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Ferret Podoplanin Is Detected by PMab-241 in Immunohistochemistry

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Podoplanin (PDPN) plays an important role in the development of many normal tissues and is expressed in various cancers. We have previously developed multiple monoclonal antibodies (mAbs) against PDPNs from a variety of animal species and characterized each of these PDPNs using the anti-PDPN mAbs. In this study, we evaluated whether these anti-PDPN mAbs possess cross-reactivity with ferret PDPN (ferPDPN) using flow cytometry. Comprehensive analysis using 17 differing anti-PDPN mAbs available for immunohistochemistry use, demonstrated that the anti-bear PDPN mAb (clone PMab-241) strongly cross-reacts with ferPDPN-overexpressed Chinese hamster ovary-K1 (CHO/ferPDPN) cells. Immunohistochemistry analysis demonstrated intense PMab-241 staining within Bowman's capsules and glomeruli of the ferret kidney, and lymphatic endothelial cells of the ferret lung. These results demonstrate that PMab-241 is suitable for the detection of PDPN in ferret tissues.

Keywords: ferret podoplanin, PDPN, immunohistochemistry

Introduction

Podoplanin (PDPN)/T1α/gp36/Aggrus is a type I transmembrane sialoglycoprotein[1] that plays essential roles in the development of the heart,[2] alveoli,[3] and lymphatic system.[4] Alongside its roles in normal tissue development, it is also expressed in some cancer cells, including brain tumors,[5] bladder cancers,[6] and squamous cell lung carcinomas.[7] PDPN has been shown to induce platelet aggregation by binding to the C-type lectin-like receptor-2 (CLEC-2)[8,9] and this dynamic between PDPN/CLEC-2 axis in platelets, is what regulates inflammation.[10] In addition, PDPN has been reported to be associated with various proteins, including galectin 8,[11] CD44,[12] heat-shock protein A9,[13] and CC chemokine ligand 21.[14] The expression of PDPN is induced by tumor promoters such as Src and TPA.[15,16] Miyashita et al. reported that PDPN activates Rho-associated coiled-coil kinase (ROCK) activity, and this specific activation by PDPN suppresses cell apoptosis.[17] This was demonstrated with a reduction in PDPN-positive cells upon treatment with a ROCK inhibitor. Sikorska et al. demonstrated the link between PDPN and the epithelial-mesenchymal transition through regulation of ezrin, radixin, and moesin expression, or matrix metalloproteinases-2 and -9.[18] This report also indicated the inhibition of the MAPK pathway resulted in a reduced level of PDPN expression in thyroid cancer cell lines. These reports demonstrate that PDPN is involved in the control of tumor cell migration, invasion, and metastasis.

PDPN is expressed in not only podocytes of kidneys, but also within type I alveolar cells of the lung and lymphatic endothelial cells of various tissues. Therefore, anti-PDPN monoclonal antibodies (mAbs) are valuable in distinguishing between lymphatic endothelial cells from vascular

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endothelial cells, or between type I alveolar cells to type II alveolar cells of the lung.[1,19] We have previously developed various anti-PDPN mAbs for human,[20–22] mouse,[20] rat,[23] rabbit,[24] dog,[25,26] bovine,[27] cat,[28] tiger,[29] horse,[30,31] pig,[32] goat,[33] alpaca,[34] Tasmanian devil,[35] bear,[36,37] whale,[38] and sheep.[39] However, anti-ferret PDPN (ferPDPN) mAbs have not yet been reported.

In this study, we investigated whether those previously reported anti-PDPN mAbs could have cross-reactivity with ferPDPN using flow cytometry and immunohistochemical analyses.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding ferPDPN plus an N-terminal MAP tag (GDGMVPPGIEDK),[40] which is recognized by an anti-MAP tag mAb (PMab-1), were subcloned into a pCAG-Ble vector (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Plasmids were transfected into CHO-K1 using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants (CHO/MAP-ferPDPN) were selected using cell sorter (SH800; Sony Corp., Tokyo, Japan).

CHO-K1 and CHO/MAP-ferPDPN were cultured in a Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). CHO/MAP-ferPDPN were cultivated in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA). The cells were cultured in an incubator at 37°C with humidity and 5% CO_2 and 95% air atmosphere.

Ferret tissues

Ferret (*Mustela putorius furo*) tissues were collected after autopsy at Yamaguchi University, and fixed in 10% neutral-buffered formalin. Then, paraffin-embedded tissue sections (4-μm thick) were made.

Flow cytometry

CHO/MAP-ferPDPN cells were harvested after short-time exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.), and were subsequently washed with 0.1% bovine serum albumin in phosphate-buffered saline (PBS). Cells were treated with primary mAbs (10 μg/mL) for 30 minutes at 4°C. The primary mAbs used were as follows: PMab-2 for rat PDPN,[23] PMab-32 for rabbit PDPN,[24] PMab-38 for dog PDPN,[25] PMab-44 for bovine PDPN,[27] PMab-48 for dog PDPN,[26] PMab-52 for cat PDPN,[28] PMab-210 for pig PDPN,[41] PMab-213 for pig PDPN,[32] PMab-219 for horse PDPN,[31] PMab-225 for alpaca PDPN,[34] PMab-231 for tiger PDPN,[29] PMab-233 for Tasmanian devil PDPN,[35] PMab-235 for goat PDPN,[33] PMab-237 for whale PDPN,[38] PMab-241 for bear PDPN,[37] PMab-247 for bear PDPN,[36] and PMab-256 for sheep PDPN.[39] After incubation with primary antibodies, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA). An anti-MAP tag mAb (clone PMab-1; 10 μg/mL) was also used for detecting N-terminal MAP tag,[40] followed by Alexa Fluor 488-conjugated anti-rat (H+L) IgG (1:2000; Cell Signaling Technology, Inc.). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp.).

Immunohistochemical analyses

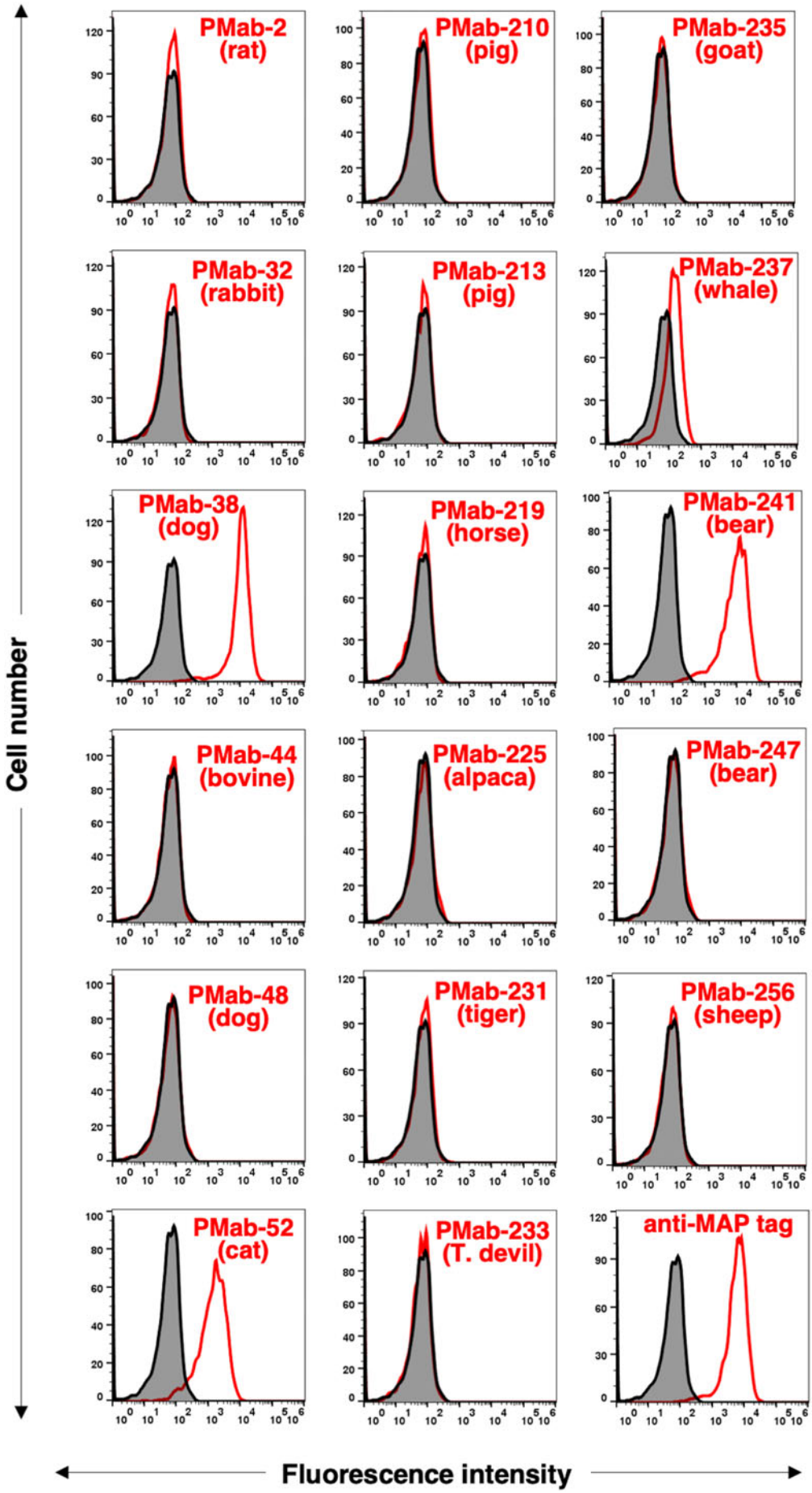
Histological sections (4-μm thick) of ferret tissue were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. After blocking histological sections with the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), sections were incubated with PMab-241 (10 μg/mL) for 1 hour at room temperature and treated with the EnVision + Kit for mouse (Agilent Technologies, Inc., Santa Clara, CA), for 30 minutes. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (Fujifilm Wako Pure Chemical Corporation). Hematoxylin and eosin (HE; Fujifilm Wako Pure Chemical Corporation) staining was also performed using the serial section of the ferret tissues.

Results

Flow cytometry analyses using anti-PDPN mAbs for ferPDPN

To begin, a stable transfectant cell line of ferPDPN with N-terminal MAP tag (CHO/MAP-ferPDPN) was produced. Cross-reactivity between CHO/MAP-ferPDPN and anti-PDPN mAbs, which are useful for immunohistochemistry, was investigated using flow cytometry. The results demonstrated that PMab-38 for dog PDPN, PMab-52 for cat PDPN, PMab-237 for whale PDPN, and PMab-241 for bear PDPN, all cross-reacted with CHO/MAP-ferPDPN cells (Fig. 1). In contrast, PMab-2 for rat PDPN, PMab-32 for rabbit PDPN, PMab-44 for bovine PDPN, PMab-48 for dog PDPN, PMab-210 for pig PDPN, PMab-213 for pig PDPN, PMab-219 for horse PDPN, PMab-225 for alpaca PDPN, PMab-231 for tiger PDPN, PMab-233 for Tasmanian devil PDPN, PMab-235 for goat PDPN, PMab-247 for bear PDPN, and PMab-256 for sheep PDPN, did not react with CHO/MAP-ferPDPN cells (Fig. 1). The positive control, anti-MAP tag mAb, reacted with CHO/MAP-ferPDPN cells as expected.

FIG. 1. Flow cytometry analyses using various anti-PDPN mAbs. Anti-PDPN mAbs (red line) or an anti-MAP tag (red line), or negative control (0.1% BSA in PBS; gray shade) were treated with CHO/MAP-ferPDPN cells at a concentration of 10 μg/mL for 30 minutes at 4°C, followed by secondary antibodies. BSA, bovine serum albumin; CHO, Chinese hamster ovary; ferPDPN, ferret PDPN; PBS, phosphate-buffered saline; PDPN, podoplanin; mAbs, monoclonal antibodies.



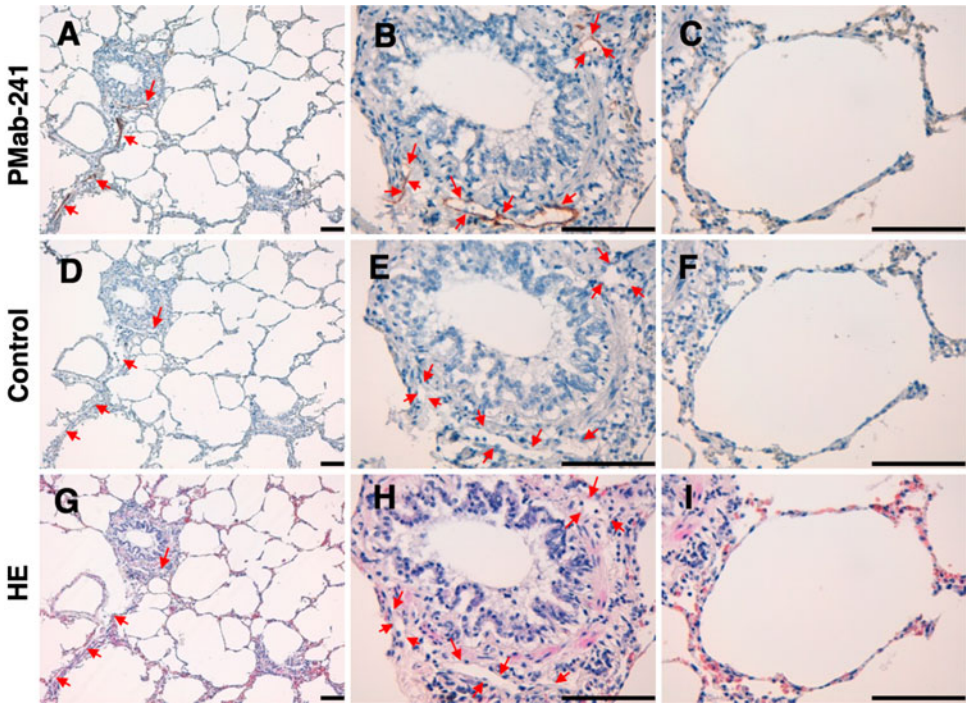


FIG. 2. Immunohistochemical analyses of ferret lung tissues using PMab-241. Histological sections of ferret lung tissues were incubated with 10 μg/mL of PMab-241 **(A–C)** or blocking buffer **(D–F)**. **(G–I)** Hematoxylin and eosin staining using the serial section of the ferret lung tissues. Scale bar = 100 μm. Red arrows: lymphatic endothelial cells.

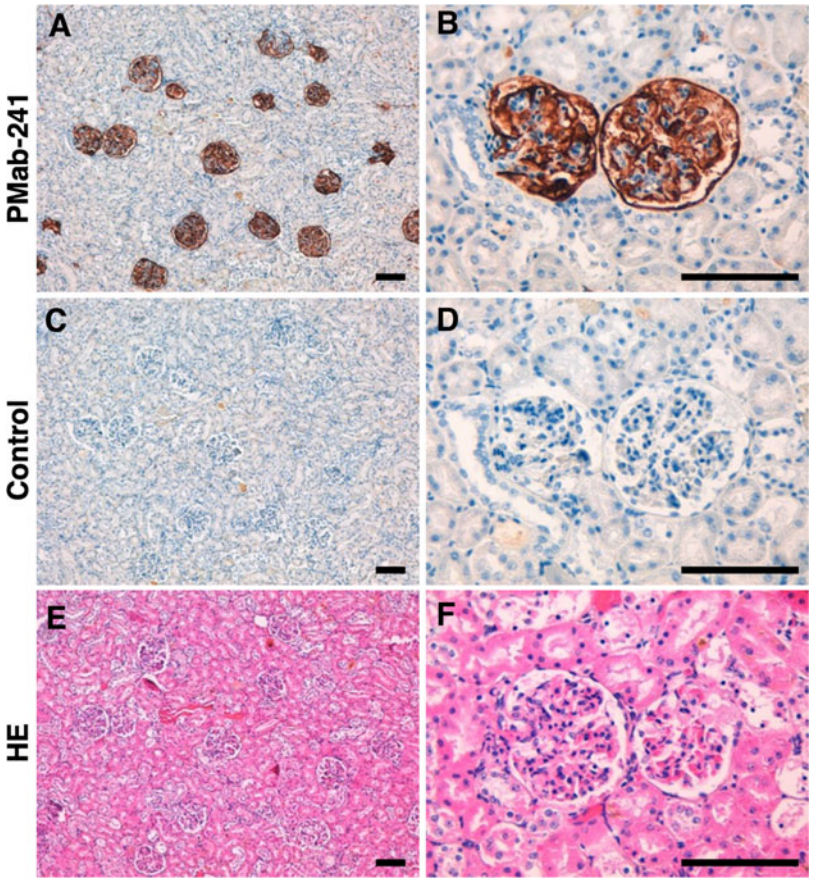


FIG. 3. Immunohistochemical analyses of ferret kidney tissues using PMab-241. Histological sections of ferret kidney tissues were incubated with 10 μg/mL of PMab-241 **(A, B)** or blocking buffer **(C, D)**. **(E, F)** Hematoxylin and eosin staining using the serial section of the ferret kidney tissues. Scale bar = 100 μm.

Among the four anti-PDPN mAbs that cross-reacted with CHO/MAP-ferPDPN cells, PMab-241 has demonstrated having the best sensitivity in immunohistochemical analyses in our previous study.[37] Therefore, PMab-241 was selected for all subsequent immunohistochemical analyses of ferret tissues.

Immunohistochemical analyses of the ferret lung using PMab-241

The expression of ferPDPN in ferret lung using PMab-241 by immunohistochemistry was next evaluated. Our previous immunohistochemical studies reported that PMab-241 reacts with lymphatic endothelial cells of the bear lung, but not with type I alveolar cells.[37] In this study, PMab-241 reacted with lymphatic endothelial cells in the ferret lung, but not with type I alveolar cells of the ferret lung (Fig. 2A–C). This result is similar to our previous findings that PMab-241 was also detectable in bear lung.[37] No staining was observed in any of the serial sections of the ferret lung when stained with the buffer negative control (Fig. 2D–F). HE staining using the serial sections of the ferret lung is shown in Figure 2G–I.

Immunohistochemical analyses of the ferret kidney using PMab-241

We next investigated whether PMab-241 could react with the ferret kidney in immunohistochemical analyses. Results demonstrate that PMab-241 stained Bowman's capsules and glomeruli of the ferret kidney (Fig. 3A, B). The serial section of the ferret kidney was not stained by the negative buffer control (Fig. 3C, D). HE staining using the serial sections was shown in Figure 3E and F.

Discussion

Ferrets have frequently been used as model species for many human diseases.[42–45] Due to having similar symptoms to humans when infected with the human influenza virus, such as runny nose and fever, they are used as animal models for human influenza preclinical evaluation and influenza vaccine efficacy studies.[42,43] Ferrets have been also used in gastritis and gastric adenocarcinoma models, both of which are related to *Helicobacter mustelae* infection.[44,45] Recently, ferrets have been utilized as model animals for the SARS-CoV-2 outbreak.[46,47] Although the wide use of ferrets for these disease models, plus others, specific anti-ferret mAbs have not yet been developed.

In this study, we have demonstrated that an anti-bear PDPN mAb, PMab-241, can detect ferPDPN by flow cytometry and immunohistochemistry. PMab-241 is the first mAb that can stain lymphatic endothelial cells of the ferret lung and glomeruli of the ferret kidney. However, type I alveolar cells in the ferret lung were not stained by PMab-241 in this study, indicating that PMab-241 reactivity in ferret exhibits tissue specificity. These results demonstrate the further need to investigate whether PMab-241 could be utilized for pathophysiological studies using ferrets in the future.

Author Disclosure Statement

No competing financial interests exist.

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