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PMab-213: A Monoclonal Antibody for Immunohistochemical Analysis Against Pig Podoplanin

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Podoplanin (PDPN) is known to be expressed in normal tissues, including lymphatic endothelial cells, renal podocytes, and type I lung alveolar cells. Monoclonal antibodies (mAbs) against human, mouse, rat, rabbit, dog, cat, and bovine PDPN have already been established; however, mAbs against pig PDPN (pPDPN) are lacking. In the present study, mice were immunized with pPDPN-overexpressing Chinese hamster ovary (CHO)-K1 cells (CHO/pPDPN), and hybridomas producing mAbs against pPDPN were identified by flow cytometric screening. One of the mAbs, PMab-213 (IgG_{2b}, kappa), could specifically detect CHO/pPDPN cells through flow cytometry and detect pPDPN through western blot analysis. K_D of PMab-213 for CHO/pPDPN was determined to be 2.1×10^{-9} M, indicating a high affinity for CHO/pPDPN. Furthermore, PMab-213 strongly stained lymphatic endothelial cells, renal podocytes, and type I lung alveolar cells through immunohistochemistry. PMab-213 is expected to be useful in investigating the function of pPDPN.

Keywords: pig podoplanin, PDPN, PMab-213

Introduction

Podoplanin (PDPN), a type I transmembrane glycoprotein, is expressed in normal tissues, including lymphatic endothelial cells, renal podocytes, and type I alveolar cells of lung.[1,2] The expression of human PDPN has been reported in several malignant tumors, including malignant brain tumors,[3–6] oral squamous cell carcinomas,[7] esophageal cancers,[8] lung cancers,[9] malignant mesotheliomas,[10,11] osteosarcomas,[12–14] chondrosarcomas,[13] and testicular tumors.[15] Moreover, the expression of PDPN is associated with malignant progression and cancer metastasis.[3,16,17] The interaction between PDPN on lymphatic endothelial cells and C-type lectin-like receptor-2 on platelets has been shown to facilitate embryonic blood/lymphatic vessel separation.[1,16,18–24]

We have previously developed monoclonal antibodies (mAbs) against human,[25] mouse,[25] rat,[26] rabbit,[27] dog,[28] cat,[29] and bovine[30] PDPNs. However, mAbs against pig PDPN (pPDPN), which are useful for immunohistochemical analysis, are lacking. Specific and sensitive mAbs against pPDPN are necessary to investigate the expression and function of pPDPN. In the present study, we immunized mice with Chinese hamster ovary (CHO)/pPDPN cells and established hybridomas that could produce mAbs against pPDPN.

Materials and Methods

Cell lines

P3X63Ag8U.1 (P3U1) and CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). The pig kidney cell line PK-15 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). pPDPN bearing an N-terminal PA16 tag (PA16-pPDPN) was inserted into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The PA16 tag consists of 16 amino acids (GLEGGVAMP-GAEDDVV).[31] CHO-K1 cells were transfected with pCAG-Ble/PA16-pPDPN using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected by limiting dilution and cultivated in a medium containing 0.5 mg/mL zeocin (InvivoGen, San Diego, CA).

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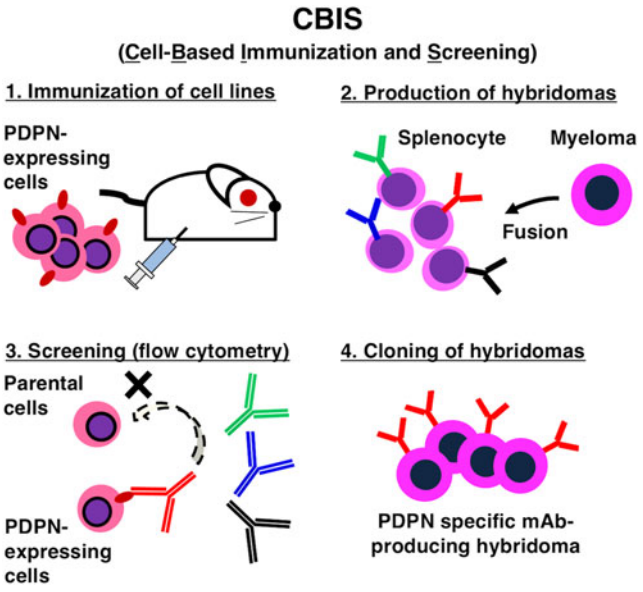

FIG. 1. Schematic illustration of CBIS. Stable transfectants expressing the protein of interest are used as an immunogen with no purification procedure. The selection of hybridomas secreting specific mAbs is performed through flow cytometry using parental and transfectant cells. CBIS, Cell-Based Immunization and Screening; mAb, monoclonal antibody.

P3U1, CHO-K1, and CHO/pPDPN cells were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and PK-15 cells were cultured in Dulbecco's Modified Eagle's medium (Nacalai Tesque, Inc.). All media were supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were grown at 37°C in a humidified environment with an atmosphere of 5% CO_2 and 95% ambient air.

Animals

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all the animal experiments described in this study. Microminipig (Fuji Micra, Inc., Shizuoka, Japan) tissues were collected at autopsy at Gifu University and fixed in 4% paraformaldehyde.

Production of hybridomas

One BALB/c mouse was immunized against CHO/pPDPN cells (1×10^8), which were administered intraperitoneally (i.p.) together with Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included an additional three immunizations followed by a final booster injection administered i.p. 2 days before spleen cells were harvested—a total of five immunizations. Subsequently, these spleen cells were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN) and the hybridomas were grown in RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). The culture supernatants were screened using flow cytometry.

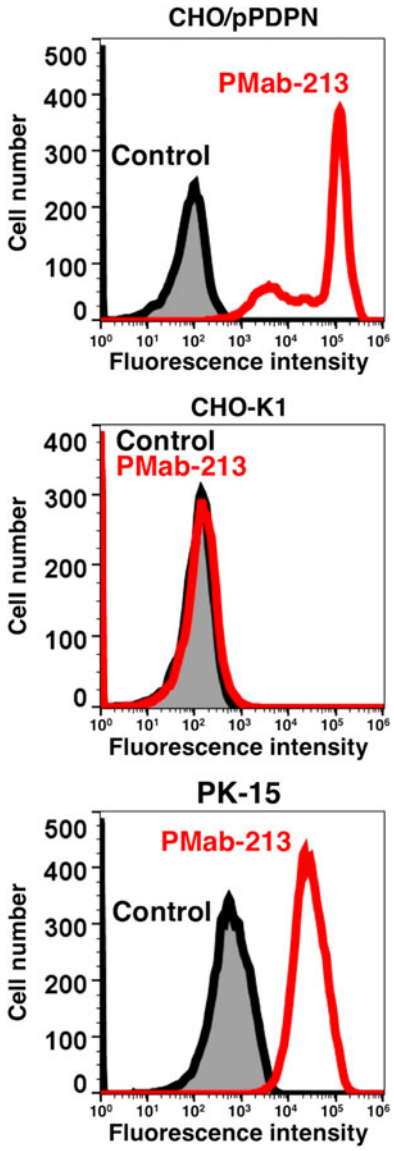

FIG. 2. Detection of pPDPN through flow cytometry using PMab-213. CHO/pPDPN, CHO-K1, and PK-15 cells were treated with PMab-213 (red line) at a concentration of 10 μg/mL or control (gray) for 30 minutes followed by incubation with secondary antibodies. pPDPN, pig podoplanin; CHO, Chinese hamster ovary.

Flow cytometry

The cells were harvested following a brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraaceticacid (Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin/phosphate-buffered saline (PBS) and treated with primary mAbs for 30 minutes at 4°C; thereafter, the cells were treated with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG, 1:2000; Cell Signaling Technology, Inc., Danvers, MA) or Oregon Green anti-rat IgG (1:2000; Thermo Fisher Scientific, Inc.). Fluorescence data were collected using SA3800 Cell Analyzers (Sony Corp., Tokyo, Japan).

Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). The proteins were

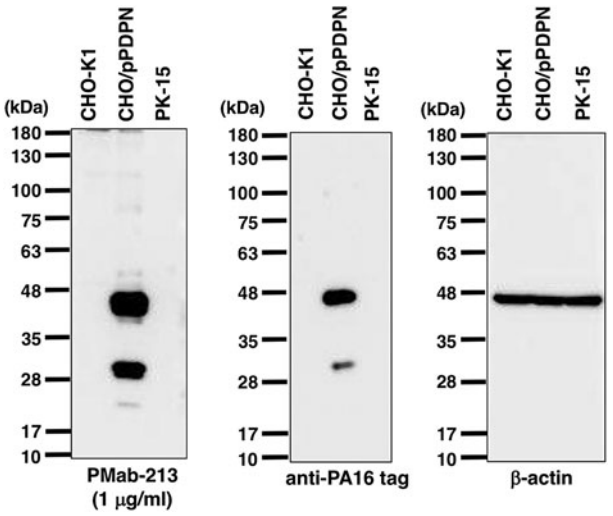

FIG. 3. Western blot analysis. Cell lysates of CHO-K1, CHO/pPDPN, and PK-15 (10 μg) were electrophoresed and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 1 μg/mL of PMab-213, 1 μg/mL of anti-PA16 tag (NZ-1), or 1 μg/mL of anti-β-actin and subsequently with peroxidase-conjugated anti-mouse or -rat IgG. IgG, immunoglobulin G.

subjected to electrophoresis on 5%–20% polyacrylamide gels (Nacalai Tesque, Inc.) and then transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), each membrane was incubated with primary mAbs, such as 1 μg/mL of PMab-213, 1 μg/mL of anti-PA16 tag (NZ-1) or 1 μg/mL of anti-β-actin (AC-15; Sigma-Aldrich Corp., St. Louis, MO) and subsequently with peroxidase-conjugated anti-mouse IgG (1:1000; Agilent Technologies, Santa Clara, CA) or anti-rat IgG (1:10,000; Sigma-Aldrich Corp.). Bands were visualized with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Determination of binding affinity using flow cytometry

CHO/pPDPN or PK-15 (2×10^5 cells) was suspended in 100 μL of serially diluted PMab-213, followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using EC800 Cell Analyzer (Sony Corp.). The dissociation constant (K_D) was obtained by fitting the binding isotherms to built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

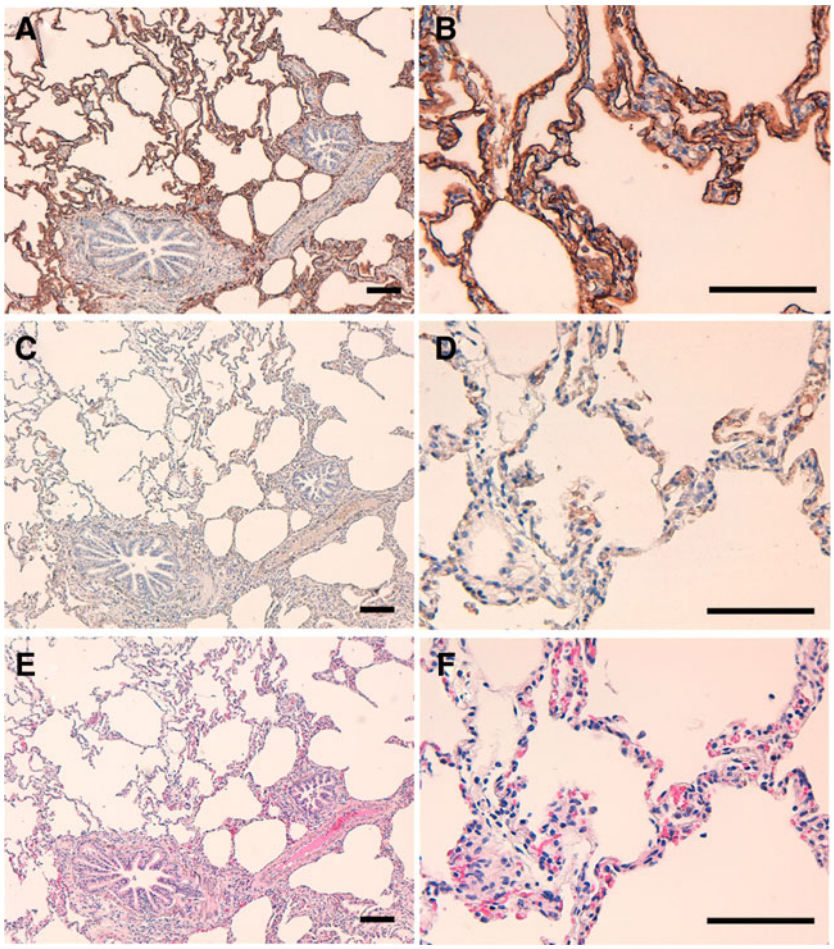

FIG. 4. Immunohistochemical analyses using microminipig lungs. Histological sections of microminipig lung tissue were incubated with 1 μg/mL of PMab-213 **(A**, **B)** or with blocking buffer **(C**, **D)**, followed by an Envision+ Kit. Hematoxylin and Eosin staining **(E**, **F)**. Scale bar = 100 μm.

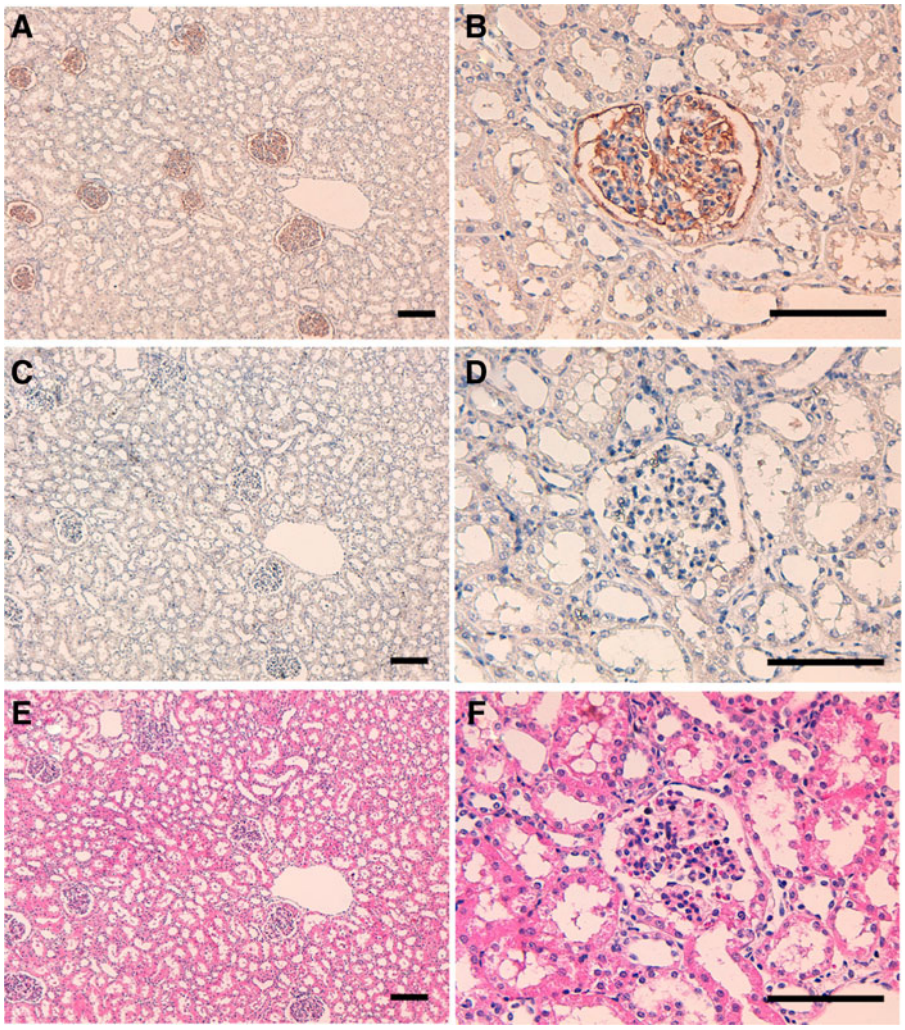

FIG. 5. Immunohistochemical analyses using microminipig kidneys. Histological sections of the microminipig kidney tissue were incubated with 1 μg/mL of PMab-213 **(A**, **B)** or with blocking buffer **(C**, **D)**, followed by an Envision+ Kit. Hematoxylin and Eosin staining **(E**, **F)**. Scale bar = 100 μm.

Immunohistochemical analyses

Tissues were processed to produce 4-μm paraffin-embedded tissue sections, which were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. These tissue sections were blocked using SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.) and incubated with PMab-213 (1 μg/mL) for 1 hour at room temperature and treated using the Envision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using Hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results and Discussion

In the present study, we employed a Cell-Based Immunization and Screening (CBIS) method to develop specific and sensitive mAbs against pPDPN to facilitate the immunohistochemical analysis of paraffin-embedded tissue sections. We have previously successfully utilized a CBIS method to establish mAbs against various types of membrane proteins, such as cat PDPN,[29] CD44,[31] CD133,[32] and PD-L1.[33] One mouse was immunized with pPDPN-overexpressing CHO-K1 (CHO/pPDPN) cells using an immunization and screening procedure (Fig. 1). Hybridomas produced were seeded into 96-well plates and cultivated for 10 days. Wells positive for CHO/pPDPN and negative for CHO-K1 were selected using flow cytometry. Moreover, PK-15 cells were used to identify antibodies that reacted with the endogenous pPDPN. Screening identified strong signals against CHO/pPDPN cells and weak or no signals against CHO-K1 cells in 54 out of 480 wells (11.3%). Of these 54 wells, 7 showed strong signal against PK-15 cells and were negative against CHO-K1 cells. PMab-213 (IgG_{2b}, kappa) was finally identified using limiting dilution from one of these wells.

PMab-213 recognized CHO/pPDPN but showed no reaction with CHO-K1 as assessed by flow cytometry (Fig. 2). PMab-213 reacted with the PK-15 pig kidney cell line, indicating that PMab-213 was able to recognize pPDPN. On the other hand, PMab-213 showed no reaction with human, mouse, rat, rabbit, dog, cat, or bovine PDPNs (Supplementary Fig. S1). Furthermore, it showed no reaction with horse, Tasmanian devil, tiger, alpaca, bear, goat, sheep, or whale

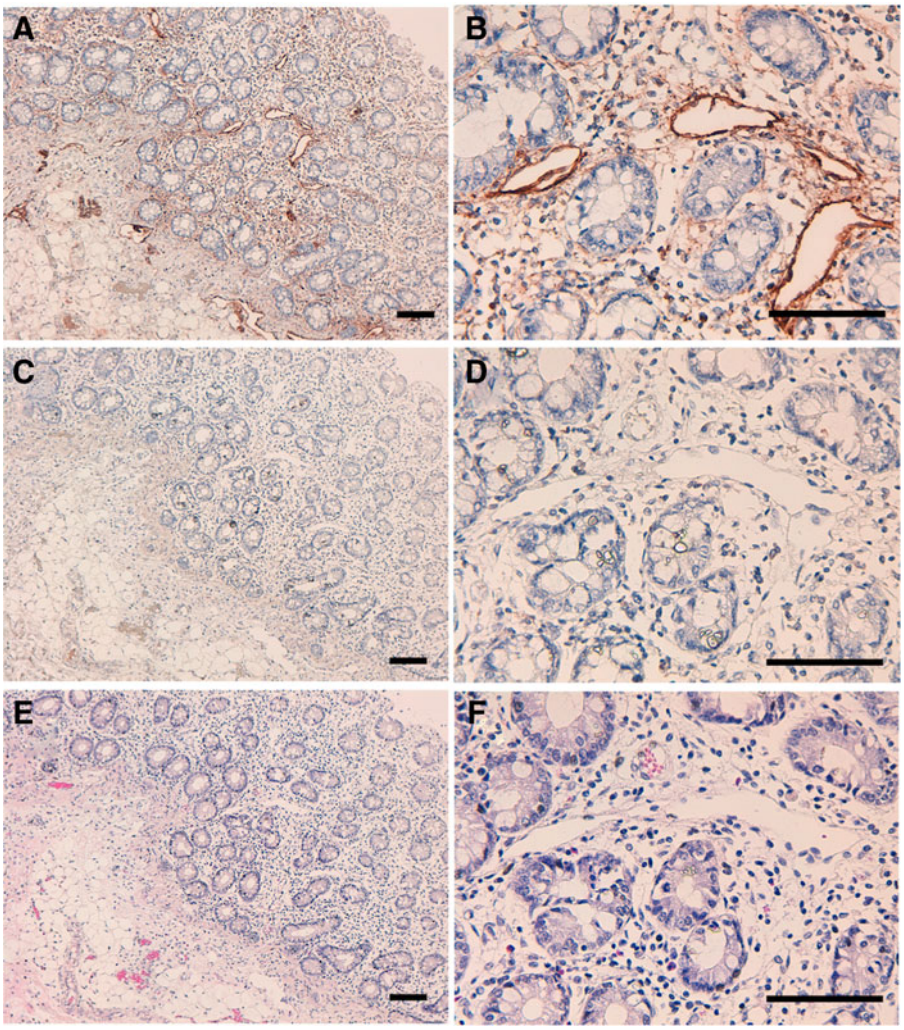

FIG. 6. Immunohistochemical analyses using microminipig colon. Histological sections of the microminipig colon tissue were incubated with 1 μg/mL of PMab-213 **(A, B)** or with blocking buffer **(C, D)**, followed by an Envision+ Kit. Hematoxylin and Eosin staining **(E, F)**. Scale bar = 100 μm.

PDPNs (Supplementary Fig. S2), indicating that PMab-213 is specific to pPDPN.

Western blot analysis performed using PMab-213 (Fig. 3) demonstrated that PMab-213 detects pPDPN as a 40-kDa band and a 25-kDa band in CHO/pPDPN cells. However, PMab-213 did not detect a 40-kDa band in PK-15 cells; this can be attributed to low expression levels of pPDPN in PK-15 cells or a low affinity of PMab-213 for PK-15 cells. A mAb against PA16 tag (NZ-1) also detected a 40-kDa band and a 25-kDa band in CHO/pPDPN cells. The 40-kDa band is a highly glycosylated form, whereas the 25-kDa band is an unglycosylated form.[6,18]

Additionally, a kinetic analysis conducted using flow cytometry assessed the interaction of PMab-213 with CHO/pPDPN and PK-15 cells. K_D of PMab-213 for CHO/pPDPN and PK-15 cells was determined to be 2.1×10^{-9} M and 1.4×10^{-8} M, respectively, indicating a high affinity for CHO/pPDPN and a moderate affinity for PK-15.

The immunohistochemical analyses revealed that PMab-213 strongly stained pulmonary type I alveolar cells (Fig. 4), renal podocytes and Bowman's capsules (Fig. 5), and lymphatic endothelial cells of the colon (Fig. 6) obtained from microminipigs; these results indicate that PMab-213 is useful for the detection of pPDPN using immunohistochemistry. The epitope of PMab-213 needs further investigation to clarify the specificity and sensitivity of PMab-213 against pPDPN.

In conclusion, we have established an mAb against pPDPN, PMab-213, which is applicable in flow cytometry, western blot, and immunohistochemical analyses. PMab-213 should prove useful for elucidating the pathophysiological functions of pPDPN in future studies.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

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