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Development of an Anti-Sheep Podoplanin Monoclonal Antibody PMab-256 for Immunohistochemical Analysis of Lymphatic Endothelial Cells

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Sensitive and specific monoclonal antibodies (mAbs) targeting podoplanin (PDPN) are needed for immunohistochemical analyses as a marker for lymphatic endothelial cells. We recently have developed anti-PDPN mAbs against many species, including human, mouse, rat, rabbit, dog, cat, bovine, pig, Tasmanian devil, alpaca, tiger, whale, goat, horse, and bear. However, anti-sheep PDPN (sPDPN) has not yet been established. In this study, we used the Cell-Based Immunization and Screening method for the development of anti-sPDPN mAbs. RAP14 tag was added to N-terminus of sPDPN, and anti-RAP14 tag mAb (PMab-2) was used to detect the expression level of sPDPN in flow cytometry and western blot. We immunized mice with sPDPN-overexpressing Chinese hamster ovary (CHO)-K1 (CHO/sPDPN) cells and screened mAbs against sPDPN using flow cytometry. One of the mAbs, PMab-256 (IgG_1, kappa), specifically detected CHO/sPDPN cells by flow cytometry and western blot. Furthermore, PMab-256 stained type I alveolar cells of lung, renal glomerulus and Bowman's capsule, and lymphatic endothelial cells of lung and colon. Our findings suggest the potential usefulness of PMab-256 for the functional analyses of sPDPN.

Keywords: podoplanin, sheep, sPDPN, monoclonal antibody

Introduction

Podoplanin (PDPN) is an *O*-glycosylated type I transmembrane protein expressed in renal podocytes, type I alveolar cells of the lung, and lymphatic endothelial cells of every organ.[1,2] It has platelet aggregation-stimulating (PLAG) domains[3] and a PLAG-like domain,[4–8] which bind to C-type lectin-like receptor-2 (CLEC-2).[9] The PDPN/CLEC-2 interaction induces platelet aggregation and separation of embryonic blood/lymphatic vessels.[10] In morphological studies, PDPN is used as an important specific marker of lymphatic vessels.[2]

We previously developed monoclonal antibodies (mAbs) against human,[11] mouse,[12] rat,[13] rabbit,[14] dog,[15] cat,[16] bovine,[17] pig,[18,19] Tasmanian devil,[20] alpaca,[21] tiger,[22] whale,[23] goat,[24,25] horse,[26,27] and bear[28,29] PDPNs using the Cell-Based Immunization and Screening (CBIS) method.[30–32] These mAbs all demonstrated usefulness for flow cytometry, western blot, and immunohistochemical analyses.

The purpose of the current study is to develop anti-sheep PDPN (sPDPN) mAbs using the CBIS method to stain lymphatic endothelial cells through immunohistochemistry.

Materials and Methods

Cell lines

P3X63Ag8U.1 (P3U1) and Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, United States). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding sPDPN (accession no.: XM_004013802.4) plus an N-terminal RAP14 tag, which is recognized by an anti-RAP14 tag mAb (PMab-2), was subcloned into a pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Plasmids were transfected using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected by limiting dilution and cultivation in medium containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc., Kyoto, Japan).

The P3U1, CHO-K1, CHO/sPDPN,[33] CHO/human PDPN (hPDPN),[3] CHO/mouse PDPN (mPDPN),[3] CHO/rat PDPN (rPDPN),[13] CHO/rabbit PDPN (rabPDPN),[14] CHO/dog PDPN (dPDPN),[15] CHO/bovine PDPN (bovPDPN),[17] CHO/cat PDPN (cPDPN),[16] CHO/pig PDPN (pPDPN),[19] CHO/horse PDPN (horPDPN),[26] CHO/tiger PDPN

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(tigPDPN),[22] CHO/alpaca PDPN (aPDPN),[21] CHO/bear PDPN (bPDPN),[19] CHO/Tasmanian devil PDPN (tasPDPN),[19] CHO/goat PDPN (gPDPN),[22] and CHO/whale PDPN (wPDPN)[23] were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc.), 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 in a humidified incubator at 37°C with atmosphere of 5% CO_2 and 95% air.

Hybridoma production

All animal experiments were performed according to relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2016MdA-153). Mice were monitored daily for health during the full 4-week duration of the experiment. Body weight loss exceeding 25% of total body weight was defined as a humane end point. Mice were euthanized by cervical dislocation, and death was verified by respiratory and cardiac arrest.

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. To develop mAbs against sPDPN, we used the CBIS method.[16,30–32] Briefly, three BALB/c mice were immunized with CHO/sPDPN cells (1×10^8) by the intraperitoneal (i.p.) route together with the Imject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunizations followed by a final booster injection administered i.p. 2 days before the harvest of spleen cells. Harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN), and the hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were screened by flow cytometry.

Flow cytometry

Cells were harvested following a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were washed with 0.1% bovine serum albumin in 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 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 the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of binding affinity by flow cytometry

CHO/sPDPN was suspended in 100 μL of serially diluted PMab-256, and Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.). The dissociation constant (K_D) was calculated by fitting the binding isotherms to built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). Proteins were then electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), membranes were incubated with 1 μg/mL of PMab-256, anti-RAP14 tag (clone: PMab-2),[13] or anti-β-actin (clone AC-15; Sigma-Aldrich Corp.,

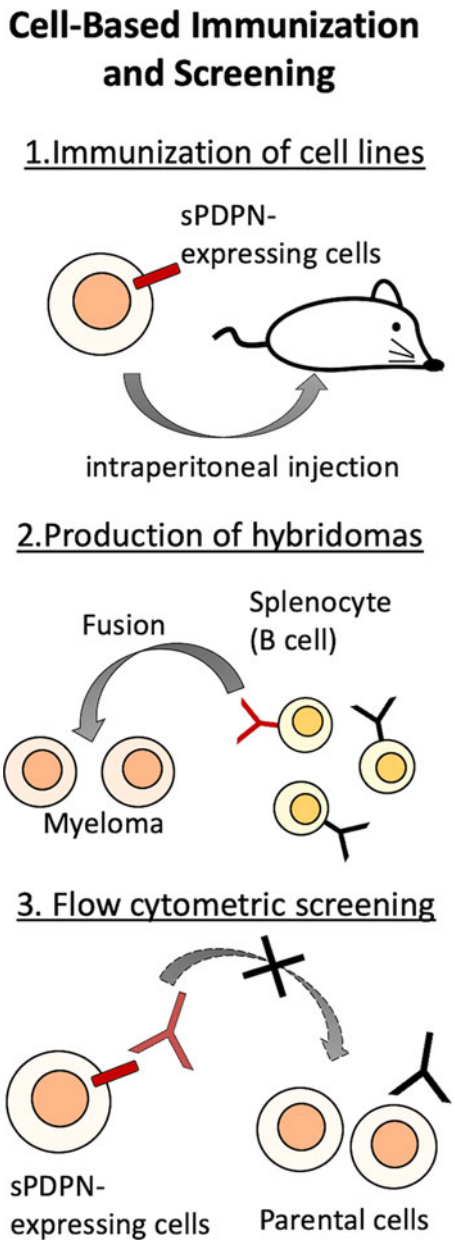

FIG. 1. Schematic illustration of the CBIS method. Stable transfectants expressing sPDPN were used as an immunogen without purification. Selection of hybridomas secreting anti-sPDPN mAbs was performed by flow cytometry using parental and transfectant cells. CBIS, Cell-Based Immunization and Screening; mAbs, monoclonal antibodies; sPDPN, sheep podoplanin.

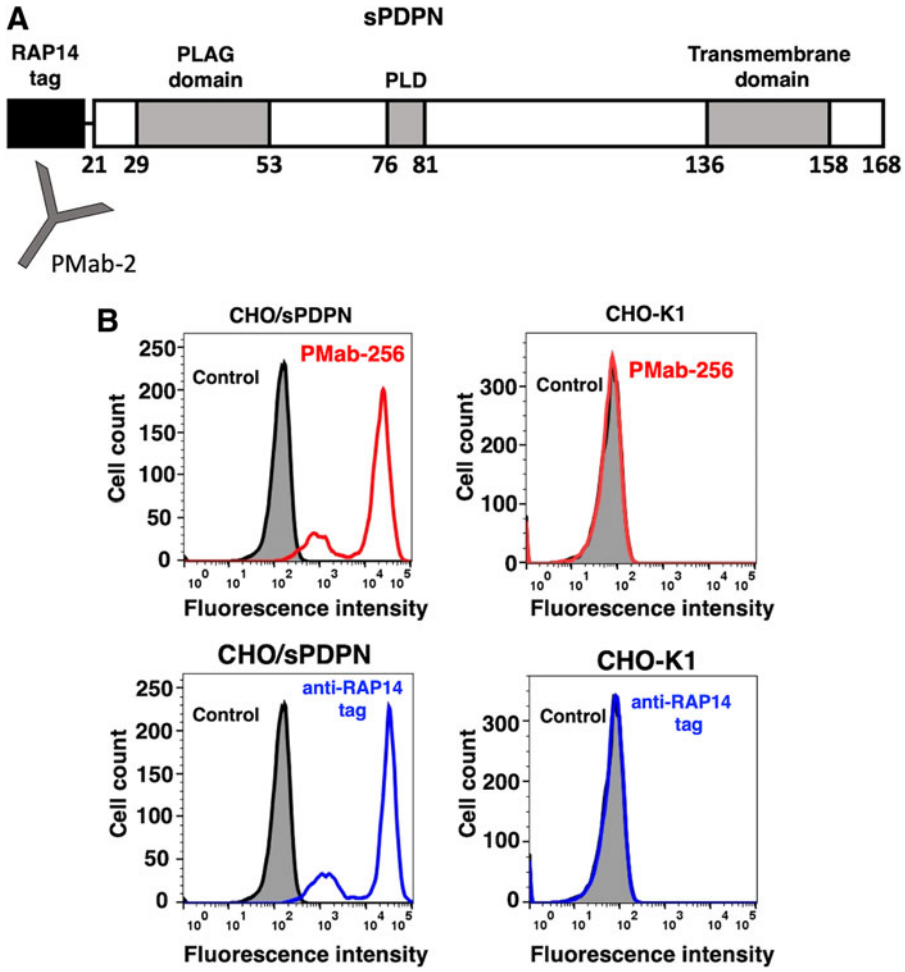

FIG. 2. Detection of sPDPN by flow cytometry using PMab-256. **(A)** Schematic illustration of sPDPN. sPDPN possesses a PLAG domain, PLD, and transmembrane domain. RAP14 was linked to the N-terminal of sPDPN. **(B)** CHO-K1 and CHO/sPDPN cells were treated with PMab-256 (red line) or anti-RAP14 tag (PMab-2; blue line) at a concentration of 1 μg/mL or 0.1% BSA in PBS (gray) for 30 minutes, followed by incubation with secondary antibodies. BSA, bovine serum albumin; CHO, Chinese hamster ovary; PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain; PBS, phosphate-buffered saline.

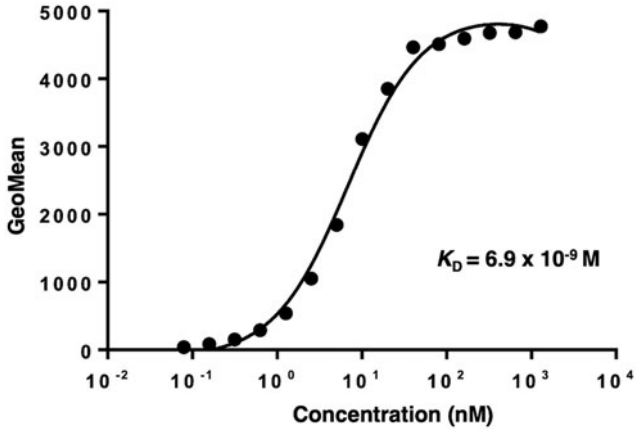

FIG. 3. Determination of binding affinity of PMab-256. CHO/sPDPN was suspended in 100 μL of serially diluted PMab-256. Alexa Fluor 488-conjugated anti-mouse IgG was then added. Fluorescence data were collected using the EC800 Cell Analyzer.

St. Louis, MO), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulin (diluted 1:2000; Agilent Technologies, Inc., Santa Clara, CA). Membranes were finally developed using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analyses

Normal sheep tissues were collected from two sheep after autopsy at Hokkaido University, fixed in 10% neutral-buffered formalin, and processed routinely to make formalin-fixed paraffin-embedded (FFPE) tissue sections as in our previous study.[33] Histological sections of 4 μm thickness were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), sections were incubated with PMab-256 (5 μg/mL) for 1 hour at room temperature and treated using Envision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3-diaminobenzidine

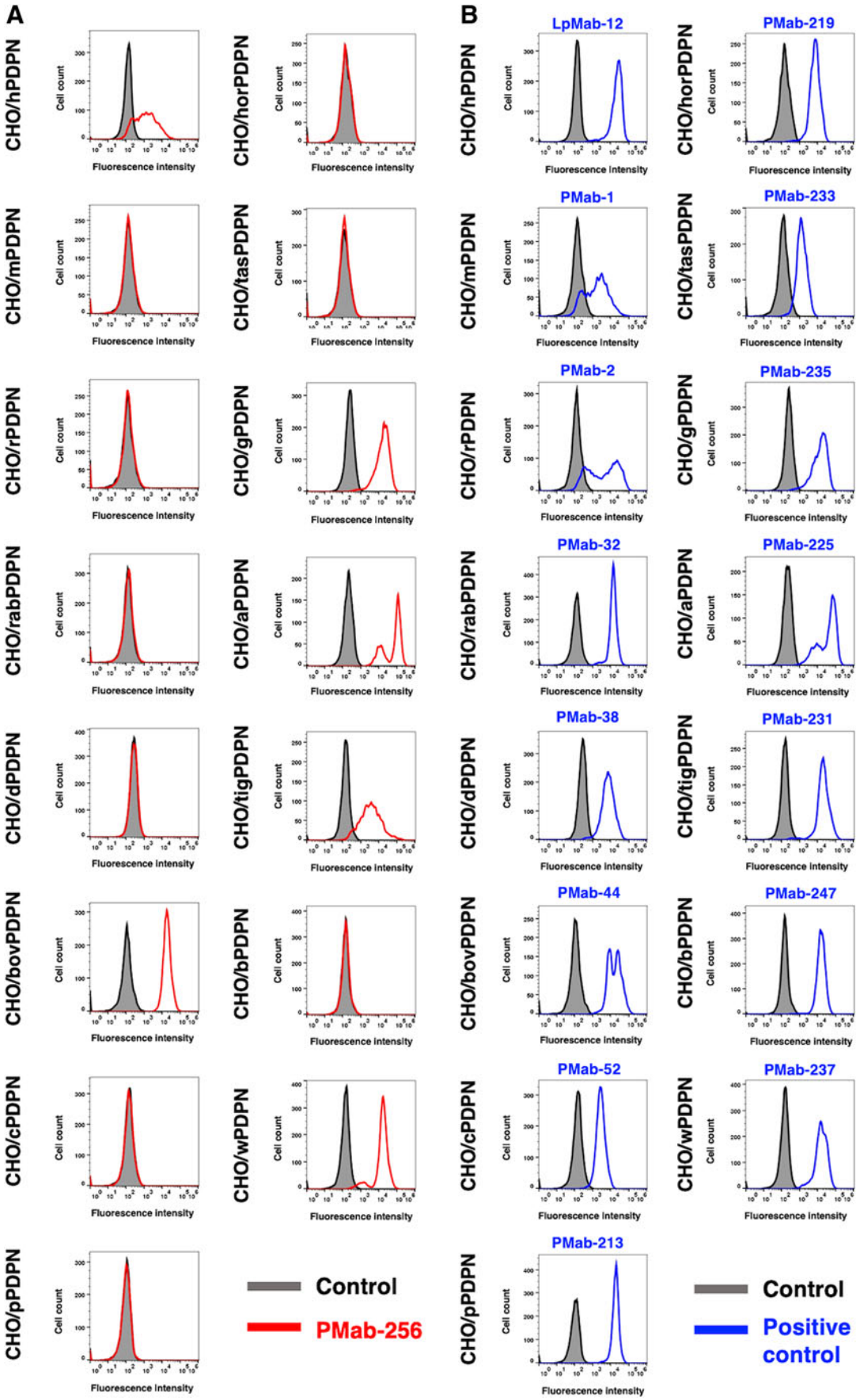

FIG. 4. Cross-reaction of PMab-256 with PDPNs of other species by flow cytometry. CHO-K1 cells transfected with PDPNs of other species were treated with PMab-256 (red line) **(A)** or each positive control (blue line) **(B)** at a concentration of 5 μg/mL or 0.1% BSA in PBS (gray) for 30 minutes, followed by incubation with secondary antibodies. PDPN, podoplanin.

tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed with hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results

Establishment of anti-sPDPN mAbs

To develop anti-sPDPN mAbs, we used the CBIS method, in which stable transfectants are used for immunization, and screening was performed using flow cytometry (Fig. 1). We immunized three mice with CHO/sPDPN cells, which overexpress sPDPN (Fig. 2A). Resulting hybridomas were seeded into 96-well plates, and CHO/sPDPN-positive and CHO-K1-negative wells were selected. This screening approach identified strong signals from CHO/sPDPN and weak or no signals from CHO-K1 in 22 of the 1440 wells (1.5%). PMab-256 (IgG_1, kappa) was finally selected using western blotting and immunohistochemistry.

Flow cytometric analyses

We performed flow cytometry using PMab-256 against CHO/sPDPN and CHO-K1. PMab-256 recognized CHO/sPDPN but not CHO-K1 (Fig. 2B). As expected, an anti-RAP14 tag mAb (PMab-2) also detected CHO/sPDPN. These results indicate that PMab-256 is useful for detecting sPDPN in flow cytometry analysis.

We next measured the apparent binding affinity of PMab-256 with CHO/sPDPN using flow cytometry. The K_D of PMab-256 for CHO/sPDPN was 6.9×10^{-9} M (Fig. 3), indicating that PMab-256 possesses high affinity for CHO/sPDPN cells.

The cross-reaction of PMab-256 with the other PDPNs, which are overexpressed in CHO-K1 cells, was then assessed. PMab-256 cross-reacted with hPDPN, bovPDPN, gPDPN, aPDPN, tigPDPN, and wPDPN (Fig. 4A). Expression levels of these PDPNs were confirmed by each specific anti-PDPN mAb (Fig. 4B).

Western blot analyses

We next investigated whether PMab-256 can be applied to western blot analysis using cell lysates of CHO-K1 or CHO/sPDPN. As shown in Figure 5, PMab-256 detected sPDPN as a 55-kDa band (N-terminal RAP14 tag + sPDPN) in CHO/sPDPN cell lysates, but this antibody did not detect any bands from CHO-K1 cells. Anti-RAP14 tag (PMab-2) as a positive control also detected a band of the same molecular weight from CHO/sPDPN. These results indicate that PMab-256 is very useful for detecting sPDPN in western blot analysis.

Immunohistochemical analyses

Finally, we investigated whether PMab-256 can be applied to immunohistochemical analysis using FFPE sheep sections. For this assessment, we selected normal tissues of sheep lung,

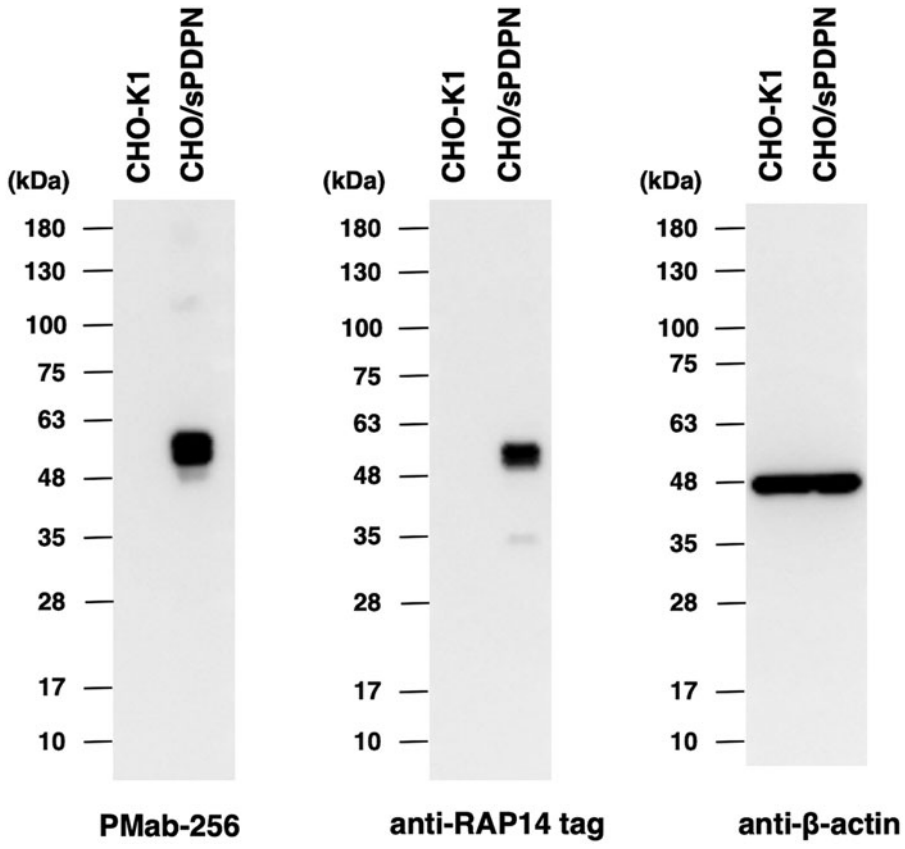

FIG. 5. Western blot analysis. Cell lysates of CHO-K1 and CHO/sPDPN (10 μg) were electrophoresed and transferred onto PVDF membranes. The membranes were incubated with 1 μg/mL of PMab-256, anti-RAP14 tag (PMab-2), and anti-β-actin and subsequently with peroxidase-conjugated anti-mouse immunoglobulin. PVDF, polyvinylidene difluoride.

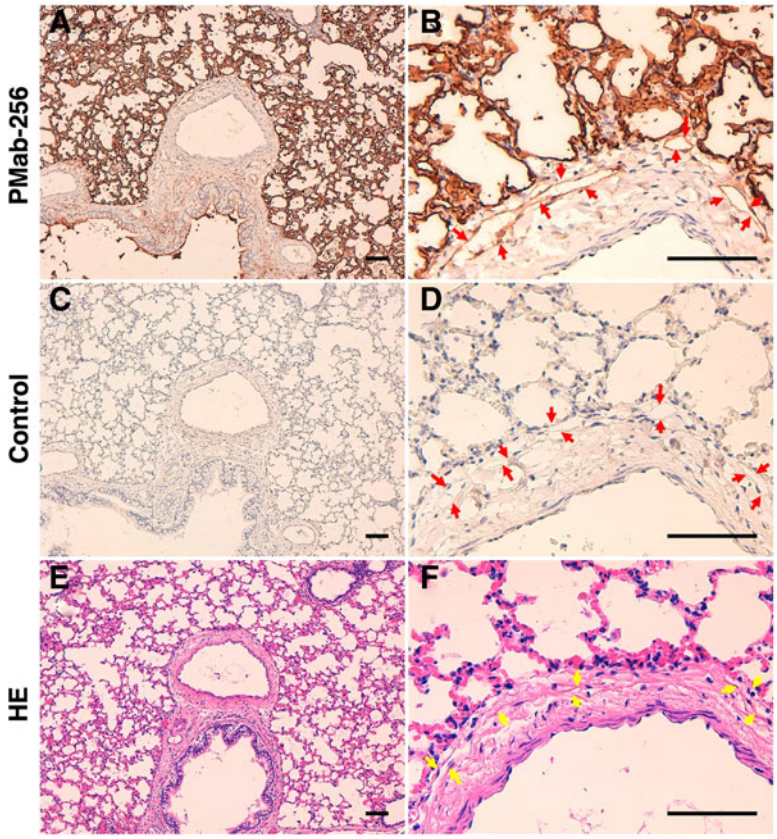

FIG. 6. Immunohistochemical analyses of the sheep lung. FFPE tissue sections of sheep lung were incubated with 5 μg/mL of PMab-256 **(A, B)** or blocking buffer **(C, D)**, followed by the Envision + Kit. **(E, F)** HE staining. Red and yellow arrows indicate lymphatic endothelial cells. Scale bar = 100 μm. FFPE, Formalin-fixed paraffin-embedded; HE, hematoxylin and eosin.

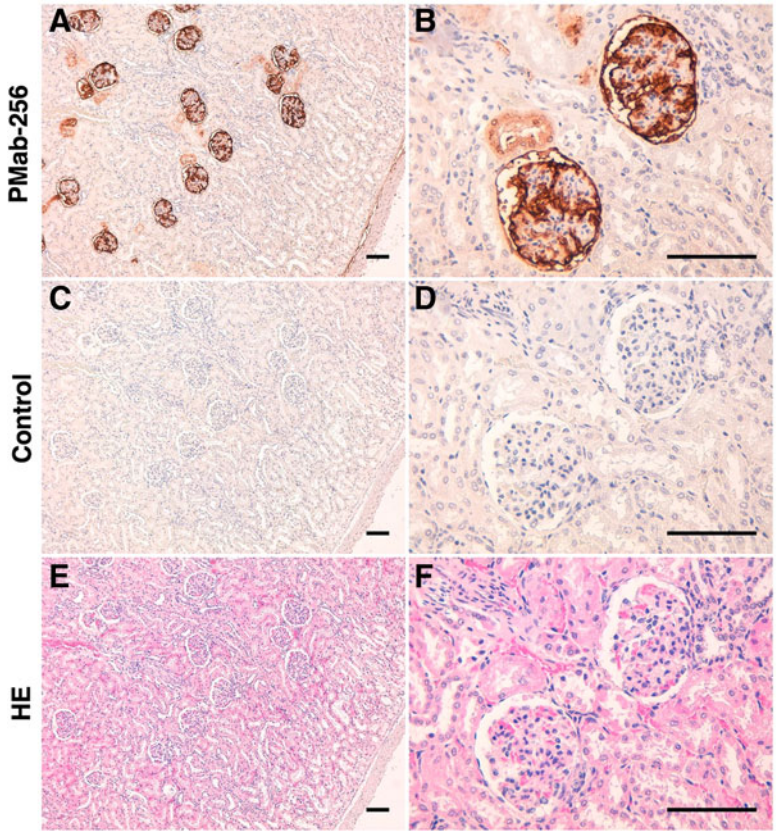

FIG. 7. Immunohistochemical analyses of the sheep kidney. FFPE tissue sections of sheep kidneys were incubated with 5 μg/mL of PMab-256 **(A, B)** or blocking buffer **(C, D)**, followed by that with the Envision + Kit. **(E, F)** HE staining. Scale bar = 100 μm.

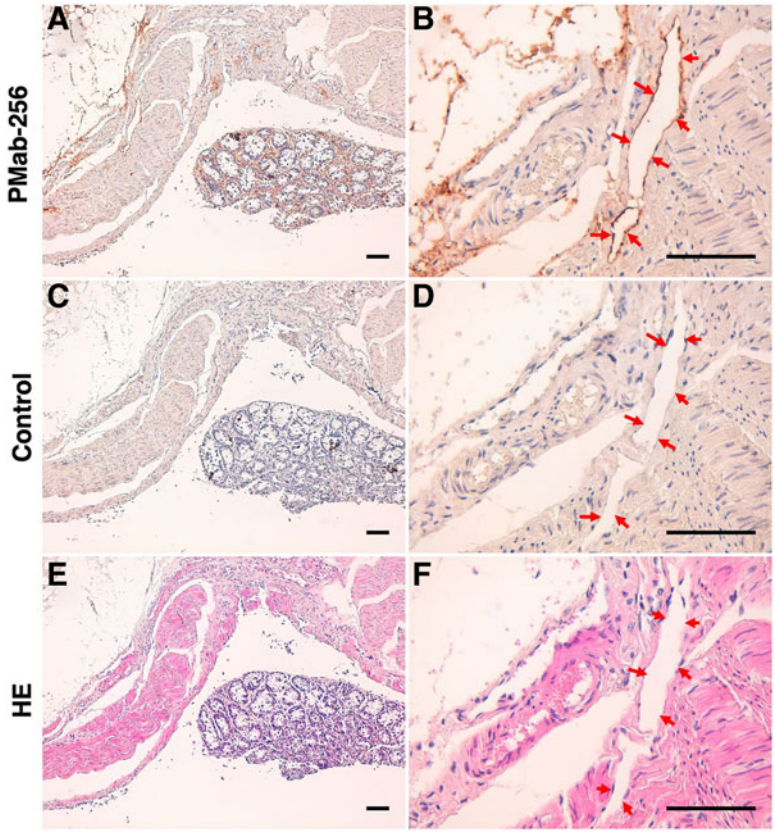

FIG. 8. Immunohistochemical analyses of the sheep colon. FFPE tissue sections of sheep colon were incubated with 5 μg/mL of PMab-256 **(A, B)** or blocking buffer **(C, D)**, followed by the Envision + Kit. **(E, F)** HE staining. Red arrows indicate lymphatic endothelial cells. Scale bar = 100 μm.

kidney, and colon, all of which have been reported to express PDPN in other species, including human,[11] mouse,[12] rat,[13] rabbit,[14] dog,[15] cat,[16] bovine,[17] pig,[18,19] Tasmanian devil,[20] alpaca,[21] tiger,[22] whale,[23] goat,[24,25] horse,[26,27] and bear.[28,29] As depicted in Figure 6, PMab-256 strongly stained type I alveolar cells within the lung and also clearly stained lymphatic endothelial cells of the lung (red arrow). PMab-256 stained renal glomerulus and Bowman's capsule (Fig. 7), although this staining pattern was not previously observed by PMab-44 (an anti-bovPDPN mAb), which cross-reacts with sPDPN.[33] The lymphatic endothelial cells (red arrow) of the colon were also clearly detected by PMab-256 (Fig. 8), indicating that PMab-256 is very useful for detecting lymphatic endothelial cells in FFPE tissues of sheep organs.

Discussion

Sheep possess anatomical and physiological structural resemblance to humans[34] and have been used as experimental animals for many studies, including the immune system,[35,36] development of the blood–brain barrier,[37] and lymphopoiesis/lymphocyte recirculation.[38] By contrast, detailed morphological studies of sheep lymphatic vessels have not been performed due to the lack of specific markers for lymphatic endothelial cells.

In our previous study, we investigated possible cross-reactions between anti-PDPN mAbs and sPDPN.[33] Flow cytometry analysis demonstrated that PMab-44 (an anti-bovPDPN mAb)[17] reacted with CHO/sPDPN. Other anti-PDPN mAbs such as anti-mouse (PMab-1),[12] anti-rat (PMab-2),[13] anti-rabbit (PMab-32),[14] anti-dog (PMab-38 and PMab-48), and anti-cat (PMab-52)[16] did not react with CHO/sPDPN, thus indicating then that only PMab-44 was useful for the detection of sPDPN. We therefore investigated the expression of sPDPN using PMab-44 in sheep lungs using immunohistochemical analyses. Sheep lungs were stained by PMab-44 when EnVision FLEX Target Retrieval Solution and a high pH were used for an antigen retrieval procedure, although they were stained weakly using PMab-44 when citrate buffer (pH 6) was used for antigen retrieval.[33] By contrast, the renal glomerulus and Bowman's capsule were not stained by PMab-44. Furthermore, lymphatic endothelial cells of the lung and colon were not stained by PMab-44. Therefore, the development of novel anti-sPDPN mAbs, which are applicable for detecting lymphatic endothelial cells in immunohistochemical analysis, is crucial.

In this study, we used the CBIS method to develop novel anti-sPDPN mAbs (Fig. 1) because we have successfully produced many mAbs against membrane proteins using the CBIS method.[18–32] We immunized mice with CHO/sPDPN cells and performed the first screening using only flow cytometry. Using this method abolishes the need for purified protein for immunization and screening, which means that we can develop mAbs more quickly compared with methods using purified proteins and ELISA. The established clone PMab-256 was characterized in this study, but two other clones, PMab-253 and PMab-260, were established at the same time. Both PMab-253 and PMab-260 were identified as

IgM class antibodies; and the sensitivities of PMab-253 and PMab-260 were less compared with PMab-256. For these reasons, we selected PMab-256 (IgG_1) for further analyses.

Although our previous study showed that PMab-44 stained only type I alveolar cells and did not stain the lymphatic endothelial cells of sheep organs, PMab-256 strongly stained lymphatic endothelial cells of the lung (Fig. 6) or colon (Fig. 8). Our recent study showed that PMab-241, one of the anti-bPDPN mAbs, reacted with lymphatic endothelial cells, but not with type I alveolar cells of the bear lung.[29] By contrast, PMab-247, the other anti-bPDPN mAb, reacted with not only lymphatic endothelial cells but also type I alveolar cells and renal podocytes,[28] suggesting that the binding epitope of each mAb is critical to distinguish PDPN-expressing lymphatic endothelial cells from other PDPN-expressing tissues. The binding epitope of PMab-256 should be determined in future studies to clarify mechanisms for which sheep lymphatic endothelial cells were strongly stained. PMab-256 may be advantageous for detailed morphological studies of sheep lymphatic vessels.

Author Disclosure Statement

No competing financial interests exist.

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