggested by in vivo studies, PSG seem to render alternatively activated antigen presenting cells that in turn polarize maternal T-cell differentiation to the TH2-type phenotype compatible with successful pregnancy [16].

The placenta is a unique organ designed to ensure the selective delivery of nutrients from the mother to the fetus, a function that is highly dependent on timely regulated adaptations of the uterine vasculature, including structural modifications to increase blood flow and the expansion of preexisting vessels through the process of angiogenesis. Administration of a single dose of antiangiogenic compounds during mouse pregnancy results in embryo resorption due to disrupted placentation [17], demonstrating the pivotal importance of vascular responses for the outcome of pregnancy. Angiogenic activity in the uterus is controlled by the coordinate interplay between stimulatory and inhibitory signals of both maternal and embryonic origin, which determine the activation status of vascular endothelial cells.

Among the embryo-derived cell lineages, GC are most likely to influence placental vascularization because of their

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[2]Correspondence: Gabriela S. Dveksler. E-mail: gabriela.dveksler@usuhs.edu
[3]Correspondence: Sandra M. Blois. E-mail: sandra.blois@charite.de
[4]These authors contributed equally to this work.

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unique colocalization with maternal blood sinuses. In addition, they produce hormones, angiogenic and vasoactive mediators, such as VEGF, proliferin, adrenomedullin, and insulin growth factor II [18–20]. Some GC functions may be mediated by their ability to produce PSG, as suggested by the prominent association of at least one PSG with the endothelial lining of vascular spaces surrounding the implantation site from Gestation Day (GD) 8.5 to 11.5 [6]. PSG23 has been demonstrated to induce the proangiogenic factors TGFB and VEGFA from a variety of cells involved in the control of pregnancy-induced vascular modifications, including macrophages and bone marrow-derived dendritic cells (DC) [21]. Although the high homology between members of the PSG family is likely to result in overlapping protein functions, it remains to be determined whether other PSG exert similar proangiogenic effects during pregnancy.

In the present study we demonstrate that PSG22, the predominant member expressed by GC in early pregnancy, is able to stimulate TGFB and VEGFA production by primary immune cells, including uterine and splenic DC, NK cells, and macrophages. In addition, we show that PSG22 binds to heparan sulfate proteoglycans. Most important, PSG22 supports angiogenesis not only by inducing growth factor expression but also by directly promoting the differentiation of capillary tubes by vascular endothelial cells. Our findings suggest that PSG22, which is detected as early as GD 5.5, as well as other members of the PSG family expressed at later times, could play an important role in driving the normal vascular modifications that occur throughout pregnancy in addition to modulating maternal immunity.

MATERIALS AND METHODS

Protein Production and Purification

The cDNA encoding for *PSG22N1A-His-Flag* (hereafter referred to as PSG22N1A) was synthesized by Genscript (Piscataway, NJ). This synthetic cDNA codes for the leader peptide, N1, and A domains of *PSG22*, along with the nucleotide sequence for six histidines (6× His) and a Flag tag (Sigma, St. Louis, MO). The *PSG22N1A-His-Flag* cDNA was subcloned into the *Eco*RI and *Xba*I sites of the pEF1/V5-His expression vector (Invitrogen, Carlsbad, CA) and transfected into dihydrofolate reductase-negative (DHFR-) CHO cells, along with the pDCHIP plasmid, which encodes the *DHFR* minigene. Prior to methotrexate selection, single cell clones were obtained by limiting dilution in a 96-well plate. Expression of the recombinant protein in single clones was confirmed by immunoblotting with the anti-Flag M2 antibody (Sigma) and by ELISA with the anti-Flag M2-peroxidase (HRP) antibody (Sigma). The clone expressing the highest levels of recombinant protein, as determined by ELISA after methotrexate selection (reaching a maximum concentration of 1.28 μM), was expanded and inoculated into a 5-kDa-molecular-weight cutoff hollow fiber cartridge (FiberCell Systems, Frederick, MD). The supernatant from the cartridge was harvested daily and kept frozen until it was processed by affinity chromatography to purify the protein. PSG22N1A was dialyzed into a 20-mM sodium phosphate buffer (pH 7.4) containing 20 mM imidazole (EMD Chemicals, Inc., Gibbstown, NJ) and purified from the harvested supernatant using a HisTrap column on the ÄKTAprime Plus system (GE Healthcare, Piscataway, NJ). Positive fractions eluted from the HisTrap column were pooled, buffer exchanged into PBS, and applied to a column packed with anti-Flag M2 agarose (Sigma). The protein was then eluted with 3× Flag peptide (Sigma), concentrated, and buffer exchanged with PBS. The purified protein was run on a SDS-PAGE gel, stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL), and quantitated against BSA standards. The level of endotoxin, a potent inducer of cytokines including TGFB in the purified proteins, was determined using the HEK-Blue LPS detection system (InvivoGen, San Diego, CA). The proteins had less than 0.3 ng/ml of *Escherichia coli* LPS, which is the limit of detection of the assay.

Cell Lines

Chinese hamster ovary epithelial (CHO-K1), CHO-pgsA-745, and mouse fibroblast L929 cells were obtained from American Type Culture Collection (Manassas, VA). Sog 9 and L929 fibroblasts were obtained from Drs. F. Tufaro and G. Cohen and were sent to us by Dr. K. Spindler (University of Michigan, Ann Arbor). Bone marrow-derived macrophages were obtained as previously reported [22]. Human B lymphocyte (Namalwa) transfectants were a gift of Dr. G. David (University of Leuven, Belgium). Sog 9 cells expressing murine CD9 were generated as previously reported [23]. All cells were maintained as previously described [23].

Primary Cells

Five- to 6-wk-old C57BL/6 mice deficient in CD9 were bred from a $CD9^{+/-}$ breeding pair obtained from Dr. Claude Boucheix (Hôpital Paul Brousse, Villejuif, France). Five- to 6-wk-old BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). Briefly, mice were injected i.p. with thioglycollate (BD Biosciences, San Jose, CA). Three days after i.p. injection, macrophages were obtained by peritoneal lavage and seeded at 1.5×10^6 cells per well in a 24-well plate. Twenty-four hours after seeding the cells, macrophages attached to wells and nonadherent cells were removed. Plating media (RPMI 1640, Gibco, Carlsbad, CA; supplemented with 2% FBS and 0.5× PS) was replaced with treatment media (RPMI supplemented with 2% fetal clone III and 0.5× PS, sterile filtered through a 0.2-μm membrane) prior to addition of protein. To obtain DC and NK cell suspensions, we isolated uterine cells from Balb/c mated C57BL/6 female mice at GD 8.5 and splenic cells from nonpregnant controls. Briefly, the whole uteri or spleens were collected, washed with sterile PBS, carefully cut into small pieces, collected in tubes containing HBSS, and digested for 20 min at 37°C under slight agitation in HBSS with 200 U/ml hyaluronidase (Sigma, Munich, Germany), 1 mg/ml collagenase (type C-2139, Sigma), 0.2 mg/ml DNase I (Boehringer Mannheim, Mannheim, Germany), and 1 mg/ml bovine serum albumin-fraction V (Sigma). The isolated cells were collected in a fresh tube through a 100-μm net (BD Biosciences, Heidelberg, Germany) and washed with RPMI 1640 containing 10% FCS (Invitrogen, Darmstadt, Germany). Trypan blue exclusion revealed that cell viability was approximately 90%. For collection of cells expressing ITGAX (also known as CD11c) or KLRB1C (also known as NK1.1), uterine or spleen suspensions were incubated for 30 min at 4°C with biotinylated hamster anti-mouse ITGAC (CD11c; BD Biosciences, Heidelberg, Germany) diluted 1:100 or biotinylated anti-mouse KLRB1C (NK1.1; BD Biosciences, Heidelberg, Germany) diluted 1:100 in labeling buffer (PBS supplemented with 2% BSA, Sigma). After washing, cells were incubated (15 min, 4°C) with streptavidin MicroBeads (#130-048-101) and processed using MACS to collect $ITGAX^+$ or $KLRB1C^+$ cells. Examination by FACS revealed that >95% of selected cells by miniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) expressed ITGAX or KLRB1C. Procedures that involved mice were approved by both university committees on animal use in research and education.

Semiquantitative RT-PCR

Total RNA was extracted from the whole implantation unit at GD 5.5, 7.5, and 10.5 with Nucleospin RNA/protein isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. In addition, embryo-free implantations unit at GD 5.5 and 7.5 were processed as described above. After DNase digestion (Invitrogen), total RNA was reverse transcribed with random primers (Invitrogen) followed by PCR amplification for *PSG22* using the forward primer 5′-GTGTTGACAATCTGCCAGAGAATCTT-3′ and reverse primer 5′-CTCCTGGGTGACATTTTGGATC-3′. PCR reactions were performed for 30 cycles with a denaturing temperature of 95°C (4 min), annealing temperature of 55°C (30 sec), and extension temperature of 72°C (45 sec) followed by final extension at 72°C (7 min). As a control, a conventional PCR for hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT*) was performed using the *HPRT* forward primer (5′-GTTGGATACAGGCCAGACTTTGT-3′) and *HPRT* reverse primer (5′-ACAGGACTAGAACACCTGC-3′; Tib-Molbiol, Berlin, Germany). The size of the expected PCR product was 179 base pairs. All amplified products were separated by agarose gel electrophoresis (1.5%) and stained with ethidium bromide. Photodocumentation was performed using the UV-transilluminator GENEFLASH (Syngene Bio Imaging, Cambridge, UK). Density of detected bands was quantified using ImageJ 1.42q program (National Institutes of Health, Bethesda, MD).

Dual Fluorescence Immunohistochemistry Localization of PSG22 and PAN-Cytokeratin in the Mouse Pregnant Uterus and Generation of PSG22 Antisera

The rabbit polyclonal anti-PSG22 antibody was generated by Genscript. Rabbits were inoculated with recombinant purified PSG22N1A-His-Flag described above under *Protein Production and Purification*. The antisera obtained were tested by ELISA and immunoblot analysis at different dilutions against recombinant proteins generated in the Dveksler laboratory, including

the N-domains only of PSG22, PSG17, and PSG23; PSG23N1A-His-Flag; PSG22N1A-His-Flag; and FLAG-Fc. The antiserum employed in the immunohistochemical staining reacted primarily with the N-domain of PSG22. No reactivity to the N-domains of PSG23 or PSG17 or the Flag epitope was detected. To determine whether the antibody could inhibit binding of PSG22 to cells and for the IHC studies, the rabbit anti-PSG22 antiserum was purified with a protein A column under manufacturer's recommendations (GE Healthcare). There was some reactivity apparent at the lower dilutions in the ELISA and at a 1:800 dilution in the Western blot analysis against the A-domains of both PSG22 and PSG23, which are similar in amino sequence.

C57BL/6 females were placed with fertile BALB/c males and the morning that a vaginal plug was observed was considered to be GD 0.5. Mice uteri were removed on GD 5.5, 7.5, and 10.5. Implantation segments (which included mesometrial and antimesometrial sides) containing endometrium and myometrium were removed and individually frozen for immunohistochemistry. Uterine cross sections (8 μm) were mounted onto silanized slides, fixed in acetone at −20°C for 10 min, and stored until use. Subsequently, sections were washed in TBS buffer and blocked with serum-free protein block (DAKO, Hamburg,Germany) for 25 min at RT. Polyclonal rabbit anti-PSG22N1A antiserum was added to the tissue samples at a 1:500 dilution and incubated overnight. After washing in TBS buffer and incubation with TRI-TC-conjugated secondary antibody (1:200; Jackson ImmunoResearch, Suffolk, UK) for 2 h at RT, the slides were washed and incubated with Alexa 488 conjugated anti-Pan Cytokeratin Ab (1:500; eBioscience, Frankfurt, Germany) overnight at 4°C. Slides were washed, incubated for 10 min with 4′,6-diamino-2-phenylindole, and mounted. Negative controls substituting the primary antibody with preimmune serum did not reveal immunoreactivity. Slides were analyzed using a confocal laser scanning microscope (cLSM 510; Carl Zeiss, Jena, Germany). Photodocumentation was performed using Zeiss LSM Image Browser software, version 4.0.0.157 (Carl Zeiss).

Solid-Phase Binding Assay

ELISA plates (96 wells) were coated overnight at 4°C with 200 μg of heparin, heparan, or chondroitin sulfate or BSA in quadruplicate. The coated plates were washed and blocked with PBS and 0.5% BSA. Purified recombinant proteins (200 ng/well in 100 μl of PBS) were applied to the coated plates and incubated overnight at 4°C. After three washes with PBS and 0.05% Tween 20, the proteins were detected with 1 ng/ml HRP-conjugated anti-Fcγ Ab (Thermo Scientific) followed by the addition of TMB substrate reagent (BD Biosciences). The reaction was stopped by the addition of sulfuric acid, and the plate was read at 450 nm in an ELISA plate reader.

ELISA and Flow Cytometry

For ELISA, cells were plated in 24-well plates (BD Biosciences) in triplicate, 1.5×10^6 cells per well (peritoneal macrophages) and 1.0×10^5 cells per well (DC and NK cells), for each treatment and incubated in a 37°C humidified incubator with 5% CO_2. The following day, cells were treated with recombinant proteins for an additional 20–24 h. After treatment, the supernatants were collected and centrifuged at 3000 rpm for 5 min to remove cell debris. For TGFB ELISA, the supernatant was activated according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). Murine VEGFA was measured by ELISA (R&D Systems, Oxon, UK). At least three different protein preparations were tested, and experiments were repeated a minimum of three times.

For FACS analysis, peritoneal macrophages were detached from the tissue culture flask by scraping and were incubated with 1 μg of Fc-block (BD Biosciences) per million cells for 15 min in FACS buffer (PBS with 2% BSA and 0.01% sodium azide). Macrophages were then incubated with 50 μg/ml of anti-CD9 mAb KMC8.8 (BD Biosciences) or isotype control mAb for 60 min. All other cells were detached from the flasks with Accutase (Innovative Cell Technologies, San Diego, CA) prior to incubation with proteins. Five micrograms of PSG22N1A or the control protein (FLAG-Fc) were added for 1 h, followed by biotin-conjugated anti-FLAG mAb (Sigma) and Streptavidin-APC (Invitrogen). All the incubations were performed on ice in 100 μl of FACS buffer, with two 5-ml washes with FACS buffer between each step. The cells were fixed in CytoFix (BD Biosciences) and analyzed in an LSR II flow cytometer (BD Biosciences). Fifty thousand events were collected, and the FlowJo software was used for postacquisition analysis.

Endothelial Tube Formation Assay

The endothelial tube formation assay was carried out using Purecol, a three-dimensional type I collagen gel (Nutacon, Leimuiden, The Netherlands), prepared in 48-well cluster tissue culture dishes as previously described [24]. After polymerization of the gel at 37°C, human dermal microvascular endothelial cells (HDMECs) were seeded at a concentration of 6×10^4 cells per well in 300 μl of endothelial growth medium MV containing 5% FCS and SupplementMix (containing heparin, EGF, and hydrocortisone; Promocell, Heidelberg, Germany). At confluence, the medium was replaced by basal medium containing 5% heat-inactivated FCS without further supplements. After 24 h, cells were treated with PSG22N1A (100 or 500 ng/ml) alone or in combination with VEGF-A (50 ng/ml). This treatment was repeated every 3 days, and photographs were taken with a phase-contrast microscope (Carl Zeiss) equipped with a digital camera (ProgResC10plus; Jenoptik, Jena, Germany). In each well, eight endothelial tubes were measured in length using the Optimas software program according to the procedure published by Kilic et al. [25]. After 3–6 days, gels were taken from the wells, fixed with 4% paraformaldehyde, and embedded in paraffin. Subsequently, cross sections were prepared and stained by calcium red for light-microscope studies in order to examine the capillary-like lumen formation.

Statistics

SPSS (SPSS, Inc., Chicago, IL) and Microsoft Excel were used for data statistical analysis. The two-tailed Student *t*-test was used to determine statistical significance in experiments comparing protein treated versus untreated cells, with a *P*-value of <0.05 as a cutoff. One-way analysis of variance (ANOVA) was used to determine statistical significance of dose-response assays with a *P*-value of <0.001 as a cutoff. RT-PCR results were analyzed using the 7500 System Sequence Detection Software version 1.2.3 (Applied Biosystems, Carlsbad, CA). FACS data were analyzed using WinList 6.0 (Verity Software House, Topsham, ME). All data are representative of at least three independent experiments.

The morphometric quantification of tubular length was performed using Optimas according to the procedure published by Kilic and coworkers [25]. Images were recorded from the collagen gel containing endothelial tubes using a Leica phase-contrast microscope equipped with a digital camera and a 20× objective. The images were then imported into the Optimas image analysis system, and the tubes were marked by lines. The length of these lines was measured automatically by the software, and the sum of the measurements of all the lines from a single well was calculated as final tubular length induced by a factor. While the absolute length of the tubes differed between experiments, the tube-forming effects of the factors used remained similar. Therefore, the measurements of the tubular length of one experiments is presented here as being representative of the results of three separate experiments. The graphic bars were created using the software Microsoft Excel.

RESULTS

Generation of Recombinant PSG22N1A and Expression of Psg22 During Pregnancy

There are 17 mouse PSG family members with different expression levels at different stages of development [4]. *Psg22* has been identified as an early expressing gene in the mouse placenta [4, 6]. Accordingly, *Psg22* mRNA was detectable in mouse uterine tissue as early as GD 5.5 and became most prominent at GD 10.5, coinciding with placental formation (Fig. 1A). In contrast, *Psg22* mRNA was undetectable when the embryo compartment was excluded from the implantation unit (Supplemental Fig. S1A; all Supplemental Data are available online at www.biolreprod.org). These results were confirmed by fluorescence immunohistochemistry analysis revealing a strong signal within the trophoblast cells in the embryonic crypt at GD 5.5. Later, on GD 7.5, PSG22 is expressed mainly in GC bordering the decidua (Fig. 1B). On GD 10.5, the PSG22 signal is observed on the placenta and also decidua basalis. To analyze the functional relevance of this early expression in vitro, we used stable transfection of DHFR-CHO cells to generate recombinant PSG22N1A protein, as described in *Materials and Methods*. The recombinant protein consists of the leader peptide, N1, and A domains of PSG22, followed by six histidines and a C-terminal Flag tag (Fig. 1C). Previous studies have demonstrated that these domains are sufficient to support PSG binding and activity [7, 14, 26]. Recombinant PSG22N1A was sequentially purified on HisTrap

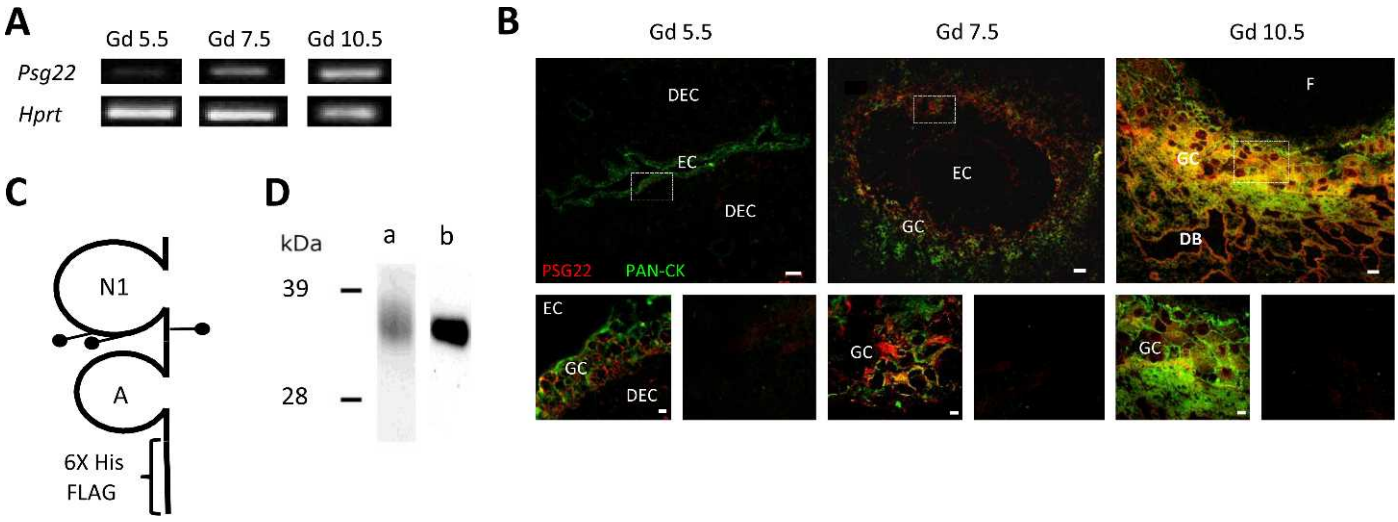

FIG. 1. *Psg22* is an early expressing gene during mouse normal pregnancy. **A**) Semiquantitative RT-PCR analysis of *Psg22* expression on whole implantation unit at GD 5.5, 7.5 and placenta tissue on GD 10.5, where bands correspond to nonconsecutive lanes visualized on the same agarose gel after ethidium bromide staining. **B**) Dual fluorescence immunohistochemistry confirmation of PSG22 and Pan-Cytokeratin (Pan-CK) expression in the mouse uterus during early pregnancy. Uterine sections were stained with PSG22 (red) and PAN-CK (green). Note that PSG22 protein was detectable as early as GD 5.5 within the trophoblast cells (GC, PAN-CK+) in the embryonic crypt (EC; left, top and bottom). On GD 7.5, PSG22 was detected on trophoblast giant cells (middle, top and bottom). Giant cells PAN-CK+ remained PSG22 positive on GD 10.5 (right, top and bottom). The photomicrographs of representative uterine sections are shown. Bars = 50 μm (top panels), 5 μm (bottom panels). No immunostaining was noted when similar sections were incubated with the preimmune serum and the TRI-TC labeled secondary antibody (right bottom). DB, decidua basalis; DEC, decidua; F, fetus. **C**) Schematic representation of PSG22N1A with the potential N-linked glycosylation sites indicated. **D**) Purification of PSG22N1A by affinity chromatography. Recombinant PSG22N1A secreted from stably transfected CHO cells was sequentially purified on a HisTrap and anti-FLAG affinity column, rendering a glycoprotein that was approximately 90% pure based on Coomassie Blue staining (lane a). This was confirmed by immunoblot analysis using an anti-FLAG mAb rendering a single band with an approximate molecular weight of 36 kDa (lane b).

and anti-Flag affinity columns, rendering a glycoprotein that was approximately 90% pure based on Coomassie Blue staining (Fig. 1D).

Recombinant PSG22N1A Induces TGFB and VEGFA in Macrophages, NK, and DC

Previous studies indicated that TGFB and VEGFA are up-regulated in macrophages in response to PSG23N1A treatment [21]. The homology between murine PSGs varies, depending on which domains are compared, but previous studies indicate that family members may have overlapping functions [5]; therefore, we tested whether PSG22 also could up-regulate TGFB and VEGFA. Macrophages treated with 30-μg/ml or higher concentrations of PSG22N1A secreted TGFB, while cells required at least 40 μg/ml of PSG22N1A before significant induction of VEGFA could be observed (Supplemental Fig. S1, B and C). Alternative activation of macrophages, which favors the production of anti-inflammatory mediators, has been linked to the ability of these cells to promote angiogenesis [26, 27]. In addition to macrophages, uterine DC and NK cells secrete VEGF and TGFB, which may be important for the proangiogenic functions ascribed to these leukocyte subsets during decidualization and placentation [28–30]. To investigate if these cells respond to PSG22N1A, we isolated DC and NK cells from GD 8.5 pregnant mice and tested the ability of PSG22N1A to induce the secretion of VEGF and TGFB by ELISA. Basal secretion of TGFB from DC and NK cells isolated from spleen and pregnant uterine tissue did not differ (Fig. 2A). Leukocytes isolated from spleen and pregnant uterine tissue up-regulated TGFB in response to 50 μg/ml of PSG22N1A, compared to control protein (FLAG-Fc). Similarly, DC and NK cells isolated from spleen and pregnant uterine tissue secreted more VEGFA in response to PSG22N1A stimulation than when treated with control protein (Fig. 2B). However, we did not observe a significant difference in TGFB or VEGFA up-regulation in cells isolated from spleen versus pregnant uteri, though there was a trend toward higher responses observed in cells isolated from uterine tissue.

PSG22N1A Binds to Syndecans and Not to the Tetraspanin CD9

Previous studies showed that PSG17 binds to the CD9 and to heparan sulfate proteoglycans (HSPG), including all four members of the syndecan family, but that PSG23 binds only to the latter [22, 23]. We sought to determine if PSG22 utilizes CD9, HSPGs, or an unknown moiety as its receptor. In binding assays, we tested whether preincubation of murine peritoneal macrophages with anti-CD9 mAb blocked binding of PGS22 to these cells, as was previously shown for PSG17 binding [26]. Cells preincubated with 50 μg/ml of the isotype control mAb (IM) had a median fluorescence intensity (MFI) of 19, and cells preincubated with the same concentration of anti-CD9 mAb had an MFI of 21, as determined by FACS analysis (Fig. 3A). Thus, our studies show that unlike PSG17, PSG22 does appear to use CD9 as its putative receptor. We then tested whether PSG22N1A binds to HSPG. To this end, we performed binding studies in cells that do not express cell surface HSPG, such as bone marrow-derived macrophages, Sog9 fibroblasts, and CHO-PSGA-745 [23, 31–33]. PSG22 did not bind to these cells, though it did bind to their counterparts, peritoneal macrophages, L929, and CHO-K1, respectively, which express HS on their cell membrane (Fig. 3B and Supplemental Fig. S1, D). In addition, PSG22N1A did not bind to Sog 9 cells transfected with CD9 (Supplemental Fig. S1, E) [23], supporting the results shown in Figure 3A, which demonstrate that PSG22 binding does not depend on CD9. To further analyze the interaction of PSG22N1A with cell surface proteoglycans, we studied binding of PSG22N1A to Namalwa

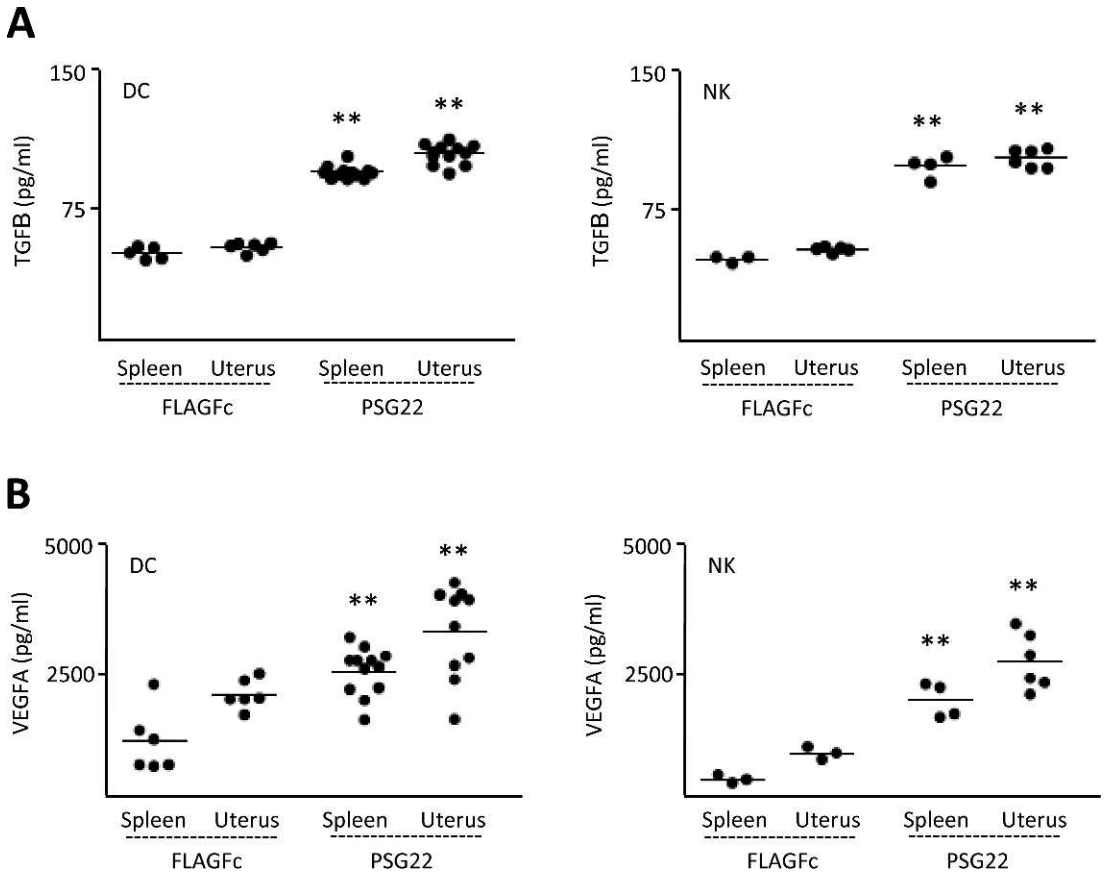

FIG. 2. PSG22N1A induces proangiogenic factor secretion by spleen and uterine DC and NK cells. **A**) Splenic and uterine DC and NK cells were isolated from mice carrying allogeneic pregnancies at GD 8.5 and nonpregnant controls and tested for proangiogenic factor secretion following stimulation with 50 μg/ml PSG22N1A. Both cell types responded to treatment with PSG22N1A by up-regulating the secretion of TGFB compared to cells treated with FLAGFc as a control protein. **B**) Analysis of VEGFA in supernatants of splenic and uterine DC and NK cells stimulated PSG22N1A and FLAGFc for 48 h. All cells were treated in triplicate wells and supernatants analyzed as separate samples. Data are presented as mean ± SEM of at least three separate experiments. $^{**}P < 0.01$ as assessed by one-way ANOVA.

cells, which express low levels of HS on their membrane, and to Namalwa cells transfected with plasmids encoding syndecans 1 to 4 [34]. PSG22N1A bound significantly better to cells expressing syndecans than to untransfected Namalwa cells ($P < 0.05$; Namalwa-transfected SD1 or SD2; $P < 0.001$; Namalwa-transfected SD3 or SD4; Fig. 3C). Solid-phase binding assays also demonstrated that PSG22 binds to heparin and heparan sulfate, but, in contrast to PSG17 and 23, no binding to chondroitin sulfate could be detected over-BSA control (Fig. 3D).

PSG22N1A Induces Capillary Differentiation In Vitro

We have previously reported that human PSG1 binding to the HS chains of syndecans induces the formation of capillary-like structures by endothelial cells [35, 36]. Because PSG22 also binds to syndecans, we hypothesized that this murine PSG might also induce endothelial tube formation. To address this possibility, we performed in vitro tube formation assays using HDMECs. Treating cells with PSG22N1A at concentrations of 100 and 500 ng/ml (Fig. 4, C and D) induced the formation of endothelial tubes, comparable to endothelial tubes observed in the positive controls that were treated with 50 ng/ml VEGFA (Fig. 4B). Endothelial tubes induced using a combination of 100 ng/ml each of PSG22N1A and VEGFA showed no significant difference (Fig. 4E) compared to those induced by VEGF alone (used as positive control). However, addition of 500 ng/ml of both recombinant protein and growth factor led to an increase in the length and network of the tubes (Fig. 4F). The morphometric quantification of tubular length using Optimas confirmed these findings (Fig. 4G). Measurements were performed in three independent experiments. In each of these analyses, the tube-forming effect of PSGs alone was comparable to that observed by VEGF alone. In cases of combined application with VEGF, PSG increased the length of VEGF-induced tubes by 55%–90%, depending on PSG concentrations used, as exemplified in Figure 4G. Light-microscope analyses on cross sections of the paraffin-embedded endothelial tubes showed that the endothelial cells formed clear capillary-like lumens within the collagen gel after stimulation with VEGF alone (Fig. 4H), 500 ng/ml of PSG22N1A alone (Fig. 4I), and VEGF plus 500 ng/ml of PSG22N1A (Fig. 4J), confirming the results observed using phase-contrast microscopy. In wells where the HDMECs were exposed to basal medium only (control), the endothelial cells remained on the top of collagen gel (Fig. 4K), and no tube formation was observed.

DISCUSSION

The success of mammalian pregnancy relies on the spatial-temporal coordination of several vascular processes at the fetal-maternal interface. These include early adaptations of the endometrial vasculature to support blastocyst implantation, as well as the expansion and de novo formation of blood vessels

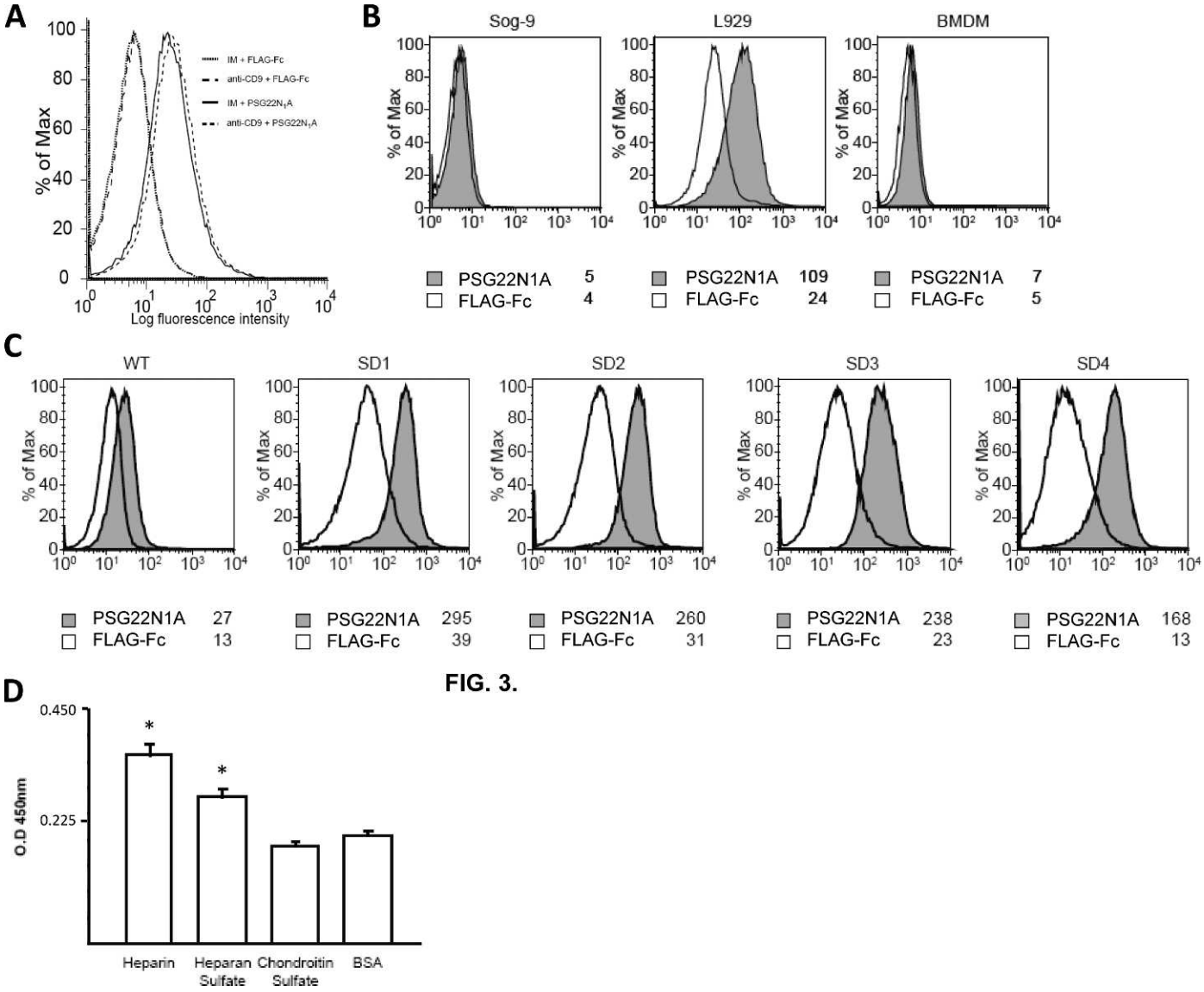

FIG. 3.

FIG. 3. PSG22 binds to plasma membrane proteoglycans, immobilized heparin, and heparan sulfate but not to chondroitin sulfate and CD9. **A**) PSG22 binding to cells is not inhibited by the anti-CD9 mAb KMC8. **B**) Sog-9, L929, and BMDM cells were incubated with PSG22N1A followed by anti-FLAG biotin and streptavidin APC. The median fluorescence intensity (MFI) of each treatment is shown. **C**) Namalwa and Namalwa cells stably transfected with syndecans 1–4 (SD1–SD4) were incubated with PSG22N1A followed by anti-FLAG biotin and streptavidin APC. The MFI of each treatment is shown. **D**) 96-well NUNC plates were coated overnight with the glycosaminoglycans indicated or with BSA as a control. After blocking, PSG22N1A was added to the wells, and following extensive washes, bound PSG22 was detected with HRP-conjugated anti-FLAG Ab. All treatments were performed in quadruplicate, and data are representative of three independent experiments. Statistical significance between treatments was determined by the Student *t*-test, and error bars represent the SEM. Asterisks represent statistically significant binding over the BSA control.

to ensure an adequate flow allowing normal placental function [20]. As defective angiogenesis and impairments in the remodeling of spiral arteries have been linked to pregnancy disorders, such as preeclampsia and intrauterine growth restriction [24, 25], the elucidation of the mechanisms controlling uteroplacental vascularization has become a major concern for reproductive medicine. In addition to trophoblast cells, many other cellular components at the fetal-maternal interface produce cytokines and growth factors involved in the control of angiogenic responses during pregnancy. Within this context, the functional studies reported here identify murine *Psg22* as a gene expressed in early pregnancy that may be important for this process not only by stimulating maternal immune cells to produce angiogenic growth factors but also by direct effects on endothelial cells to promote vascular expansion and development. A proposed model of the mechanism by which PSG22 may exert its proangiogenic function is shown in Figure 5.

We observed that treatment of murine macrophages with recombinant PSG22 increased the production of TGFB but not the proinflammatory mediator TNFA and IL6 [13–15]. Secretion of anti-inflammatory cytokines by decidual antigen presenting cells, which was observed in the present study, has been linked to the ability of these cells to promote fetal tolerance and angiogenesis. The phenotype and function of peritoneal macrophages is determined by signals from the local microenvironment, and though the cells used in this study are likely to differ from those residing at the fetal-maternal interface, our results showed an increased VEGFA production, suggesting that PSG may represent one of the environmental cues to promote the polarization of macrophages toward a proangiogenic cell type.

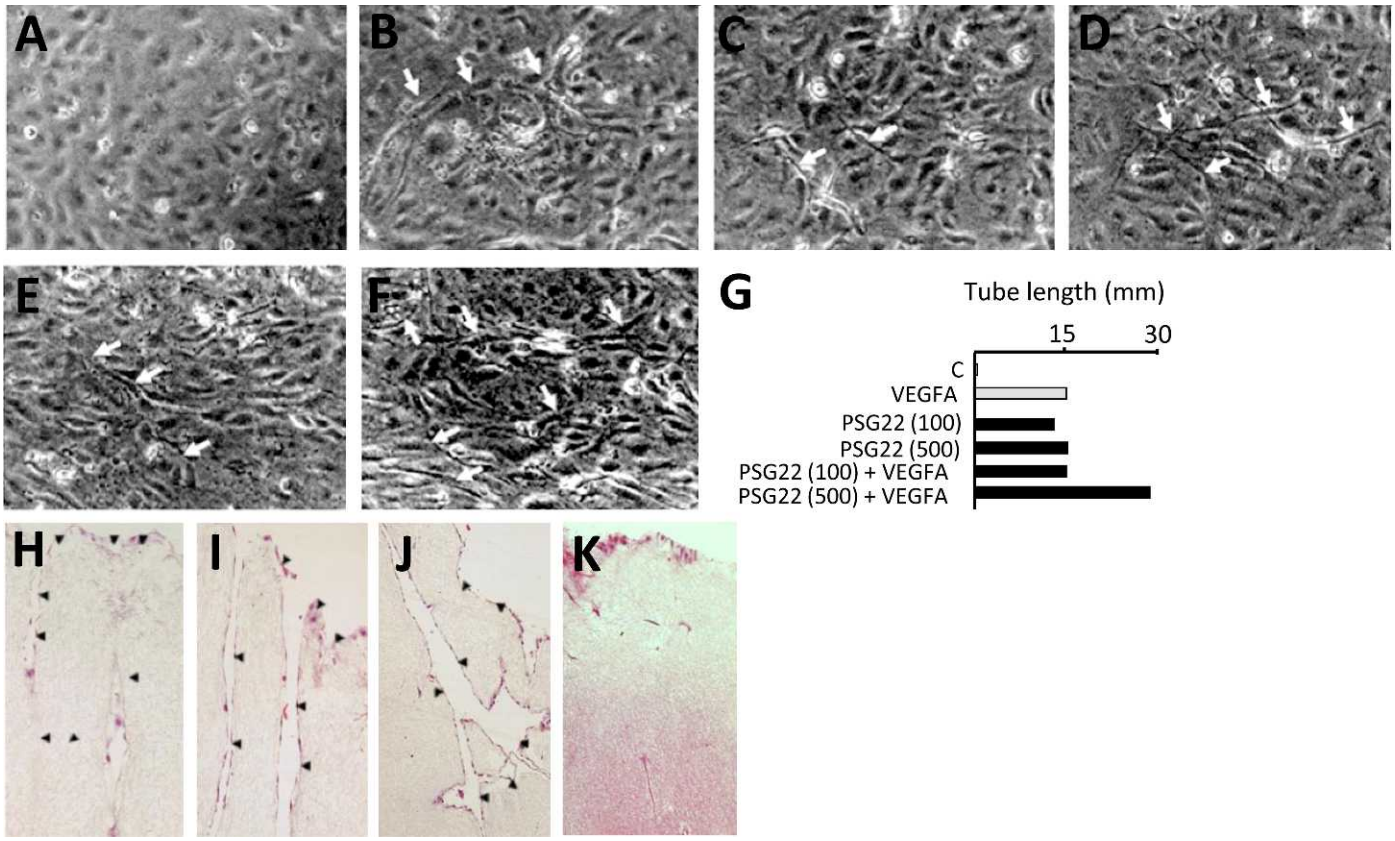

FIG. 4. Endothelial tube formation in response to murine PSG22. **A**) In the negative control, where human dermal microvascular endothelial cells (HDMECs) were exposed to basal medium only, no cell cords or capillary-like endothelial tubes are seen. **B**) In contrast, endothelial cells treated with VEGFA (50 ng/ml), used as positive control, show tube formation (white arrows) as expected. **C** and **D**) The application of PSG22 at concentrations of 100 and 500 ng/ml induces endothelial tubes (white arrows), respectively. **E**) Combined application of PSG22 and VEGFA at PSG concentration of 100 ng/ml induces endothelial tubes of comparable length and network as by VEGFA alone. **F**) PSG at 500 ng/ml plus VEGFA increases the length and network of tubes as marked by arrows. **G**) The quantification of the endothelial tube length shows that PSG alone induce endothelial tubes in comparable length to those induced by VEGF alone. Combined application of VEGF PSG22 increases the VEGF-induced tube length to 89.6% at 500 ng/ml in comparison to VEGF alone, and combined application of VEGF with PSG23 at 100 ng/ml increases the VEGF-induced tube length to 59.9%. **H**–**K**) Cross sections of paraffin-embedded endothelial tubes. Black arrowheads mark the top of the collagen gels, and cells remaining there are not involved in the tube formation. Under stimulation with VEGFA (positive control), HDMECs invade the gel and from capillary-like channels (**H**; arrowheads). Similarly, also under stimulation with PSG22 at 500 ng/ml alone, HDMECs form capillary channels (**I**; arrowheads). Application of PSG22 (500 ng/ml) + VEGFA leads to capillary-lumen formation (**J**; arrowheads) with remarkably high number of branching. **K**) Except some single cells, no lumen formation is visible in the control where HDMECs treated with basal medium only. Sections are stained with calcium red. Original magnification ×300.

Cumulative evidence suggests that, in addition to their potential involvement in the regulation of maternal immune tolerance, uterine DC [29, 37] and NK cells [28–30, 38] are important for the vascular adaptations induced during implantation and placentation. The proangiogenic functions of DC and NK cells during pregnancy might also result from their unique phenotype shaped by specific signals derived from the uterine milieu. In the present study, PSG22 was found to promote the secretion of VEGFA and TGFB by DC and NK cells isolated both from the spleen of nonpregnant mice and from GD 8.5 uterine mucosa exerting an effect similar to that described for murine PSG23 and human PSG1 in immune cells, though this is the first time that the effect of a PSG has been tested in NK cells [13, 21]. We previously showed that PSG23 induced TGFB and VEGFA, similarly to the present results on uterine cells; however, the results with PSG1 showed no significant effects on VEGFA secretion in human monocyte-derived DC [35]. These observations highlight tissue-specific differences in the proangiogenic potential of DC. As the experiments described in this work were the first to be conducted on DC and NK cells isolated from the uterus, PSG now emerge as a possible tissue-specific signal stimulating the proangiogenic functions ascribed to these cell subsets. DC accumulate in the mouse uterus shortly after implantation [39], where they have been shown to promote the recruitment and maturation of NK cell precursors concomitant with the decidualization of the endometrial stroma [29, 40]. The regulatory functions of both cell subsets might be important for the vascular processes driving implantation and early placentation. Indeed, DC depletion has been shown to cause implantation failure linked to impairments on uterine NK cell differentiation and defective decidual angiogenesis and placental functions [29]. The early expression of PSG22, detectable as early as GD 5.5, together with its proangiogenic effects, suggests that this protein may play an important role modulating the ability of DC and NK cells to induce the early vascular adaptations required for successful implantation and placentation. Importantly, these results raise the possibility that human PSGs, which also are detectable at very early stages of gestation [13, 41], exert similar functions that could be relevant to the etiology of pregnancy disorders arising from defective decidual vascular remodeling, notably small-for-gestational age fetuses and preeclampsia. Although the direct involvement of PSG-mediated mechanisms in these syndromes needs to be

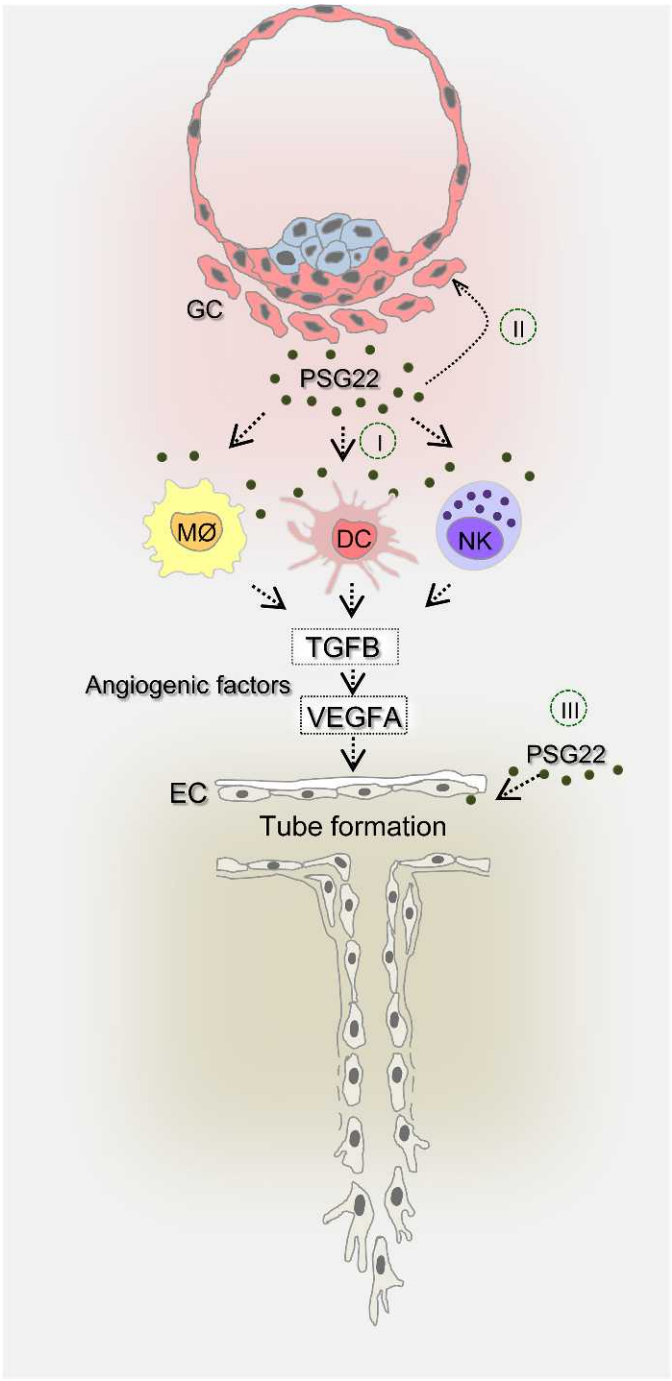

FIG. 5. Proposed mechanism of PSG22-induced angiogenesis in mouse pregnancy. During the implantation process in mice, the blastocyst trophoblast giant cells (GC) secrete PSG22 into the decidualizing environment. This results in the production of TGFB and subsequently VEGFA by different leukocytes, such as macrophages (MØ), dendritic cells (DC), and natural killer cells (NK) (**I**). In addition, PSG22 could induce secretion of angiogenic factors in trophoblast cells (**II**). The increased availability of proangiogenic factors, together with a possible direct interaction of PSG22 (**III**) with endothelial cells (EC), promotes endothelial tube formation. The induction of angiogenesis by PSG22 may contribute to the delicate process of new maternal blood vessel formation during implantation in mice.

investigated further, the potential of these proteins as predictive markers already has been suggested by different studies associating decreased serum PSG levels with suboptimal pregnancy outcomes [11, 42].

The tetraspanin CD9 has been shown to mediate the secretion of anti-inflammatory cytokines by PSG17 in macrophages [7]; however, PSG22 and PSG23 do not bind to CD9, and the mechanism of induction of TGFB by these PSG remains to be investigated. Recently, we determined that human PSG1 binding to syndecans on endothelial cells mediates its ability to induce tube formation. Which of the four syndecans expressed in endothelial cells is involved in the PSG1-mediated tube formation is yet to be examined [36]. Here we show that murine PSG22, which differs considerably in its amino sequence from human PSG1 but like PSG1 induces endothelial tube formation, binds to cell surface proteoglycans, including all four syndecans. These observations indicate that, while there are some differences in placentation between humans and rodents, the function of the human and murine PSG examined thus far is highly conserved, further supporting the suitability of the murine model to study the functions of PSG in vivo.

In summary, the present study demonstrated that *Psg22* is a gene expressed early in mouse pregnancy with the ability to induce the secretion of proangiogenic factors in immune cell types, including uterine DC and NK cells. These cells emerge as novel PSG targets relevant to decidual and placental angiogenesis. Most important, PSG22 not only induced angiogenic growth factor expression but also functioned as a proangiogenic factor by directly affecting vascular endothelial cells. By linking immunoregulation and angiogenesis, PSG emerge as potential pivotal mediators that could influence the outcome of pregnancy by playing important roles in establishing a tolerogenic environment that promotes the survival of the allogeneic fetus and an adequate vascular network to support fetal growth.

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