The Ambiguous beta-cell : On the loss of human pancreatic beta-cell identity

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CHAPTER 6

GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AGONISTS PREVENT LOSS OF β-CELL IDENTITY IN HUMAN ISLET CELL AGGREGATES

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ABSTRACT

Loss of insulin-secreting β-cell mass and function is central in the pathophysiology of type 2 diabetes (T2DM). We previously reported that mature human β-cells can lose identity and spontaneously convert into α-cells following islet cell dispersion and reaggregation *ex vivo* and that this conversion may be involved in β-cell failure in T2DM. Activation of the GLP-1 receptor is involved in β-cell differentiation and can prevent apoptosis. Therefore, we investigated whether activation of the GLP-1 receptor plays a role in maintenance of a β-cell phenotype. Using β-cell specific lineage tracing, we show that GLP-1 receptor agonists can prevent loss of human β-cell identity, as characterized by a higher percentage of insulin^{+}GFP^{+} out of GFP^{+} cells after 7 days of reaggregation. Incretin treatment was accompanied by an induction of endogenous Pax4 gene expression. The protective effects of GLP-1 signaling on human β-cells were mimicked by overexpression of hPax4 in human islet cell aggregates. Our results indicate a novel potential role for incretin-based therapies, targeting the maintenance of mature β-cells, possibly through induction of Pax4 gene expression.

INTRODUCTION

Dysfunction of insulin-secreting β-cells and loss of pancreatic β-cell mass are central in the development of type 2 diabetes (T2DM) (1). Reduced β-cell mass may result from increased loss via apoptosis or reduced cell renewal via proliferation or neogenesis (2). But β-cell loss could also arise due to dedifferentiation as recent data shows that adult murine β-cells depleted of FoxO1 lose their identity and even convert into α-cells under conditions of metabolic stress (3). Moreover, oxidative stress, associated with β-cell failure in T2DM, lowers the expression of transcription factors such as MafA and Nkx6.1 that promote insulin gene transcription (4). We previously used a lineage tracing approach to show that human β-cells can spontaneously convert into α-cells following human islet cell dispersion and reaggregation *ex vivo* (5). The β- to α-cell transition was marked by β-cell degranulation and glucagon-positive cells expressing β-cell transcription factors (Nkx6.1 or Pdx1), a similar phenotype as observed in the islets of the genetically manipulated FoxO1 model (3;5). In addition, we and others found that cells with such a mixed phenotype (glucagon$^+$/insulin$^+$ bihormonal cells or glucagon$^+$-cells co-expressing β-cell transcription factors) are more prevalent in donor pancreas from subjects with T2DM, indicating that β-cells may lose their identity during the progression of diabetes (6-9). Therefore, ways to prevent or reverse loss of β-cell identity may provide novel therapeutic opportunities in T2DM.

During embryologic development, endocrine cell types are formed from a common Neurogenin3-expressing progenitor (10-12). The subsequent segregation of the α- and β-cell lineages depends on the expression of specific transcription factors with Arx and Pax4 playing a central diverging role. Arx null mutant mice do not contain α-cells, while mice that lack Pax4 show large numbers of α-cells at the expense of β- and δ-cells (13). We have previously shown that knockdown of Arx can prevent loss of human β-cell identity *ex vivo* (5). In line with these observations, overexpression of Pax4 *in vivo* can protect mouse β-cells against streptozotocin induced hyperglycemia (14). The role of Pax4 in human β-cell (de)differentiation is unclear.

The incretin hormone glucagon-like peptide-1 (GLP-1) is a gut-derived peptide that enhances glucose-induced insulin secretion, and GLP-1 receptor agonists (GLP-1RAs) are currently used in clinical care. Moreover, GLP-1RAs were used in differentiation protocols for culture of stem and progenitor cells to obtain insulin-producing cells (15;16). It is known that GLP-1 can also reduce glucagon secretion and prevent β-cell apoptosis (17), and that GLP-1 induces Pax4 expression in human isolated islets (18). In this study, we investigated the effects of GLP-1RAs and Pax4 on the maintenance of human β-cell identity.

MATERIALS & METHODS

Human islet isolation and cell culture

Human islet isolations were performed in the Good Manufacturing Practice facility of our institute according to a modified protocol originally described by Ricordi et al. (19). Pancreatic tissue was used in our study if the pancreas could not be used for clinical

pancreas or islet transplantation, according to national laws, and if research consent was present. Islet cells were dispersed and left to reaggregate in microwell plates after lentiviral transduction (5). In short, islets were dispersed into single cells by adding 0.025% trypsin solution containing 10 mg/mL DNase (Pulmozyme, Genentech) at 37°C while pipetting up and down for 6–7 min. The islet cell suspension was plated onto 3% agarose microwell chips containing 2,865 microwells/chip with a diameter of 200 μm/microwell (20). Seeding of ~3*10^6 cells per chip resulted in spontaneous reaggregation of ~1,000 islet cells/ microwell. Islet cell aggregates and intact human islets (control) were cultured in CMRL 1066 medium (5.5 mmol/L glucose) containing 10% FCS, 20 mg/mL ciprofloxacin, 50 mg/mL gentamycin, 2 mmol/L L-glutamin, 0.25 mg/mL fungizone, 10 mmol/L HEPES, and 1.2 mg/mL nicotinamide, in the presence or absence of 10 nM exendin-4 (Sigma) while medium was refreshed every 24-48 hours.

Lentivirus vectors

pTrip-RIP405Cre-ERT2-DeltaU3 (RIP-CreERT2) and pTrip–CMV-loxP-Neo-STOP-loxP-eGFP-DeltaU3 (CMVstopGFP) were kindly provided by P. Ravassard (21). pTrip vectors were produced as third-generation lentivirus vectors by adding a Tat-expressing vector (gift from B. Berkhout, Amsterdam) to the regular helper plasmids and produced as previously described (22). Vector pcDNA3.1:pcDNAPax4 encoding human Pax4 (hPax4) was kindly provided by K. Nanjo (23). Human Pax4 cDNA was subcloned in a pRRL lentivirus vector (CMV-hPax4) (22). For lineage tracing, transduction was performed overnight as previously described and exendin-4 was added following overnight lentivirus incubation (5). In case of hPax4, a second round of transduction was performed for 8 hours during the following day. 4-hydroxy-tamoxifen (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 1 mmol/L in the evening. After overnight incubation, the medium was refreshed and cells were seeded on the microwell. The start of reaggregation represents day 0 in our experiments.

RNA isolation and quantitative PCR

Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR was performed on a Light Cycler 480-II Real-time PCR system (Roche). Fold induction was calculated using delta CT method with human β-actin as housekeeping gene. Taqman probes were used for glucagon (Hs00174967_m1), insulin (Hs00355773_m1), Arx (Hs00292465_m1) and Pax4 (Hs00173014_m1).

Immunofluorescence staining

Formalin-fixed islet cell aggregates were washed in PBS and spun down at high speed in fluid agar. Agar-containing cell pellets were embedded in paraffin. Blocks were cut into 4-μm sections. Primary antibodies against insulin (1:200; Linco), glucagon (1:200; Vector and Invitrogen), Ki67 (1:200; BD Pharmingen), and green fluorescent protein (GFP) (1:500; Roche and Molecular Probes) were used. DAPI (Vector) was used as nuclear

counterstaining. Secondary antibodies were TRITC–anti–guinea pig (1:400; Jackson) and Alexa Fluor 488-, 568-, and 647 anti-mouse or anti-rabbit when appropriate (1:1,000). Apoptosis was assessed by TUNEL assay (Roche). Sections were examined using confocal microscopy. Staining was quantified as percentage of positive cells per total cell number, counting at least 750 cells per donor for each condition.

Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Statistical significance of differences between two groups were determined by an unpaired Student's t test. One-way ANOVA followed by Bonferroni multiple comparisons test was used if more groups were compared. $P < 0.05$ was considered statistically significant.

RESULTS

GLP-1RA treatment prevents β-cell dedifferentiation in human islet cell aggregates

Treatment with the GLP-1RA exendin-4 showed a significantly higher proportion of GFP^+ cells expressing insulin after 1 week compared to aggregation without exendin-4 treatment (25.3 ± 4.3% vs. 39.8 ± 4.8%, untreated versus GLP-1RA treated, $P < 0.05$, Fig. 1A,B). As the lineage tracing system was induced by a tamoxifen pulse, this indicates that loss of β-cell identity was prevented rather than new β-cells generated. Immunolabeling showed that the overall number of proliferating (Ki67) and apoptotic (TUNEL) cells was low (<1% and <2%, respectively), and did not differ between both groups (Fig. 2). The proportion of $glucagon^+GFP^+$ out of GFP^+ cells was not significantly lower in the exendin-4 treated aggregates (Fig 3A,B).

Pax4 overexpression prevents β-cell dedifferentiation in human islet cell aggregates

Exendin-4 treatment induced a 2-fold higher *Pax4* gene expression in aggregates after 7 days of culture compared to intact cultured islets or aggregates without exendin-4 treatment (Fig. 4A). Since endogenous *Pax4* gene expression is normally low or even absent in mature β-cells (24), we hypothesized that increased *Pax4* expression following exendin-4 treatment induced protection against loss of β-cell identity. To test whether the protective effect of GLP-1 could be mimicked by human Pax4 protein overexpression (hPax4OE), we transduced islet cells using the lentiviral vector CMV-hPax4. Pax4 overexpression resulted in a 50% increase in *insulin* gene expression after 7 days of reaggregation (Fig. 4B). Gene expression of the α-cell transcription factor *Arx* and hormone *Glucagon* was significantly lower following hPax4OE (Fig. 3C). Using β-cell lineage tracing, a significantly higher proportion of GFP^+ cells expressing insulin was observed in hPax4OE aggregates (Fig. 4C). $Glucagon^+GFP^+$ cells were observed in both groups, and 2 out of 3 donors showed a distinct decrease in the percentage of double positive cells (Fig. 3D).

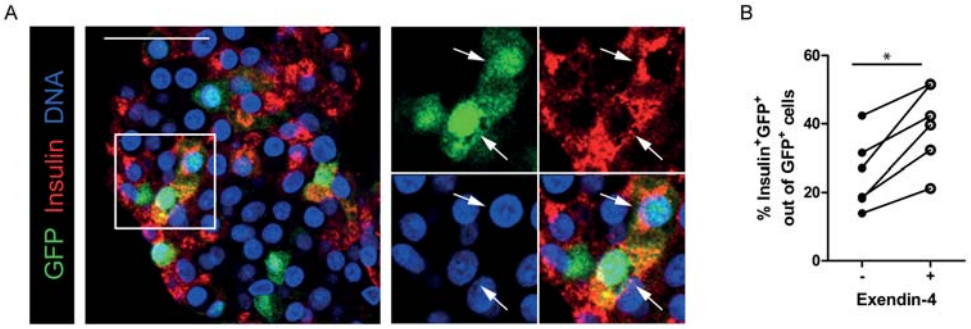

Figure 1. GLP-1RA administration yields a higher percentage of insulin$^+$GFP$^+$ cells. *A:* Representative immunostaining for insulin (red) and GFP (green) of a human aggregate after 7 days treatment with 10 nM GLP-1RA exendin-4. The right panel highlights the presence of Insulin$^+$GFP$^+$ cells (arrows). *B:* Quantification of the percentage of insulin$^+$GFP$^+$ out of all GFP$^+$ cells in 6 donors. Data derived from the same donors (with or without exendin-4 treatment) are indicated by connecting lines. * $P < 0.05$, scale bar: 50 μm.

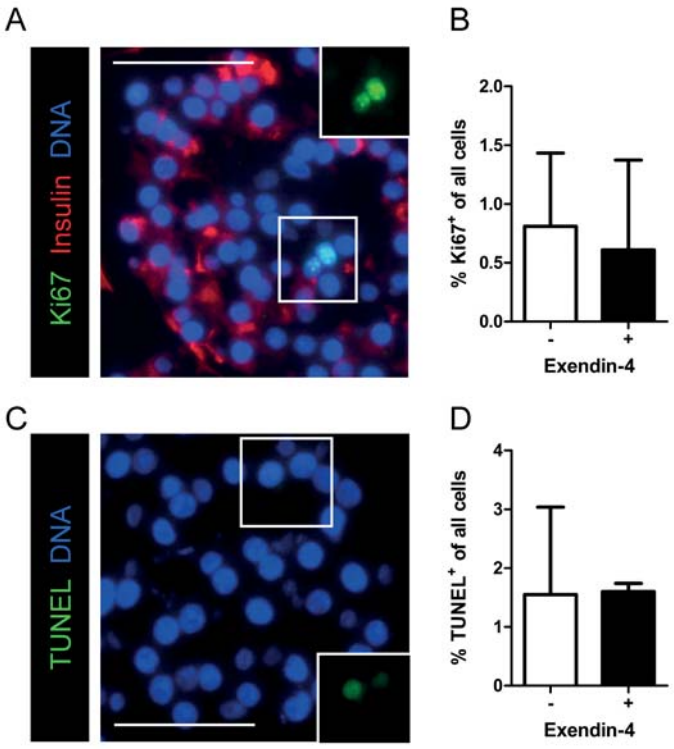

Figure 2. No difference in the proportion of proliferating (Ki67+) or apoptotic (TUNEL+) cells following treatment with exendin-4. *A:* Representative image of a human aggregate after 7 days reaggregation, immunostained for insulin (red) and Ki67 (green), inset shows two Ki67$^+$ cells. *B:* Quantification of the percentage of Ki67$^+$ cells out of all cells with or without GLP1-RA treatment. *C:* Representative image of TUNEL labelling (green). *D* Quantification of the percentage of TUNEL$^+$ cells out of all cells (n = 2, mean ± SD). Scale bars: 50 μm.

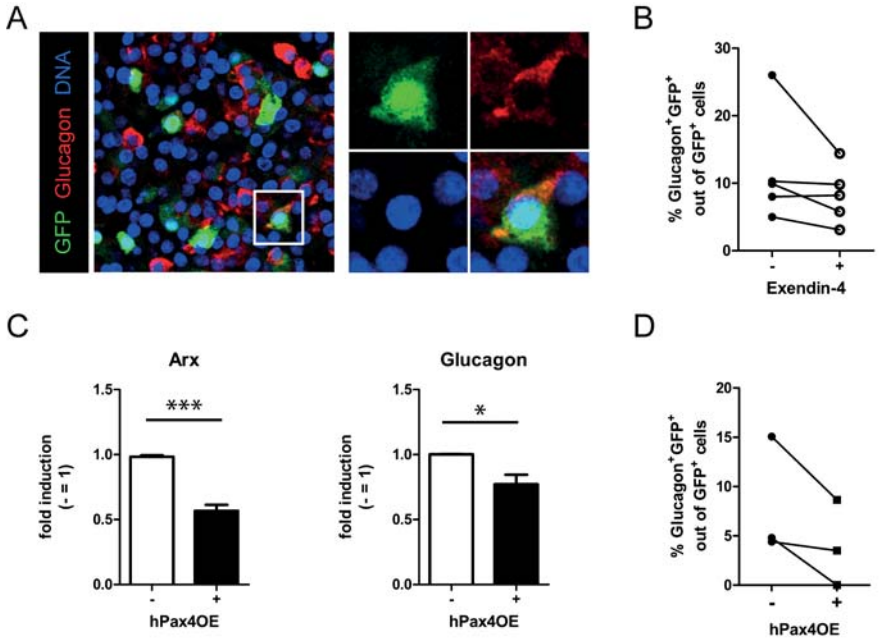

Figure 3. Human Pax4 overexpression lowers expression of α-cell genes *ARX* and *glucagon* but GLP1-RA or hPax4OE do not affect the proportion of glucagon$^+$GFP$^+$ cells. *A:* Immunostaining for glucagon (red) and GFP (green). *B:* Percentage of glucagon$^+$GFP$^+$ cells out of GFP$^+$ cells following exendin-4 treatment (n = 5, data from the same donors are indicated by connecting lines). *C:* hPax4OE in human islet cell aggregates results in a lower gene expression of the α-cell genes *ARX* and *glucagon* compared to controls. * $P < 0.05$, *** $P < 0.001$ (n = 6, mean ± SEM). *D:* Quantification of glucagon$^+$GFP$^+$ cells out of GFP$^+$ cells following hPax4OE (n = 3, data from the same donors are indicated by connecting lines).

DISCUSSION

We recently showed that loss of β-cell identity can occur in subjects with T2DM *in vivo* (7) and also in human pancreatic islets *ex vivo* following dispersion and reaggregation (5). Using our *ex vivo* culture system as a model for loss of human β-cell identity, we now add that both activation of the GLP-1 receptor as well as genetic overexpression of human Pax4 can promote the maintenance of β-cell identity.

GLP-1RAs are used in the clinic to improve glycemic control in patients with type 2 diabetes, mainly by stimulating glucose-dependent insulin secretion (25). Here, we show that activation of the GLP-1 receptor by exendin-4 can prevent loss of β-cell identity (characterized by a higher proportion of insulin$^+$GFP$^+$ out of GFP$^+$ cells after reaggregation) in human islet cell aggregates under normal glucose culture conditions. It has previously been shown that GLP-1 receptor signaling under normoglycemic conditions can increase insulin sensitivity and β-cell function (26) and can improve β-cell protection under lipid stress (27). The observation that approximately 50% of GFP$^+$ cells were insulin negative is in agreement with our previous finding that a large proportion of the cells is degranulated (5). Furthermore, β-cell conversion into α-cells still occurred since the proportion of

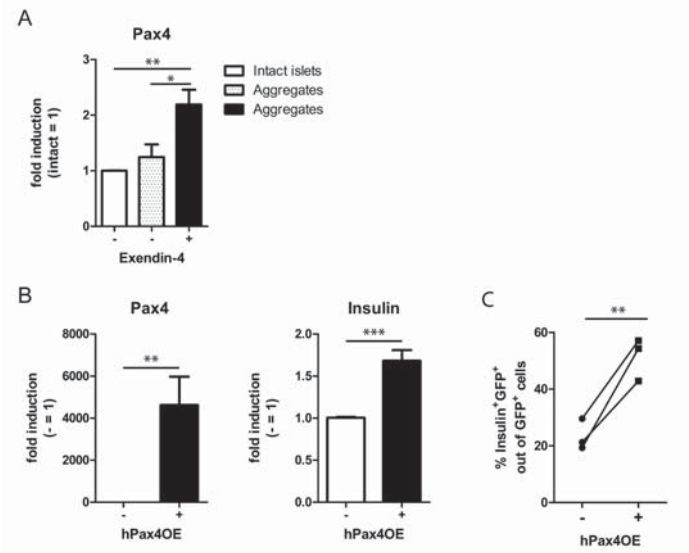

Figure 4. hPax4OE mimicks the effect of exendin-4 on human islet cell aggregates. *A:* Administration of 10 nM exendin-4 showing higher *PAX4* gene expression compared to aggregates or intact islets that were cultured for 7 days in the absence of exendin-4 (crude Ct-values ranging from 30-35 cycles). *B:* Lentivirus-mediated overexpression of human Pax4 (hPax4OE) in human islet cell aggregates results in higher *PAX4* and *insulin* gene expression after 7 days reaggregation (n = 6, mean ± SEM). *C:* hPax4OE shows a higher proportion of insulin$^+$GFP$^+$ out of GFP$^+$ cells as analysed by immunostaining (n = 3, data from the same donors are indicated by connecting lines). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

glucagon$^+$GFP$^+$ cells was not significantly changed. This lack of significance may be due to the small number of experiments, or it may indicate that GLP-1 receptor activation lowers the number of 'empty' GFP$^+$ cells (neither expressing glucagon nor insulin) by stimulating hormone expression. Altogether, our data suggest a novel benefit of GLP-1 receptor activation by providing maintenance of a β-cell phenotype.

GLP-1RA treatment in our study resulted in elevated *Pax4* gene expression levels. It was previously shown that inhibition of phosphatidylinositol-3 kinase (PI3-kinase) blunted this GLP-1 mediated effect on Pax4 (18). This suggests that the effect of GLP-1 on Pax4 is mediated by signaling via the epidermal growth factor receptor that activates PI3-kinase and AKT downstream (28). Whereas GLP-1RA treatment did not significantly affect α-cell markers (data not shown), Pax4 overexpression strongly diminished *Arx* and *Glucagon* gene expression. Furthermore, while GLP-1RA treatment had little effect on the proportion of glucagon$^+$GFP$^+$ cells, Pax4 overexpression showed a protective effect in 2 out of 3 donors. This difference may be explained by the higher levels of Pax4 expression that result from overexpression compared to the modest increase after GLP-1RA treatment.

Pax4 plays a pivotal role in the restriction of endocrine progenitors towards a β-cell fate in mice (29). In adult human β-cells, overexpression of mouse Pax4 has been shown to stimulate β-cell expansion and survival (30), while β-cell specific overexpression of wild-type Pax4, but not of mutated inactive Pax4, protected against streptozotocin-induced

apoptosis (14). Moreover, genetic polymorphisms in the human Pax4 gene have been associated with type 2 diabetes (23;31). We now add that hPax4 prevents loss of human β-cell identity, using a combination of hPax4 overexpression and β-cell lineage tracing. These data together suggest that Pax4 not only plays a crucial role in development, but is also involved in the protective response of β-cells against environmental (metabolic or inflammatory) stress (32). Interestingly, Pax4 gene expression is low or even absent in mature human islets (24), but is upregulated in a subset of subjects with T2DM (18). In light of our study, this could reflect activated protection against β-cell dedifferentiation.

Thus, we show that overexpression of Pax4 and GLP-1 receptor activation can prevent loss of human β-cell identity in an *in vitro* model of human islet cell reaggregation. We thereby identify a previously unknown function of these molecules, targeting maintenance of mature β-cells. Future research should investigate further by which mechanisms GLP-1 maintains β-cell identity.

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