Equine pregnancy-specific glycoprotein CEACAM49 secreted by endometrial cup cells activates TGFB

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Abstract

In early equine pregnancy, a highly invasive trophoblast cell subpopulation, the chorionic girdle cells, invade the endometrium and form endometrial cups (EC). These cells express classical MHC molecules, thereby stimulating a humoral and cellular immune response, resulting in a massive accumulation of maternal CD4+ and CD8+ T cells around the EC. Nevertheless, no immediate destruction of endometrial cups by maternal lymphoid cells occurs, presumably due to immune tolerance. Although the environment of EC is rich in TGFB and in FOXP3+, CD4+ T cells, the mechanisms leading to tolerance have not been elucidated. Recently, we discovered that equine trophoblast cells secrete pregnancy-specific glycoproteins (PSGs). Since human and murine PSGs activate latent TGFB, we hypothesized that equine PSGs may have a similar activity. We performed plasmon surface resonance experiments to show that equine PSG CEACAM49 can directly bind to the latency-associated peptide (LAP) of both TGFB1 and TGFB2. We then found that the binding of CEACAM49 leads to the activation of TGFB1 as determined by both ELISA and cell-based assays. Furthermore, the activation of TGFB is a unique function of PSGs within the human CEA family, because CEACAM1, 3, 5, 6, 8 do not activate this cytokine. This finding further strengthens the classification of CEACAM49 as an equine PSG. Based on our results, we hypothesize that activation of latent TGFB in the EC environment by equine PSGs secreted by invasive trophoblast cells, could contribute to the generation of regulatory T cells (Tregs) to maintain immune tolerance.

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Introduction

Pregnancy-specific glycoproteins (PSGs) are secreted members of the carcinoembryonic antigen (CEA) family, which are expressed by trophoblast cells (Kammerer & Zimmermann 2010, Rattila *et al.* 2019). In contrast, the second group of the human CEA family, the carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are primarily membrane-anchored glycoproteins, which are widely expressed in different human tissues (Beauchemin & Arabzadeh 2013). However, due to alternative splicing and proteolytic cleavage CEACAMs may be released from cells and reach the circulation (Naghibalhossaini & Ebadi 2006, Beauchemin & Arabzadeh 2013). While CEACAMs exist in all analyzed mammalian species, PSGs are found in mammals which have a hemochorial placenta, including primates (Streydio *et al.* 1988, Watanabe & Chou 1988, Zhou & Hammarstrom 2001), rodents (Chan *et al.* 1988, Rudert *et al.* 1992, Kromer *et al.* 1996) and microbats

(Kammerer *et al.* 2017). This suggests that the intimate contact of fetal trophoblast cells with maternal blood supports PSG evolution. However, more recently, we identified PSG-like CEACAMs in the horse (Aleksic *et al.* 2016, Missbach *et al.* 2018). From the 16 CEACAM1-related genes in the horse, 8 are PSG-like genes, of which 4 were found to be expressed in equine trophoblast cells (Aleksic *et al.* 2016). mRNA encoding CEACAM49 was detected in conceptus tissues and in cultured chorionic girdle cells but was neither detected in cells from the uterus nor in maternal blood cells, thus showing a fetal-cell specific expression pattern (Aleksic *et al.* 2016).

The horse has an epitheliochorial placenta, although a subset of equine trophoblast cells invade the endometrium, and form a unique structure called the endometrial cups (Allen *et al.* 1973, Antczak *et al.* 2013). T cells of the mare recognize but do not kill endometrial cup cells for about 100 days of pregnancy (de Mestre *et al.* 2010). This suggests that endometrial cup cells are protected from T cell attack by a yet

unknown tolerance mechanism (de Mestre *et al.* 2010). Interestingly, endometrial cups are surrounded by a large number of lymphoid cells. These cells were found to be enriched in FoxP3-expressing regulatory T cells (Tregs) (de Mestre *et al.* 2010). Transforming growth factor beta (TGFB) plays a critical role in the generation and immunosuppressive function of Tregs (Hadaschik & Enk 2015). Therefore, it is not surprising that a high content of TGFB was previously found in the placenta of different species, and specifically in the area surrounding the endometrial cups of the equine placenta (Lea *et al.* 1995, Lennard *et al.* 1995).

TGFB is a member of a gene family composed of 33 genes in mammals (Morikawa *et al.* 2016). Three of them belong to the TGFB subgroup (TGFB1, TGFB2, TGFB3) and all are synthesized as a precursor protein, consisting of a signal peptide, a prodomain and the mature polypeptide (Derynck & Budi 2019). After cleavage of the signal peptide, the polypeptide is further processed by proteases to separate the prodomain (termed latency-associated polypeptide (LAP)) from the mature polypeptide, which remains non-covalently associated (Derynck & Budi 2019). While TGFB homodimers are associated with LAP they cannot bind to their receptors and exist in a latent/inactive state termed the small latent complex (SLC) that is secreted from cells or binds to the plasma membrane-associated glycoprotein-A repetitions predominant (GARP) (Stockis *et al.* 2009). The SLC can also bind to the latent TGFB binding proteins (LTBPs) forming the large latent complex (LLC), which then binds to extracellular matrix (Robertson *et al.* 2015). In either case, TGFB has to be activated to be able to bind to its receptor (Shi *et al.* 2011, Lienart *et al.* 2018). The TGFB receptor complex is formed by oligomerization of two type 2 serine/threonine kinase receptors (TBR2) and two type I receptors (TBR1) (Derynck & Budi 2019). Activation of the TGFB receptor leads to SMAD protein translocation to the nucleus and induction of their target genes transcription (Derynck & Budi 2019). Therefore, activation of latent TGFB has been implicated as a major regulatory mechanism of TGFB function (Annes *et al.* 2003). Different mechanisms for latent TGFB activation have been described, including extremes of pH, proteases, and surface integrins (Robertson & Rifkin 2016). Which mechanisms are important for TGFB activation in the placenta or around EC remain unknown.

We found that members of the human and murine PSG family, which are expressed in increasing concentrations as pregnancy progresses, can activate latent TGFB1 (Moore & Dveksler 2014, Ballesteros *et al.* 2015, Warren *et al.* 2018). This is of interest, since PSGs are highly expressed by syncytiotrophoblast cells which represent the fetal frontline at the feto-maternal interface. Thus, the fetus by itself may regulate the availability of active TGFB in its immediate vicinity.

This may be of particular relevance with respect to the immune suppressive function of TGFB, which may lead to the generation of Tregs at the feto-maternal interface. Even more intriguing is the finding that human and murine PSGs share the ability to activate TGFB, despite their different structures and their independent evolution (Warren *et al.* 2018). This indicates that TGFB activation is a pivotal function of PSGs.

Therefore, we hypothesized that activation of TGFB in the human is specific to PSGs within the CEA family and that, PSGs from other species, such as the horse, may also be able to activate latent TGFB. Indeed, we show in this report that human PSG1 binds to LAP of TGFB1 and TGFB2 and that this interaction is PSG-specific, since none of the human CEACAMs (CEACAM1, 3, 5, 6, 8) can bind to LAP of TGFB1 or TGFB2. We further demonstrate that the equine PSG, CEACAM49, can activate latent TGFB, like human PSGs. Given the fact that TGFB is abundant at EC and the leucocyte infiltrate demarking the EC contains a large amount of FoxP3 expressing T cells, our findings support the hypothesis that equine EC cells control the activity of maternal immune cells by regulating the availability of active TGFB through a PSG-dependent mechanism.

Materials and methods

Generation and purification of recombinant proteins

CEACAM49 and PSG1-V5His production and purification were performed as previously described (Shanley *et al.* 2013), using transient transfection of HEK-293 cells (Freestyle™ 293 cells) with CEACAM49 and PSG1 coding sequences (CDS) cloned into the pTT3 expression vector. The full-length cDNA of CEACAM49 was cloned from endometrial cup tissues, as previously described (Aleksic *et al.* 2016). The pTT3-CC49 construct was generated by PCR amplifying the CEACAM49 CDS from pRc/CMV-ecaCEACAM49 incorporating 5' EcoRI and 3' HindIII restriction sites using the primers, ecaCEACAM49For: 5'-gGAATTCaccatgcaatcacctca and ecaCEACAM49Rev: 5'-cAAGCTtggtctgtaactgggg. The PCR amplified band was blunt cloned into PSTBlue1 and sub-cloned into pTT3 using EcoRI and HindIII restriction enzymes, in frame with a V5-His tag as previously described (Shanley *et al.* 2013). Recombinant proteins were purified from cell culture medium 72 h post-transfection using a Qiagen Ni-NTA resin. Imidazole (Sigma) was added to the culture medium to a final concentration of 10 mM to reduce non-specific binding. Ni-NTA resin was added to the medium at a ratio of 1 mL resin suspension (corresponding to 0.5 mL resin bed volume) to 100 mL medium. The medium and resin were then batch bound overnight on a rotating wheel at 4°C. The medium and resin mix was then passed through a disposable polypropylene column (Thermo Fisher Scientific) and the resin was washed with 500 mM NaCl, 20 mM NaH₂PO₄, pH6 (wash buffer). Protein was then eluted from the column with increasing concentrations of imidazole in wash buffer. Fractions containing recombinant PSG1 or CEACAM49 were then pooled and passed through a new column containing

0.5 mL Ni-NTA resin, the flow-through collected, and bound protein eluted with 1 mL 50 mM imidazole followed by three 1 mL 200 mM imidazole fractions. The flow-through and three 200 mM fractions were pooled and concentrated to a volume of 1 mL using a Millipore Amicon Ultra Ultracel 10K centrifugal filter (Millipore). For size exclusion chromatography (SEC) 1 – 4 mg of purified recombinant protein was applied to a HiLoad 16/60 Superdex S200 prep grade column in a 1 mL volume with a flow rate of 1 mL/min using the AKTA explorer system in PBS. Determination of the 1 mL fractions to pool was based on chromatogram peaks and the resultant protein solution was further concentrated.

Human LAP of TGFB1 and LAP of TGFB2 were synthesized as a gene string (Thermo Fisher Scientific) and cloned directly into HindIII/NotI cut pcDNA4.0 (Thermo Fisher Scientific). The extracellular domains of CEACAM5 was PCR amplified from HG11077 CH (Sino Biological) and cloned into XmaI/NotI cut pSG160 (Bonsor *et al.* 2015). The three proteins had a 6xHis tag at the C-terminus and were expressed in HEK cells grown in FreeStyle 293 supplemented with GlutaMAX and gentamicin (Thermo Fisher Scientific) by transfections of the plasmids with polyethyleneimine. Cells were grown for 5 days at 37°C with 8% CO₂. The proteins were directly purified from the clarified supernatant using a 5-mL HisTrap Excel column and further purified on a Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5. Recombinant CEACAMs were generated as previously described with minor modifications (Singer *et al.* 2014). Plasmids encoding CEACAM1, CEACAM3, CEACAM6 and CEACAM8 containing an Fc-tag at their C-terminus were generously provided by Esther Klaile (University Hospital Jena, Germany) and Bernhard Singer (University of Essen, Germany). The plasmids were transfected into Expi293 cells following the manufacturer's recommendations and the supernatants were harvested 6 days post-transfection (Thermo Fisher Scientific). PSG1-Fc was generated from the supernatant of a stably transfected CHO-K1 single-cell clone established in our laboratory that was grown in a hollow fiber cartridge bioreactor (FiberCell Systems, Frederick, MD, USA) as previously described (Ballesteros *et al.* 2015). The Fc control protein which comprises the CH2 and CH3 domains of the human IgG1 heavy chain and the hinge region was generated by transfecting the pFuse-hlgG1e3-Fc2 plasmid (InvivoGen, CA, USA) into ExpiCHO cells following the manufacturer's recommendations for the high titer protocol and the supernatants were harvested 10 days post-transfection (Thermo Fisher Scientific). Clarified supernatants were loaded onto a MabSelect protein A column (GE Healthcare) equilibrated with PBS and the proteins were eluted with 0.1 M glycine pH 2.7 and neutralized immediately with 1 M Tris buffer pH 8.0. All proteins were buffer exchanged into PBS and concentrated using Amicon Ultra-15 10K MW cut off centrifugal filter units (Millipore).

All expressed CEACAMs are composed only of the extracellular domains. The transmembrane and the cytoplasmic tails or the GPI linkage sequences are not included in the cDNAs utilized to express these proteins.

Protein concentrations were determined by absorbance at 280 nm with adjustment for their respective extinction coefficients, following their separation on NuPAGE 4–12% Bis Tris gels with

MOPS running buffer and visualization with GelCode Blue. The proteins were prepared by adding NuPAGE LDS sample buffer and sample reducing agent, which contains DTT. All of these reagents were purchased from Thermo Fisher Scientific.

Activation of the small latent complex (SLC) of TGFB1 by PSGs

To detect active TGFB1, we performed two assays as previously described using triplicate samples for each condition tested (Ballesteros *et al.* 2015). In the cell-free system assay, proteins were incubated with 50 ng/mL of SLC (R&D Systems) at 37°C for 1 h in a final volume of 0.1 mL of PBS in siliconized tubes. The samples were then transferred to wells of a 96-well Nunc Maxisorb plate that had been coated overnight with recombinant human TGFB receptor 2-Fc (R&D systems) and blocked with PBS-1% BSA. After 2 h, the plates were washed, and active TGFB1 was detected with a biotinylated antibody specific for mature TGFB1 (BAF240, R&D systems). In the second assay, bioactive TGFB was determined using the TGFB responsive plasminogen activator inhibitor-1 reporter mink lung epithelial cells line (TMLEC) provided by Dr Rifkin (New York University, NY, USA) following incubation of the proteins with 50 ng/mL of SLC (R&D Systems) at 37 °C for 1 h in a final volume of 0.1 ml of serum-free DMEM supplemented with 0.1% insulin-transferrin-sodium selenite (ITS) (Sigma-Aldrich) in siliconized tubes, which was then added to the cells for 16–18 h (Abe *et al.* 1994). After this time, the cells were lysed and luciferase was measured in a GloMax Discover system (Promega). To verify that the observed increase in luciferase was the result of TGFB binding to its receptor in the TGFB reporter cells, the experiments were carried out in the presence of 5 µM of the TGFB receptor 1 kinase inhibitor SB431542 (R&D Systems) as previously described (Blois *et al.* 2014). Two-fold dilutions of active TGFB1 (R&D Systems) from 2000 to 62.5 pg/mL were used as standards to determine the linearity of the signal in these assays. Controls included SLC incubated with the same volume of PBS (vehicle) or a protein consisting of the Fc portion of human IgG1, which undergoes glycosylation due to the presence of two N-glycosylation sites. We observed no differences between these two controls in any of our assays.

Binding of CEACAMs and PSG1 to LAP-TGFB1 and LAP-TGFB2

For LAP binding assays, wells (Nunc Maxisorb) were coated overnight with either recombinant human LAP-TGFB1 or LAP-TGFB2 and blocked with PBS-2% BSA. The wells were washed three times (PBS/0.05% Tween-20) and the samples to be tested were added and incubated for 4 h at room temperature. The wells were washed again and incubated with HRP-labeled anti-human Fc antibody (Thermo Fisher Scientific) for 1 h. After three final washes, binding of the antibody was visualized with TMB substrate.

Surface plasmon resonance (SPR) experiments were performed in a BIAcore 3000 instrument (GE Healthcare) at a flow rate of 10 µL/min and 25°C using HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005%, v/v

Surfactant P20) as running buffer. Purified recombinant LAP of TGFB2 or LAP of TGFB1 diluted in 10 mM sodium acetate pH 4.0 buffer were immobilized onto a CM5 sensor chip (GE Healthcare) using standard amine coupling chemistry. A control surface where no protein was immobilized was used as control to correct for background and potential non-specific binding. PSG1 or CEACAM49 were injected over a range of concentrations (20 μ M to 30 nM) for 180 s followed by a 240 s dissociation period. To regenerate the surface after each protein injection, a solution containing 10 mM Glycine-HCl pH 2.0 was injected for 30 s. Real-time data were analyzed using the BIAevaluation 4.1 software (GE Healthcare), and kinetic profiles were fitted using a global 1:1 binding algorithm to estimate the association (k_a) and dissociation (k_d) constant, and the affinity (KD) of the interaction. Drift correction was added for the interactions of CEACAM49 with LAP of TGFB1 (maximum drift of 0.052 RU) and LAP of TGFB2 (maximum drift of 0.103 RU) to correct for baseline drift. Real-time sensorgrams were aligned using the BIAevaluation 4.1 software (GE Healthcare) and final figures were generated in Prism8 (GraphPad).

Phylogenetic analyses and datasets

Phylogenetic analyses based on nucleotide and amino acid sequences were conducted using MEGA X (Kumar *et al.* 2018). Sequence alignments were performed using Muscle implemented in MEGA X. Phylogenetic trees were constructed using the maximum likelihood (ML) method with bootstrap testing (500 replicates) and the Tamura-Nei substitution model. The following Ensembl releases were used to analyze CEACAM loci: human, GRCh38.p13; mouse, GRCm.p6; horse, EquCab3.

Statistical analysis

Activation of latent TGFB1 is reported as the mean fold increase over control $\pm$ s.e. One-way ANOVA and the post hoc Tukey tests for pairwise comparisons were used for statistical analysis. Data groups passed the Shapiro-Wilk test for normality and the Brown-Forsythe test for equal variances. Significant differences between binding of PSG1-Fc and control to LAP of TGFB1 or LAP of TGFB2 were calculated using the Student's *t*-test. Data were analyzed using version 8 of GraphPad Prism (GraphPad Software) and a *P* value of <0.05 was considered statistically significant.

Ethics approval and consent to participate

In the present study, we did not use human or animal tissues. In addition, we did not perform animal experiments.

Results

Activation of TGFB1 is specific for PSGs within the human CEA family

We have previously shown that all human PSGs activate latent TGFB1 and that this activity is preserved whether the protein has an Fc or a V5His tag as observed with

Figure 1 Generated recombinant CEACAMs and PSG1. The proteins with their different tags are schematically shown above the gel. Recombinant CEACAM1-Fc, CEACAM3-Fc, CEACAM5-His, CEACAM6-Fc, CEACAM8-Fc, CEACAM49-V5His, and PSG1-V5His were separated on 4–12% SDS-PAGE gels under denatured and reducing conditions and stained with GelCode blue.

PSG1 (Blois *et al.* 2014, Warren *et al.* 2018). In addition, we observed that while more than one PSG domain can activate latent TGFB1, the B2 domain is the most efficient (Ballesteros *et al.* 2015). Since B domains are also found in human CEACAMs we wondered whether human CEACAMs can also activate latent TGFB1. Therefore, we expressed recombinant human CEACAMs with 6xHis (CEACAM5) or an Fc-tag (CEACAM1, 3, 6 and 8) and compared their activity to PSG1 with a 6xHis and a V5 tag at the C-terminus. The purity of all proteins was confirmed by staining with GelCode Blue following separation on 4–12% SDS-PAGE gels (Fig. 1).

We then used these proteins to test their ability to activate latent TGFB1 using two different assays: an ELISA (Fig. 2A) and a cell-based assay (data not shown). Both assays gave qualitatively similar results. As shown in Fig. 2A, only PSG1 significantly activated TGFB1,

Figure 2 Activation of latent TGFB1 by members of the human CEACAM family and by human PSG1. (A) 280 nM of CEACAM (CC) 1, 3, 5, 6 and 8 or PSG1 were incubated with 50 ng/mL of SLC for 1 h at 37°C in PBS. The concentration of active TGFB1 was determined as indicated in the materials and methods section and expressed as fold activation over Fc protein control designated as 1. Statistical analysis was performed with one-way ANOVA followed by Dunnett's pairwise test. **** $P < 0.0001$ ($n = 3$). (B) Phylogenetic analysis of human PSG B domains and selected human, murine and equine CEACAM B domains were performed using amino acid sequences, the ML method and the MEGA X software. CC1 Eca, CEACAM1 from horse (*Equus caballus*); CC1 Mmu, CEACAM1^a from mouse (*Mus musculus*).

but although some CEACAMs have B domains (Fig. 1), none of the CEACAMs was able to activate latent TGFB1 at the concentrations tested (Fig. 2A). Most strikingly, even CEACAM5 which has three B domains did not reproducibly activate latent TGFB1. This suggests that fundamental differences exist between B domains of human PSGs and CEACAMs. Indeed, phylogenetic analysis showed that the amino acid sequences of PSG B domains and the B domains of human, murine and equine CEACAMs were clearly separated with each forming an individual cluster. The cluster of human PSG sequences has relatively short branches compared to the branches in the CEACAM cluster, indicating either concerted evolution of PSG B domains or a more recent PSG duplications compared to CEACAMs (Fig.2B).

Equine PSG CEACAM49 binds to LAP of TGFB1 and TGFB2

We reported that human and murine PSGs interact with LAP of TGFB1 and that human PSGs use the IgC-like B domain to bind to LAP while murine PSGs used the N domain (Ballesteros *et al.* 2015, Warren *et al.* 2018). Equine PSGs are extremely small consisting of only a single IgV-like domain (Aleksic *et al.* 2016), thus the interaction could only take place via the N domain similar to the interaction of latent TGFB1 with murine PSGs. Previously, we showed that PSG1 binds to the commercially available LAP-TGFB1 (Ballesteros *et al.* 2015). Now, we asked if human PSG1 can also bind to LAP-TGFB2. Because LAP-TGFB2 is not commercially available, we generated this protein for binding studies. Plates were coated with LAP-TGFB1 or LAP-TGFB2 and incubated with PSG1-Fc or the control Fc-fusion protein (Ballesteros *et al.* 2015). PSG1-Fc binds to both LAP-TGFB1 and LAP-TGFB2 while the control Fc protein did not, demonstrating that human PSG1 can bind not only to LAP-TGFB1 as previously observed but also to LAP-TGFB2 (Fig. 3A). We performed similar experiments with the other CEACAMs with an Fc-tag (CC1, CC3, CC6 and

CC8). Consistent with their inability to activate TGFB described previously, none bound to LAP-TGFB1 and LAP-TGFB2 over the Fc control protein (data not shown). Because some of the proteins we generated have a His tag rather than the Fc-tag, we could not utilize ELISA to study their interaction as the His tag is also present in LAP-TGFB2. Therefore, we studied the potential binding with the LAP peptides using surface plasmon resonance, which also allowed us to determine the kinetics and the affinity of the interactions.

We generated a biosurface with immobilized LAP-TGFB1 or LAP-TGFB2 and injected CC49 or PSG1 for 3 min. After a dissociation phase of 3 min, the LAP surfaces were regenerated with glycine buffer to reach the baseline level and start a subsequent experimental cycle as indicated in Material and Methods and depicted in Fig. 3B. We first analyzed the binding affinity of human PSG1-V5His to LAP-TGFB2 using a Biacore 3000 instrument (GE Healthcare). As shown in Fig. 3E and Table 1, PSG1-V5His binds to LAP-TGFB2 with an affinity of 100 nM, which is about 40 times higher than the affinity of this protein for the LAP-TGFB1 (Warren *et al.* 2018). Next, we tested if CEACAM49 interacts with LAP of either TGFB1 or TGFB2. CEACAM49 binds to both LAP peptides in a micromolecular range with a KD of 10 μ M to LAP-TGFB1 and a KD of 26 μ M to LAP-TGFB2 (Fig. 3B, C, D and Table 1). Thus, CEACAM49 binds with a slightly lower affinity to the LAP peptides than previously determined for the interaction of human PSG1 and TGFB1 (Table 1). The half-lives of CEACAM49-LAP β 1 and CEACAM49-LAP β 2 complexes was 67.6 min and 71.8 min, respectively while human PSG1 forms complexes with TGFB2 with a half-life of 14.4 min (Table 1).

Equine CEACAM49 activates TGFB1

After demonstrating that CEACAM49 binds to LAP-TGFB1 with similar binding characteristics as human PSG1, we investigated if CEACAM49 is also able to activate the latent TGFB1. To evaluate this, we incubated SLC (LAP-

Figure 3 Binding of CEACAM49 and PSG1 to the LAP of TGFβ1 and LAP of TGFβ2. (A) PSG1 or Fc control protein were added to wells previously coated with LAP of TGFβ1 or LAP of TGFβ2. (B) Representative SPR sensorgram of the interaction of CC49 at 0.2 mM with immobilized LAP of TGFβ2. The beginning of the CC49 injection, association, dissociation, and regeneration phases are shown. A surface without immobilized protein was used as a control for potential non-specific binding to the biosensor surface and was subtracted from the binding of CC49 to the LAP of TGFβ2 coated surface. (C) Sensorgrams of the interaction of CC49 with the LAP of TGFβ1. Serial dilutions of CC49 ranging from 20.0 to 1.2 mM were injected during 3 min over a CM5 biosensor chip with immobilized LAP of the TGFβ1. SPR sensorgrams for each protein concentration are shown as grey lines while the fitted data are shown as black lines. (D) Sensorgrams of the interaction of CC49 with the LAP of the TGFβ2 represented as in B. (E) Sensorgram of the interaction of PSG1 with the LAP of the TGFβ2 represented as in B. The specific concentration utilized for each sensorgram is indicated. **** $P < 0.0001$

TGFβ1) with CEACAM49-V5His for 1 h at 37°C and then determined the amount of activated TGFβ1 using an ELISA-based protocol with the TGFβ receptor II as capture as previously described (Blois *et al.* 2014, Ballesteros *et al.* 2015, Warren *et al.* 2018). We found that CEACAM49 activated the SLC of TGFβ1 in a concentration-dependent manner (Fig. 4A). In previous experiments, PSG1 at 140 nM induces approximately a 1.5 increase over the control in the luciferase signal in the luciferase reporter assay (Warren *et al.* 2018). To confirm these results, we also performed a cell-based functional assay. Again, we observed a concentration-dependent activation of the SLC of TGFβ1 as indicated by the increase in luciferase expression in the reporter cells (Fig. 4B). To demonstrate the specificity of the reaction, we incubated the cells with the TGFβ1 receptor I inhibitor SB431542 or the vehicle control (DMSO) together with CEACAM49. The

increase in luciferase activity, which is readout of active TGFβ signaling in these reporter cells, was blocked in the presence of the TGFβ receptor I inhibitor, while the vehicle control had no effect (Fig. 4C). These experiments strongly suggest that CEACAM49 not only binds to the SLC but also activates TGFβ. Thus human, murine and equine PSGs function as TGFβ activators. Unfortunately, because the SLC of TGFβ2 is not commercially available and we were unable to generate it, we could not perform these experiments with latent TGFβ2.

Synthetic relationship and exon composition suggest convergent evolution of equine, murine and human PSGs

Given the similar functionalities of equine PSGs with murine and human PSGs (this study (Aleksic *et al.*

Table 1 Kinetics constants and affinity of the interaction of CEACAM49 (CC49) with the LAP of TGFβ1 and of TGFβ2 and of PSG1 with the LAP of TGFβ2.

Interaction	k_a /M/s (mean $\pm$ S.D.)	k_d /s (mean $\pm$ S.D.)	KD (μ M)	Rmax (mean $\pm$ S.D.)	χ^2	$t_{1/2}$ (min)
CC49 – LAP-TGFβ1	$(1.7 \pm 0.06) \times 10^3$	$(1.7 \pm 0.7) \times 10^{-4}$	10	55.2 ± 1.3	3.3	67.6
CC49 – LAP-TGFβ2	$(0.6 \pm 0.03) \times 10^3$	$(1.6 \pm 0.5) \times 10^{-4}$	26	129.0 ± 4.8	1.2	71.8
PSG1 – LAP-TGFβ2	$(8.8 \pm 0.10) \times 10^3$	$(0.8 \pm 0.1) \times 10^{-3}$	0.1	268.0 ± 3.6	3.8	14.4
*PSG1 – LAP-TGFβ1	$(1.4 \pm 0.30) \times 10^3$	$(6.1 \pm 0.4) \times 10^{-3}$	4.4	44.2 ± 0.5	3.0	1.9

The SPR sensorgrams shown in Fig. 3C, D and E for the interaction of CEACAM49 with the LAP of TGFβ1 and of TGFβ2 and of PSG1 with the LAP of TGFβ2 were fitted using a 1:1 Langmuir binding model for the estimation of the association and dissociation rates (k_a and k_d , respectively), and affinity ($KD = k_a/k_d$). The maximum response (Rmax), χ^2 (χ^2) values for the fitted data and the complex half-time life ($t_{1/2}$) are also indicated.

*Published in Warren *et al.* (2018).

Figure 4 CEACAM49 activates the small latent complex (SLC) of TGFB1. (A) CEACAM49 at the indicated concentrations or PBS were incubated with SLC for 1 h at 37°C in PBS. The TGFB1 concentration was determined by ELISA and represented as fold increase over the PBS control. All treatments were made in triplicate wells. (B) CEACAM49 at the indicated concentrations or PBS (control) were incubated with SLC for 1 h at 37°C in DMEM-0.1% ITS and then added to TMLEC reporter cells. The treated TMLECs were incubated for 18 h and then lysed. The luciferase activity in the lysate was analyzed with a GloMax luminometer. (C) The TMLEC assay described in A was performed in the presence of the specific TGFB receptor I inhibitor SB431542 or DMSO (vehicle control). ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

2016)), the question arises as to whether a common ancestor of equine PSGs with human or rodent PSGs can be inferred. Comparison of the loci of the *CEA* gene families in equine and murine genomes suggests that PSGs evolved independently in horses and mice, since the location of equine *PSGs* is close to the *CEACAM* genes, similar to human *PSG* genes, and not in a separated locus as murine *Psg* genes, which are located between *Pglyrp1* and *Npas1* (Fig. 5A). Next, we compared the exon arrangement of equine *PSG* genes with that of human *PSG* genes (Fig. 5B). Equine *PSG* genes are composed of a single IgV-like domain exon and a single IgC-like domain exon. The IgC-like domain exon is similar to the A2 exon of *CEACAM1*. In contrast, human *PSG* genes have three IgC-like domain encoding exons. Two of them have sequence similarity with the A1 exon of *CEACAM1* and one has similarities to the B-domain encoding exon of *CEACAM1*. Thus, equine and human *PSG* genes contain distinct IgC-domain exons which suggest that no common ancestor of equine and human *PSGs* existed (Fig. 5B).

Discussion

In this report, we show that the recently identified equine PSG CEACAM49 can directly bind to LAP of TGFB1 and TGFB2 with similar affinity and binding parameters resulting in a half-life time of the CEACAM49-LAP complex of approximately 1 h. The affinity of CEACAM49 for LAP of TGFB1 is in the same range as previously found for human *PSGs* (Ballesteros *et al.* 2015, Warren *et al.* 2018). On the other hand, the affinity of the interaction of CEACAM49 with LAP of TGFB2 is 250 times weaker than that of PSG1 mainly due to the slower association observed with CEACAM49. We further demonstrate by ELISA and a cell-based functional assay that binding of CEACAM49 to the SLC leads to TGFB1 activation. In the experiments presented, we used human LAP of TGFB1 and TGFB2 and the small latent complex of human

TGFB1 as their equine counterparts are not available. It is important to note that equine TGFB1 and TGFB2 have a 92% and 99% identity to their human counterparts, respectively.

The ability to activate latent TGFB is a PSG-specific function within the human *CEA* family, since none of the tested human *CEACAMs* were able to activate TGFB1. This indicates that activation of latent TGFB is a newly acquired function for the *CEA* family following the evolution of *PSGs*.

Surprisingly, there is no evidence suggesting that a common ancestor of *PSGs* from different mammalian lineages exist. In contrast, comparison of the *PSG* gene locus and the exon composition suggest that *PSGs* evolved independently in various mammalian lineages, although further analysis is needed to understand the evolution of *PSGs* in different mammalian lineages. If *PSGs* evolved independently at least three times in mammals, common functions of *PSGs* in different species are the result of convergent evolution.

Convergent evolution requires a strong selective pressure (Laayouni *et al.* 2014); therefore, our finding indicates that the regulation of TGFB activity is a key function of *PSGs*. Remarkably, the TGFB superfamily is a master regulator in the placenta, playing pivotal roles in tissue remodeling, trophoblast invasion, neovascularization as well as in the regulation of immune responses (Jones *et al.* 2006). In addition to TGFB activation, we have previously identified the inhibition of platelet aggregation to be a common function of human, murine and equine *PSGs* (Shanley *et al.* 2013, Aleksic *et al.* 2016). Moreover, at least one human and murine *PSG* were found to play a role in angiogenesis (Lisboa *et al.* 2011, Blois *et al.* 2012) as well as in the induction of immunosuppressive cytokines including TGFB by immune cells such as monocytes and dendritic cells (Motran *et al.* 2003, Martinez *et al.* 2012, Falcon *et al.* 2014). All of these activities attributed to *PSGs*, together with the activation of TGFB, contribute to ensure a successful pregnancy that may provide the

Figure 5 Syntenic relationship of the *CEACAM/PSG* gene loci and exon composition of *PSG* genes in the horse, mouse, and human genomes suggest independent evolution of *PSG* genes in the horse. (A) The syntenic chromosome regions from human, horse and mouse are shown. Arrowheads represent genes with their transcriptional orientation. *CEACAM* genes are indicated in yellow and *PSG* genes in red, the orthologous *CEACAM* genes in blue and marker genes in black. Names of *CEACAM1*-like genes with ITIM/ITSM motif-encoding exons are shown in red. Clockwise oriented arrows symbolize inversion events of the regions between the two *CEACAM1*-like gene clusters. Inversions relative to the human gene order are shown. Nucleotide numbering of the chromosomes starts at the telomere of the short arms. The scale indicated by dots is 1 Mbp unless interrupted by slanted lines. C, *CEACAM/Ceacam*; chr, chromosome; P, *PSG/Psg*. (B) Exon arrangement of *PSG* genes in humans, horses and mice. The exon types are indicated by differently colored boxes. Leader sequences (L) are shown as gray, IgV-like domain exons as red (N), IgC-like domain exons as light blue (A1 type), green (B type) and blue (A2 type) and 3'-sequences as black boxes.

necessary strong selective pressure to drive convergent evolution of PSGs in different mammalian lineages.

Despite all the functional similarities of PSGs in different mammalian species, there are fundamental differences between the hemochorial placenta of humans and mice and the placenta of equids. In the horse, the vast majority of the placenta is of the epitheliochorial type (Allen *et al.* 1973). Only a minority of trophoblast cells invade the endometrium, where they form the endometrial cups (Allen *et al.* 1973). These structures are unique in equids and have not been found in other mammals (Antczak *et al.* 2013). Although these structures are surrounded by a large amount of lymph vessels, which allow the transport of hormones, cytokines and other secreted products of the EC cells to the circulation, these trophoblast cells are not in direct contact with the

blood of the mare (Antczak *et al.* 2013). Nevertheless, invading chorionic girdle cells and EC cells secrete PSGs in the horse. In humans, the main source of PSGs is the syncytiotrophoblast, which is in direct contact with maternal blood. Thus, tissue penetration of PSGs in the horse placenta is much more challenging than for PSGs in the hemochorial placentas of the human and mouse. We, therefore, hypothesize that the nanoscale size (single IgV-like domain, which is one-tenth of the size of an IgG antibody) of equine PSGs are an adaptation to their high requirements on tissue penetration (Aleksic *et al.* 2016).

The horse is the only mammalian species in which a strong immune response to paternal antigens can be regularly detected. This may be because invading trophoblast cells (chorionic girdle cells) express, at least temporarily, conventional MHC class I molecules on

the cell surface, therefore presenting semi-allogeneic antigens to the immune system of the mare. Consequently, maternal lymphoid cells migrate to EC and build a kind of demarcating zone at the edge of the EC. Nevertheless due to unknown mechanisms, the lymphoid cells do not attack and destroy EC until around day 130 of pregnancy (Antczak *et al.* 2013). More recently, it was described that the lymphocyte infiltrate consists of predominantly T cells and that a high frequency of these cells express FoxP3, a marker of Tregs (Noronha & Antczak 2010). Differentiation and maturation of Tregs is favored by TGFB and we determined that the two PSGs examined, PSG1 and PSG9, induced the generation of Tregs following activation of naïve T cells with anti-CD3 and anti-CD28 antibodies and addition of IL-2 in a TGFB dependent manner (Jones *et al.* 2019). Tregs are essential for suppressing destructive alloantigenic immunity during pregnancy in mice (Zenclussen 2006, Tsuda *et al.* 2019). In humans, Tregs were decreased in peripheral blood and decidua in women with miscarriages and an impaired suppressive capability of Tregs in recurrent miscarriage cases was also observed (Tsuda *et al.* 2019). Furthermore, early pregnancy loss correlated with a low level of naturally occurring Tregs in mares (Aurich *et al.* 2014). Taken together, these observations indicate that equine PSGs, like human and murine PSGs, may play a role in the generation of Tregs via activation of TGFB, and therefore are important to maintain immune tolerance during pregnancy.

Declaration of interest

Tom Moore is on the editorial board of *Reproduction*. Tom Moore was not involved in the review or editorial process for this paper, on which he is listed as an author. The other authors have nothing to disclose.

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Author contribution statement

R K conceived the study, did biocomputing and manuscript writing. A B performed experiments and graphed the data. D B generated and purified recombinant proteins. J W performed experiments and graphed the data. J M W performed experiments. T M supervised protein production and aided in the manuscript writing. G D designed experiments and aided in the manuscript writing. All authors participated in the design of the study and read and approved the final version.

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