Activation of GLP-1 and gastrin signalling induces in vivo reprogramming of pancreatic exocrine cells into beta cells in mice

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Abstract

Aims/hypothesis Lineage conversion of non-beta cells into insulin-producing cells has been proposed as a therapy for the cure of diabetes. Glucagon-like peptide-1 (GLP-1) and its derivatives can induce beta cell neogenesis in vitro and beta cell mass expansion in vivo, but GLP-1 signalling has not been shown to regulate cell fate decisions in vivo. We therefore tested the impact of GLP-1 receptor (GLP1R) expression on beta cell differentiation in vivo.

Methods Mice overexpressing GLP1R in pancreatic exocrine cells were generated by Cre-mediated recombination in sex-determining region Y-box 9 (SOX9)-expressing cells and then treated with exendin-4 and/or gastrin. Histological analysis was performed to detect cellular reprogramming from the exocrine lineage into insulin-producing cells.

Results Whereas no newly generated beta cells were detected in the mice treated with exendin-4 alone, treatment with gastrin only induced the conversion of exocrine cells into insulin-producing cells. Furthermore, the overexpression of GLP1R, together with gastrin and exendin-4, synergistically promoted beta cell neogenesis accompanied by the formation of islet-like clusters. These newly generated beta cells expressed beta cell specific transcription factors, such as pancreatic and duodenal homeobox 1 (PDX1), NK6 homeobox 1 (NKX6.1) and musculoaponeurotic fibrosarcoma oncogene family A (MafA). These mice showed no histological evidence of pancreatitis or pancreatic dysplasia in their acini and had normal plasma amylase levels.

Conclusions/interpretation Activation of GLP-1 and gastrin signalling induces beta cell neogenesis in the exocrine lineage without any deleterious pancreatic changes, which may lead to a potential therapy to cure diabetes by generating surrogate beta cells.

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Keywords Beta cell neogenesis · Gastrin · GLP-1 · GLP-1 receptor

Abbreviations

CELA1	Chymotrypsin-like elastase family member 1
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
β-Gal	β-Galactosidase
GLP-1	Glucagon-like peptide-1
GLP1R	GLP-1 receptor
IRES	Internal ribosomal entry site
MafA	Musculoaponeurotic fibrosarcoma oncogene family A
NEUROG3	Neurogenin 3
NKX6.1	NK6 homeobox 1

PDX1	Pancreatic and duodenal homeobox 1
PTF1A	Pancreas-specific transcription factor 1a
SOX9	Sex-determining region Y-box 9

Introduction

To date, insulin-producing cells have been generated from various differentiated cell types in adult organs, such as the liver, gut and pancreas, as well as from embryonic stem cells and induced pluripotent stem cells, which may lead to future cell therapies for the cure of diabetes. Owing to their shared developmental origins, non-beta cells in the pancreas, such as acinar cells and alpha cells, have attracted significant attention as potential candidates that might be easily reprogrammed into beta cells [1–3].

Ectopic expression of pancreas-specific transcription factors, such as pancreatic and duodenal homeobox 1 (PDX1), neurogenin 3 (NEUROG3) and musculoaponeurotic fibrosarcoma oncogene family A (MafA), has been shown to efficiently induce the reprogramming of non-endocrine pancreatic cells into other cell types, including insulin-producing cells [1, 4, 5]. Gene transfer methods may not be practical for clinical use owing to safety concerns, such as immune responses and random integration of the transferred gene into the genome. However, alternative methods for inducing beta cell reprogramming using humoral factors, such as incretins and growth factors, have recently received a large amount of attention [6].

Among the many humoral factors that regulate pancreas development, glucagon-like peptide-1 (GLP-1) and its analogue, exendin-4, have been reported to affect beta cell differentiation under in vitro and ex vivo conditions [7–9]. In addition, previous in vivo studies showed that the co-activation of GLP-1 and gastrin signalling increased the number of beta cells in mouse models of diabetes [10–12]. Thus, although the activation of GLP-1 signalling plays a role in beta cell mass maintenance, it remains unclear whether or not the in vivo activation of GLP-1 signalling directly induces beta cell neogenesis. To address this question, we applied genetic strategies based on a Cre/loxP-based lineage tracing system and generated a mouse model overexpressing GLP-1 receptor (GLP1R). Whereas no newly generated beta cells were detected by the activation of GLP-1 signalling alone, GLP-1 was able to facilitate beta cell neogenesis initiated by gastrin, although the reprogramming efficiency in our model was not as high as that of other mouse models [1, 13]. It is noted that activation of GLP-1 signalling did not induce pancreatitis and pancreatic dysplasia, suggesting that these safety concerns, which have been reported in previous clinical cases and in vivo studies of incretin-based therapies [14–16], were abated in our models. The present findings point to possible future therapies for diabetes via these signalling pathways.

Methods

Animals *NEUROG3*-Timer, *Sox9*-CreER and *ROSA26-lacZ* reporter mice (R26R) were generated as described previously [17–19]. The transgenic construct CAG-CAT-*Glp1r* was assembled using a 2.9-kb CAG-CAT fragment cleaved from pCAG-CAT-*lacZ* [20], a 1.4-kb mouse *Glp1r* sequence obtained from mouse pancreas cDNA, a 1.3-kb internal ribosomal entry site (IRES)-*egfp* sequence cleaved from pAAV-Syn-AlstR- IRES2-*egfp* (plasmid #14895, Addgene, Cambridge, MA, USA) and a 0.2-kb fragment of the bovine growth hormone cassette gene. The 5.8-kb fragment of the CAG-CAT-*Glp1r* transgene was purified by electrophoresis and injected into fertilised eggs of B6D2F1 (BDF1) mice (Japan SLC, Hamamatsu, Japan). A total of 16 lines of CAG-CAT-*Glp1r* mice were generated and four lines highly expressing enhanced green-fluorescent protein (eGFP) were selected for the subsequent studies described herein. All animal procedures were approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine. Transgenic mice were used after genotyping without randomisation. Most experiments were not blind. See electronic supplementary material (ESM) Methods for further details.

Sorting endocrine progenitors The methods of *NEUROG3*-Timer mice generation, pancreatic cell dispersion and flow cytometry were as previously described [19, 21]. Briefly, pancreases from E17.5 *NEUROG3*-Timer embryos were treated with 0.05% trypsin/0.53 mmol/l EDTA (Invitrogen, Carlsbad, CA, USA) at 37°C for 5 min and inactivated by the addition of FBS. A MoFlo cell sorter (Dako Cytomation, Carpinteria, CA, USA) was used for sorting endocrine progenitors and other cell populations.

Induction of exogenous GLP1R expression Tamoxifen (Sigma-Aldrich, St Louis, MO, USA) was prepared at 20 mg/ml in corn oil. For lineage tracing, *Sox9*-CreER;*ROSA26-lacZ* reporter strains or *Sox9*-CreER;CAG-CAT-*Glp1r* mice were injected subcutaneously with tamoxifen at 4 mg/20 g body weight at the age of 6 weeks, five times over a 2 week period. Four weeks after the first tamoxifen injection, the mice were injected with exendin-4 (100 µg/kg; Sigma-Aldrich) and/or human gastrin (1 mg/kg; Abbotec, San Diego, CA, USA) intraperitoneally daily for 2 weeks, and then killed for further analyses at the age of 12 weeks. To inhibit GLP-1 signalling continuously, exendin-9-39 (150 pmol/kg/min; Sigma-Aldrich) was infused continuously using a mini-osmotic pump (Alzet, model 2002; Durect, Cupertino, CA, USA), which delivers the solution for up to 14 days.

Histology and immunostaining Tissues were fixed in 4% paraformaldehyde in PBS at 4°C then washed in PBS, immersed in sucrose solution and embedded in Tissue-Tek (OCT Compound, Sakura, Japan) or processed routinely for paraffin embedding. Frozen and paraffin blocks were sectioned at 6 µm thickness and immunostained as previously described [22]. Although we used some representative images for histology, all data were used for quantification. See [ESM Methods](#) for further details.

TaqMan real-time PCR Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen, Valencia, CA, USA) and reverse-transcribed at 42°C for 30 min using the Verso cDNA synthesis kit (Thermo Fisher, Rockford, IL, USA) according to the manufacturer's protocol. The expression levels of *Glp1r* (Mm00445292_m1), chymotrypsin-like elastase family member 1 (*Cela1*) (Mm00712898_m1) and pancreas-specific transcription factor 1a (*Ptf1a*) (Mm00479622_m1) mRNAs were quantified by TaqMan Real-Time PCR Assays (Applied Biosystems, Foster City, CA, USA), and normalised to glucuronidase, beta (*Gusb*, Mm00446953_m1). Data are expressed as mean±SE.

Measurement of cAMP activity Pancreatic tissues were collected from the mutant mice and control littermates at the age of 12 weeks. After homogenisation by polytron (Kinematica, Lucerne, Switzerland) in 0.1 M HCl, cAMP concentration (in supernatant fractions) was measured using a cAMP Direct Immunoassay Kit (#ab65355, Abcam, Cambridge, MA, USA).

Amylase activity Plasma samples were collected from 12- and 24-week-old transgenic mice and stored at −20°C until use. Plasma amylase activity was measured using an Abaxis VetScan VS2 chemistry analyser (Abaxis, Union City, CA, USA).

Statistical analyses Statistical analyses were performed using the JMP Statistical Discovery Software 9.0 (SAS Institute, Cary, NC, USA). Comparisons of two samples were performed by unpaired two-tailed *t* tests. Multiple groups were analysed by one-way ANOVA with a multiple comparison test and the Tukey–Kramer's post-hoc test was used to compare different treatments in two mouse strains. A value of $p < 0.05$ was considered to indicate a statistically significant difference between two groups. Data are presented as the mean±SE.

Results

GLP-1 receptor is expressed in pancreatic endocrine progenitors Since GLP-1 and/or its analogue has been shown to induce beta cell differentiation from non-beta cells [7, 8], we

hypothesised that GLP1R functions in pancreatic progenitors as well as in beta cells. To investigate the expression levels of *Glp1r* mRNA in endocrine progenitors during development, *NEUROG3*-expressing endocrine progenitors were sorted by fluorescence-activated cell sorting (FACS) from *NEUROG3*–Timer embryos, in which upstream and downstream sequences of human *NEUROG3* drive the reporter protein DsRed-E5 that shifts its fluorescence peak from green to red over time. This enables the separation of endocrine progenitors from the more differentiated endocrine cells within a 6-h time window [19]. Transcriptome analysis by FACS revealed that *Glp1r* mRNA is expressed in green-fluorescent endocrine progenitors as well as in red-fluorescent mature endocrine cells (Fig. 1a, b), although the expression level of *Glp1r* is lower in the early progenitors compared with the islet cells from 4-week-old C57BL/6 mice, which includes more differentiated, but not fully mature, beta cells. These data suggest that GLP1R signalling pathway may function during pancreas development before beta cell specification.

Generation of transgenic mice that overexpress GLP-1 receptor Since the expression of GLP1R is restricted to only a proportion of exocrine cells at much lower levels than that in beta cells [23], and *Glp1r* is expressed in pancreatic endocrine progenitors (Fig. 1a, b) and in newly generated beta cells in embryonic pancreases [24], we hypothesised that increased GLP-1 signalling may be able to induce the reprogramming of pancreatic exocrine cells into beta cells. To investigate this hypothesis, we generated a transgenic mouse line (CAG-CAT-*Glp1r*) in which ectopic expression of *Glp1r* is highly induced following Cre-mediated recombination (Fig. 2a). The *egfp* reporter gene was placed downstream of *Glp1r* cDNA via an IRES element, so that the exogenous GLP1R-expressing cells are labelled as green-fluorescent cells and their cell fates can be traced. The CAG-CAT-*Glp1r* mice were bred with the *Sox9*-CreER mice to generate double mutant *Sox9*-CreER;CAG-CAT-*Glp1r* (exo-*Glp1r*) mice. As a control, we used a reporter mouse model (*Sox9*-CreER;Rosa26-*lacZ*, exo-*lacZ* mice), which were generated by crossing *Sox9*-CreER mice with ROSA26-*lacZ* reporter strains (Fig. 2a) [17, 18]. Because the transcription factor sex-determining region Y-box 9 (SOX9) is expressed in pancreatic ductal cells during adulthood, exo-*Glp1r* and exo-*lacZ* mice treated with tamoxifen during adulthood can mark duct cells and their descendant acinar cells as eGFP- and β-galactosidase (β-gal)-expressing cells, respectively, whereas endocrine cells are never labelled [18].

When Cre-mediated recombination was induced with tamoxifen in exo-*Glp1r* and exo-*lacZ* mice from 6 weeks of age, and immunostaining against eGFP and β-gal was performed 6 weeks after the first tamoxifen treatment, GFP- and β-gal-expressing cells were observed in ductal and acinar cells. The efficiency of Cre-mediated recombination of exo-*Glp1r*

Fig. 1 GLP1R is expressed in pancreatic endocrine progenitors. **(a)** Pancreases from *NEUROG*-Timer embryos were dissected at embryonic day (E) 17.5 and sorted by FACS into four populations in a time-dependent manner. **(b)** The sorted cell populations were analysed by real-time PCR for *Glp1r*. Isolated islets from C57BL/6 mice at the age of 4 weeks (W) were analysed as a control. Expression levels of *Glp1r* were normalised to that of β -glucuronidase ($n=3$ in each group)

mice was comparable with that of *exo-lacZ* mice (percentage of eGFP- and β -gal-expressing cells among acinar cells: $51.8\% \pm 1.6\%$ and $53.2\% \pm 5.1\%$, respectively, $p=0.83$; among ductal cells: $46.9\% \pm 2.6\%$ and $47.6\% \pm 2.8\%$, respectively, $p=0.88$; Fig. 2b–m). Consistent with the immunostaining results for eGFP, *Glp1r* mRNA levels and cAMP concentrations in whole pancreases were significantly higher in *exo-Glp1r* mice than in *exo-lacZ* mice (Fig. 2n, o).

No beta cell neogenesis was observed by the activation of GLP-1 signalling alone Although several reports have shown that GLP-1 and its derivatives can induce the generation of insulin-producing cells from pancreatic non-endocrine cells in vitro [7, 10] or can increase beta cell mass in vivo [25, 26], there is no direct evidence using genetic lineage tracing demonstrating that beta cell neogenesis is induced by the activation of GLP-1 signalling alone. To investigate whether or not the activation of GLP-1 signalling can affect the conversion of exocrine cells into beta cells, exendin-4 or PBS was administered to *exo-Glp1r* and *exo-lacZ* mice daily for 2 weeks (Fig. 3a). Double immunostaining against insulin and β -gal for *exo-lacZ* could not detect insulin/ β -gal double-positive cells in the acinar area (Fig. 3b–e), consistent with a previous report showing no beta cell neogenesis during adulthood [18]. Furthermore, treatment of *exo-Glp1r* mice with exendin-4 or PBS did not result in any insulin/eGFP double-positive cells in the acinar area (Fig. 3f–i). Thus, activation of GLP-1 signalling failed to induce beta cell neogenesis in these mouse models.

Gastrin initiates beta cell neogenesis and activation of GLP-1 signalling enhances its effect Gastrin is known to be expressed in the developing pancreas whereas it is not

expressed in adult pancreas [27, 28], suggesting its possible role in pancreatic endocrine development. To investigate whether or not gastrin can induce beta cell neogenesis in the adult pancreas, *exo-lacZ* and *exo-Glp1r* mice were treated with gastrin and exendin-4 using the same protocol as above (Fig. 4a). Treatment with exendin-4 and gastrin significantly

Fig. 2 Generation of transgenic mice that overexpress GLP1R. (a) Schematic representation of the *Sox9-CreER;CAG-CAT-Glp1r* (exo-*Glp1r*) mouse line and the exogenous expression of *Glp1r* after Cre-mediated recombination. *Sox9-CreER;Rosa26-lacZ* (exo-*lacZ*) mice were used as a control. (b–m) Immunofluorescent staining for (b, j) eGFP, (f) β -gal, (c, g, k) insulin, (d, h) DAPI, (l) *Dolichos biflorus* agglutinin (DBA) and (e, i, m) merged images in exo-*Glp1r* and exo-*lacZ* mice showed that eGFP and β -gal were expressed in acinar and duct cells, but not in the islets 6 weeks after the first tamoxifen injection. The efficiency of Cre-mediated recombination was comparable between exo-*Glp1r* and exo-*lacZ* mice. (j, l, m) Arrows indicate pancreatic ducts positive for eGFP. Scale bars, 100 μ m. (n) Expression levels of *Glp1r* mRNA in the pancreases of exo-*lacZ* and exo-*Glp1r* mice were quantified by TaqMan real-time PCR. * $p < 0.05$; $n = 4$. (o) cAMP concentrations in the pancreases of exo-*lacZ* and exo-*Glp1r* mice. * $p < 0.05$; $n = 4$. White bars, exo-*lacZ*; black bars, exo-*Glp1r*

increased the percentage of beta cells and beta cell mass without affecting pancreatic weights (Fig. 4b–d). Immunostaining against insulin and β -gal detected insulin/ β -gal double-positive cells in the acinar area when exo-*lacZ* mice were treated with gastrin alone (Fig. 4e–h). These results suggest that gastrin administration on its own induced beta cell neogenesis, although the efficiency of neogenesis was too low to affect total beta cell mass. Furthermore, co-administration of gastrin and exendin-4 significantly increased the number of insulin/ β -gal double-positive cells compared with administration of gastrin alone (3.81 ± 0.45 and $1.79 \pm 0.60/\text{cm}^2$ pancreas, respectively, $p < 0.05$; Fig. 4i). However, exo-*Glp1r* mice treated with gastrin demonstrated a

significantly larger number of insulin/eGFP double-positive cells than insulin/ β -gal cells in exo-*lacZ* mice treated with gastrin (3.40 ± 0.41 and $1.79 \pm 0.60/\text{cm}^2$ pancreas, respectively, $p < 0.05$; Fig. 4i). Furthermore, co-injection of exendin-4 with gastrin dramatically increased the number of newly generated beta cells in exo-*Glp1r* mice, to 4.4-times higher than that in the exo-*lacZ* mutants (16.7 ± 1.7 and $3.81 \pm 0.45/\text{cm}^2$ pancreas, respectively, $p < 0.01$; Fig. 4i). In addition, injection of both gastrin and exendin-4 elicited a 5-times greater increase in newly generated beta cells compared with gastrin injection alone (16.7 ± 1.7 and $3.40 \pm 0.41/\text{cm}^2$ pancreas, respectively, $p < 0.01$; Fig. 4i). It is noted that islet-like-clusters consisting of two or more insulin-producing cells were observed in exo-*Glp1r* mice treated with exendin-4 and gastrin (Fig. 4j–n), but not in the other treatment groups. However, continuous inhibition of GLP-1 signalling using the GLP1R antagonist exendin-9-39, decreased the number of newly generated beta cells in mice treated with gastrin (Fig. 4o), suggesting that gastrin induces beta cell neogenesis at least partially through the activation of GLP-1 signalling. Taken together, these data suggest that the endogenous and genetic activation of GLP-1 signalling in the presence of gastrin enhances the reprogramming of non-endocrine cells into insulin-expressing cells, and this effect is synergistically increased by pharmacological activation by exendin-4.

Newly generated insulin-producing cells express beta cell specific transcription factors To characterise newly differentiated insulin-expressing cells derived from exocrine cells, fluorescent immunostaining was performed with antibodies against pancreas-specific transcription factors. In the pancreas of exo-*Glp1r* mice treated with exendin-4 and gastrin, >80% of the insulin/eGFP double-positive cells expressed NK6 homeobox 1 (NKX6.1), PDX1 and MafA (85.1%, 86.4% and 84.1%, respectively), which are pancreas-specific transcription factors that play essential roles in beta cell differentiation and maturation (Fig. 5a–e') [29–33]. These neogenic insulin-producing cells did not express other endocrine hormones (glucagon, somatostatin and pancreatic polypeptide) or an exocrine enzyme (amylase; data not shown). These findings suggest that the activation of GLP-1 and gastrin signalling induces not only insulin transcription but also a certain level of beta cell maturation.

Activation of incretin signalling does not induce pancreatitis or pancreatic dysplasia The activation of incretin signalling was reported to possibly be associated with pancreatitis and pancreatic dysplasia both in humans and animal models, although recent studies support the safety of incretin-based drugs [14–16, 34–36]. To test whether or not increased GLP-1 signalling in the pancreas results in undesired pathologies, pancreases from exo-*Glp1r* mice were examined microscopically. As shown in ESM Fig. 1a, there was no histological evidence of pancreatitis or pancreatic dysplasia

Fig. 3 No beta cell neogenesis was observed by the activation of GLP-1 signalling on its own. (a) Schematic of the experimental design for detecting beta cell neogenesis based on the Cre-loxP system. (b–i) Immunofluorescent staining for (b) β -gal, (f) eGFP, (c, g) insulin, (d, h) DAPI and (e, i) merged images in exo-*lacZ* mice treated with PBS and exo-*Glp1r* mice treated with exendin-4. All β -gal- and eGFP-positive cells were negative for insulin. Scale bar, 50 μ m

Fig. 4 Gastrin initiates beta cell neogenesis and activation of GLP-1 signalling enhances its effect. **(a)** Schematic of experimental design for detecting beta cell neogenesis. **(b–d)** Measurement of **(b)** total beta cell (insulin-positive) area per pancreas (i.e. beta cell proportion), **(c)** pancreatic weights and **(d)** beta cell mass of *exo-lacZ* and *exo-Glp1r* mice that were treated with exendin-4 (Ex-4) and/or gastrin (Gast). * $p < 0.05$; $n = 4–6$. Beta cell mass is the product of the beta cell proportion and pancreatic weight. **(e–h)** Immunofluorescent staining for **(e)** β -gal, **(f)** insulin, **(g)** DAPI and **(h)** merged images in *exo-lacZ* mice treated with gastrin alone. Double-positive cells for β -gal and insulin were observed, suggesting that exocrine-to-beta reprogramming was induced. **(i)** The number of insulin/eGFP (or β -gal) double-positive cells (i.e. newly generated beta cells) in the pancreases of the indicated mice. ND, not detected; * $p < 0.05$; $n = 4–6$. **(j)** Islet-like clusters were observed only in *exo-Glp1r* mice treated with exendin-4 and gastrin. While 56.9% of insulin/eGFP double-positive cells remained as single cells, 25.0% and 18.1% formed clusters with 2 and ≥ 3 insulin-positive cells, respectively. **(k–n)** Representative figures of islet-like clusters in *exo-Glp1r* mice treated with exendin-4 and gastrin, stained with **(k)** eGFP, **(l)** insulin, **(m)** DAPI and **(n)** a merged image. **(k)** Arrowheads indicate newly formed beta cells stained for both insulin and eGFP; arrows indicate acinar cells negative for eGFP. **(o, p)** The number of newly generated beta cells in the pancreases of **(o)** *exo-lacZ* and **(p)** *exo-Glp1r* mice treated with gastrin, together with or without exendin-9-39. * $p < 0.05$; $n = 3$. Scale bar, 50 μ m

Fig. 5 Newly generated insulin-producing cells express beta cell specific transcription factors. Immunofluorescent staining for (a, f, k, p, u, a') eGFP, (b, g, l, q, v, b') insulin, (c, h) PDX1, (m, r) NKX6.1, (w, c') MafA and (e, j, o, t, y, e') merged images in the pancreases of *exo-Glp1r* mice. Insulin/eGFP double-positive cells expressed (a–e) PDX1, (k–o) NKX6.1 and (u–y) MafA. (f–j, p–t, a'–e') Pancreatic islets are shown as positive controls. (a, k, u) The dashed lines outline the clusters of newly formed beta cells. Scale bars, 50 μ m

in exendin-4 and gastrin-treated *exo-Glp1r* or *exo-lacZ* mice at 12 weeks of age. Immunostaining for Ki67 revealed comparable cell proliferation in the ducts and acini between the mutant mice and control littermates (ESM Fig. 1b, c). In addition, there was no significant increase in plasma amylase levels in the mutant mice (ESM Fig. 1d). Furthermore, TaqMan real-time PCR analysis showed no significant alterations in mRNA levels for elastase-1 (*Cela1*) and *Ptf1a*, a transcription factor that functions in acinar cells (ESM Fig. 1e, f) [37, 38]. In addition, pancreases of 24-week-old mutant mice revealed no abnormal findings in haematoxylin staining and no elevation of plasma amylase levels compared with C57BL/6 controls (data not shown). Thus, the activation of GLP-1 and gastrin signalling did not induce pancreatitis or pancreatic dysplasia, even with the ectopic activation of GLP-1 signalling in acinar cells.

Discussion

Although it has been reported that the activation of GLP-1 and gastrin signalling can induce beta cell neogenesis in vitro and increase beta cell mass in vivo [10, 25, 26, 39], there have been no direct in vivo studies of cell lineage analysing whether or not the activation of these pathways induces the cellular reprogramming of non-endocrine cells into insulin-producing cells. Here, we demonstrate direct in vivo evidence that gastrin administration alone initiates beta cell neogenesis within exocrine cells and that the concurrent activation of GLP-1 signalling synergistically enhances this effect, although the reprogramming efficiency in our model was lower than that of other reported strategies [1, 13].

GLP-1 and its derivatives, such as exendin-4 and liraglutide, have been shown in clinical studies to effectively improve glycaemic control with minimal risk of

hypoglycaemia, and basic research has uncovered several mechanisms of their diabetes-protective effects on beta cells, such as the stimulation of insulin secretion, increase in beta cell replication and inhibition of beta cell apoptosis [40]. Based on the findings that GLP1R is detected in early endocrine progenitors of *NEUROG3*-Timer embryos (Fig. 1a, b), newly generated beta cells of Insulin-Timer embryos [24] and in acinar cells of the human adult pancreas [23], we hypothesised that GLP-1 may function in non-beta cells, including acinar and duct cells, and we activated the GLP-1 signalling pathway by the administration of exendin-4, a long-acting GLP1R agonist, and by overexpressing GLP1R in exocrine cells. Whereas in vitro studies have shown that GLP-1 and exendin-4 induce beta cell differentiation in the pancreatic tumour cell line AR42J [7, 9], our present study revealed that exendin-4 itself fails to initiate beta cell neogenesis in SOX9-lineage exocrine cells in vivo, even after the overexpression of GLP1R (Fig. 3f–i). SOX9-positive cells and their descendants are known to lose their capacity to differentiate into endocrine cells, even after in vivo regeneration experiments such as partial pancreatectomy and pancreatic duct ligation [18]. Thus, at least in our model, the activation of GLP-1 signalling alone is insufficient to reprogram SOX9-lineage cells into beta cells. Further optimisation of the dose and timing of administration of GLP-1 analogues is required for inducing beta cell neogenesis.

In contrast to the results of exendin-4 treatment, our mouse model directly demonstrated that beta cell neogenesis is induced by gastrin alone. Gastrin is expressed in the developing pancreas, particularly in endocrine populations [27, 28]. Although several reports have shown that combination therapy with gastrin and growth factors, such as EGF and TGF- α , increased beta cell mass [41–45], there was no evidence that gastrin could induce beta cell neogenesis as distinguished from beta cell replication. To our knowledge, this is the first report that a pharmacological dose of gastrin on its own initiated beta cell neogenesis independently of other humoral factors, such as EGF and GLP-1. Our results, taken together with previous reports, suggest that the activation of gastrin signalling combined with other humoral factors might provide a new therapeutic option that could be used to increase beta cell mass.

Recently, a clinical study of combination therapy with sitagliptin and lansoprazole in patients with type 1 diabetes failed to slow the progressive loss of insulin secretion [46]. This negative result might be due to an insufficient increase of GLP-1 or gastrin concentrations by these drugs. However, our direct approach with a GLP-1 analogue and gastrin induced the cellular reprogramming into beta cells, although the efficiency was not sufficient to cure streptozotocin-treated diabetic mice (data not shown). Further studies for optimising drug regimens could help to increase beta cell mass through beta

cell neogenesis, which would lead to a cure for those diabetic patients who have lost most of their beta cells.

In the present study, *Sox9*-CreER mice were used to induce Cre-mediated recombination in SOX9-expressing cells, which resulted in the exogenous expression of GLP1R in duct cells and acinar cells [18]. Therefore, there are a number of possibilities for the origin of newly generated insulin-producing cells; namely, SOX9-expressing progenitors, differentiated duct cells or acinar cells. Several groups have demonstrated that acinar cells can be reprogrammed into beta cells under various conditions [1, 13, 47]. To clarify the origin of the newly generated insulin-producing cells, further studies should be performed using other Cre-expressing lines that induce Cre-mediated recombination specifically in acinar cells, such as Elastase1-CreER [48] and *Ptf1a*-CreER mice [47].

The mouse model overexpressing GLP1R helped us not only to investigate the beneficial effects of GLP-1 on beta cell differentiation, but also to evaluate possible pancreatic risks related to incretin therapy. The present data showed no obvious evidence of histological abnormalities or elevated amylase levels in GLP1R-overexpressing pancreases at the age of 24 weeks, 18 weeks after the induction of exogenous GLP1R in SOX9-lineage cells. Further careful observations for longer periods are required to apply our in vivo findings with this mouse model to human patients. In addition, it would be of interest to overexpress GLP1R in other tissues by crossing CAG-CAT-*Glp1r* mice with other Cre-expressing mouse lines, in order to test the physiological and/or pathological roles of GLP-1 signalling in various cell types in which its function remains controversial, such as pancreatic alpha cells [49] and thyroid C cells [50].

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