Endotoxin detection and removal from affinity-purified podoplanin-Fc

Endotoxin levels in purified podoplanin-Fc samples were determined using the PyroGene Recombinant Factor C system (Lonza, Walkersville, MD) and contaminations removed using Detoxi-Gel columns (Pierce, Rockford, IL). All proteins were sterile filtered and stored at 4°C.

Silver staining and gel filtration

Silver staining was done according to the procedure of Blum [1], with an additional incubation in 0.2% periodic acid for 1 hour at 4°C after the fixation step. Gel filtration chromatography was performed on an ÄKTApurifier FPLC system (GE, Little Chalfont, UK), using a Superdex 200 10/300 GL column (GE) and phosphate-buffered saline (PBS) as elution buffer.

Enzyme-linked immunosorbent assay (ELISA)

96-well ELISA plates were coated with 10 μg/ml anti-human Fc antibody (Sigma, St. Louis, MO) in 100 mM carbonate buffer, pH 8.8, and then blocked with 5% nonfat dry milk in PBS. Serum and podoplanin-Fc from HEK 293 cells, diluted with 5% bovine serum albumin in PBS, were applied to the plate for 2 hours at room temperature. Podoplanin-Fc was detected using rabbit anti-human podoplanin antibody (2 μg/ml; Cellsciences, Canton, MA) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10000 dilution; GE), followed by BM Blue POD substrate (Roche Diagnostics, Mannheim, Germany).

REFERENCES

1. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987;8:93-99.

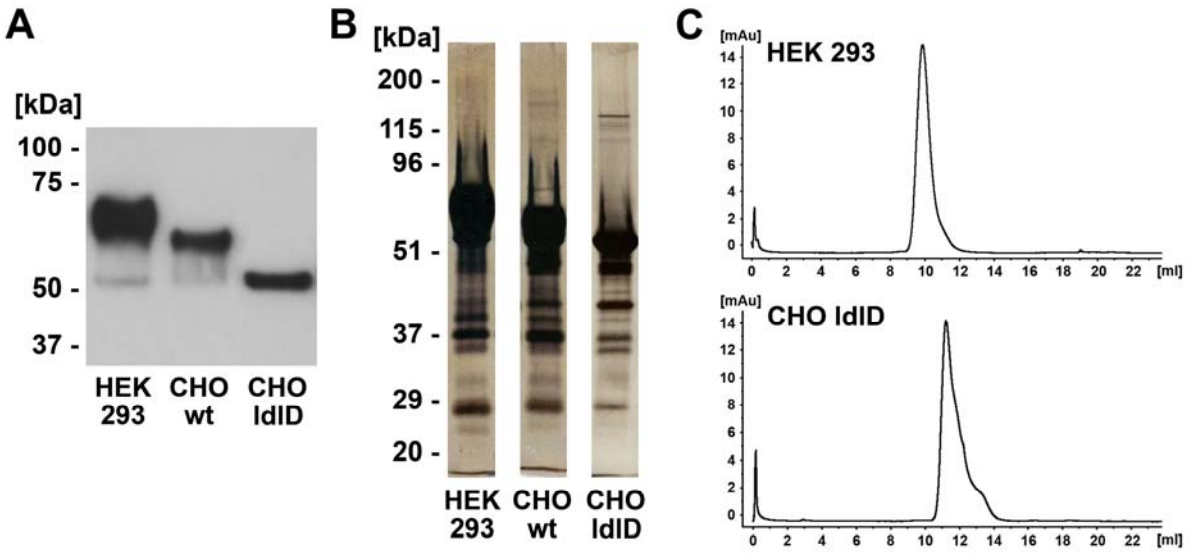

Figure S1. Production of pure, dimeric podoplanin-Fc glycoforms. (A) Different podoplanin-Fc glycoforms were produced in mammalian cell lines, purified from serum-free cell supernatants by protein A affinity chromatography, and immunoblotted with anti-human podoplanin antibody (D2-40). The three podoplanin-Fc glycoforms differ in their electrophoretic mobilities, reflecting their different molecular weights due to different glycosylation patterns. The protein produced by HEK 293 cells is the most extensively glycosylated and the protein produced by CHO ldlD cells is unglycosylated (also verified by glycosidase digestions; not shown). (B) Purified podoplanin-Fc (3 μg) was resolved by 10 % SDS PAGE and visualized by silver staining. For all three glycoforms, podoplanin-Fc is the dominant species and no major protein contaminants are detected. Also, no proteins other than human podoplanin and human IgG_1 were identified by mass spectrometric analysis of purified podoplanin-Fc (not shown). (C) Purified podoplanin-Fc (30 μg) was analyzed by gel filtration on a Superdex 200 10/300 GL column. The elution of a single species reveals that podoplanin-Fc is present exclusively in the desired dimeric form.

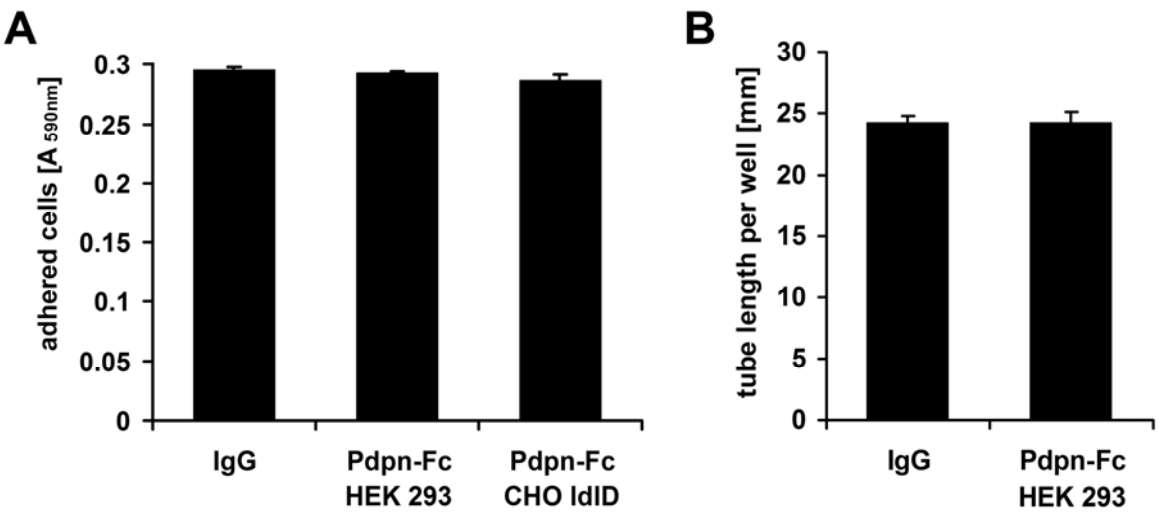

Figure S2. Podoplanin-Fc does not affect functions of HUVECs *in vitro*. (A) HUVECs were allowed to adhere to type I collagen-coated cell culture plates for 45 min in the presence of 0.5 µM podoplanin-Fc glycoforms or human IgG. Attached cells were stained with crystal violet and the absorbance of subsequently re-solubilized dye was measured at 590 nm. (B) Tube formation by HUVECs was assessed in type I collagen gels containing 0.5 µM podoplanin-Fc, produced in HEK 293 cells, or human IgG. The length of tube-like structures was determined after 8 hours in three 5x magnified images per well. Data represent mean +/- SEM (n=3).

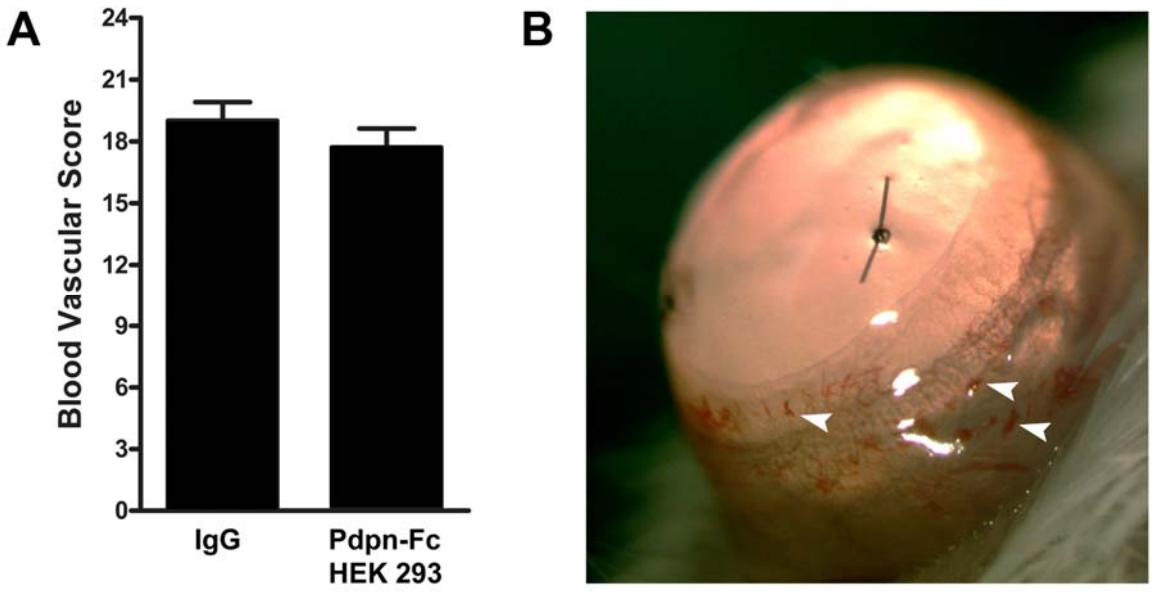

Figure S3. Podoplanin-Fc does not inhibit hemangiogenesis in inflamed mouse corneas *in vivo* but appears to induce blood coagulation. (A) Summary of corneal blood vascular scores from slit-lamp bio-microscopic examination, after two weeks of treatment with human IgG or podoplanin-Fc (100 µg, every second day injected into subconjunctival space). No significant difference was detected between the IgG and podoplanin-Fc treatment groups. Data represent mean +/- SEM (n_{IgG}=10, $n_{Pdpn\text{-}Fc}$=7). (B) Representative slit-lamp picture of the ocular surface demonstrating signs of potentially embolized blood vessels (white arrowheads) after one week of podoplanin-Fc treatment.