Characterisation of Ppy-lineage cells clarifies the functional heterogeneity of pancreatic beta cells in mice

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Abstract
Aims/hypothesis Pancreatic polypeptide (PP) cells, which secrete PP (encoded by the Ppy gene), are a minor population of pancreatic endocrine cells. Although it has been reported that the loss of beta cell identity might be associated with beta-to-PP cell-fate conversion, at present, little is known regarding the characteristics of Ppy-lineage cells.
Methods We used Ppy-Cre driver mice and a PP-specific monoclonal antibody to investigate the association between Ppy-lineage cells and beta cells. The molecular profiles of endocrine cells were investigated by single-cell transcriptome analysis and the glucose responsiveness of beta cells was assessed by Ca2+ imaging. Diabetic conditions were experimentally induced in mice by either streptozotocin or diphtheria toxin.
Results Ppy-lineage cells were found to contribute to the four major types of endocrine cells, including beta cells. Ppy-lineage beta cells are a minor subpopulation, accounting for 12–15% of total beta cells, and are mostly (81.2%) localised at the islet periphery. Unbiased single-cell analysis with a Ppy-lineage tracer demonstrated that beta cells are composed of seven clusters, which are categorised into two groups (i.e. Ppy-lineage and non-Ppy-lineage beta cells). These subpopulations of beta cells demonstrated distinct characteristics regarding their functionality and gene expression profiles. Ppy-lineage beta cells had a reduced glucose-stimulated Ca2+ signalling response and were increased in number in experimental diabetes models.
Conclusions/interpretation Our results indicate that an unexpected degree of beta cell heterogeneity is defined by Ppy gene activation, providing valuable insight into the homeostatic regulation of pancreatic islets and future therapeutic strategies against diabetes.
Data availability The single-cell RNA sequence (scRNA-seq) analysis datasets generated in this study have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE166164 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166164).
Introduction

The islets of Langerhans consist of alpha, beta and delta cells and a fourth type of islet cell, namely, pancreatic polypeptide (PP) cells. PP cells are located at the periphery of the islets and secrete PP [1–3], encoded by the Ppy gene. PP is a member of the neuropeptide Y (NPY) family of peptides, which also includes peptide YY (PYY) and NPY, all reported to be involved in appetite regulation [4, 5]. However, the precise physiological functions of these peptides, including their roles in glucose homeostasis,
remain poorly understood. A previous study demonstrated that Ppy-lineage cells were indispensable for the differentiation of a substantial fraction of endocrine cells by the diphtheria toxin (DT)-induced ablation of Ppy-lineage cells [6]. However, owing to the lack of specificity of the available PP antibody and the fidelity of the Ppy promoter used, the precise roles of Ppy-lineage cells have not yet been clarified.

Beta cell failure is the main hallmark of type 2 diabetes and it has been reported that inactivation of pancreatic and duodenal homeobox 1 (encoded by Pdx1) in mature beta cells results in the loss of beta cell identity and a beta-to-alpha cell fate conversion in vivo [7, 8]. Moreover, a reduction in the gene dose of Pdx1 by heterozygous deletion of its evolutionarily conserved enhancer region caused an increase not only in the number of alpha cells but also in the number of PP cells in the pancreatic islets of adult mice, together with insufficient beta cell development [9]. In another report, Rip-Cre-mediated deletion of Abcc8 in mice caused a sustained increase in intracellular Ca2+ concentrations, whereas beta cells underwent a fate switch to PP cells [10]. These findings suggest that unhealthy beta cells may shift not only towards alpha cells but also towards a PP cell fate.

Previous reports have highlighted the functional heterogeneity of beta cells. Many of these reports have particularly focused on the immature population of beta cells. For instance, they characterised immature beta cells located at the islet periphery, which are called ‘virgin beta cells’ [11], and low GLUT2-expressing immature beta cells with robust proliferative potential and resistance to streptozotocin (STZ)-induced cytotoxicity [12–16]. Other reports described Wnt-signal-regulated Fltp (also known as Cfap126)-positive beta cells [17] or NPY-positive immature beta cells [18]. The characterisation of beta cell subpopulations from the viewpoint of functionality and plasticity is especially important in the field of diabetes treatment for the further identification of beta cell sources with robust regenerative potential.

Methods

Animals The study protocol was reviewed and approved by the Committee for Institutional Animal Care and Experimentation at Gunma University. All animals were housed in specific pathogen-free barrier facilities, maintained under a 12 h light/dark cycle, fed standard rodent food (CLEA Japan, Tokyo, Japan) and had access to water ad libitum. Male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used in all experiments, except for the single-cell RNA sequence (scRNA-seq) analysis and Ca2+ imaging experiments, in which male B6.Cg-Tg(Ins1-EGFP)1Hara/J (MIP-GFP) mice (stock number 006864; The Jackson Laboratory, Bar Harbor, ME, USA; https://www.jax.org/strain/006864) [19], which have a mixed background of CD-1 and C57BL/6J, were used. Ppy-Cre knockin mice were established previously (12-week-old male mice, weighing 23–26 g, were used), with the protein-coding region of Ppy in exon 2 being precisely replaced with that of NLS-Cre [20]. Ppy-DTA knockin mice, in which the diphtheria toxin A (DTA)-coding sequence was inserted in the Ppy locus, were also established by CRISPR/Cas9-mediated genome editing (12-week-old male mice, weighing 23–26 g, were used). Ins-TRI (C57BL/6-human INS promoter-DTR TRECK) transgenic mice [21] have been described previously. B6.129X1-Gt(ROSA)26Sor tm14(CAG-tomato)Hze/J (Rosa26-YFP) mice (stock number 006148; The Jackson Laboratory; https://www.jax.org/strain/006148) and B6;129S6-Gt(ROSA)26Sor tm14(CAG-tomato)Hze/J (Rosa26-tdTomato) mice (stock number 007908; The Jackson Laboratory; https://www.jax.org/strain/007908) were used as reporter mice. Randomisation and blinding were not carried out in this study. Mice outside of the indicated weight range were excluded from the experiments. We repeated all experiments at least twice, except for scRNA-seq analysis, which was validated by additional immunohistochemistry. To perform the IPGTT, 2 g/kg of glucose was injected intraperitoneally into mice and blood glucose levels were measured at the indicated times.

Immunohistochemistry and cell quantification Harvested pancreases were divided into the head and tail and fixed in 4% vol./vol. paraformaldehyde at 4°C overnight. Then, pancreases were immersed in sucrose solution for 24 h prior to embedding in O.C.T compound (Sakura Finetek Japan, Osaka, Japan). Frozen pancreas blocks were sectioned at 14 μm thickness and immunostained. The following primary antibodies were used at the stated dilutions: guinea pig anti-insulin (INS; 1:100; DAKO, Glostrup, Denmark; catalogue no. IR002); mouse anti-glucagon (GCG; 1:1000; Abcam, Cambridge, UK; catalogue no. ab10988); rabbit anti-GCG (1:1000; Abcam; catalogue no. ab92517); rabbit anti-somatostatin (SST; 1:1000; Peninsula Laboratories, San Carlos, CA, USA; catalogue no. T-4103); mouse anti-PP (1:1000; IBL, Gunma, Japan; catalogue no. 23-2D3 [20]); chicken anti-green fluorescent protein (GFP; 1:1000; Abcam; catalogue no. ab13970); rabbit anti-chromogranin-A (1:100; Abcam; catalogue no. ab15160); rabbit anti-GLUT2 (1:200; Abcam; catalogue no. ab54460); rabbit anti-urocortin 3 (UCN3; 1:500; Phoenix Pharmaceuticals, Burlingame, CA, USA; catalogue no. H-019-29); rat anti-tetraspanin 8 (TSPAN8; 1:50; R&D Systems, Minneapolis, MN, USA; catalogue no. MAB6524); rabbit anti-folate receptor 1 (FOLR1; 1:100; Thermo Fisher Scientific, Waltham, MA,
KRB buffer. Cells were loaded with 2 mol/l Fura 2-AM prior to injection, and a single dose of 200 mg/kg was injected intraperitoneally into 6-week-old male (18–22 g). DT was dissolved in PBS prior to injection and a single dose of 100 ng/kg was injected intraperitoneally into 6-week-old male Ppy-Cre;Rosa26-YFP;Ins-TR1 mice. Mice were killed at 7 days or 5 weeks after the injection.

**Ca²⁺ imaging** Islets were isolated from the pancreases of 8- to 10-week-old MIP-GFP;Ppy-Cre;Rosa26-tdTomato mice (22–25 g), as described previously [23]. Isolated beta cells were seeded onto poly-L-lysine-coated glass-bottomed dishes (MatTek Corporation, Ashland, MA, USA) 24 h before observation. Cells were washed with modified KRB buffer. Cells were loaded with 2 μmol/l Fura 2-AM (Dojindo, Kumamoto, Japan) and 0.01% vol./vol. Cremophor EL (Sigma-Aldrich) in a humified incubator (95% air and 5% CO₂ at 37°C) for 30 min. After washing with modified KRB, the cells were visualised using an Olympus UPlanAPO 10× water objective lens (Olympus). To obtain fluorescence images, the AQUACOSMOS/ASHURA 3CCD-based fluorescence energy transfer imaging system (Hamamatsu Photonics, Tokyo, Japan) was used. The ratio of 340/380 nm fluorescence was calculated and values were normalised to F0.

**Statistical analysis (other than for scRNA-seq analysis data)** Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Data were expressed as means ± SEMs. Statistical comparisons between two groups were performed by the two-tailed Student’s t test, and one-way ANOVA followed by the Bonferroni post hoc test was used for comparisons between groups. A p value of less than 0.05 was considered to indicate a statistically significant difference between groups.

**scRNA-seq analysis** Islet cells were isolated from an 8-week-old male MIP-GFP;Ppy-Cre;Rosa26-tdTomato mouse. Cell quality was checked under a microscope before loading cells onto the chip, and cell viability was confirmed to be 84%. Cells that met one or more of the following three criteria, including doublets, were excluded from further analysis: (1) cells with 200 or less, or 8000 or more detected genes per cell; (2) cells with 80,000 or more unique molecular identifiers (UMIs) per cell; and (3) cells with 5% or more mitochondrial gene UMIs / the number of total gene UMIs per cell. Isolated single cells were loaded for scRNA-seq analysis using the Chromium System (10x Genomics, Pleasanton, CA, USA), following the manufacturer’s protocol of Single Cell 3′ Library kit v3.1. RNA libraries were sequenced on a Nova Seq6000 platform (Illumina, San Diego, CA, USA) with the following sequencing parameters: 28 bp read 1; 8 bp index 1; 91 bp read 2.

**Analysis of scRNA-seq data** Sequencing data were aligned to the mouse genome, Genecode release M25/GRCm38.p6 with GFP and tdTomato sequences, and UMI-collapsed with the Cell Ranger (v3.1.0) pipeline (10x Genomics).

For pre-processing of data, the Seurat (v3.1) R package for quality control filtering was used. Genes that were detected in at least three cells, and cells with more than 200 detected genes were selected. In addition, only cells with less than 8000 detected genes, less than 5% of mitochondrial genes, and less than 80,000 detected UMIs were retained.

Normalisation was performed using the global-scaling normalisation method, which normalises gene expression measurements for each cell by total gene expression, and then multiplies them by 10,000, and finally log-transforms the results. The variable genes were identified using the ‘vst’ method. Data were scaled to regress out the cell cycle score, the number of UMIs and the percentage of mitochondrial gene expression.

Based on the extracted variable genes, dimensionality reduction was performed by principal component analysis. A total of 50 dimensions were used for whole-cell analysis and 75 dimensions for beta cell analysis. Cell clustering was performed according to the Shared Nearest Neighbors method and was visualised in two-dimension by uniform manifold approximation and projection (UMAP).

Differentially expressed genes (DEGs) were identified by comparing the expression levels of the cluster cells with all other cells. For the analysis of statistical significance, the likelihood ratio test was performed by modelling as a two-part generalised regression model using model-based analysis of single-cell transcriptomics [24], and the Bonferroni method was used for multiple comparison adjustment. The
mean fold change of expression was calculated by the following formula:

\[ \text{mean fold change} = \frac{\text{expression in treated group}}{\text{expression in control group}} \]
**Legend**

- **a**: Diagram of the Ppy gene showing Exon 1 and Exon 2 (coding region) with ATG and DTA.
- **b**: Fluorescence images of PP, CHGA, and Merge for Ppy-DTA.
- **c**: Fluorescence images of GCG and INS for WT and Merge.
- **d**: Fluorescence images of GCG and INS for Ppy-DTA and Merge.
- **e**: Bar graph showing GCG+ cells/total islet cells (%) for WT and Ppy-DTA.
- **f**: Fluorescence images of SST and INS for WT and Merge.
- **g**: Fluorescence images of SST and INS for Ppy-DTA and Merge.
- **h**: Bar graph showing SST+ cells/total islet cells (%) for WT and Ppy-DTA.
- **i**: Graph showing Beta cell mass (mg) for WT and Ppy-DTA.
- **j**: Graph showing Blood glucose (mmol/l) over time (min) for WT and Ppy-DTA.

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Fig. 2 Analysis of Ppy-DTA mice. (a) Diagram of genotype of Ppy-DTA knockin mice. (b) Immunohistochemical analysis revealed almost complete deletion of PP cells in the head of the pancreas of adult Ppy-DTA mice. Representative images of $n = 4$ mice. CHGA, chromogranin-A. Scale bar, 50 μm. (c, d) Immunohistochemical analysis of INS$^+$ and GCG$^+$ cells in the head of the pancreas of adult WT mice (e) and Ppy-DTA mice (d). Representative images of $n = 4$ mice. Scale bars, 50 μm. (e) Ratio of GCG$^+$ cells to total islet cells in adult WT vs Ppy-DTA mice ($n = 4$). (f, g) Immunohistochemical analysis of INS$^+$ and SST$^+$ cells in the head of the pancreas of adult WT mice (f) and Ppy-DTA mice (g). Representative images of $n = 4$ mice. Scale bars, 50 μm. (h) Ratio of SST$^+$ cells to total islet cells in adult WT vs Ppy-DTA mice ($n = 4$). (i) Beta cell mass in the head of the pancreas of Ppy-DTA mice vs WT mice ($n = 6$). (j) Results of IPGTT performed on adult WT mice and Ppy-DTA mice. Data are shown as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, (two-tailed Student’s $t$ test)
corresponding to the Ppy-Cre-mediated activation of the tdTomato gene. Signals of tdTomato were detected not only in PP cells but also in alpha and delta cells (Fig. 3f). Therefore, the above data are consistent with the results that a significant fraction of alpha and delta cells have a history of Ppy activation (Fig. 1g).

To gain further insight into the heterogeneity of beta cells, transcriptomic characterisation of INS+ beta cells containing clusters beta-1, -2 and -3 was performed (see Fig. 3a). Refined clustering of beta cells demonstrated the presence of seven clusters (Fig. 3g-l, ESM Fig. 2b). In total, 24.6% of beta cells expressed Ppy mRNA and, importantly, such cells were enriched in clusters 4, 6 and 3, rather than being scattered among all beta cells, which is consistent with the enrichment of tdTomato mRNA (Fig. 3k,l). In cluster 3, however, the enrichment of Ppy and tdTomato mRNA was highly heterogeneous, in contrast to the homogeneous and clear enrichment of Ppy and tdTomato mRNA in clusters 4 and 6 (violin plots in Fig. 3k,l). For this reason, clusters 4 and 6 were annotated as Ppy-lineage beta cells and clusters 0, 1, 2 and 5 were annotated as non-Ppy-lineage beta cells in this study, and they were subjected to further analysis, including DEG and Gene Ontology (GO) analyses.

DEG analysis between Ppy-lineage and non-Ppy-lineage beta cells (ESM Table 3) demonstrated that Ppy-lineage beta cells show an upregulation of some of the signature genes for PP cells, including Tspan8, Fohr1, Sppl and Ppy in addition to Ppy, indicating that these beta cells share molecular characteristics with canonical PP cells. Moreover, these beta cells showed the downregulation of key genes for beta cell maturation and INS secretion (e.g. Slc2a2, Ucn3, Ins1, Ins2, Mafa, Nkx6.1 [also known as Nkx6-1], Neurod1, G6pc2, Sytl4 and Ero1lb [also known as Ero1b]; ESM Table 3). GO analysis of the DEGs identified the upregulation of several cell pathways, including the cell proliferation pathway, and the downregulation of the transport and INS secretion pathway in Ppy-lineage beta cells compared with non-Ppy-lineage beta cells (Fig. 3m,n).

**TSPAN8 is a marker for Ppy-lineage beta cells**

Next, to validate the molecular profiles of Ppy-lineage beta cells shown by scRNA-seq analysis at the protein level, we performed immunohistochemical analysis of some of the upregulated and downregulated markers extracted from DEG analysis and compared them in Ppy-lineage and non-Ppy-lineage beta cells. Among the markers analysed, we found that TSPAN8 was specifically expressed in Ppy-lineage beta cells. TSPAN8 is a member of the tetraspanin transmembrane protein family and has been reported as a PP-cell signature gene [26]. Approximately 40% of Ppy-lineage beta cells expressed TSPAN8, and, importantly, no expression was observed in non-Ppy-lineage beta cells (Fig. 4a).

As a negative marker, we found a low expression of GLUT2/Slc2a2 in Ppy-lineage beta cells at the islet periphery (Fig. 4b). We also observed a lower expression of UCN3, a beta cell maturation marker, in Ppy-lineage beta cells compared with non-Ppy-lineage beta cells (Fig. 4c). These immunohistochemical results further confirm the PP-cell-like molecular characteristics and immaturity of Ppy-lineage beta cells indicated by scRNA-seq analysis.

**Ppy-lineage beta cells demonstrate reduced glucose-stimulated Ca2+ responses**

Our scRNA-seq analyses demonstrated lower expression of a group of genes implicated in beta cell maturation and INS secretion in Ppy-lineage beta cells compared with non-Ppy-lineage beta cells (ESM Table 3). We hence postulated that Ppy-lineage beta cells may demonstrate decreased glucose responsiveness. To assess this, islets from 8- to 10-week-old MIP-GFP;Ppy-Cre;Rosa26 tdTomato mice were dispersed into single cells and then the increase in intracellular Ca2+ concentrations ([Ca2+]i), which is the final trigger of INS exocytosis, was measured in isolated Ppy-lineage (GFP+) and non-Ppy-lineage (GFP– tdTomato–) beta cells during exposure to basal (2.8 mmol/l) and high (25 mmol/l) glucose concentrations (Fig. 5a). Single-cell Ca2+ imaging demonstrated that 25 mmol/l glucose increased [Ca2+]i in both beta cell types (Fig. 5b). The peak Ca2+ response was smaller in Ppy-lineage beta cells than in non-Ppy-lineage beta cells (Fig. 5c,d). Accordingly, the AUC of [Ca2+]i was significantly decreased in Ppy-lineage beta cells. A similar increase in [Ca2+]i was observed in both beta cell types when cells were depolarised by a 40 mmol/l K+ stimulation (Fig. 5e). Taken together, the results of our physiological studies demonstrated that Ppy-lineage beta cells show a smaller glucose-stimulated Ca2+ response, supporting our molecular findings.
Ppy-lineage beta cells with low GLUT2 expression become dominant after STZ administration to mice

To characterise the behaviour of Ppy-lineage beta cells in diabetes, a single dose of 200 mg/kg STZ was administered to 6-week-old male Ppy-Cre;Rosa26-YFP mice. Mice were killed after 7 days and their pancreatic tissue was analysed. Mice became hyperglycaemic shortly after STZ injection and Ppy-lineage beta cells became relatively dominant (Fig. 6a,b), accounting for 48.8% of the remaining beta cells. TSPAN8+ INS+ beta cells also became dominant (ESM Fig. 3a–c), consistent with the results shown in Fig. 4a. However, the induction of hyperglycaemia by the administration of DT to 6-week-old male Ppy-Cre;Rosa26-YFP;Ins-TR1 mice, in which beta cells can be ablated by the administration of DT acting on the DT receptors that they express, did not change the proportion of Ppy-lineage beta cells among the remaining beta cells 7 days after DT administration (Fig. 6c,d). Considering that STZ is transported into beta cells via GLUT2, the increased population of Ppy-lineage beta cells in the islets of mice with STZ-induced diabetes is thought to correspond to the Ppy-lineage beta cells with low expression of Slc2a2/GLUT2 observed by scRNA-seq analysis and immunohistochemistry. Intriguingly, Ppy-lineage beta cells became dominant also in Ppy-Cre;Rosa26-YFP;Ins-TR1 mice 5 weeks after DT administration (Fig. 6e,f), suggesting that Ppy-lineage beta cells are more resistant to prolonged hyperglycaemia than non-Ppy-lineage beta cells.

Discussion

In this study, lineage tracing and scRNA-seq analysis using lineage tracers were performed to characterise PP cells and Ppy-lineage cells. YFP and tdTomato were detected in alpha and delta cells, in addition to beta cells (Figs 1c–e, 3f), suggesting that a substantial fraction of endocrine cells have a history of Ppy gene activation. Indeed, scRNA-seq analysis of adult WT islets showed that a subpopulation of alpha, beta and delta cells express Ppy mRNA, and the coexpression of PP and other endocrine hormones in these cells was confirmed at the protein level (ESM Fig. 1a–c). The substantial reduction of alpha and delta cell numbers in Ppy-DTA mice (Fig. 2c–h) also supports the idea that activation of the Ppy gene occurs in these endocrine cell types.

Ppy-lineage cells can contribute to all four major types of endocrine cells. The results of cell-lineage tracing may simply reflect the heterogeneity of beta cells, in which a subpopulation of beta cells express Ppy. Beta cell heterogeneity has attracted much attention and provides additional insight into the homeostatic regulation of islet function in the progression and treatment of diabetes. ‘Virgin beta cells’ [11] showed similar characteristics to the Ppy-lineage beta cells, with low levels of GLUT2 expression and their localisation at the islet periphery. However, these beta cells were shown to be induced via alpha-to-beta transdifferentiation, as assessed by Gcg-Cre-mediated lineage tracing. Therefore, ‘virgin beta cells’ were considered as a neogenic niche, a continuous

![Fig. 4](image-url)
supply of cells provided from alpha cells. There are reports of other beta cell subpopulations showing low levels of GLUT2 expression, robust proliferative capacity and resistance to STZ-induced cytotoxicity [12–16], possibly sharing the characteristics of Ppy-lineage beta cells shown in the present study (i.e. immaturity and impaired function confirmed by the low expression of GLUT2 and UCN3 and a low glucose-stimulated Ca\(^{2+}\) response). However, these previous studies lacked analysis using a molecular marker that identifies specific types of beta cells, and we propose that Ppy gene expression is a candidate for such a marker.

Additional analysis of the pathophysiological characteristics of Ppy-lineage beta cells in diabetic conditions using Ppy-CreERT2 knockin mice, which we generated together with Ppy-Cre knockin mice (data not shown), is expected to provide further useful information. However, as the pulse-and-chase labelling efficiency of these mice (particularly in their Ppy-lineage beta cells) is low, it is difficult to analyse the dynamics of Ppy-lineage beta cells within a specific time window in these mice. This insufficient labelling efficiency of Ppy-lineage beta cells might be owing to suppressed activity of the Ppy promoter during their differentiation into beta cells. The establishment of tools and techniques for detecting low levels of Ppy gene transcription in endocrine cells within a specific time window will resolve this limitation of our present study.

In this study, we found that 40% of the Ppy-lineage beta cells express TSPAN8 (Fig. 4a), a member of the tetraspanin transmembrane protein family that is mainly expressed in the gastrointestinal tract in both mice and humans [27]. Tspan8 has been reported as a PP-cell signature gene [26] that is highly expressed in human pancreatic ductal progenitors [28]. Other PP-cell signature markers, such as PP (ESM Fig. 1a), FOLR1 and SPP1 (ESM Fig. 4a,b), were rarely merged.

**Fig. 5** Comparison of glucose-stimulated Ca\(^{2+}\) influx between Ppy-lineage and non-Ppy-lineage beta cells. (a) Diagram of the method of identification of Ppy-lineage and non-Ppy-lineage beta cells using two reporters, GFP and tdTomato. (b) Ca\(^{2+}\) response to high-glucose stimulation in non-Ppy-lineage beta cells (green cells; n = 223) and Ppy-lineage beta cells (yellow cells; n = 84) from a total of n = 10 mice (n = 5 mice per group). Fura2-ratio is the ratio of 340/380 nm fluorescence. (c, d) Representative time course of Ca\(^{2+}\) responses in green cells (n = 111 cells) (e) and yellow cells (n = 44 cells) (d) from n = 1 mouse at glucose concentrations of 2.8 mmol/l (G2.8) and 25 mmol/l (G25). (e) Ca\(^{2+}\) response to 40 mmol/l K\(^{+}\) stimulation in non-Ppy-lineage beta cells (green cells; n = 62) and Ppy-lineage beta cells (yellow cells; n = 49) from a total of n = 8 mice (n = 4 mice per group). Data are shown as the mean ± SEM. **p < 0.01, (two-tailed Student’s t test)
with Ppy-lineage beta cells [26, 29]. Some recent reports showed that TSPAN8 regulates cell proliferation, invasion and metastasis in various types of tumours, including pancreatic adenocarcinoma [30]. Further investigation of the physiological role of TSPAN8 in Ppy-lineage beta cells will also clarify the physiological role of Ppy-lineage beta cells.

**Fig. 6** Characteristics of Ppy-lineage beta cells under hyperglycaemic conditions. (a) Immunohistochemical analysis of YFP + INS cells (arrowheads) in the head of the pancreas of Ppy-Cre;Rosa26-YFP mice 7 days after 200 mg/kg STZ injection. Scale bar, 50 μm. (b) Ratio of YFP + INS + cells to total INS + cells in the head of the pancreas of Ppy-Cre;Rosa26-YFP mice 7 days after 200 mg/kg STZ injection compared with control mice (n = 4). Control mice were treated with citrate buffer. (c) Immunohistochemical analysis of YFP + INS + cells (arrowheads) in the head of the pancreas of Ppy-Cre;Rosa26-YFP;Ins-TR1 mice 7 days after 100 mg/kg DT injection. Scale bar, 50 μm. (d) Ratio of YFP + INS + cells to total INS + cells in the head of the pancreas of Ppy-Cre;Rosa26-YFP;Ins-TR1 mice 7 days after 100 mg/kg DT injection compared with control mice (n = 4). Control mice were treated with PBS. (e) Immunohistochemical analysis of YFP + INS + cells (arrowheads) in the head of the pancreas of Ppy-Cre;Rosa26-YFP;Ins-TR1 mice 5 weeks after 100 mg/kg DT injection. Scale bar, 50 μm. (f) Ratio of YFP + INS + cells to total INS + cells in the head of the pancreas of Ppy-Cre;Rosa26-YFP;Ins-TR1 mice 5 weeks after 100 mg/kg DT injection compared with control mice (n = 4). Control mice were treated with PBS. Ctrl, control mice. Data are shown as the mean ± SEM. **p < 0.01 (two-tailed Student’s t test)
particularly those associated with their proliferative characteristics. Regarding cell proliferation, DEG analysis identified the upregulation of cell proliferation markers in Ppy-lineage beta cells (e.g. Jun, Junb, Fyn, Fgfr1, Pdgfb and Regl). Of these enriched genes, Pdgfb and Regl are of particular interest, as they have been reported to regulate beta cell proliferation during ageing and in some models of diabetes [31–34]. The upregulation of these genes associated with beta cell proliferation indicates that Ppy-lineage beta cells are a promising therapeutic target in diabetes.

In summary, we found an unexpected degree of beta cell heterogeneity while investigating the characteristics of Ppy-lineage cells. High-resolution single-cell transcriptome analysis demonstrated that this subpopulation of beta cells shows unique functional characteristics and gene expression profile. We can speculate that Ppy-lineage beta cells with low levels of GLUT2 and UCN3 expression may be generated by the pancreas to survive conditions of metabolic stress under hyperglycaemia, at the expense of glucose-induced INS secretion. Identification of this unique subpopulation of beta cells is expected to provide valuable insight into the homeostatic regulation of islet function and contribute towards the development of novel therapeutic strategies to cure diabetes.

Supplementary Information The online version contains peer-reviewed but unedited supplementary material available at https://doi.org/10.1007/s00125-021-05560-x.

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Data availability The scRNA-seq analysis datasets generated in this study have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE166164 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166164). Other datasets are available from the corresponding author upon reasonable request.

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Contribution statement YF and TF conceived and designed the study. TF, YN, AF and TS performed the animal experiments and acquired the data. AH, KN, MS, KK, TMi, MT, MM and TMs contributed to resource preparation and data acquisition. TF and YF wrote the original draft. YN, AF, TS, AH, KN, MS, KK, TMi, MT, MM, TMs, TY and HW interpreted the data and revised the manuscript for valuable intellectual content. All authors gave final approval of the version to be published. YF is the guarantor of this work, has full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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