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PYY in the Expanding Pancreatic Epithelium

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Gut peptide YY (PYY) plays an important role in regulating metabolism and is expressed during the ontogeny of the pancreas. However, its biological role during endocrine cell formation is not fully understood, and its role, if any, during pancreatic regeneration in the adult has not yet been explored. The knowledge of factors involved in beta cell renewal in adult animals is clearly relevant for the design of treatment strategies for type 1 diabetes. We therefore sought to determine if observations during fetal pancreas formation also apply to pancreatic growth in adult animals. Indeed, we have found marked expansion of the PYY-expressing population during pancreatic regeneration. In addition, we demonstrate the presence of cells co-expressing PYY and the critical pancreatic transcription factor pancreatic duodenal homeobox1 (PDX-1). Interestingly, these cells also co-expressed specific islet hormones during pancreatic development and re-growth, suggesting a developmental relationship. Furthermore, we have found that PYY can act in concert with IGF-1 to stimulate cellular responsiveness in pancreatic epithelial cells in vitro. Our data suggest that PYY may be a mediator of islet cell development, as well as a cofactor for growth factor responses, not only during fetal pancreas formation but also during regeneration in adult animals.

Key Words: PYY; pancreas; regeneration; neogenesis.

Introduction

The gut and pancreas share a common embryological origin, with the pancreas arising from an outpocketing of the gut endoderm near the future duodenum *(1)*. Therefore, it is not surprising that pancreatic and intestinal cell lineage commitment requires a number of the same transcription and growth factors *(2–12)*. Indeed, the gut and pancreatic programs can be cross-committed during normal development. For example, expression of the transcriptional regulator Ptf-1 converts intestinal cells to pancreatic progenitors *(13)*. In addition, glucagon-like peptide 1 (GLP 1) exposure converts intestinal epithelial cells into insulin-producing cells *(14)*. These studies suggest that a parallel developmental relationship exists between the endocrine cells of the intestine and pancreas.

Gut peptide YY (PYY) is known to play a very important role in regulating metabolism *(15–18)*. Examination of the ontogeny of colonic endocrine cells reveals that PYY is the first hormone to appear during foregut development. Between embryonic d 16.5 and 18.5, PYY-expressing cells have been found to co-express glucagon, cholecystokinin, substance P, serotonin, secretin, neurotensin, gastrin, or somatostatin *(19)*. This implies that PYY expression is a frequent occurrence during colonic endocrine cell differentiation. Interestingly, PYY immunoreactivity is also identified in the earliest endocrine cells in the fetal mouse pancreas (d 9.5 pc), and is co-expressed with each islet hormone during development, suggesting that pancreatic endocrine cells arise from a common PYY-producing progenitor cell *(20)*, parallel to the observations in the gut.

IFN-γ transgenic mice provide a convenient model for studying islet neogenesis during pancreatic regeneration. In this mouse strain, the islet cells constitutively express IFN-γ under the control of the insulin promoter. The pancreata of these mice undergo spontaneous ductal hyperplasia and islet neogenesis during their adult life *(21,22)*. Furthermore, studies of this transgenic mouse reveal the presence of numerous intermediate cell types in the duct epithelium of the pancreas, including cells simultaneously exhibiting both duct and exocrine phenotype or endocrine and exocrine features, as well as single endocrine cells containing multiple islet hormones *(21–23)*. The ability to promote pancreatic tissue renewal in adult animals could provide an avenue for treatment of type 1 diabetes. Therefore, the identification of growth factors and hormones that are involved in regeneration is critical if this end is to be achieved. Given its prominence and potential significance during fetal pancreas formation, we hypothesized that PYY might likewise

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be relevant to adult tissue renewal. In this report, we investigated this hypothesis, using the IFN-γ transgenic mouse model. Importantly, our results suggest a role for PYY during endocrine cell renewal and growth factor responsiveness during pancreatic regeneration in adult animals.

Results

PYY Co-expressing Endocrine Cells Are Significantly Increased in the Transgenic Fetal Pancreas

PYY is the earliest detectable islet hormone and is present in all early appearing islet cell types *(20,24,25)*. Its expression is downregulated after birth *(20)*, and, by adulthood, PYY is no longer expressed in beta cells *(20)*. Given its prominence during fetal pancreas formation in normal mice and the importance of defining factors involved in endocrine cell formation in adult models of growth, we sought to determine if PYY expression was elevated during pancreatic regeneration in adult animals. We studied IFN-γ mice, because substantial new endocrine cell formation occurs during adult life in these animals. We first explored PYY expression during pancreas formation in order to determine if IFN-γ transgenic and non-transgenic embryos exhibited differences in their patterns of PYY expression. We chose to look at embryos at E16, a point during development at which PYY expression is prominent in normal mice and which occurs after the secondary transition has begun at E13, leading to an exponential increase in endocrine cell formation. We did indeed detect significant expression of PYY in the pancreatic epithelium of IFN-γ embryos (data not shown). We characterized PYY-expressing cells further by combining PYY immunostaining with that of the endocrine hormones. Interestingly, although no significant difference was observed in the number of PYY/insulin double positive cells in the embryonic E16 IFN-γ transgenic pancreas compared to the congenic NOD fetal pancreas, we found significantly increased numbers of PYY-positive cells that also expressed either somatostatin, glucagon, or PP in the IFN-γ fetal pancreas compared to the NOD fetal pancreas (Table 1). This suggests that specific populations of PYY-expressing pancreatic endocrine cells preferentially accumulate during fetal pancreatic development in the transgenic strain, an observation that might be relevant to the propensity toward ductal and endocrine re-growth in this strain and which would implicate PYY as a mediator of endocrine cell expansion.

PYY and Its Receptor Are Expressed During Pancreatic Regeneration

Next, we investigated whether the expression of PYY was prominent during islet neogenesis in the adult IFN-γ transgenic pancreas. We examined additional relevant gut endocrine hormones (GIP and GLP) for comparison. Our results show that during pancreatic regeneration, PYY, as well as GIP and GLP, are strongly expressed in the ducts and in periductal regions of the transgenic pancreas (Fig. 1B). Ductal expression of these factors is not observed in the NOD pancreas (Fig. 1A), although they are expressed in islets. Interestingly, PYY positive cells co-expressing insulin or glucagon are more abundant in ducts and islets in the IFN-γ pancreas compared to the NOD pancreas (Fig. 2 and Table 1), suggesting that these PYY-expressing populations may participate in the activation or growth of endocrine cells during regeneration. No significant difference was found between the IFN-γ and NOD mice in the percentage of PYY-expressing cells also expressing somatostatin or PP (Fig. 2 and Table 1). Additionally, we found PYY positive cells that also express GIP or GLP localized in the ducts and islets of the transgenic pancreas, as well as in the NOD and IFN-γ fetal pancreas, whereas we were not able to find these double positive cell populations in the adult NOD pancreas (Fig. 2 and Table 1). This suggests that during pancreatic regeneration, we observe an expansion of cells co-expressing PYY, along with other gut-associated molecules.

In order to exert its effects, PYY acts through its receptor. Therefore, we expect the PYY receptor to be present in responsive tissues. Indeed, the PYY/NPY receptor (Y1R) is expressed in the developing pancreas *(26)*. To determine whether pancreatic cell populations in adult mice can respond directly to PYY, we asked whether expression of Y1R occurred in the adult NOD pancreas and in the IFN-γ transgenic pancreas. We used RT-PCR for this purpose because we were not able to obtain high-quality specific staining using the commercially available Y1R antibodies. Although we did not do quantitative determinations of receptor message levels, we found that Y1R mRNA was expressed at higher levels in the IFN-γ pancreas compared with syngeneic pancreas (Fig. 3), suggesting that cells present in the regenerating pancreas are indeed responsive to PYY.

PYY and PDX-1 Are Co-expressed in the IFN-γ Transgenic Pancreas

As described previously and above, we observed prominent PYY expression during pancreatic development and regeneration, suggesting a role for this molecule in islet cell formation. Because PDX-1 is a critical pancreatic transcription factor for pancreatic development, we asked whether its expression coincided with that of PYY. Interestingly, we found that PDX-1 was expressed in PYY positive cells in both the NOD (35.7%) and the IFN-γ transgenic (44.8%) embryonic pancreas at E16 (Figs. 4A,B, Table 1). In contrast, in the NOD adult pancreas, only 3.4% of the PYY cells co-expressed PDX-1 (Fig. 4C, Table 1). However, we found that 40.7% of PYY-positive cells co-expressed PDX-1 in the IFN-γ transgenic pancreas (Fig. 4D, Table 1, $p < 0.01$).

In addition, a small subset of PYY-positive cells that co-expressed PDX-1 and either insulin, glucagon, or somatostatin were observed in the fetal NOD pancreas, as well

Table 1
Analysis of Immunohistochemical Staining in the NOD and IFN-γ Embryonic and Adult Pancreas

	Embryonic pancreas (E16)		Adult Pancreas (8–9 wk old)	
Double/triple staining	NOD (%)	IFN-γ (%)	NOD (%)	IFN-γ (%)
PYY + In	4.3 (5/117)	3.6 (4/111)	0 (0/82)**	28.6 (207/724)**
PYY + Glu	19.1 (18/94)**	57.4 (27/47)**	44.7 (46/103)**	71.8 (229/319)**
PYY + PP	16.8 (19/113)*	45.6 (36/79)*	15.3 (29/190)	34.2 (12/35)
PYY + Som	29.8 (25/84)*	57.9 (22/38)*	19 (8/42)	41 (32/78)
PYY + GIP	30.8 (8/26)	27 (10/37)	0 (0/52)**	58.2 (53/91)**
PYY + GLP	38.7 (12/31)	24 (6/25)	0 (0/54)**	65.6 (61/93)**
PYY + PDX	35.7 (15/42)	44.8 (30/67)	3.4 (4/118)**	40.7 (57/140)**
PYY + PDX + In	1.9 (1/53)	1.5 (1/66)	0 (0/41)	8.8 (4/45)
PYY + PDX + Glu	6.7 (3/45)	8.3 (5/60)	0 (0/30)	9.5 (2/21)
PYY + PDX + Som	4.2 (2/48)	6.3 (4/63)	15 (6/40)	17.4 (8/46)
PYY + PDX + PP	N/A	N/A	7.7 (6/77)	9 (4/44)
PYY + IGFR	N/A	N/A	60 (24/40)	86.1 (143/166)
PYY + Glu + In	6.2 (1/16)	5.8 (1/17)	0 (0/598)	0 (0/318)

The percentages of double and triple positive cells within the total PYY positive cell numbers from the fluorescence staining studies were compared by the *t* test. *$p < 0.05$; **$p < 0.01$.

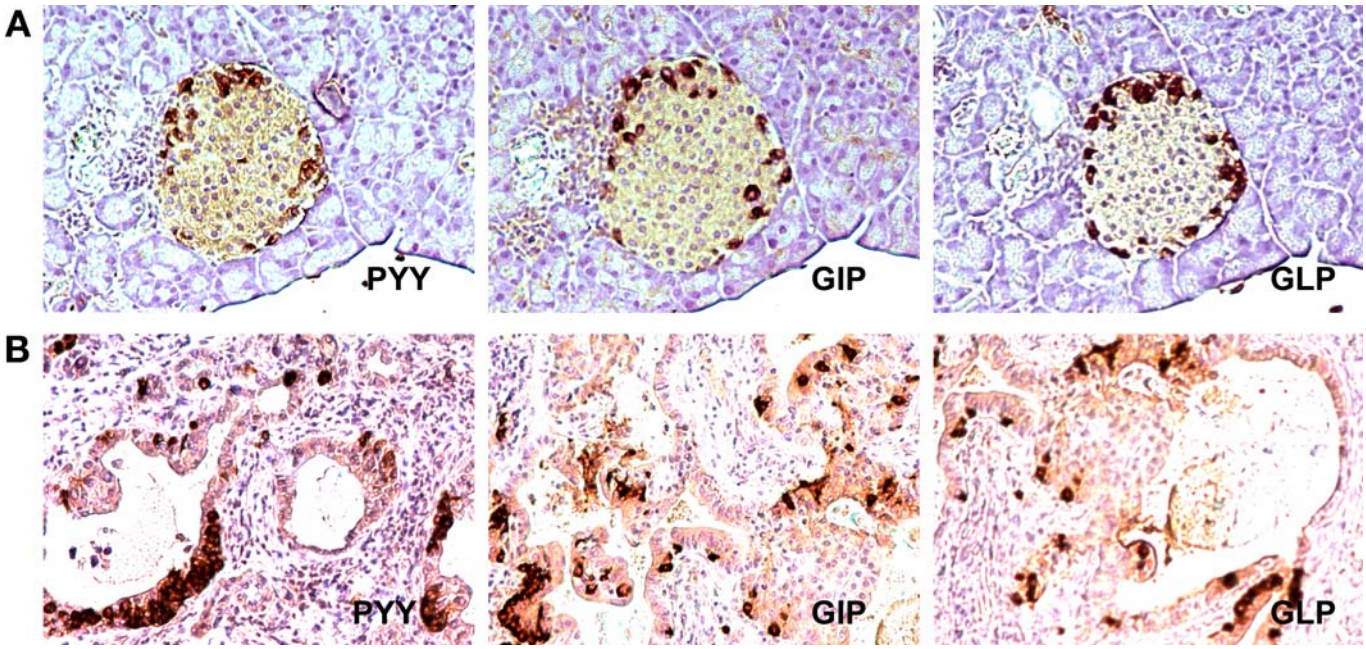

Fig. 1. Gut peptide expression in NOD and IFN-γ regenerating pancreas. Immunohistochemistry of adult NOD (**A**) and IFN-γ transgenic (**B**) pancreas sections utilizing DAB as a chromagen. The analysis reveals that gut peptides (PYY, GIP, GLP) are extensively expressed in the ducts and periductal regions of the IFN-γ pancreas (**B**) compared with the same staining in the NOD pancreas (**A**). Note that the immunoreactivity of these proteins is mainly localized in the periphery of islets in the NOD pancreas. Original magnification: 20×.

as in the fetal transgenic pancreas (Figs. 5A–F, Table 1). In the adult IFN-γ pancreas, PYY/PDX-1/insulin, PYY/PDX-1/glucagon, PYY/PDX-1/somatostatin, and PYY/PDX-1/PP triple positive cells were observed, whereas only PYY/PDX-1/somatostatin and PYY/PDX-1/PP triple positive populations were observed in the adult NOD pancreas (Figs. 5G–L, Table 1). These results suggest that during pancreatic regeneration, PYY/PDX-1/insulin and PYY/PDX-1/glucagon cells, found in both the developing and regenerating pancreas, might contribute to the formation of new endocrine cell populations.

PYY Acts in Concert with the Pancreatic Growth Factor IGFR-1 to Activate Epithelial Cells

Proliferation of the intestinal epithelium can be stimulated by a number of different growth factors and hormones, including insulin-like growth factor-1 (IGF-1) and PYY *(27)*. These factors have been linked previously, with evi-

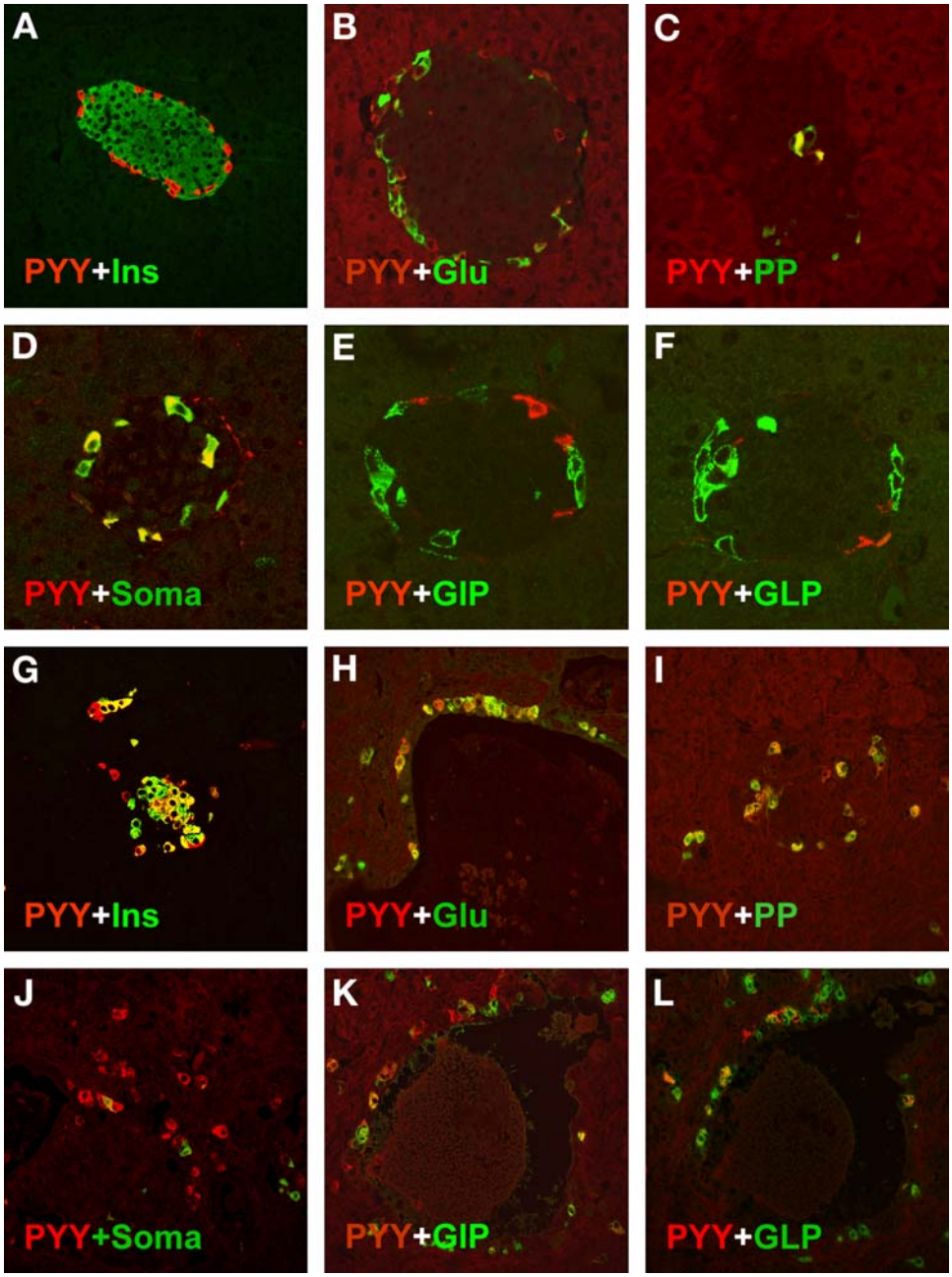

Fig. 2. PYY immunofluorescence staining in the adult pancreas. The NOD (**A–F**) and IFN-γ (**G–L**) pancreas were stained with antibodies to PYY/insulin (**A,G**), PYY/glucagon (**B,H**), PYY/PP (**C,I**), PYY/somatostatin (**D,J**), PYY/GIP (**E,K**), PYY/GLP (**F,L**). Double positive staining is indicated by the yellow color. Original magnification: **D–F**, 60×. others: 40×.

dence to suggest that IGF-1 can enhance PYY expression. Because we observed that pancreatic regeneration is characterized by the accumulation of PYY-expressing cells, and given the potential for PYY and IGF-1 to promote cellular growth responses, we sought to determine if these factors could act in concert to facilitate the growth of the pancreatic epithelium. Therefore, we first performed double immunostaining for insulin-like growth factor receptor 1 (IGFR-1) and PYY in order to determine if IGF-1 responsiveness is also linked to PYY expression. Importantly, we observed that IGFR-1 was extensively expressed in the ducts and in the islets of the IFN-γ transgenic pancreas, as well as in the NOD islets. We observed that many of the PYY-expressing cells co-expressed IGFR-1 in the IFN-γ transgenic pancreas, as well as in the NOD pancreas (Fig. 6, Table 1).

IGF-1 binding to the IGF-1 receptor signals into the nucleus and mitochondrion via the mitogen-activated protein kinase (MAPK) pathway; Y1R has been shown to link MAPK phosphorylation and cell growth as well *(28)*. Therefore, to investigate whether PYY and IGF-1 can act in con-

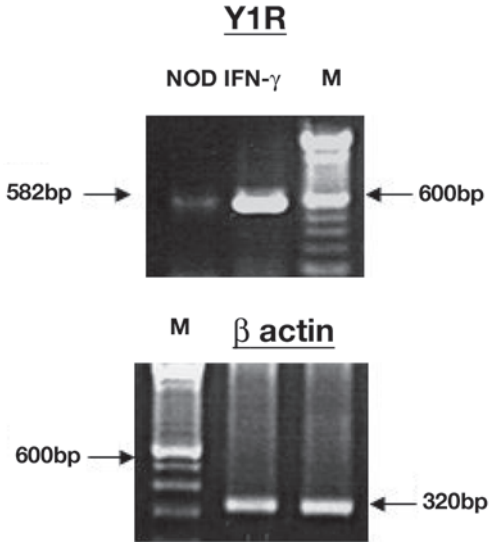

Fig. 3. RT-PCR analysis of the Y1 receptor. RT-PCR reactions for the detection of Y1 receptor mRNA were carried out using RNA isolated from either NOD or IFN-γ pancreas. Expression of the Y1 receptor in the NOD and IFN-γ pancreas is given in the upper panel. Control RT-PCR reactions to determine levels of β actin expression for comparative purposes in the NOD and IFN-γ RNA samples are given in the lower panel (the NOD sample is

cert to enhance cellular responses, we performed in vitro stimulation experiments. Because we have no direct way of isolating the PYY-expressing population from the pancreas, we created a PYY-expressing pancreatic epithelial cell line for our in vitro studies utilizing a mouse pancreatic epithelial cell line (LTPA CRL-2389). LTPA cells were derived from a spontaneous pancreatic adenocarcinoma and form ductular structures when injected into nude mice *(29)*. This cell line was chosen since it is pancreatic in origin and it expresses the Y1R mRNA (data not shown).

For these analyses, we transfected a PYY-containing plasmid into the LTPA cells to create a stable cell line. The expression of PYY was verified by immunohistochemical and RT-PCR analysis (Fig. 7). Subsequently, we treated the PYY-expressing LTPA cell line and the control non-transfected LTPA cell line with IGF-1 (10 n*M*). To test for cellular activation, we assessed the levels of pMAPK following IGF-1 stimulation by Western blotting, normalizing the pMAPK levels to actin to control for variations in loading. At the zero time point, we observed a slight increase (1.3-fold) in pMAPK levels in PYY-transfected cells compared to the control non-transfected cells, as might be expected since PYY has been shown to signal through pMAPK. In addition, whereas we observed a slight stimulation (1.3-fold) in pMAPK activity 2 min following IGF-1 treatment in the control cells, we observed significantly increased pMAPK activity 2 min (2.7-fold) and 5 min (2.1- fold) following IGF-1 treatment in the cells that express PYY (Fig. 8). Therefore, these data indicate that PYY and IGF-1 can act in concert to promote cellular responses in pancreatic epithelial cells.

Discussion

Our results show that PYY is expressed in increasing numbers of cells within the IFN-γ transgenic fetal and adult regenerating pancreas, suggesting a role for this peptide in endocrine cell formation and renewal. Our data also reveal that PYY/PDX-1 co-expressing cells are increased during pancreatic growth in the IFN-γ transgenic pancreas, suggesting that these cells might represent an expanded population of endocrine progenitor cells that are involved in pancreatic regeneration. We have also shown that PYY is frequently expressed in cells potentially responsive to IGF-1 (expressing IGFR-1), which mediates enhanced activation of MAPK in the presence of PYY.

PDX-1 expression is first initiated when the foregut endoderm commits to a pancreatic fate at embryonic d 9.5 in mice and is required for the specification of islet, acinar, and ductal tissues *(5,30–33)*. When PDX-1 is removed from mice by targeted mutagenesis, the embryos develop without a pancreas *(30)*. PDX-1 is expressed early in the whole pancreas and is later restricted to the islets in adults *(32)*. Interestingly, PDX-1 expression is induced in the epithelium of several models of pancreatic regeneration *(34,35)*, and it is also required for the development of intestinal endocrine cells *(5,31,36)*. Kahan and colleagues *(37)* reported that a subset of PDX-1 positive cells also express PYY in ES cell cultures at early time points before the appearance of mature islet hormones, suggesting that double positive PDX-1/PYY expressing cells may be islet precursor cells *(37)*. However, the existence of these cells, which we observe during fetal pancreas formation, have not previously been described in embryonic and adult pancreata. In addition, PYY and PDX-1 were co-expressed with islet hormones. Interestingly, PYY/PDX-1/insulin or glucagon triple positive cells are only co-expressed in cells in the fetus or adult IFN-γ transgenic pancreas. Therefore, we hypothesize that these PYY triple positive cells may represent an endocrine progenitor population that is involved in pancreatic endocrine maturation during islet neogenesis.

Interestingly, Schonhoff and colleagues reported that intestinal and pancreatic endocrine cells develop normally in the absence of peptide YY, with the exception of pancreatic polypeptide (PP) cells, indicating that peptide YY expression is not required for terminal differentiation of all islet cell types *(38)*. While these results indicate that PYY is dispensable for normal pancreas formation, it is possible that other factors could compensate for the loss of PYY during development. Furthermore, their data suggest that most beta cells and the majority of alpha cells are not descendants of the triple positive PYY/glucagon-positive/insulin-positive cells that appear during early pancreatic neogenesis *(38)*. Our data in the IFN-γ transgenic mouse support

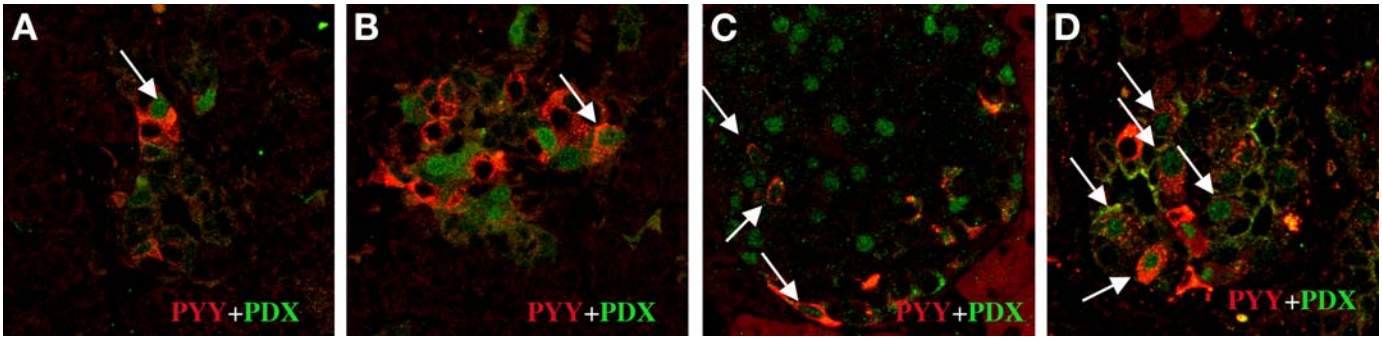

Fig. 4. Accumulation of PYY/PDX-1 cells during pancreatic development and regeneration. Immunofluorescence staining shows that PYY (indicated by red cytoplasmic staining) is expressed in PDX-1 positive cells (indicated by green nuclear staining) in the embryonic pancreas of NOD (**A**) and IFN-γ transgenic (**B**) fetuses at E16, as well as in the adult NOD pancreas (**C**) and in the adult regenerating pancreas (**D**). Arrows indicate double positive cells. Original magnification: 60×.

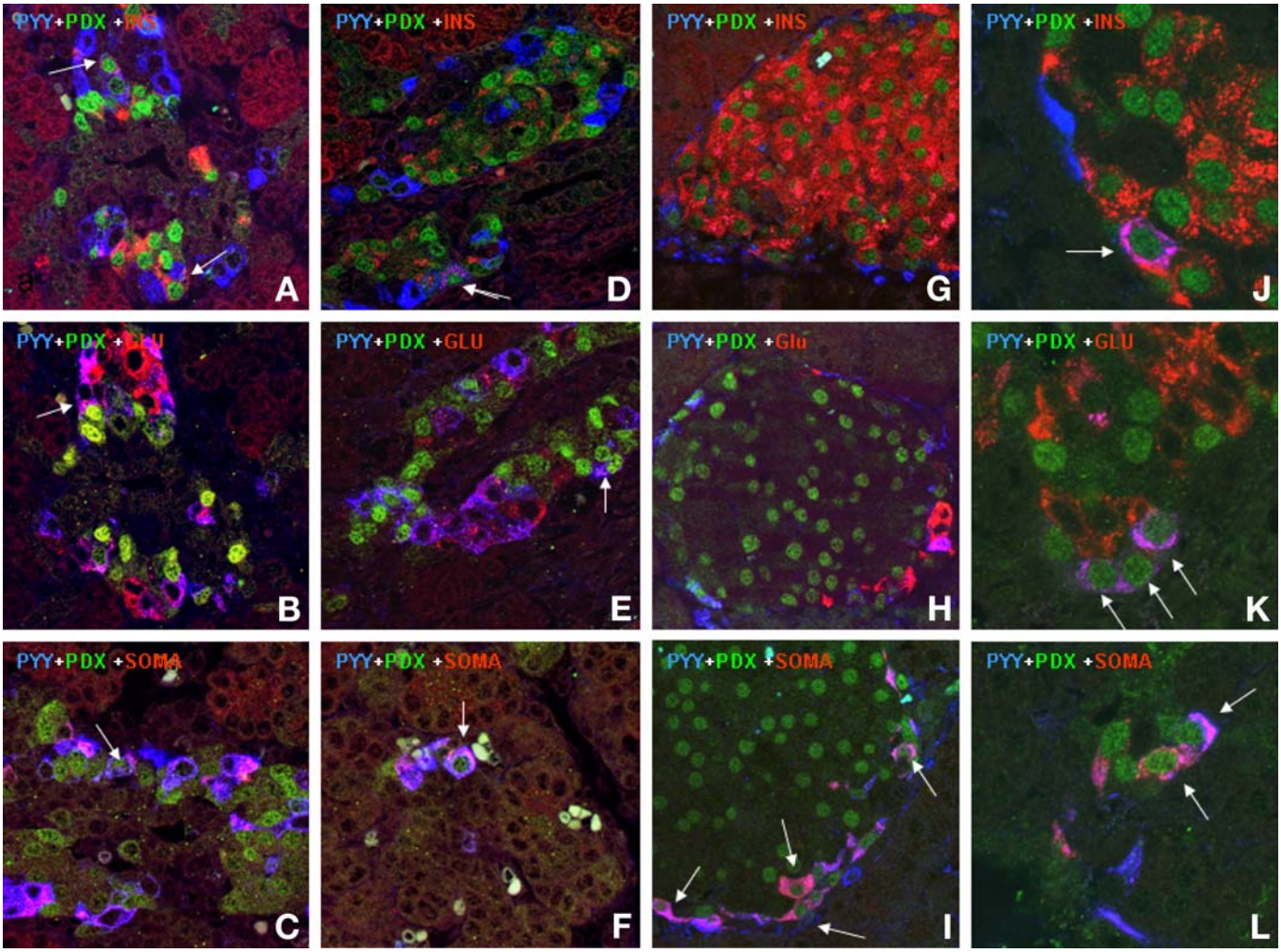

Fig. 5. Accumulation of PYY/PDX-1/islet hormone-expressing cells during pancreatic development and regeneration. NOD fetal (**A–C**), IFN-γ fetal (**D–F**), adult NOD (**G–I**), and adult IFN-γ (**J–L**) pancreas were stained and assessed by immunofluorescence utilizing antibodies to PYY/ PDX-1/insulin (**A,D,G,J**); PYY/ PDX-1/glucagon (**B,E,H,K**); PYY/ PDX-1/ somatostatin (**C,F,I,L**). Blue color reflects PYY staining, red color reflects insulin, glucagon, or somatostatin staining, and green color reflects PDX-1 nuclear staining. The purple color reflects cytoplasmic double positive staining (red plus blue). Arrows indicate triple positive cells. There were no PYY/PDX-1/insulin and PYY/PDX-1/glucagon triple positive cells observed in the adult NOD pancreas (**G**) and (**H**), respectively. Original magnification: **A–I**, 40×; **J–L**, 60×.

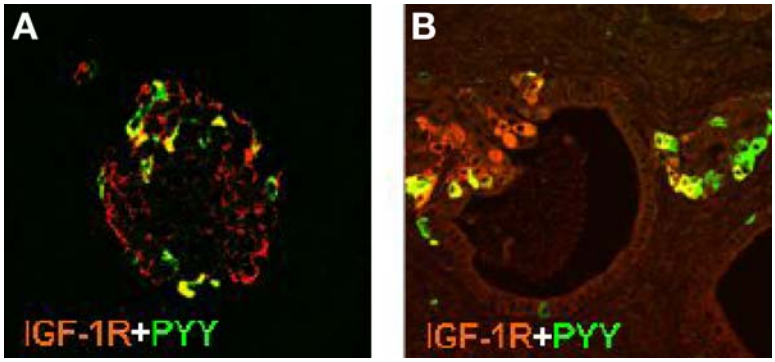

Fig. 6. PYY and IGFR-1 are co-expressed in the pancreas. Immunofluorescence double staining of IGFR-1 and PYY in the NOD (**A**) and IFN-γ pancreas (**B**). Original magnification: 40×.

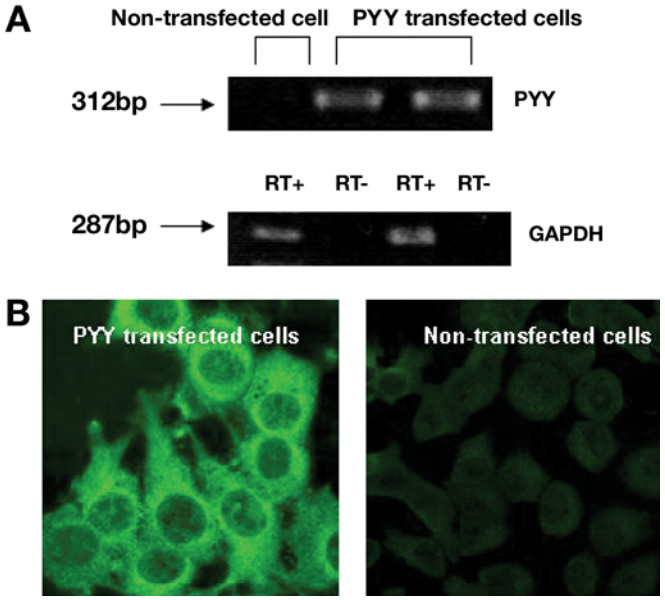

Fig. 7. Expression of PYY mRNA and protein in PYY-transfected epithelial cells. (**A**) RT-PCR analysis indicates PYY mRNA expression (top panel) in the transfected cells (duplicate loading is shown) but not in the non-transfected cells; control RT-PCR reactions for GAPDH mRNA (bottom panel) in the presence or absence of reverse transcriptase (RT) are shown for the non-transfected (left two lanes) and PYY-transfected (right two lanes) samples. (**B**) Immunofluorescence staining for PYY in the PYY transfected cells (left panel) and the control non-transfected cells (right panel). Original magnification: 60×.

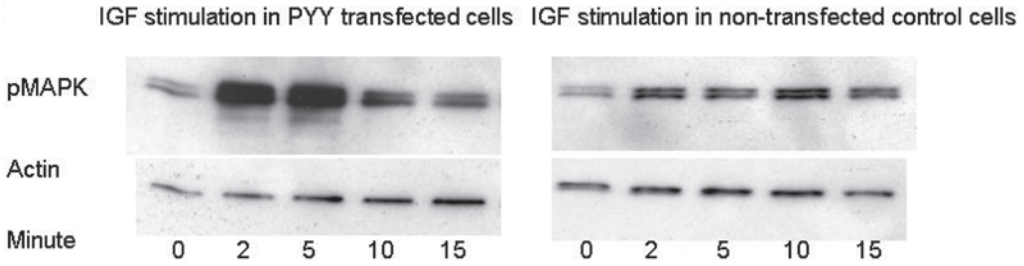

Fig. 8. pMAPK activation is enhanced by IGF-1 stimulation in PYY transfected cells. Western blot analysis utilizing antibodies directed against pMAPK and actin in the PYY transfected and the non-transfected pancreatic epithelial cell line LTPA CRL-2389 following IGF-1 stimulation 2, 5, 10, or 15 min. These data are representative of three independent experiments.

this finding, because we do not observe this cell type during adult pancreatic regeneration (Table 1).

We also found abundant PYY/insulin, PYY/GIP, and PYY/GLP-1 co-expressing cells in the IFN-γ transgenic pancreas; these cell types are normally observed only in the embryonic pancreas. Interestingly, it was reported recently that the combination treatment of EGF and the gastrointestinal peptide gastrin induces neogenesis of human islet beta cells from pancreatic ductal cells and increases the functional beta cell mass *(39)*. Therefore, it is possible that gut

peptide production may play a role in the differentiation of pancreatic endocrine cells.

IGF-1 treatment was reported to increase steady-state levels of PYY mRNA in a dose-related manner *(40)*. Similarly, it was also reported that growth hormone (GH) *(41)* and IGF- 1 can upregulate gastrointestinal PYY gene expression *(40)*. Interestingly, PYY stimulates growth in Y1R-expressing gut epithelial cells that are dependent on EGFR TK activity *(28)*. It is possible that PYY may prime cells to become responsive to differentiation-inducing growth factors. Kuribayashi and Ahmad showed that a similar pathway is required for IGF-1 stimulation of the MAPK family member ERK, which also requires EGFR TK activity *(42, 43)*. Therefore, increased MAPK activity in cells expressing the PYY gene may be mediated by collaboration between the IGF-1 signaling pathway and the G protein–coupled peptide PYY signaling pathway.

In summary, our work demonstrates the relationship between cells expressing PYY and pancreatic progenitor cells during pancreatic duct cell expansion and islet regeneration in adult animals. PYY-expressing cells may be important intermediates in the epithelial transition toward the pancreatic endocrine cell lineage. Our data also reveal that PYY may play a role in promoting pancreatic epithelial cell responsiveness to growth factors, such as IGF-1. The identification of critical hormones and growth factors that can facilitate new beta cell formation in adults is important as we search for strategies to treat type 1 diabetes, and utilization of this knowledge may be important for developing protocols that regulate pancreatic endocrine cell growth and differentiation.

Materials and Methods

Mice

Transgenic mice expressing IFN-γ in the pancreatic β cells in the non-obese diabetic (NOD) mouse background have been previously described *(21,23)*. NOD mice were imported from Jackson Labs in 2002, and are currently being bred at TSRI. All fetal NOD and fetal IFN-γ mice were E16. Adult NOD and IFN-γ transgenic mice were females, 8–9 wk of age. None of the animals used were diabetic. All work was performed under strict accordance with the TSRI animal care and use committee.

Immunohistochemistry and Immunofluorescence Staining

Tissues were fixed in Bouin's fixative and processed into serial paraffin sections using routine procedures. Three to five different embryos or adults of each genotype were analyzed per antibody. The primary antibodies were obtained from commercial sources: guinea pig anti-PYY (Accurate Chemical & Scientific, Westbury, NY); rabbit anti-GLP-1, rabbit-anti-GIP, rabbit-anti-PP, and rabbit anti-somatostatin (PhoenitidePeptide, Belmont, CA); rabbit anti-glucagon and rat-anti-BrdU (Dako, Carpentaria, CA); mouse anti-insulin (Research Diagnostics, Flanders, NJ); rabbit anti-PDX-1 (Chemicon, Temecula, CA). We have performed extensive studies screening for any cross-reactivity of these primary antibodies and have not observed any (see below). Secondary antibodies for DAB staining and for immunofluorescence staining were purchased from Vector Labs (Burlingame, CA). Antibodies were used at the concentrations recommended by the manufacturers. Slides were analyzed using a Bio-Rad MRC1024 laser scanning confocal microscope attached to a Zeiss Axiovert S1000TV microscope. Images were collected using Bio-Rad's Laser Sharp (2000) software plus Image J (NIH) software. For PYY/PDX-1/PP triple staining, PDX-1 DAB staining was performed first, followed by PYY and PP fluorescence double staining. The DIC (differential interference contrast) image and fluorescence image were merged using Image J V1.33 software. The controls utilized included slides incubated without primary antibodies, and the patterns of DAB staining were carefully compared with the patterns obtained using the fluorescently tagged secondary antibodies. For the triple staining studies, slides from consecutive sections were stained with each antibody alone, to compare the staining pattern with the co-localization slides. Furthermore, the experiments were repeated varying the sequence of addition of the primary antibodies and the specific fluorescent tags for each antigen marker. The results were carefully compared to screen for any cross-reactive staining artifacts. Each combination was repeated at least three times. In control studies, using serial section analysis and confocal microscopy, we also confirmed that the PYY antibody does not cross-react with NPY.

Plasmid Vector Construction

Total RNA extracted from intestinal tissue was reverse transcribed using the Superscript II kit (Invitrogen) and oligo-dT. The PYY coding sequence (GenBank accession number NM_145435) was amplified by PCR with the PCR Master Mix (Promega). The sense primer was GCTGCTAT GGTGGCGGTGCGC and the reverse primer was TCTTC ACCACTGGTCCAAACC. PCR conditions were: 95°C 5 min; 25 cycles (92°C 30 s; 45°C 30 s; 72°C 30 s); 72°C 10 min. PCR yielded a 312 bp fragment. The amplified product was cloned into the pcDNA3.1/V5-His©TOPO©TA expression plasmid (Invitrogen), according to the manufacturer's recommendations. The resulting positive clones were sequenced on both strands to verify that the PYY coding sequence was intact.

Cell Culture and Transfection

The mouse adenocarcinoma pancreatic epithelial cell line LTPA CRL-2389 *(44)* was purchased (ATCC, Manassas, VA). Cells were cultured at 37°C under air/CO_2 in ATCC medium: Eagle's minimum essential medium with 2 m*M* L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 m*M* non-essential amino acids,

1.0 m*M* sodium pyruvate, 10% fetal bovine serum, 1% (W/V) streptomycin and penicillin G. The day before transfection, cells were seeded onto 10 cm Petri dishes to achieve 50–80% confluence after overnight incubation. The cells were transfected with 24 μg of plasmid DNA complexed with 60 μL Lipofectamin® (Invitrogen). Stable transfectants were isolated from clones picked after 10 d of selection in medium containing 0.5 mg/mL G418.

Detection of Transcribed Genes by RT-PCR

Total RNA was extracted from vector containing control cells or cells transfected with PYY, or from IFN-γ transgenic or NOD pancreas using Trizol (Invitrogen). Samples were digested with RNase-free DNase I (Clontech) to eliminate genomic DNA. Reverse transcription was performed using oligo-dT primers with the Superscript II kit (Invitrogen). The following primer pairs were used: β-actin primers (Genbank accession number M12481) (forward, 5'TGC AGGAAGAAGGAGATTACTGCG3'; reverse, 5'AAAA CGCAGCTCAGTAACAGTCC3') to yield a 320 bp fragment. Y1 primers (Genbank accession number NM 010934) (forward, 5'GCTTCTTCTCTGCCCTTYGTG3'; reverse, 5'RGTCTCGTAGTCRTCGTCTCG3') to yield a 582 bp fragment. GAPDH primers (forward, 5'GGTGCTGAGTA TGTCGTG3'; reverse, 5'CTTCTGGGTGGCAGTGAT3') to yield a 287 bp fragment. The conditions for PCR were: 95°C 5 min; 30 cycles (92°C 30 s; 56°C 30 s; 72°C 30 s); 72°C 10 min.

Western Blot Analysis

Freshly cultured cells of the PYY-transfected and non-transfected epithelial cell line LTPA were lysed in RIPA buffer containing 20 m*M* Tris pH 7.5, 1 m*M* EDTA, 140 m*M* NaCl, 1% Nonidet P-40, 1 m*M* orthovanadate, 1 m*M* phenylmethylsufonyl fluoride, and 10 μg/mL aprotinin. The cell lysates were solubilized in Laemmli's buffer and boiled for 5 min and the proteins were resolved on a 12% SDS-PAGE gel. The proteins were subsequently transferred to nitrocellulose membranes and immunoblotted with rabbit anti-phosphorylated 42/44 MAP Kinase (1:1000, Cell Signaling Technology, Beverly, MA), followed by a horseradish peroxidase–conjugated anti-rabbit IgG (1:2000; Cell Signaling Technology, Beverly, MA). Immunoreactive proteins were visualized by enhanced chemiluminescent (ECL) substrate to detect HRP according to the manufacturer's instruction (Pierce, Rocktord, IL). Membranes were stripped with NaOH (0.8 g in 100 mL water) and reblotted with the mouse mAb to actin (ICN Biomedical, Auroa, OH) to confirm equal protein loading. The data were quantitated by using NIH Image J software analysis.

IGF Stimulation and pMAPK Assay

The PYY cDNA-transfected cells and non-transfected control cells (1×10^5) were plated in 24 well plates to achieve 80% confluence. They were serum starved overnight and then stimulated with 10 ng/mL recombinant human IGF-1 (Research Diagnostics, Flanders, NJ) for 0, 2, 5, 10, and 15 min at 37°C. The cells were then lysed with RIPA buffer as described in the preceding section, after which they were solubilized on ice, and the proteins were resolved on a 12% SDS-PAGE gel.

Statistical Analysis

The percentages of double and triple positive cells within the total number of PYY positive cells were compared by the Student's *t*-test.

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