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ORIGINAL

Development of monoclonal mouse antibodies that specifically recognize pancreatic polypeptide

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Abstract. Pancreatic polypeptide (PP) is a 36-amino acid peptide encoded by the *Ppy* gene, which is produced by a small population of cells located in the periphery of the islets of Langerhans. Owing to the high amino acid sequence similarity among neuropeptide Y family members, antibodies against PP that are currently available are not convincingly specific to PP. Here we report the development of mouse monoclonal antibodies that specifically bind to PP. We generated *Ppy* knockout (*Ppy*-KO) mice in which the *Ppy*-coding region was replaced by Cre recombinase. The *Ppy*-KO mice were immunized with mouse PP peptide, and stable hybridoma cell lines producing anti-PP antibodies were isolated. Firstly, positive clones were selected in an enzyme-linked immunosorbent assay for reactivity with PP coupled to bovine serum albumin. During the screening, hybridoma clones producing antibodies that cross-react to the peptide YY (PYY) were excluded. In the second screening, hybridoma clones in which their culture media produce no signal in *Ppy*-KO islets but detect specific cells in the peripheral region of wild-type islets, were selected. Further studies demonstrated that the selected monoclonal antibody (23-2D3) specifically recognizes PP-producing cells, not only in mouse, but also in human and rat islets. The monoclonal antibodies with high binding specificity for PP developed in this study will be fundamental for future studies towards elucidating the expression profiles and the physiological roles of PP.

Key words: Islet, Pancreatic polypeptide (PP), Peptide YY, Neuropeptide Y, Monoclonal antibody

PANCREATIC POLYPEPTIDE (PP) is a 36-amino acid peptide produced and secreted by PP cells (also called F cells or γ cells) of the islets of Langerhans. In mice, PP is encoded by the *Ppy* gene, which is located on chromosome 11. Immunogenicity of PP is localized to a specific cell type in the periphery of islets in a variety of species [1-4]. Compared with other islet endocrine cell types, such as α and β cells, the roles of PP cells in glucose homeostasis remain largely unknown to date. PP is a member of the NPY family of peptides, which also includes peptide YY (PYY) and neuropeptide Y (NPY), all of which are involved in appetite regulation [5, 6]. The idea that PP may be a satiety factor arose from the observation that PP secretion is markedly decreased in children with Prader-Willi syndrome [7, 8]. Importantly,

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Abbreviations: PP, pancreatic polypeptide; KO, knockout; PYY, peptide YY; NPY, neuropeptide Y; NLS, nuclear localization signal; ZFN, zinc finger nucleases; KI, knock-in; ELISA, enzyme-linked immunosorbent assay; ISH, *in situ* hybridization; HAT, hypoxanthine-aminopterin-thymidine.

intravenous infusion of bovine PP reduced food intake both in healthy individuals [9] and patients with Prader-Willi syndrome [10, 11]. The forced expression of PP in transgenic mice reduced food intake and body weight partially due to the reduced rate of gastric emptying [12]. Wortley *et al.* recently generated and characterized mutant mice deficient in two related genes, *Ppy* and *PYY*. Whereas mice deficient in *PYY* demonstrated body weight gain, *Ppy*-null mice had a normal body weight, making it difficult to conclude the physiological role of PP in body weight regulation *in vivo* [13].

Besides body weight regulation, the NPY family of peptides have attracted attention in the field of developmental biology of the endocrine pancreas. A previous study reported that PYY was an early embryonic marker of endocrine cells and in the adult it is found in δ, α, and PP cells, but not in β cells [14]. Another report proposed that PP marks embryonic precursors common to PP and β cells, as a toxin driven by the PP promoter eliminated not only PP but also β cells in adult islets [15]. However, these findings should be interpreted with caution, as the close sequence similarity in amino acids makes it difficult to precisely distinguish among PP, NPY, and PYY. Indeed, overlapping reactivity of antibodies for PYY, PP, and NPY has been reported [16-18]. Thus, an antibody with specificity exclusively for PP is essential for the characterization of PP-producing cells. Here we report the generation of monoclonal mouse antibody with exclusive binding specificity for PP, which effectively works in immunohistochemistry of tissue from mouse, rat, and human.

Materials and Methods

Animal experiments

The protocol for animal experiments in this study was approved by the Ethics Review Committees of Animal Experimentation of Juntendo University, Gunma University, Saitama Medical University, and Hirosaki University.

Human specimens

For human pancreas staining, human tissues were obtained from Tokushima University and used with written informed consent and institutional review board approval. These tissue samples were those obtained from diabetic patients and nondiabetic controls during surgery for the pathological diagnosis of adenocarcinoma.

Generation of Ppy^{Cre} mice

The donor plasmid *pPPY/NLS-Cre* contained a nuclear translocation signal (NLS)-cre and three SV40 polyadenylation signals (a kind gift from Dr. Gu [19]). The targeting strategy is shown in Fig. 1. To target the mouse *Ppy* gene (gene ID: 19064), zinc finger nucleases (ZFN) technology has been used. ZFN plasmid design and validation was performed by CompoZr ZFN technology (Sigma-Aldrich, St. Louis, MO, USA). The ZFN were designed to cut the [TAGGTA] sequence located at the 3' end of exon 2 (Fig. 1A). The *Ppy*-targeting ZFN and the knock-in (KI) donor plasmid were injected into embryos, which were subsequently implanted into pseudopregnant female mice. A total of 81 pups were born.

Founder identification and breeding

Tail or toe biopsies were used for genomic DNA extraction and analysis, as described previously [20]. Primer sets flanking the 5' target site (P1 and P2 in Fig. 1) and the 3' target site (P3 and P4) used for screening were as follows: P1: 5'-ACTCCCTTCCAAGTGGCTTT-3'; P2: 5'-AATTGGACACCTTCCTCTTCTTC-3'; P3: 5'-GCTGGAAGATGGCGATTAGT-3'; and P4: 5'-GGAGC ACAACTGCAAAGTCA-3'. A founder (#76) was selected and bred with wild-type (WT) mice to obtain heterozygous animals, and sibling mating of heterozygotes resulted in homozygous $Ppy^{Cre/Cre}$ ($Ppy^{-/-}$) mice.

PCR genotyping

DNA analysis of litters was performed by extracting tail snip genomic DNA prepared in 500 μL lysis buffer (200 mM NaCl, 100 mM Tris-HCl, 5 mM EDTA, 0.25% Tween-20) with 1 mg Proteinase K (Sigma-Aldrich) overnight at room temperature, followed by incubation at 60°C to complete tissue lysis. DNA was obtained by standard techniques with 2-propanol, 70% ethanol, and DNA stored in TE buffer (10 mM Tris-HCl and 1 mM EDTA) at 22°C. For PCR, 0.5 μL of DNA was mixed with 9.35 μL H_2O, 10 μL EmeraldAmp MAX PCR Master Mix (Takara, Tokyo, Japan), and 0.05 μL of each primer (P5 [forward] 5'-AGGAAGGTGGTTGATGCTGG-3', P6 [reverse for WT] 5'-CTGGGTACATTGGCTCC AGG-3', P7 [reverse for mutant] 5'-CAGCGTTTTCG TTCTGCCAA-3'). PCR conditions were 95°C for 2 minutes, followed by 35 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a fixed cycle at 72°C for 5 minutes. This PCR resulted in amplification of a 269-bp DNA fragment for the wild-type *Ppy* allele and a 766-bp DNA fragment for the Ppy^{Cre} allele (Fig. 1C).

In situ hybridization

The pancreata of C57BL/6 WT and $Ppy^{Cre/Cre}$ mice were dissected, fixed with G-Fix (Genostaff, Tokyo, Japan), and then embedded in paraffin. *In situ* hybridization was performed with the ISH Reagent Kit (Genostaff, Tokyo, Japan) according to manufacturer's instructions. Tissue sections (6 μm) were hybridized with digoxigenin-

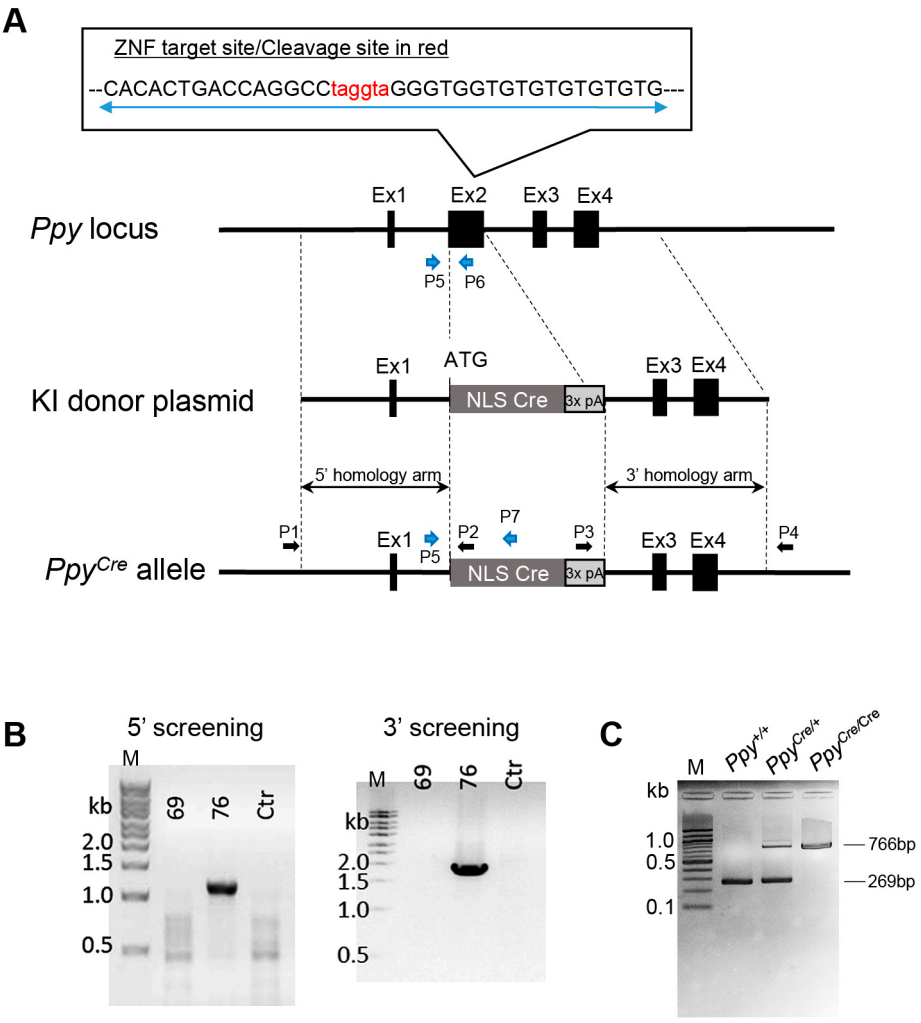

Fig. 1 Gene targeting for *Ppy*^{cre} knockin mice

(A) Schematic diagram of the ZFN target site is shown (top). Targeting of the *Ppy* gene to generate *Ppy*^{Cre} mice. The protein-coding region of *Ppy* was precisely replaced with that of NLS-Cre. Primers (P1–P4), used for screening of targeted mice. (B) PCR screening of targeted mice. Representative results of 5'-screening (P1/P2) and 3'-screening (P3/P4) PCR reactions in randomly integrated (#69) or correctly targeted (#76) clones are shown. (C) PCR genotyping of *Ppy*$^{+/+}$, *Ppy*$^{cre/+}$, and *Ppy*$^{cre/cre}$ mice using primers that distinguish *Ppy*^{Cre} from the *Ppy* WT allele (P5, P6 and P7).

labeled antisense RNA probes at 60°C for 16 hr. (*Ppy*: GenBank Accession number NM_008918.1, position 42–226; *insulin 1*: GenBank Accession number NM_008386.3, position 239–462.) The signal was detected using NBT-BCIP, an alkaline phosphate color substrate. Sense RNA probes were also used for negative controls (data not shown). After the *in situ* hybridization, the same sections were incubated with 0.2 µg/mL of anti-PP antibody (32-1A8) at 4°C overnight, and PP immunoreactivity was visualized using DAB solution. For immunohistochemistry, as a second staining after *in situ* hybridization, endogenous peroxidase was blocked with 0.3% H_2O_2 in PBS for 30 min, followed by incubation with Block A (Nichirei, Tokyo, Japan). The sections were incubated with 0.2 µg/mL of anti-PP antibody at 4°C overnight. After treatment with Block B (Nichirei) for 10 min at RT, they were incubated with Simple stain mouse MAX-PO(M) (Nichirei) for 10 min at RT as the secondary antibody reaction. Peroxidase activity was visualized by diaminobenzidine (Dojindo, Kumamoto, Japan). The sections were mounted with G-Mount (Genostaff).

Generation of monoclonal antibodies against PP

Monoclonal anti-PP antibodies were developed by Cell Engineering Corporation (Osaka, Japan) and the specific recognition of PP by three clones (23-2D3, 32-1A8, and 32-1C3) was confirmed. The mouse PP antigen: CAPLEPMYPGDYATPEQMAQYETQLRRYINTLTRPRY (amino acids [aa] 30–65, underlined) (Sigma-Aldrich) was synthesized for immunization. The mouse PYY antigen: CAKPEAPGEDASPEELSRYYA

SLRHYLNLVTRQRYGK (aa 30–65, underlined) (Sigma-Aldrich) was synthesized for the enzyme-linked immunosorbent assay (ELISA). The N-terminal cysteine residue added to the PP fragment and PYY fragment enabled coupling to the carrier protein, maleimide-activated keyhole limpet haemocyanin (Thermo Fisher Scientific, Waltham, MA, USA). ELISA for PYY was used for negative selection of hybridoma clones cross-reacting to PYY. The coupling reaction was performed according to the instructions provided by the supplier. $Ppy^{Cre/Cre}$ (*null*) mice were immunized with the PP peptide and monoclonal antibodies were obtained based on the lymph node method established by Sado *et al.* [21, 22]. The PP peptide (125 μg) was used as the antigen. Hybridoma cells were cloned in HAT selection medium (hybridoma SFM medium [Thermo Fisher Scientific]; 10% fetal bovine serum; 10% BM Condimed H1 (Roche Applied Science, Mannheim, Germany); 100 mM hypoxanthine; 0.4 mM aminopterin; and 1.6 mM thymidine). Hybridoma cells were screened 7 days post-fusion by using ELISA. Clones positive for PP with no cross-reactivity for PYY were selected. Positive clones were subcloned and rescreened by immunohistochemistry based on specific signals from the $Ppy^{+/+}$islets with no signals observed from the $Ppy^{Cre/Cre}$ islets.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 wells) were coated by filling with 50 μL/well of 100 mM carbonate buffer (pH 9.5) containing 50 ng/well of human PP, NPY, mouse/rat PYY or human PYY (Phoenix Pharmaceuticals, CA, USA) overnight at 4°C. The plates were subsequently washed with PBS and blocked with 200 μL/well of 0.1% (w/v) bovine serum albumin (BSA) in PBS overnight at 4°C. After washing twice with PBST, 50 μL/well antibodies serially diluted with 1% BSA in PBST were added into the wells of the coated microtiter plates, and the plates were incubated at 37°C for 30 min. After washing 4 times with PBST, 50 μL of anti-mouse IgG (H+L) goat IgG Fab'-HRP (Immuno-Biological Laboratories, Gunma, Japan) was added to each well and incubated at 37°C for 30 min. The wells were washed with PBST 4 times, and then 100 μL of freshly prepared o-phenylenediamine dihydrochloride solution was added to each well as a substrate. The wells were then incubated for 15 min at room temperature. The reaction was terminated by adding 100 μL of 1N H_2SO_4. Absorbance of the solution in each well at 490 nm was measured using a microplate reader (E-Max; Molecular Devices, San Jose, CA, USA).

Immunofluorescence staining

Mouse or rat pancreata were fixed overnight at 4°C in 4% paraformaldehyde. Tissues were washed for 24 hours with PBS, incubated in Holt's gum sucrose solution for a few days, embedded in O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and cryosectioned. Primary antibodies used were monoclonal mouse anti-pancreatic polypeptide (32-1A8; 0.2 μg/mL, 23-2D3; 0.2 μg/mL), rabbit anti-human pancreatic polypeptide [1:1,000, RRID: AB_92383, catalog no. AB939 (Lot number: NG2069349, designated as NG) EMD Millipore (Darmstadt, Germany); 1:20 (manufacturer's recommendation), RRID: AB_92383, catalog no. AB939 (Lot number: NMM1584014, designated as NMM) Millipore; two different batches of the Millipore antibody with the same catalog no. were used] and guinea pig anti-insulin (Envision FLEX-Insulin, catalog no. IR002; Ready to use, Dako-Agilent, Santa Clara, CA). For immunofluorescence staining with a mouse primary antibody on mouse tissue sections, the tissue sections were blocked with Mouse IgG Blocking Kit (MKB-2213-1, Vector Laboratories Inc. - Maravai LifeSciences, Burlingame, CA, USA). After washing sections in 1% Triton X-100 in PBS, they were incubated in a solution containing fluorescent-conjugated Alexa 488 [(RRID: AB_2534117) to detect insulin; (RRID: AB_2535792) to detect PP, 1:2,000 each] or Alexa 594 [(RRID: AB_2534073) to detect PP, 1:2,000] secondary antibodies (Thermo Fisher Scientific) for 60 min. Samples were counterstained with the nuclear dye DAPI (Polysciences, Inc., Warrington, PA, USA). The sections were then examined using confocal microscopy (Zeiss LSM 710: Carl Zeiss, Oberkochen, Germany and IX81: Olympus, Tokyo, Japan).

Results

Generation of the Ppy^{Cre} allele

A pair of ZFNs were confirmed to cleave the target site located at the 3' end of exon 2 of *Ppy*, as shown in Fig. 1A. Following microinjection and embryo transfer, 81 pups were born. One founder (#76) had positive PCR amplification of a sequence spanning the *NLS-Cre-3 x polyA* sequence, and positive amplification in all 6 sets of PCRs (3 in the 5'-flanking region and 3 in the 3'-flanking region) designed to amplify an integrated knock-in fragment (two primer sets, P1/P2 and P3/P4, among the six are shown in Fig. 1). Founder #76 had no amplification of the donor plasmid backbone, thus excluding the possibility of random integration (data not shown). The genomic sequence of the Ppy^{Cre} allele spanning 2-kb of the 5'-flanking region, the open-reading frame of $Ppy^{NLS\text{-}Cre\text{-}3\ x\ polyA}$ and 0.8 kb of the 3'-flanking region was further verified.

Validation of the function of the Ppy^{Cre} allele by in situ hybridization

To validate whether *Ppy*^{Cre} is a functionally null allele of *Ppy*, *Ppy*$^{Cre/Cre}$ mice were generated by intercrossing *Ppy*$^{Cre/+}$ mice. *In situ* hybridization confirmed that the mRNA for *insulin 1* was similarly and abundantly expressed by islets of WT and *Ppy*$^{Cre/Cre}$ mice (Fig. 2). Whereas *Ppy* mRNA was clearly detected in the mantle of WT islets, its expression was completely absent from *Ppy*$^{Cre/Cre}$ islets (Fig. 2), indicating that *Ppy*^{Cre} is a functionally null allele.

Generation of anti-PP monoclonal antibodies and their binding specificities

Next, mouse monoclonal antibodies against PP were generated by immunizing *Ppy*$^{Cre/Cre}$ (*Ppy*$^{-/-}$) mice with the mouse PP peptide. After two rounds of screening (see Materials and Methods), three independent hybridoma clones (23-2D3, 32-1A8, and 32-1C3) were obtained. Whereas mRNA of the NPY family members (*Ppy*, *PPY*, and *NPY*) was all expressed in WT mouse islets (data not shown), most of the cells labeled by a monoclonal anti-PP antibody (32-1A8) in the islet mantle region were colocalized with *Ppy* mRNA (Fig. 2I, J), suggesting specific binding of the monoclonal antibody to cells producing the *Ppy* gene product. The specific binding of the monoclonal antibodies to PP and the absence of cross-reactivity to PYY and NPY were further confirmed by ELISA (Fig. 3).

Validation of PP antibodies in Ppy-KO tissues

Next, epitope specificity of the antibodies was verified using pancreatic tissue from *Ppy*-KO mice (Fig. 4). Signals detected by 32-1A8 (Fig. 4A) in WT islets were completely abolished when the *Ppy* gene was disrupted (Fig. 4D), further demonstrating specific binding of the monoclonal antibody to PP. Essentially the same results were obtained when the two other monoclonal antibodies, 23-2D3 (Fig. 4G, J) and 32-1C3 (data not shown), were tested. AB939 (batch NG), a commercially available polyclonal antibody against PP (Table 1) detected exactly the same cells labeled by the monoclonal antibody 32-1A8, suggesting that AB939 (batch NG) specifically recognizes PP-producing cells in the WT context (Fig. 4A–C). It is of special interest, however, that in *Ppy*-KO mouse islets, AB939 (batch NG) still labeled many cells which were located in the mantle of the islets (Fig. 4E). Therefore, it is highly likely that AB939 (batch NG) recognizes peptides other than PP. On the other hand, when AB939 with a different product number, designated as batch NMM was used, positive cells detected by AB939 in the context of *Ppy-KO* were significantly decreased compared with AB939 (NG)

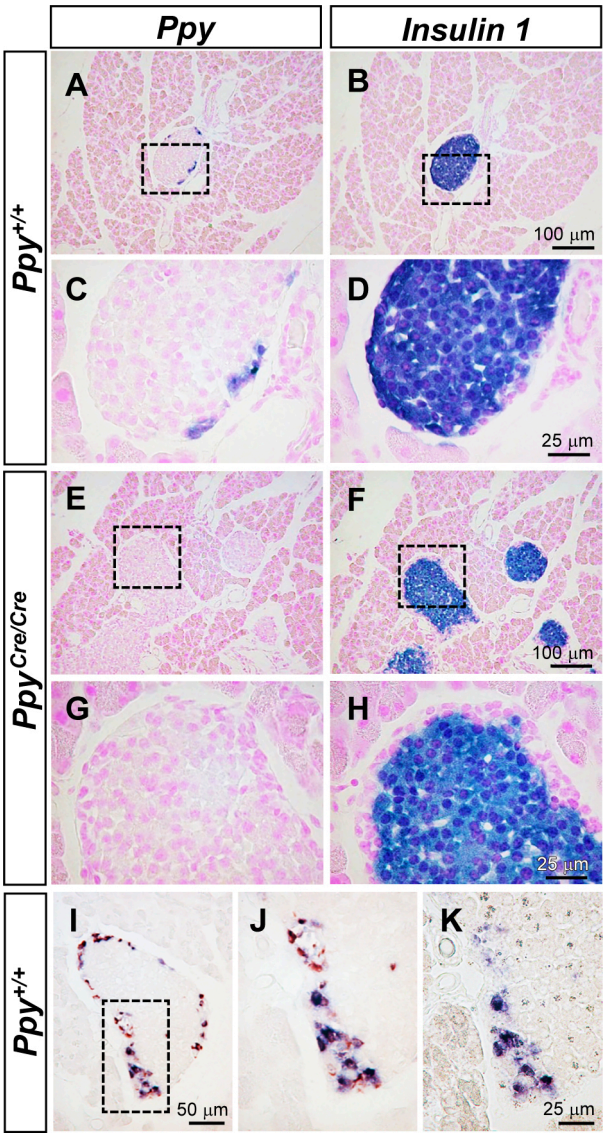

Fig. 2 Validation of *Ppy*$^{Cre/Cre}$ mice and binding specificity of the anti-PP monoclonal antibody

Pancreatic sections from 8-week-old WT C57BL/6J mice were hybridized with antisense riboprobes against *Ppy* (A, C) and *insulin 1* (B, D). Pancreatic sections from 8-week-old *Ppy*$^{Cre/Cre}$ mice were hybridized with antisense riboprobes against *Ppy* (E, G) and *insulin 1* (F, H). C, D, G, and H are magnified pictures of A, B, E, and F, respectively. I–K. Pancreatic sections from 8-week-old C57BL/6J mouse were hybridized with a *Ppy* antisense riboprobe (blue), followed by immunostaining with the monoclonal anti-PP antibody 32-1A8 (brown signals in I and J). J show magnified pictures of the region surrounded by the dashed rectangle in I.

(compare Fig. 4E and 4K), suggesting batch-to-batch variation in the binding specificity of AB939 against PP.

Characterization of monoclonal PP antibodies in human and rat tissues

Next, we investigated whether these monoclonal anti-

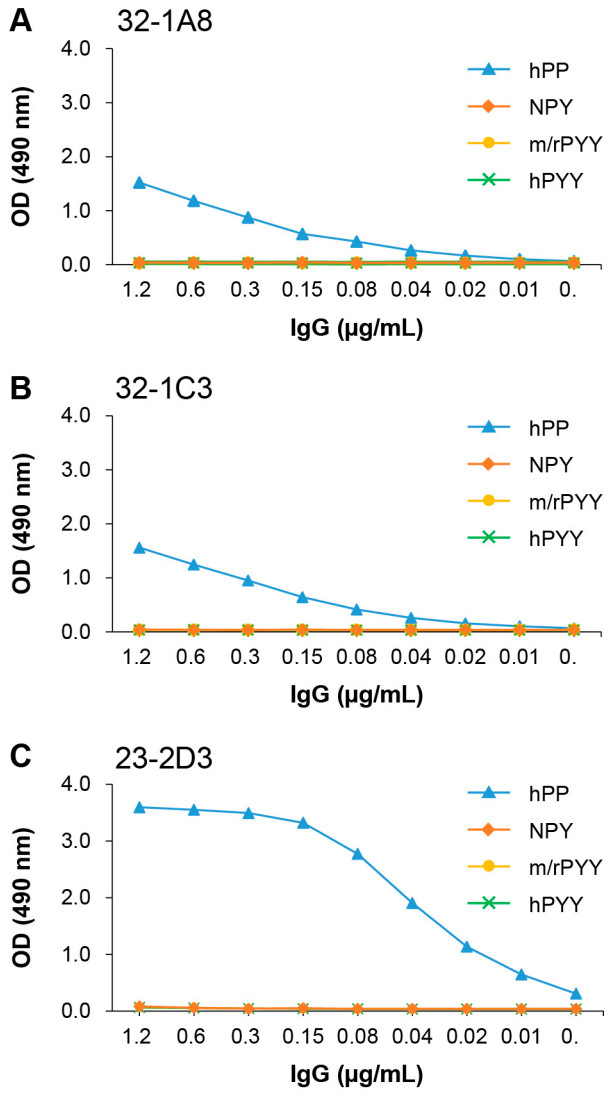

Fig. 3 Validation of the binding specificities of anti-PP antibodies by ELISA

The reactivity of anti-PP monoclonal antibodies to PP, NPY, and PYY was tested by ELISA. Three different anti-PP monoclonal antibodies, (A) 32-1A8, (B) 32-1C3, and (C) 23-2D3, were reacted to each peptide fixed on microtiter plates. Bound antibodies were detected with an HRP-labeled anti-mouse antibody by absorbance at 490 nm. m/rPYY represents mouse and rat PYY, which have the same amino acid sequence.

bodies are able to detect PP cells also in human tissues. Human pancreatic tissues that were surgically removed during pathological analysis of possible pancreatic adenocarcinoma were immunostained (Fig. 5). As shown in Fig. 5A, the 32-1A8 antibody clearly labeled cells located in the peripheral region of the islets (labeled in red), that were distinct from insulin-producing β cells (labeled in green). We also confirmed that the 23-2D3 antibody was also able to label PP cells in human pancreatic tissues (Fig. 5G). The monoclonal antibodies were further characterized in rat pancreatic tissues. Whereas the monoclonal antibody 32-1A8 did not produce any signals in islet samples of 10–week-old Wistar rats, 23-2D3 clearly labeled cells located at the periphery of islets (Fig. 5D, J), demonstrating that 23-2D3 is the only antibody that is capable of detecting PP cells in mice, rats, and humans.

Discussion

It is intriguing that AB939 (batch NG), a commercially available antibody labeled many cells located in the periphery of *Ppy*-KO mouse islets (Fig. 4E) Therefore, it was concluded that AB939 (NG) recognized proteins other than PP. Given the highly conserved amino acid sequence between PP and PYY, it is likely that the antigens detected by AB939 (NG) in the absence of PP are those of PYY. A possible explanation may be that PP cells concomitantly express PP and PYY in the adult pancreas, as previously reported [16, 23]. It is of special note however that the same antibody with a different batch [AB939 (NMM)] appears to be more specific to PP. The observed batch-to-batch variation of AB939 is intrinsic to polyclonal antibodies [24]. Thus, monoclonal antibodies which detect only single epitope are more reliable to reproduce consistent results.

Information on the major anti-PP antibodies that have been used in immunohistochemistry to date are summarized in Table 1. They are all polyclonal antibodies raised in rabbits or guinea pigs. Among them, the antibody produced by Millipore/Linco (RRID: AB_433709) is the only one in which its binding specificity to PP was validated by a rigorous method using *Ppy*-deficient mice, as demonstrated in the present study [13]. Unfortunately, this PP-specific antibody is no longer available. Other antibodies have no solid validation in their binding specificity against PP. Here we provide rigorous evidence that the mouse monoclonal antibodies 23-2D3, 32-1A8, and 32-1C3 are specific to PP, by ELISA and immunostaining using *Ppy*-KO mouse tissues. Hybridoma clones enable a limitless supply of antibodies with the same binding specificity. In this respect, we believe that the monoclonal antibodies against PP, as well as the Ppy^{Cre} mice established in this study, will be valuable research resources in the field of islet biology.

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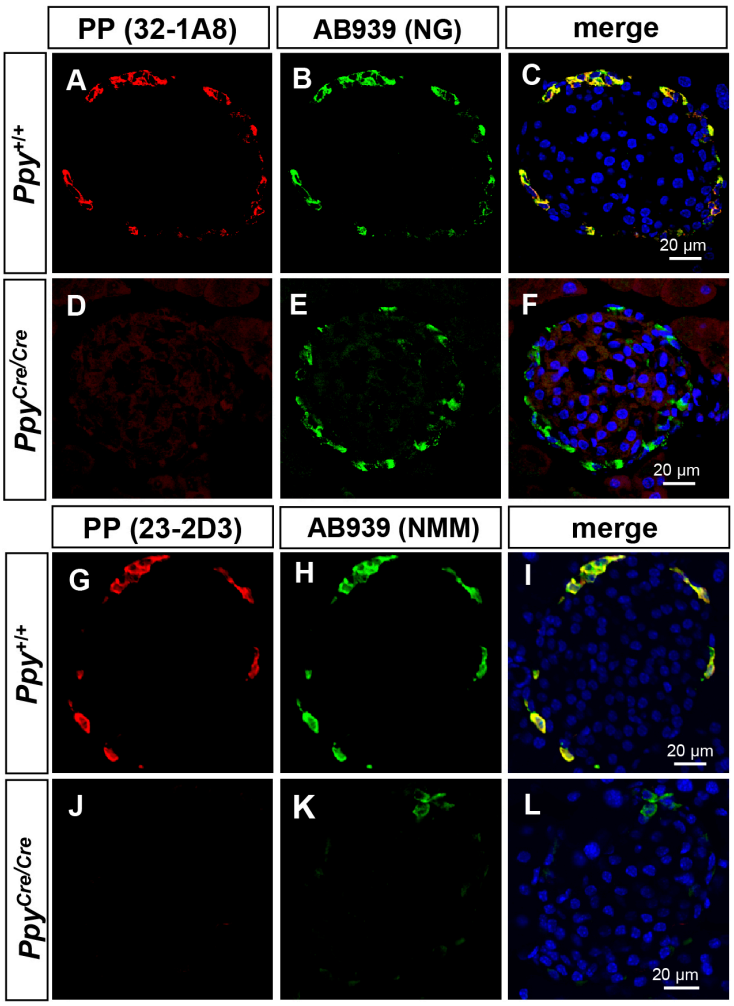

Fig. 4 Characterization of monoclonal mouse anti-PP antibodies and a commercially available polyclonal anti-PP antibody

(A, B, G, H) Pancreatic sections from 8-week-old C57BL/6J mice were immunostained with the indicated antibodies against PP. C shows a merged image of A and B. I shows a merged image of G and H. (D, E, J, K) Pancreatic sections from 8-week-old $Ppy^{Cre/Cre}$ (*null*) mice immunostained with the indicated antibodies against PP. F is a merged image of D and E. L is a merged image of J and K.

Table 1 List of the major anti-PP antibodies used in the literature

Antibody Name	Catalog no	RRID	Validation of binding specificity	Reference
(1) DAKO rabbit anti-human PP	A0619	—	Not determined	[25-28]
(2) Thermo Fisher Scientific rabbit anti-PP	PA1-36141	AB_2169061	Not determined	[29]
(3) Pierce rabbit anti-PP [same antibody as (2)]	PA1-36141	—	Not determined	[30]
(4) Millipore guinea pig anti-rat PP	4041-01	AB_433709	No positive cells in *Ppy null*	[26]
(5) Linco guinea pig anti-rat PP [same antibody as (4)]	Not specified		No positive cells in *Ppy null*	[13, 28]
(6) Millipore rabbit anti-human PP	AB939	AB_92383	Positive cells in *Ppy null*	[31], this study
(7) Millipore rabbit anti-human PP	4040-01	AB_1977273	Not determined	[32]
(8) Novus Biologicals goat anti-human PP	NB100-1793	—	Not determined	[33-35]
(9) Peninsula Laboratories rabbit anti-human PP	T-4088	—	Not determined	[35]
(10) Everest Biotech goat anti-PP	EB06805	AB_2169058	Not determined	[36]
(11) Abcam goat anti-human PP	ab77192	AB_1524152	Not determined	[37]
(12) Innovative Research rabbit anti-human PP	18-0043	AB_140259	Not determined	[38]

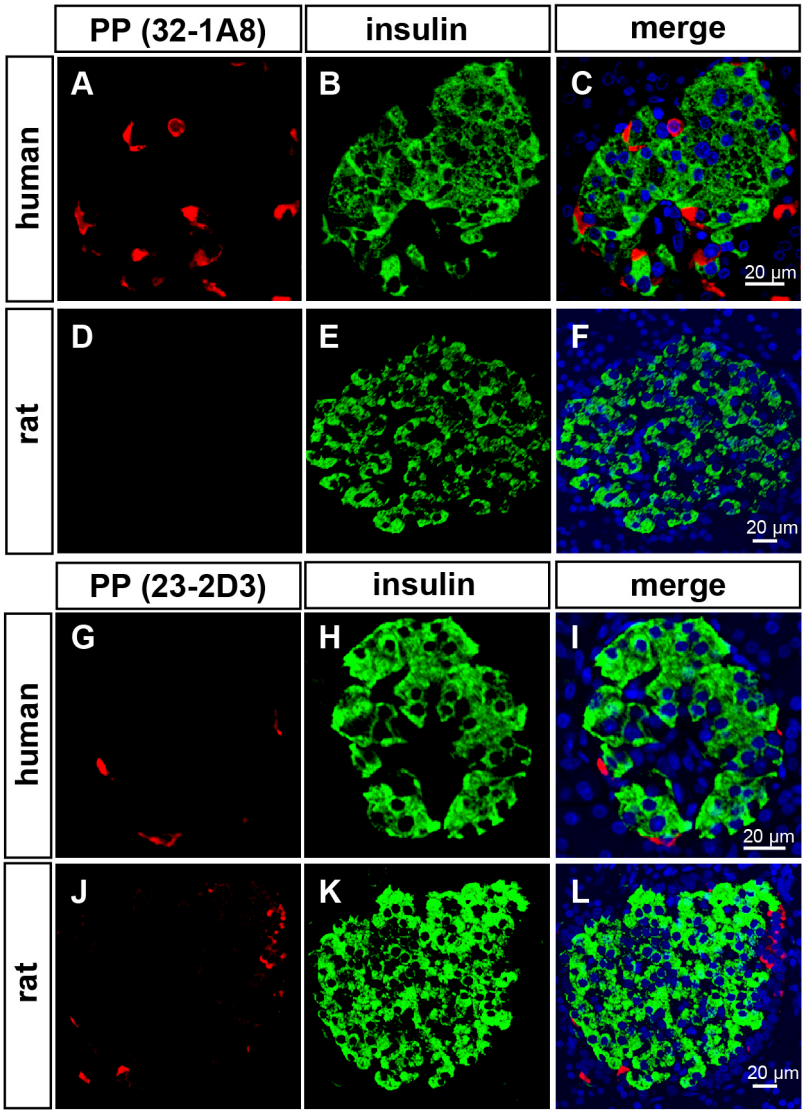

Fig. 5 Immunostaining of human and rat islets with monoclonal mouse antibodies against PP

Cells labeled with the indicated monoclonal anti-PP antibodies (A, C, G, and I; red) were found in the periphery of human islets. Insulin-positive β cells are shown in green (B, C, H, and I). Nuclei were counterstained with DAPI in blue (C). Stained cells were detected by the antibody 23-2D3 (J and L; red) in Wistar rat islets, but they were not detected by the antibody 32-1A8 (D and F).

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Disclosure

The authors declare that they have no conflicts of interest associated with this study.

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