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Trophoblast expression and function of pregnancy-specific glycoproteins (PSGs) in the horse

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3 Abstract

4

Pregnancy-Specific Glycoproteins (PSGs) are secreted members of the carcinoembryonic 5 antigen cell adhesion molecule (CEACAM) family, which are expressed by trophoblast cells. 6 PSGs may modulate maternal immune and platelet responses during pregnancy. Until now 7 PSGs are only found in species which have a highly invasive (hemochorial) placentation 8 including humans, mice and rats. Surprisingly, analyzing the CEACAM gene family of the 9 10 horse, which has a less invasive epitheliochorial placenta, we identified equine CEACAM 11 family members that seem to be related to PSGs of rodents and primates. We identified seven genes that encode secreted PSG-like CEACAMs. Phylogenetic analyses indicate that they 12 evolved independently from an equine CEACAM1-like ancestor rather than from a common 13 PSG-like ancestor with rodents and primates. Significantly, expression of PSG-like genes 14 (CEACAM44, CEACAM48, CEACAM49, CEACAM55) was found in trophoblast cells such 15 as purified chorionic girdle cells and endometrial cup cells. Chorionic girdle cells are highly 16 invasive trophoblast cells which invade the endometrium of the mare where they form 17 endometrial cups andare in close contact with maternal immune cells. Therefore despite some 18 19 fundamental differences the microenvironment of invasive equine trophoblast cells has striking similarities to the microenvironment of trophoblast cells in hemochorial placentas, suggesting 20 that equine PSG-like CEACAMs and rodent and primate PSGs have undergone convergent 21 22 evolution as an adaption to a highly specific microenvironment. This is supported by our finding that, similar to certain rodent and human PSGs, equine PSG-like CEACAM49 has anti-platelet 23 activity. Our results have implications for understanding the evolution of PSGs and their 24 functions in maternal-fetal interactions. 25

1 Introduction

2

3 The placenta has pivotal functions in facilitating nutrient exchange between mother and fetus and in protecting the fetus from the maternal immune system (Hemberger 2013). This implies that there may 4 be common placenta-specific genes expressed in all placental mammals. However, the composition of 5 placenta-specific genes appears to be highly species-specific, partly due to the expansion of different 6 7 gene families in different mammalian lineages (Rawn & Cross 2008). Indeed, placentas are quite diverse 8 among mammals in their structure, endocrine function and immunology. For example, the extent of 9 invasion of the uterus by placental trophoblast cells can range from no invasion (as in epitheliochorial 10 placentation) to very extensive invasion (as in hemochorial placentation) where fetal trophoblast cells are in direct contact with maternal blood (Moffett & Loke 2006). 11

12

In the highly invasive placentas of the human and laboratory animals such as mice, rats and rabbits, potent tolerance mechanisms are required to avoid deleterious maternal immune and thrombotic responses to the semiallogeneic fetus (von Rango 2008). Many mechanisms have been proposed to explain the maternal-fetal immune tolerance since Medawar and colleagues first discussed the idea of maternal-fetal tolerance (Trowsdale & Betz 2006, Schumacher *et al.* 2014).

18

19 One protein family which is thought to be involved in the regulation of the immune and platelet 20 responses at the maternal-fetal interface is the Pregnancy-specific glycoprotein family (PSG). PSGs 21 were originally isolated from the circulation of pregnant women (Bohn 1971). In humans, the 22 concentration of PSGs in the bloodstream increases exponentially until term, thereby comprising the 23 most abundant fetal proteins in maternal blood (Lin et al. 1974). PSGs are placentally secreted proteins 24 and part of the carcinoembryonic antigen (CEA) family, which by itself is a member of the immunoglobulin superfamily. The CEA gene family comprises a group of "conserved" CEACAMs, and 25 a larger group of CEACAM1 paralogs. The "conserved" CEACAMs include CEACAM1 itself, 26 CEACAM16, CEACAM18, CEACAM19 and CEACAM20 (Kammerer & Zimmermann 2010). The PSG 27 genes belong to the CEA-related cell adhesion molecule 1 (CEACAM1) paralogs of the CEA gene 28

1 family. PSGs are secreted CEACAMs expressed in a predominantly trophoblast-specific manner.

2

3 PSGs are one of the most rapidly evolving protein families in humans (Zebhauser et al. 2005, Chuong et al. 2010). Probably as a consequence, PSGs differ considerably in structure between primates and 4 rodents. Human PSGs are composed of one N-terminal immunoglobulin variable (IgV)-like domain (N 5 domain) followed by two to three Ig constant (IgC)-like domains of two different types (named A and 6 7 B), whereas rodent PSGs contain 2 - 9 consecutive N domains followed by one IgC-like domain 8 (McLellan et al. 2005). In addition, the expansion of PSG genes took place in two different loci in higher 9 primates and rodents (Kammerer & Zimmermann 2010). There exist 11 and 17 different PSG genes in 10 humans and mice, respectively. Recently it was demonstrated that human PSGs are expressed in trophoblast cells due to the activation of various transcription factors including Sp1 (specificity protein 11 1), KLF4 (Krüppel-like transcription factor 4) and RXRa (retinoid X receptor alpha) (Camolotto et al. 12 13 2010, Hubert et al. 2010). If this is also the case for rodent PSGs is not known.

14

15 Low PSG levels in maternal circulation are associated with certain pregnancy complications such as intrauterine growth retardation, preeclampsia and spontaneous abortion (Towler et al. 1977, Karg et al. 16 17 1981, Silver et al. 1993, Arnold et al. 1999). This may be due to loss of the immunoregulatory and anti-18 thrombotic functions of PSGs. PSGs induce monocytes to synthesize anti-inflammatory cytokines and 19 promote alternative macrophage activation which correlates with the shift from Th1- to Th2-mediated 20 immunological responses (Wessells et al. 2000, Snyder et al. 2001, Motran et al. 2003). In addition 21 elevated PSG levels are correlated with improved symptoms of rheumatoid arthritis (Fialova et al. 1991) 22 and multiple sclerosis (Bebo & Dveksler 2005). PSGs also exhibit anti-thrombotic activity by binding 23 integrin α IIb β 3 and inhibiting the platelet – fibrinogen interaction (Shanley *et al.* 2013). To our current 24 knowledge, PSGs evolved exclusively in mammals with hemochorial placentation. In cattle (epitheliochorial placenta) and dogs (endotheliochorial placenta), PSGs did not evolve (Kammerer et al. 25 2004, Kammerer et al. 2007). 26

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28 However, here we show that PSGs evolved in the horse. The horse has an epitheliochorial placenta,

1 which represents the least invasive form of placentation. However, a subpopulation of trophoblast cells (chorionic girdle cells) differentiates to a highly invasive phenotype (Allen et al. 1973, Lunn et al. 1997). 2 3 Around day 35 post-ovulation these binucleated, well-differentiated, eCG-secreting cells invade the endometrium. and build the endometrial cups which reach their maximum size and hormone-secreting 4 capacity between day 60 and 70 of gestation (Allen 2001). During invasion, chorionic girdle cells 5 express conventional MHC class I antigens and are recognized but not initially destroyed by the maternal 6 7 immune system (Donaldson et al. 1990, Noronha & Antczak 2010). The endometrial cups have a restricted 8 lifespan and the cells die around days 80-100 of pregnancy, although the exact mechanisms that lead to their 9 destruction remain poorly understood. In this report, we show that equine PSGs are expressed in Chorionic girdle cells as well as in endometrial cups and that equine PSGs have a similar functional activity to 10 11 human and rodent PSGs. Future work is in progress to examine the immunomodulatory functions of 12 equine PSGs.

13

14 Material and Methods

15 *Cells and tissues*

Different equine tissue samples including salivary gland, trachea, lung, kidney, renal pelvis, skin, fat, pancreas, vein, liver, spleen, lymph node, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, ovary, uterus, cervix, udder, oviduct, bladder, vulva were collected from freshly slaughtered healthy horses and flash-frozen in liquid nitrogen. Peripheral blood mononuclear cells (PBMCs) were isolated from blood, which was collected for diagnostic purpose, of healthy horses by density-gradient centrifugation through Ficoll-Paque 1.077 g/l (GE Healthcare, Freiburg, Germany).

22

Stimulation of PBMC with 200 U/ml human IL-2 for the indicated time was performed at a
concentration of 5 x 10⁵ cells/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS "Gold";
PAA Laboratories, Coelbe, Germany), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin,
non-essential amino acids and 1 mM sodium pyruvate (GIBCO/Invitrogen, Karlsruhe, Germany).
Endometrial cup tissue samples were obtained from a 65 days pregnant, sedated mare by transcervical
hysteroscopy via an endoscopy biopsy forceps under visual control. The procedure was performed to

1 initiate the abortion of the unwanted fetus. Tissue samples were immediately transferred to RNAlater (Qiagen, Hilden, Germany). Relevant tissue collection was approved by animal use committee of local 2 3 authorities (LALLF Rostock; 7221.3-2.1-011/13). Generation of tissue from earlier stages of pregnancy 4 and isolation and culture of chorionic girdle trophoblast cells has been previously described (Cabrera-Sharp et al. 2014). In brief, mares aged 3-7 years were maintained at the Royal Veterinary College and 5 6 animal care was performed in accordance with the Animals (Scientific Procedures) Act 1986 guidelines 7 set by the Home Office and the Ethics Committee of the Royal Veterinary College. The reproductive 8 cycle of 5 mares was manipulated, and pregnancies were established using semen from 3 stallions. 9 Conceptuses were recovered by nonsurgical uterine lavage with established methods between days 31 10 and 34 of pregnancy. Conceptuses were microdissected into chorionic girdle, allantochorion, chorion, yolk sac, bilaminar omphalopleure, and fetus and tissue snap frozen in liquid nitrogen. To gain a pure 11 population of trophoblast cells, strips of chorionic girdle were placed into DMEM, and the chorionic 12 13 girdle trophoblast cells were gently removed from basement membrane and underlying avascular mesodermal cell layer and cultured as per published methods (de Mestre et al. 2008). Tissues from 4 14 15 independent pregnancies and trophoblast cells isolated from an additional 5 independent pregnancies 16 were used with the gestational age and passage number (0-3) of the cultured cells indicated in the figure 17 legend.

18

19 Identification and prediction of equine CEACAMs

In order to identify all genes of the equine CEA gene family cluster we screened both the whole genome shot gun sequence (WGS) database at NCBI (http://www.ncbi.nlm.nih.gov) and the ensemble genome build at (http://www.ensemble.org) by running respective BLAST search tools using nucleotide sequences of all CEACAM exons of human, mouse, dog and cattle. Exon/Intron structures were predicted manually.

25

26 Reverse transcription-polymerase chain reaction analysis

Total RNA extraction from tissue was performed using the RNeasy kit (Qiagen, Hilden, Germany).
RNA was isolated from cultured trophoblast cells using RNAbee (Amsbio, Abingdon, United

1 Kingdom). RNAbee was added directly to the culture well and cells scraped before removal to an Eppendorf tube and RNA extracted as described by the manufacturer. 500ng to one microgram of total 2 3 RNA was used for cDNA synthesis by reverse transcription (RT) using the AMV Reverse Transcriptase 4 (Promega, Mannheim, Germany). The RT product was amplified by polymerase chain reaction (PCR) with Taq polymerase (Fermentas, thermos Scientific, VWR International Germany GmbH, Darmstadt, 5 Germany). After an initial denaturation step at 95°C for 45 s, 35 PCR cycles (denaturation: 95°C, 30 s; 6 7 annealing: 60°C, 1 min; extension: 72°C, 1.5 min) and a final extension step at 72°C for 15 min were 8 performed. Primers were designed according to our sequence predictions based on sequences identified 9 in various NCBI data bases and based on newly identified sequences in this work. All primers used are 10 summarized in Table 1. Eight microliters of each PCR were analyzed by electrophoresis on a 1.8% agarose gel and visualized by ethidium bromide staining. Real Time quantitative Polymerase Chain 11 Reaction (qPCR) was performed with Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo 12 Scientific). An initial denaturation at 95°C for 10 min to activate Maxima Hot Start Taq DNA 13 polymerase, was followed by 40 cycles of a two-step cycling amplification (95°C for 15 s, 60°C for 1 14 15 min). The relative abundance of transcripts was calculated in arbitrary units (AU) using the formula 2^{35-cq} (AU), where cq represents the quantification cycle. 16

17

18 *cDNA cloning*

Primers used for amplification of full-length cDNAs (CEACAM46^a CEACAM46b CEACAM47, CEACAM49 CEACAM55) are summarized in table 1. These primers introduced *Hin*dIII or *Xho*I and *Xba*I or *Eco*RI restriction sites into the PCR products corresponding to the 5'- and 3'-ends of the mRNA, respectively. The full-length cDNA was digested with *Hind*III or *Xho*I and *Xba*I or *Eco*RI and cloned into the pRc/CMV or pEGFP-N3 expression vector. Plasmid DNA isolated from various clones were analyzed by PCR and sequencing. Nucleotide sequencing was performed with the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Weiterstadt, Germany).

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27 Phylogenetic analyses

28 Phylogenetic analyses based on nucleotide and amino acid sequences were conducted using MEGA

version 5. The Neighbor-Joining (NJ) method with bootstrap testing (100 or 1000 replicates) and
 Poisson correction was applied.

3

4 CEACAM49 and PSG1 protein production and platelet assay

Protein production and purification was done as described (Shanley et al. 2013) using transient 5 transfection of pTT3CC49 and pTT3PSG1 vectors, respectively, into HEK-293 cells (Freestyle™ 293 6 7 cells). The pTT3CC49 (pTT3CEACAM49) construct was generated by PCR amplifying the CEACAM49 CDS from pRc/CMV-ecaCEACAM49 incorporating 5' EcoRI and 3' HindIII restriction 8 9 sites using ecaCEACAM49For: 5'-gGAATTCaccatgcaatcaccctca the primers, and ecaCEACAM49Rev: 5'-cAAGCTtggctctgtaactgggg. The PCR amplified band was blunt cloned into 10 11 PSTBlue1 and sub-cloned into pTT3-B using *Eco*RI and *Hind*III restriction enzymes.

12

13 To produce recombinant PSG1 and CEACAM49 proteins, endotoxin-free plasmid DNA was purified from bacterial cultures using the Endofree Plasmid Maxi Kit (Qiagen). All subsequent steps were carried 14 15 out using confirmed endotoxin-free reagents and tissue culture flasks. The DNA was transiently transfected into Freestyle 293-F cells (Invitrogen) using Freestyle MAX reagent (Invitrogen). The 16 17 Freestyle 293-F cells were grown in suspension in Freestyle 293 Expression Medium, by shaker culture, 18 to a density of 1×10^6 cells per ml. The plasmid DNA was diluted in OptiPRO serum-free medium at a 19 ratio of 1µg DNA in 20µL OptiPRO for every 1mL of cells. Freestyle MAX reagent was also diluted in OptiPRO at the same ratio (1µL Freestyle MAX reagent in 20µL OptiPRO per milliliter of cells). The 20 21 diluted DNA and Freestyle MAX reagent were then combined, mixed gently, and incubated at room 22 temperature (RT) for 20min. The mixture was added to the cell suspension and the cells were cultured 23 for a further 72h. The culture was then centrifuged at 200g for 5min at RT to separate the protein-24 containing medium from the cells, and the medium was frozen in aliquots at -80°C.

Recombinant proteins were purified from cell culture medium using Qiagen Ni-NTA resin. Imidazole (Sigma) was added to the culture medium to a final concentration of 10mM to reduce non-specific binding. Ni-NTA resin was added to the medium at a ratio of 1ml resin suspension (corresponding to 0.5mL resin bed volume) to 100mL medium. The medium and resin were then batch bound overnight

1 on a rotating wheel at 4°C. The medium and resin mix was then passed through a disposable polypropylene column (Pierce, Thermo Fisher Scientific) and the resin was washed with wash buffer 2 3 (500mM NaCl, 20mM NaH2PO4, pH6) until the absorbance at 260nm reduced to 0. Protein was then eluted from the column with increasing concentrations of imidazole in wash buffer, usually 4×1.5 ml 4 50mM fractions, 5×1.5mL 200mM fractions, 4×1.5ml 300mM fractions and 3×1.5ml 500mM fractions. 5 Fractions containing recombinant PSG1 or CEACAM49 were then pooled and passed through a new 6 7 column containing 0.5ml Ni-NTA resin, the flow-through collected, and bound protein eluted with 1mL 8 50mM imidazole followed by three 1mL 200mM imidazole fractions. The flow-through and three 9 200mM fractions were pooled and concentrated to a volume of 4-6mL using a Millipore Amicon Ultra 10 Ultracel 10K centrifugal filter (Millipore). The concentrate was then dialyzed against three changes of 2L of phosphate-buffered saline (PBS) at 4°C. The protein was then further concentrated to a volume of 11 1–2mL depending on the starting volume of culture medium. Protein was quantified by Bradford Assay 12 or UV Spectroscopy, checked by polyacrylamide gel electrophoresis, tested for LPS contamination 13 (Limulus Amebocyte Lysate QCL-1000; Cambrex BioScience, Germany), aliquoted, and frozen at 14 15 -80°C. For size exclusion chromatography (SEC), 1–4mg purified recombinant protein was applied to a HiLoad 16/60 Superdex S200 prep grade column in a 1mL volume with a flow rate of 1mL/min using 16 17 the Akta explorer system in PBS. Determination of 1mL fractions to pool was based on chromatogram 18 peaks. Resultant protein solution was concentrated. TGF β 1 contamination level was determined by 19 enzyme-linked immunosorbent assay (ELISA).

20 Preparation of human platelets

Platelets were collected into 0.15vol/vol acid-citrate dextrose (ACD; 75mM trisodium citrate, 124mM 21 22 dextrose and 38mM citric acid) anticoagulant and washed. Briefly, blood was centrifuged at 150g for 23 10min at RT. In order to avoid any contamination from the buffy coat, ~ 0.5 mL of the platelet-rich 24 suspension above the buffy coat layer was left behind in the centrifugation tube. Platelet-rich plasma 25 (PRP) was then acidified to pH6.5 with ACD and prostaglandin E₁ (PGE₁, 1mM) was added. The platelets were pelleted by centrifugation at 750g for 10min at RT. The supernatant was removed and the 26 27 platelet pellet was gently resuspended in 130mM NaCl, 3mM KCl, 10mM trisodium citrate, 9mM NaHCO₃, 6mM dextrose, 0.9mM MgCl₂, 0.81mM KH₂PO₄ and 10mM Tris pH7.4 (JNL buffer). Platelet 28

count was adjusted to 3×10⁸per mL using a Sysmex XE K-1000 counter (Toa Medical Electronics Co.
 Ltd, Kobe, Japan). Platelets were allowed to stand at RT for 45min to let PGE₁dissipate. Calcium
 chloride (CaCl₂, 1.8mM) was added to platelets immediately before use.

4 Platelet integrin binding assay

5 Platelet integrin interaction assay was done as described (Shanley et al. 2013). Briefly, 10µL of 6 2.5mg/mL Oregon green-labeled Fibrinogen (OgFg; Invitrogen) was added to 20µL aliquots of 7 washed platelet suspension along with the indicated concentrations of PSG1 or CEACAM49 in 8 successive tubes. Dose-response curves were produced for all proteins using serial dilutions of 1:2 in 9 the range 200 to 12.5µg/mL. All experiments were run in duplicate at least three times. The platelet 10 suspension was vortexed and allowed to stand at RT for 10min before the addition of 3.4µM thrombin 11 receptor activating peptide (TRAP, Bachem, UK), a dose known to produce a 50% maximal response as measured by aggregometry in pilot studies. Assay tubes were incubated at RT for a further 10min. 12 13 The reaction was stopped by addition of 1mL ice-cold buffer. In a separate series of experiments, 250nM U46619 (Tocris Bioscience, UK), 10µM ADP (Bio/Data Corporation, UK), or 25µM 14 15 epinephrine (Bio/Data Corporation, UK) were used to activate platelets. The association of OgFg with 16 platelets was detected using a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, 17 NJ, USA). Data acquisition and analysis were performed with the Cell Quest program. Platelet 18 populations were gated, and histograms of mean fluorescence were generated for each sample. 19 Statistical analysis was performed on the geometric scale.

20 **Results**

21 The equine CEA gene family cluster

The complete equine CEA gene family cluster was identified as described in "materials and methods". Surprisingly, only genes orthologous to *CEACAM1*, *CEACAM16* and *CEACAM19* exist; none orthologous gene to *CEACAM18* and *CEACAM20* could be identified. On the other hand a large number (30) of CEACAM1-related leader and N domain exons evolved in the horse genome. Further analysis indicated that 17 CEACAM1-related genes appear to be functional (named *CEACAM1*, *CEACAM41*-*56*) and 13 of the CEACAM1-related genes appear to be pseudogenes (named *CEACAMps1-13*) (data not shown). Based on the most recent assembly of the horse genome (EquCab2) these genes are split into two loci by the insertion of a chromosomal region flanked by *LIPE* and *CD79A*. Similar numbers
 of *CEACAM1*-related genes were only found in species which contain *PSG* genes. This finding
 prompted us to further analyze the possible structure of the CEACAM1-related CEACAMs of the horse.

5 Multiple equine CEACAMs do not contain a transmembrane region

It is assumed that the main structural difference of CEACAMs concerning their function is based on the
mode of their membrane anchorage. Already known CEACAMs are either type I transmembrane
proteins, GPI-anchored or secreted proteins. With very few exceptions secreted CEACAMs are PSGs.

To predict the structure of equine CEACAMs we identified exons of CEACAM1-related genes and 10 11 determined their open reading frames. Eight CEACAM genes contain an exon which codes for a transmembrane domain (TM exon), two of the TM exons contain a stop codon, which shortens the 12 13 cytoplasmic part of the encoded domain and one has a mutation leading to a disabled splice acceptor side, indicating that this exon is not part of the processed mRNA (Fig. 2). None of the pseudogenes 14 15 contains a TM exon (data not shown). In one gene (CEACAM41) the TM exon is followed by 4 exons together encoding a cytoplasmic domain (Cyt exons) possessing an immunoreceptor tyrosine-based 16 17 activation motifs (ITAM) like motif. Four genes (CEACAM1, 42, 43, 50) contain Cyt exons which code for one immunoreceptor tyrosine-based inhibition motif (ITIM) and one immunoreceptor tyrosine-based 18 19 switch motif (ITSM). In CEACAM42, however, the first Cyt exon has no functional splice donor site. 20 No indications were found for the existence of GPI-anchored equine CEACAMs. Remakably, seven 21 genes (CEACAM44, 46, 47, 48, 49, 52, 55) lack a transmembrane domain encoding exon and are 22 composed of a leader, a N domain and an IgC domain (A2-type) exon. In all these genes the third exon 23 contains a stop codon. Since all equine CEACAMs contain a leader exon the gene products without a 24 transmembrane domain are assumed to be secreted proteins (Fig. 2).

25

26

27 Phylogeny of CEACAMs in the horse

28 Phylogenetic trees were constructed from nucleotide sequences of the TM-, IgV-, and IgC-exons. Two

1 types of TM exons were identified. One (CEACAM41) is connected with Cyt exons encoding an ITAMlike motif and six which were related to the TM exon typically found in CEACAM1 genes which have 2 3 ITIM/ITSM-encoding exons (Fig. 3A). We have previously reported that IgV-like domains of 4 CEACAMs with ITAM-containing supposedly activating cytoplasmic tails are closely related to IgVlike domains of inhibitory CEACAMs forming receptor pairs (Kammerer and Zimmermann 2010). 5 Again in the present study we found that the IgV-like domain of the activating CEACAM41 is most 6 7 similar to the IgV-like domains of the inhibitory receptors (CEACAM1, and CEACAM43) (Fig. 3B). 8 Surprisingly, the IgV-like domains of secreted CEACAMs did not form one group as previously 9 recognized for the IgV-like domains of PSGs in humans and rodents. In the horse two different groups 10 of N domains of the secreted CEACAMs could be identified. The first is a sister group to the membrane bound CEACAMs and the second, including CEACAM47, CEACAM48 and CEACAM49, forms a 11 group separated from all other CEACAM1-related CEACAMs. These findings may indicate that two 12 13 different groups of secreted CEACAMs exist which may have different functions (Fig. 3B). The arrangement of the IgC-like domains of membrane bound CEACAMs is as in CEACAM1, starting with 14 15 an A1 type domain followed by a B domain and an A2-type IgC-like domain. Interestingly, all secreted CEACAMs contain an A2-type IgC-like domain exon, which comprises a stop-codon and the 3'-16 17 untranslated region.

18

19 Generation of secreted CEACAMs in the horse

20 Secreted CEACAMs in the horse belong to the CEACAM1-related CEACAMs as previously found in humans and rodents. They are, however, not found in all mammalian species (Kammerer & 21 22 Zimmermann 2010). During evolution, secreted CEACAMs could have been generated by duplication 23 of membrane anchored CEACAM1. Different truncations may account for the loss of membrane 24 anchorage of these CEACAMs in different species. Based on genomic data, generation of secreted CEACAMs in the horse was caused either by the introduction of one or more stop codons into the A2 25 exons or by mutation of a splice donor site of A2 domain exons. This interpretation was verified by 26 sequencing cloned cDNAs generated from equine mRNAs (Fig. 4). In order to get experimental support 27 for the prediction that CEACAMs without membrane anchorage are secreted, we selected four of them 28

and generated plasmids which encode CEACAM-GFP fusion proteins and transfected HEK-293 cells
with these plasmids. As shown in Fig. 5, fusion proteins were detected in the cytoplasm of the cells in a
diffuse staining pattern (Fig. 5B). Fusion proteins could further be detected in the culture supernatant of
transfected cells and the amount of fusion proteins correlated with the time of culture consistent with
these proteins being actively secreted by transfected cells (Fig. 5C).

6

7 Secreted CEACAMs are expressed by trophoblast cells

8 Next we identified tissues in which secreted equine CEACAMs are expressed. We generated gene 9 specific primers for CEACAM44, 46, 47, 48, 49, 52, 55 and analyzed their expression by RT-PCR in 10 29 different equine tissues (see "Materials and Methods"). PSG-like CEACAMs were detected in a restricted number of tissues including, Endometrial cups, freshly isolated and IL-2-stimulated PBMC, 11 lymph node, salivary gland, tongue and ovary (Fig. 6). CEACAM46 was only detected in peripheral 12 13 blood mononuclear cells and CEACAM52 was not detected in any of the tissues tested. Five secreted CEACAMs (CEACAM44, 47, 48, 49, 55) were expressed in trophoblast cells or placental tissues at one 14 15 or more stages of pregnancy with expression patterns differing between CEACAMs both spatially and temporally (Fig. 7). CEACAM44 was strongly expressed in day 34 conceptus tissues (chorionic girdle, 16 17 allantochorion and chorion) as well as day 31, 32 and 34 cultured chorionic girdle trophoblast (all 18 passage 0) and endometrium. CEACAM47 was only weakly expressed in day 32 and day 34 freshly 19 isolated and cultured trophoblast cells but not in day 34 conceptus tissues or day 65 endometrial cups. 20 CEACAM48 was weakly expressed in day 32 cultured trophoblast and day 65 endometrial cups but again not in any of the day 34 conceptus tissues. CEACAM49 was strongly expressed in day 34 21 22 chorionic girdle and cultured day 34 chorionic girdle trophoblast cells (both passage 0 and 3) and 23 expression was maintained in day 65 endometrial cups. CEACAM55 was only expressed in day 65 24 endometrial cup and at a low level in PBMC of some horses. Although day 65 endometrial cup tissue may be infiltrated with lymphocytes, it is plausible that CEACAM55 expression detected in the 25 endometrial cup tissue was due to the trophoblast cells as opposed to the expression by infiltrating 26 lymphocytes since CEACAM46 which is preferentially expressed by leucocytes was not detected in 27 endometrial cups (Fig 7. And data not shown). Together, these results provide supporting data that 5/7 28

3 To determine whether CEACAM44 and/or CEACAM49 expression was enriched in trophoblast cells, 4 we then quantified their expression using qRT-PCR in matched day 34 conceptus tissue (n=3), cultured purified chorionic girdle cells (n=5), endometrium (n=3) and PBMCs (n=3). Highest expression of 5 CEACAM44 was noted in the purified cultured passage 0 day 32 and 34 chorionic girdle trophoblast 6 7 cells and day 34 chorionic girdle tissue (Fig.7). CEACAM49 expression was very specific to trophoblast 8 tissue or cultured trophoblast cells. No significant expression was found in endometrial tissue or PBMC. 9 In contrast to CEACAM44, CEACAM49 was expressed to a similar amount in all three different 10 trophoblast tissues (chorionic girdle, chorion and allantochorion).

11

12 Horse PSG-like CEACAM49 has similar anti-platelet activity to human PSG1

13 CEACAM49 turned out to be expressed in both the chorionic girdle cells and the endometrial cups, indication that this PSG-like CEACAM may have a pivotal function. To assay the functionality 14 15 CEACAM49 was subcloned from pRcCMV-ecaCEACAM49 into the pTT3 expression vector incorporating a C-terminal V5-6xHis tag (Shanley et al. 2013). This allowed the generation and 16 17 purification of CEACAM49 protein by transient transfection of the human HEK cell line using the 18 Freestyle[™]293 expression system. Eluted and dialyzed aliquots of CEACAM49 from a nickel affinity 19 chromatography column were combined and further purified by size exclusion chromatography to 20 remove unidentified high molecular weight bands and contaminating TGFB1 (Ballesteros et al. 2015) (Fig. 8A-C). To determine whether CEACAM49 exhibits similar functionality to human PSG1, the 21 22 CEACAM49 protein was applied to a fluorescent fibrinogen-platelet interaction assay as described 23 (Shanley et al. 2013). CEACAM49 inhibited fibrinogen binding to TRAP-activated platelets in a dose-24 dependent manner similar to human PSG1 (Fig. 8D, E).

25

26

27 Discussion

28 In this study, we have characterized the CEA gene family of the horse. Unlike all other mammals

1 previously investigated, we found no evidence of conserved orthologs of human CEACAM18 and CEACAM20 in the horse genome (Kammerer & Zimmermann 2010), consistent with the findings of a 2 3 recently published independent study (Pavlopoulou & Scorilas 2014). Nevertheless, other CEACAM1-4 related CEACAM genes in the horse were identified and expanded significantly to probably 17 functional genes and 13 pseudogenes. Such a high number of CEACAMs has previously been found only in species 5 which have an expanded PSG gene cluster (Kammerer & Zimmermann 2010). However, no orthologous 6 7 relationship based on synteny or common structure could be identified for the horse PSG-like 8 CEACAMs, indicating their independent evolution from a horse CEACAM1-like ancestor rather than a 9 common PSG-like ancestor. The horse PSG-like CEACAMs are very different from PSGs in other 10 species being composed solely of a single IgV-like N domain. Furthermore, there is significant variation in the predicted number of glycosylation sites between the equine family members, much more than is 11 evident in the N or N1 domains of primate and rodent PSGs, respectively. This variation could reflect a 12 13 diversification in function among horse PSG family members.

14

15 Until now PSGs have only been identified in mammals which have a hemochorial placenta e.g., higher primates and rodents. Hemochorial placentas are characterized by direct contact of fetal cells with the 16 17 maternal blood and immune system. Therefore, potent tolerance mechanisms are required to prevent 18 destruction of fetal cells. Since PSGs have the capacity to modulate maternal immune responses 19 (Martinez et al. 2012, Martinez et al. 2013, Falcon et al. 2014) we previously suggested that 20 maintenance of immune tolerance is an important selective force in the expansion of *PSG* gene clusters (Kammerer & Zimmermann 2010). The horse has, similar to swine and cattle, an epitheliochorial 21 22 placenta in which fetal cells are separated from the maternal immune system. In addition, the recruitment 23 of maternal immune cells into epitheliochorial placentas is very limited compared to hemochorial 24 placentas (Carter & Enders 2013). However, the horse is exceptional in that in this species, the specialized chorionic girdle cells of the conceptus invade into the endometrium of the mare to form the 25 endometrial cups. The dndometrial cups are recognized by maternal T-cells due to their expression of 26 MHC class I antigens. A large number of CD4+ and CD8+ T-cells infiltrate the endometrial stroma at 27 the edge of endometrial cups (de Mestre et al. 2010), and by day 60–70, these cells move into the cup. 28

Over the next 30 days or so, neutrophils, macrophages, and eosinophils also invade into the main area of the cup. The cups become necrotic and are eventually sloughed between days 100 and 140 in most mares. Although the immune cells are present, there is evidence that they do not attack the cup cells, and the mechanism of the demise of the cups is currently unclear. However, despite such a close contact with maternal immune cells, semiallogeneic fetal cells are not attacked, indicating that powerful tolerance mechanisms are at work (Noronha & Antczak 2010).

7

8 Several immune tolerance mechanisms during pregnancy were recently identified in primates and 9 rodents, including downregulation of classical MHC class I antigens, upregulation of indoleamine 2,3-10 dioxygenase (IDO) which causes a local depletion of tryptophan, and upregulation of CD95L (Fas ligand) that promotes apoptosis of activated lymphocytes (Trowsdale & Betz 2006). Additional poorly 11 characterized secreted factors are likely to participate in tolerance induction during pregnancy 12 13 (Trowsdale & Betz 2006). PSGs may represent such factors inducing immunotolerance and thromboregulation, in human and mice (Moore & Dveksler 2014, Ballesteros et al. 2015). Thus, we 14 15 speculate that equine PSG-like CEACAMs expressed by infiltrating chorionic girdle trophoblast and endometrial cup cells may have a similar function controlling activity of T cells located at the borders 16 17 of endometrial cups, and perhaps on peripheral T cells. Indeed, peripheral blood lymphocytes from 18 pregnant mares have a reduced capacity to develop into effective cytotoxic T cells (Baker et al. 1999). 19 PSGs have the potential to be present in maternal blood, as one of the major secretions of endometrial 20 cups is equine chorionic gonadotropin, which is found at very high levels in the blood of pregnant mares while the cups persist (Allen 2001). Although additional work is needed to verify putative 21 22 immunoregulatory functions of PSGs, in this study, we did confirm that PSG-like equine CEACAM49, 23 similar to human and mouse PSGs, inhibits the interaction between activated platelets and fibrinogen 24 (Shanley et al. 2013). An anti-platelet activity may be beneficial for endometrial cup function because at the base of endometrial cups there are many newly formed lymph and blood vessels to support 25 constant circulation in this immature capillary bed (Antczak et al. 2013). In addition, PSGs have been 26 shown to bind other integrins, besides platelet integrin (Moore & Dveksler 2014), and it is possible that 27 this also occurs in the horse. Therefore, a PSG-integrin interaction may have additional yet unknown 28

benefits for the function and/or survival of endometrial cups. Thus equine PSGs have important
functional similarities with human and mouse PSGs indicating that they were generated independently
by convergent evolution.

4

There is probably no recent common PSG ancestor from which the PSGs in different species evolved. 5 Lack of synteny suggests that PSGs evolved independently in different mammalian species. As a 6 7 consequence PSGs structurally differ between primates, rodents and perissodactyls. Nevertheless, molecular interaction of PSGs with integrins is found in all mentioned orders. This striking similarity 8 9 may be explained by an interaction via the N-terminal IgV-like domain which is present in all types of 10 PSGs. Interaction with integrins could be part of the core functional requirements for the selection of 11 PSGs gene expansion. Whether regulation of immunity belongs also to these core functions, has to be 12 confirmed in further studies. Remarkably, the horse is an exceptionally well suited animal model to 13 address such a question, since mares mount a strong systemic (antibody) and local (cellular) immune response to paternal antigens (Noronha & Antczak 2010). This naturally occurring immune recognition 14 15 will be beneficial for the determination of the immune regulatory function of PSGs. Whatever the core functions of PSGs are, there must be an extremely strong selection pressure to select for the expansion 16 17 of the PSG cluster in disparate species either for diversification and functional fine tuning or for increase 18 of the dose of highly similar proteins. Among the 12 protein-coding genes with the highest level of 19 enriched expression in human placenta compared to other tissues are eight PSG genes (Uhlen et al. 20 2015). A better understanding of PSG function in horses may have important implications for the 21 elucidation of the role of PSGs in human pregnancy.

22

23 Availability of supporting data

Sequences of cloned equine CEACAMs were submitted to GenBank and have the following accession
numbers: Eca_CEACAM46a, KT124640; Eca_CEACAM46b, KT124641; Eca_CEACAM47
KT124642; Eca_CEACAM49, KT124643; Eca_CEACAM55, KT124644.

2 Competing interests

3 The author(s) declare that they have no competing interests.

4

5 Abbreviations

6 CEA, carcinoembryonic antigen; CEACAM, carcinoembryonic antigen-related cell adhesion molecule;
7 Cyt, cytoplasmic domain; GPI, glycosylphosphatidyl inositol; ITAM, immunoreceptor tyrosine-based
8 activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; ITSM, immunoreceptor
9 tyrosine-based switch motif; PSG, pregnancy-specific glycoprotein; TM, transmembrane domain.

10

11 Authors' contributions

DA and LB did most of the experiments. SM, JH, WW and JR did additional experiments. JH and VCS
collected tissues and JH performed the biopsy of the endometrial cups. RO'R conducted platelet studies.
WZ,TM and AdM analyzed data and participated in manuscript writing. RK conceived the study, did
biocomputing and manuscript writing. All authors participated in the design of the study, and read and
approved the final version.

17

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- 60

1 Figure Legends

2 Figure 1 - Genomic arrangement of the extended Leukocyte Receptor Complex and the



3 CEACAM/PSG locus in the horse.

5 The CEACAM/PSG cluster is located on horse chromosome 10 within the extended leukocyte receptor 6 complex (LRC). The extended LRC is limited on both ends by the genes coding for adaptor proteins and 7 the Leukocyte receptor complex, respectively. Loci containing genes of the CEACAM1-related 8 CEACAMs are shown in yellow and the locus containing the conserved CEACAM genes are shown in blue. Lower part: Genomic organization of horse CEA gene family loci. Arrowheads represent genes 9 10 with their transcriptional orientation. CEACAM pseudogenes are shown in yellow and named in black. Putatively intact CEACAM genes are shown in orange and named in bold (secreted CEACAMs in red 11 12 and membrane anchored in black). SIGLEC genes and marker genes are shown as black arrowheads. 13 The scale indicated by dots is 1 Mbp unless interrupted by slanted lines. The horse Ensembl/NCBI release EquCab2 was used. Nucleotide numbering of the chromosomes starts at the centromere. CCps, 14 15 CEACAM pseudogenes; CC, CEACAM; chr, chromosome.

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4

Figure 2 – Prediction of CEA gene family members from sequence analyses of the horse genome. 1 Exon arrangement of equine CEACAM1-related genes. The exon types are indicated by differently 2 3 colored boxes. Leader exons are shown as gray, IgV-like domain exons as red, IgC-like domain exons 4 as blue and transmembrane domain exons as black boxes. The exons encoding the cytoplasmic domain 5 with an ITIM or an ITAM motif are shown in yellow and green, respectively. The presence of nonsense 6 mutations, deletions/insertions in exons causing reading frame shifts are indicated by asterisks. Two 7 genes with intact ITIM/ITSM were identified and named CEACAM1 and CEACAM43. No leader exon 8 could be identified for CEACAM51 because of a sequence gap in the publicly available genomic 9 sequences. The genes are arranged in the order and orientation as found on horse chromosome 10. C1-



13 Figure 3 - Phylogeny of equine CEACAMs.



Phylogenetic trees were constructed from transmembrane domain exon (A), IgV-like domain exon (B)
and IgC-like domain exon (C) nucleotide sequences from CEACAM members of the horse using the
UPGMA method (MEGA 6.0 software). The reliability of a phylogenetic tree was assessed using the
Bootstrap test applying 500 replicates. The statistical support for selected nodes is shown. Bootstrap
values >50 are shown. CEACAM46a and CEACAM46b are putative allelic CEACAM46 variants.
Boxes group CEACAMs with the indicated properties.

1 Figure 4 – Mechanisms for generation of secreted CEA family members in the horse.

	Exon	2_Exc	on 3		10			20)			30			40			50)			60		
CEACAM52	GAC	TAC	CCA	GTG	TTA	AAC	CCC	TTC	ATC	CCA	GTC	ACC	ልጥል	CCA	CAT	AAC	GAC	CCC	ACT	CAC	ACA	ACA	TAA	CCA
CEACAM55	TAC	T AG	TCA	GTG	TCA	AAG	CCC	TCC	ATC	CAA	GTC	AGC	TTA	GGA	CAT	AAG	GAC	CCC	AGI	CAA	AGA	ACA	TAA	GGA
CEACAMAA	TAC	AG AG	CCA	GTG	TAA	AAG	CCC	TCC	ATC	TCA	GTC	AGC	አሞአ	CCA	CAT	AAA	GAC	CCC	AGT	CAC	AGA	አሞአ	TAA	GGA
CEACAM44	TAC	A AG	CCA	GTG	TCA	AAG	CCC	TCC	ATC	TCA	GTC	AGC	777	CCA	CAT	AAG	GAC	CCC	AGI	CAC	CCA	ATA	TAA	GGA
CEACAM46	101 707	A AG	CCA	GTG	TCA	AAG	CCC	TCC	ATC	TGA	GTC	AGC	ΔΤΔ	GGA	CAT	AAG	GAC	CCC	DDT	CAA	GGA	222	TAA	GAA
CEACAM47	CAC	A AG	CCA	CTC	TCA	AAC	CCC	TCC	ACT	CCA	ATC	GTC	AAA	ACA	CGT	AAA	CAC	ACC	ACA	TAC	ATC	ACA	TAA	CCA
CEACAM48	TGG	A AG	TCT	GCA	CCT	CAC	ACA	AAG	CAA	GAC	ACT	ATC	CAA	GGA	CAA	TAC	AAC	CAT	CAC	CAT	ACA	CCC	CAT	GIG.
CEACAM49	TAC	A AT	CCA	GGG	TCA	AAT	ACC	TCC	ATT	GGA	ATA	ACT	GTA	ATA	CAT	AAA	GAC	CCC	AGT	TAC	AGA	GCC	TAA	GGA
CEACAM46	TAT	A AG	CCA	GTG	TCA	AAG	TCC	TCC	ATC	CGA	GTC	AGC	АТА	GGA	CAT	AAG	GAC	CCC	AAT	CAA	GGA		AAA	GGA CCC
CERTORITION		CAT	GGT	CCT	GAC	CTG	CTT	CAC	AAA	CAA	CAC	CAG	GAT	CTC	CAT	CCA	GTG	GTT	CTT	CAA	TGA		1 11 11 1	0011 000
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CEACAM56	GTG	A CT	GAG	TGA	TTT	GTC	TCT																	
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6 from which the structure was verified by cloning are underlined. Stop codons are shown in red.

7 Homologous codons of other CEACAMs representing non-stop codons are indicated in blue. The

8 mutated splice donor site of CEACAM56 is indicated in green.

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1 Figure 5: PSG-like CEACAMs are secreted by eukaryotic cells.



2

3 (A) Schematic illustration of the proteins used in the study. CEACAM46a, CEACAM46b, 4 CEACAM47, and CEACAM55 were expressed in human cells as GFP fusion proteins. (B) Expression 5 of CEACAM46a-eGFP fusion protein by transfected and sorted HEK-293A cells. GFP fusion proteins 6 are only present in the cytoplasm not in the nucleus as wild type GFP or at the cell surface as membrane 7 anchored CEACAMs. (C) Western blot analysis to detect secreted CEACAM-eGFP fusion proteins in 8 the culture supernatant of transfected HEK-293A cells. Supernatant of untransfected HEK-293A cells 9 was used as control. Upper panel: the amount of fusion proteins in the culture supernatant increased with time of culture. For positive control of eGFP detection cell extracts of eGFP transfected HEK-10 11 293A cells were used. Lower panel: All constructs accumulate in the culture medium. CC46a, 12 CEACAM46_eGFP; CC46b, CEACAM46b_eGFP; CC47, CEACAM47_eGFP; CC55, 1 CEACAM55_eGFP; Sup, supernatant; UB, unspecific binding; FP, fusion protein;

2

Figure 6 - Horse PSG-like CEACAMs are preferentially expressed in PBMC and endometrial
cups.



5

6 Total RNA was isolated from various equine tissues as described in "Material and Methods". Biopsies 7 of endometrial cups were taken on day 65 of pregnancy. Lymphocytes were isolated from peripheral 8 blood of healthy horses some of these lymphocytes were cultured in the presence of recombinant human 9 IL-2 for 14 or 19 days (d). In additional 25 equine tissues ("Materials and Methods") none of these 10 mRNAs were detected. Gene-specific primers used in these experiments are summarized in Table 1. 11 Asterix indicate bands of the expected size.



1 Figure 7 – Horse PSG-like molecules are expressed in trophoblast cells.

3 A. Qualitative RT-PCR analysis of expression of secreted CEACAMs in day 34 conceptus tissues (n=3), 4 cultured day 31-34 chorionic girdle trophoblast cells either isolated without prior passaging (P0) or 5 isolated after 3 passages (P3) (n=5), non-pregnant endometrium (n=4) and PBMC isolated from nonpregnant mares (n=3). Amplicons were generated using primers specific for equine CEACAM44, 46, 6 7 47, 48, 49, 55, and β-ACTIN (as control) mRNA, and all bands observed in all tissues with each primer 8 set are of the correct predicted size (table 1). CG=chorionic girdle, AC=allantochorion, EM=Endometrium, H_20 = no template control. B. and C. Real time qRT-PCR expression of 9 10 CEACAM44 (B) and CEACAM49 (C) mRNA in three independent day 34 conceptus tissues, chorionic girdle (CG), chorion (Ch), allantochorion (AC) as well as cultured day 32 and 34 chorionic girdle 11 trophoblast cells (n=2). Data points represent the mean +/- the SE of two experimental replicates. 12

13 Figure 8 – Horse PSG-like CEACAM49 has similar anti-platelet activity to human PSG1.



equine CEACAM49 applied to a Superdex 200 prep grade size exclusion column with red box indicating 2 fractions that were pooled and concentrated. (B) Coomassie stained 12% polyacrylamide gel with 8 µg 3 4 of indicated protein per lane. (C) TGF^β1 ELISA of SM and post-SEC equine CEACAM49 preparations. 5 (D) FACS analysis showing fluorescent Oregon Green Fibrinogen (OGFg) binding to resting and thrombin receptor activating peptide (TRAP)-activated human platelets with, and without, 200 µg/ml 6 human PSG1 or equine CEACAM49 pre-treatment. (E) Graphs of dose-dependent inhibition of OGFg 7 8 binding to activated human platelets, relative to untreated controls, by human PSG1 and equine CEACAM49. n = 3; One-way ANOVA; **, P<0.01; ***, P<0.001. SM, starting material; SEC, size 9 10 exclusion chromatography.

1 Tables

2 Table 1: Gene-specific oligonucleotides for expression analyses and cDNA cloning of horse CEA

3 gene family members

gene	oligonucleotide sequence	location of primers (exon)	size of PCR product (bp)
CEACAM1	For: TGCATCATATAAGATAGGCCCAG Rev: AGTGAGAGTCCTCTTGTCCAGG	N domain A1 domain	367
CEACAM41	For: CATTGCATGTGATAGAGCGAC Rev: CGTCCTTCTGTTCTGTGACTGT	N domain A2 domain	246
CEACAM42	For: AGGGGAAGGAATAGATCCCG Rev: GAGTCCTGTTGTCCGAGGATAG	N domain A1 domain	392
CEACAM43	For: CCCATCAAGAAATTGTGTCCT Rev: ATGTTAACACTACAGGGTCCCTG	N domain A1 domain	276
CEACAM44	For: GCTGTTGTAGGGACCGATGTTA Rev: CCTCCTTCCTGATGATGTGTGT	N domain A2 domain	503
CEACAM45	For: GCGATAGGGCAACAAGAAATTAT Rev: CATGTTAACACTACAGGGTCCTCA	N domain A1 domain	254
CEACAM46	For: AGTCCCACCCAATGGTATCC Rev: CCCAAGTATTGCCCCTTCTGT	N domain A1 domain	525
CEACAM47	For: ACAGACCAAGTCCCAAAACC Rev: TTACGTCTTTTGACGATTCCAG	N domain A1 domain	256
CEACAM48	For: GACCAGCTCGCAAACAAA	N domain A2 domain	279

	AACAAGCTTCTTATCTGGCATTT		
CEACAM49	For: TGGAGCACGTCCACATAAAC	N domain	232
	Rev: GGAGGTATTTGACCCTGGATT	A2 domain	
CEACAM50	Rev: GACAGCTTCAGCCAGGTCCTA	A1 domain	453
CEACAM51	ND	NA	NA
CEACAM52	For: ATGCTGCTGCAGGGGATA Rev: CATCCTCCCTCCTGACACAT	N domain A2 domain	508
CEACAM53	For: TTCAAAGGGGAAATAGATTCCA	N domain	394
	Rev: GAGTCCTGTTGTCTGGGGGAC	A1 domain	
CEACAM54	For: ATCAGTCCCTGGCTTCAGA	N domain A domain	409
CEACAM55	For: CCCTACTAGTCACGAGGAAGAAC Rev: CATCCTCTCGGTCAGTCACA	N domain A2 domain	222
CEACAM46a	For: TCTTCTCACAGAGGGGAGGA Rev: GGGCCAGACTCATATTTCACA	5'UTR 3'UTR	777
CEACAM46b	For: TCTTCTCACAGAGGGGAGGA Rev: GGGCCAGACTCATATTTCACA	5'UTR 3'UTR	777
CEACAM47	For: CCAGGCTCTTCTCACAGAGG Rev: CGTCTTTTGACGATTCCAGTG	5'UTR 3'UTR	588
CEACAM49	For: CCAAGCTCTTCTCACAGACG Rev: GCGGCTCTGTAACTGGGGTCTT	5'UTR 3'UTR	600
CEACAM55	For: GGGCATAAGCTCTTCTCACG Rev: GTATATGTGGAGCTCTCCAA	5'UTR 3'UTR	714

ND, not done; NA, not applicable; For: forward primer; Rev: reverse primer