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Research article

Direct suppression of human islet dedifferentiation, progenitor genes, but not epithelial to mesenchymal transition by liraglutide

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A B S T R A C T

β-cell dedifferentiation has been accounted as one of the major mechanisms for β-cell failure; thus, is a cause to diabetes. We study direct impacts of liraglutide treatment on *ex vivo* human dedifferentiated islets, and its effects on genes important in endocrine function, progenitor states, and epithelial mesenchymal transition (EMT). Human islets from non-diabetic donors, were purified and incubated until day 1 and day 4, and were determined insulin contents, numbers of insulin (INS$^+$) and glucagon (GCG$^+$) cells. The islets from day 3 to day 7 were treated with diabetic drugs, the long acting GLP-1 receptor agonist, liraglutide. As observed in pancreatic islets of type 2 diabetic patients, *ex vivo* dedifferentiated islets showed more than 50% reduced insulin contents while number of glucagon increased from 10% to about 20%. β-cell specific genes: *PDX1, MAFA,* as well as β-cell functional markers: *GLUT1 and SUR1,* were significantly depleted more than 40%. Notably, we found increased levels of glucagon regulator, *ARX* and pre-glucagon transcripts, and remarkably upregulated progenitor expressions: *NEUROG3* and *ALDH1A* identified as β-cell dysfunction markers in diabetic models. Hyperglucagonemia was often observed in type 2 patients that could lead to over production of gluconeogenesis by the liver. Liraglutide treatments resulted in decreased number of GCG$^+$ cells, increased numbers of GLP-1 positive cells but did not alter elevated levels of EMT marker genes: *ACTA2, CDH-2, SNAIL2,* and *VIM*. These effects of liraglutide were blunted when *FOXO1* transcripts were depleted. This work illustrates that *ex vivo* human isolated islets can be used as a tool to study different aspects of β-cell dedifferentiation. Our novel finding suggests a role of GLP-1 pathway in beta-cell maintenance in FOXO1-dependent manner. Importantly, dedifferentiated islets *ex vivo* is a useful model that can be utilized to verify the actions of potential drugs to diabetic β-cell failure.

1. Introduction

β−cell dysfunction is at the core of pathogenesis in type 2 diabetes [1]. In type 2 diabetes, the understanding of mechanisms of β−cell failure could afford a novel approach to preserving metabolic balance. Developing therapies are focused on increasing β-cell number either by increasing β-cell proliferation or reducing β-cell apoptosis [2, 3]. Our previous work indicates that β-cell dedifferentiation, not apoptosis, is the main cause of β-cell failure, and that dedifferentiated β-cells are prone to converting into non-β endocrine cells that exacerbate the progression of diabetes [4]. In animal models of diabetes and human diabetic patients,

diabetic islets show reduced insulin content and insulin secretion in response to glucose, and increased numbers of glucagon cells [1, 2, 3]. Biochemistry analysis of diabetic mouse islets illustrates loss of the key metabolic transcription factor, FoxO1, β-cell functional markers, and particularly the rise of progenitor markers: Neurog3 that normally only express during fetal development of the pancreas, and Aldh1A3, a stemness marker that elevates with diabetic progression [5]. β−cell dysfunction could commence since *in utero* [6] or develop subsequent to metabolic and physiological stress [4]. Our previous experiments provide proof-of-concept that loss of β-cell function is not simply due to β-cell exhaustion, but rather to β-cells reprogramming to a progenitor-like

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stage. β-cell dedifferentiation also occurs when cultured islets *ex vivo* but it was thought to involve genes important in β−cell function while the endocrine islets undergo epithelial-mesenchymal transition (EMT) [7] or loss of β−cell maintenance [8]. In our lineage tracing study during beta-cell dedifferentiation and conversion of islet endocrine cells [4], we observed evidence of epithelial mesenchymal transition such as immunoreactive Nestin$^+$, Vimentin$^+$ and glucagon$^+$ cells [4] while other works have shown EMT gene expressions elevated in cultured human islets [7, 9]. Whether or not *ex vivo* human islet dedifferentiation share molecular mechanisms of diabetic β−cell dedifferentiation has not been clearly elucidated.

To improve the pathophysiology of T2DM and ameliorate hyperglycemia, incretin-targeting therapies involved with glucoincretin glucagon-like peptide -1 (GLP-1) have become more popular in recent years [10]. At organism level, GLP-1 treatments improve insulin release in response to glucose [11, 12], reduced glucagon secretion [13], and blunted appetite [14, 15]. Liraglutide, with a 13 h half-life, and resistant to human dipeptidyl peptidase-4 (DPP-4), emerges as one of the most effective anti-diabetic drugs [16]. In a long-term treatment studies on T2DM patients, pancreatic islets have shown improved β−cell mass and preserved glucagon number [17]. At cellular level, the effects of liraglutide on beta-cell apoptosis [18, 19], proliferation [20], and β−cell neogenesis [21] have been examined but not on mechanisms of β−cell dedifferentiation, and potential conversion to non- β−cells, that could cause diabetic β−cell cell failure. Several studies have reported involvement of FoxO1 as an effector of β−cell response to liraglutide in preserving β−cell mass and function in diabetic conditions [18, 20, 22] As our previous works [4, 5] identified FoxO1 to play a major role in β−cell dedifferentiation in mouse models. Here we evaluate the direct impact of liraglutide treatment on dedifferentiated human islets and study the mechanistic effects on gene expressions of β−cell function markers, of alpha-cell related genes, of β−cell dedifferentiation markers, and of EMT markers. Our data verify similarities between *in vivo* diabetic islet dedifferentiation, and *ex vivo* islet dedifferentiation, and expand our mechanistic understanding of liraglutide effects, and importantly support the use of *ex vivo* islet dedifferentiation as a model for antidiabetic drug testing.

2. Materials and methods

2.1. Human islet isolation and quality

Human pancreatic tissues from non-diabetic donors were provided by Ramathibodi hospital, Bangkok, Thailand. The islets were obtained from 8 individuals whose aged 20–55 years old and were both male and female. The specific individual demographic data do not permit any identification of the donors. Informed consents were obtained according to the submitted guidelines. Ethical approval for the usage of human islets was granted by the Human Research Ethics Board at the Ramathibodi Hospital Mahidol University. Islet isolation was performed using non-enzymatic method as described in [23] Pancreatic tissues were cleaned by standard practice, then mixed and incubated with approximately 35–40 ml of RPMI 1640 medium supplemented with 600 mM glucose in 20 min at room temperature. The minced pancreatic tissue was washed and switched to zero-glucose RPMI medium. To minimize cellular toxicity from histopaque, the supernatant phase was filtered using a nylon mesh with a pore size of 440-μm to remove non-islet tissues and 70-μm strainer to retain islets [24]. To recover isolated islets were cultured on the 40-μm strainer with RPMI 1640 medium supplemented with 10% FBS, 5.6 mM glucose, 3% penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.25 μg/ml amphotericin B. We measured the quality of islets by ADP/ATP ratio using ApoGlow™ kit (Cambrex Bio Science) [25]. Unlike the conventional viability tests, the ADP/ATP energy status

offers a good predictor of *in vivo* clinical transplantation outcome. Briefly, 30 islets were washed with PBS 3 times and then mixed with 50μL of nucleotide-releasing agent for 10 min at room temperature. Subsequently, 10μL of nucleotide-monitoring reagent was supplemented in the solution. The ATP levels were read-out as relative light units (RLU) using a Luminometer. After that, 10 μL of ADP converting agent was added to convert the ADP in the solution to ATP and the RLUs were measured. Consequently, the ADP/ATP ratio of the islets were calculated. The ADP/ATP ratio of isolated islets were established as an effective predictor of clinical outcome in vivo transplantation; thus, the ADP/ATP ratio has been a more informative assay for islet quality predictor performance [25]. The quality islets were then cultured in RPMI as mentioned at 5.6mM glucose for 24 h then changed to the media at 16.7mM glucose and replenished with the new media every other day.

2.2. Insulin and protein contents

We washed purified islets with D-PBS 3 times, and then applied RIPA buffer containing protease inhibitors as previously described [25]. To determine protein content of the lysate, we used the Bio-Rad DC protein assay and we measured insulin content using ELISA kit (Abnova).

2.3. Imaging flow cytometry

Intact islets were dissociated with 0.25% trypsin for 10 min at 37 °C and fixed with 4% paraformaldehyde for 20 min and the cells were subsequently permeabilized with perm/wash buffer (BD). Intracellular staining was carried out in perm/wash buffer containing 2% goat serum with anti-insulin (Sigma MABF2117), anti-GLP1 (Abcam ab23468), anti- CHGA (Novusbio NB120-15160), and anti-glucagon (R&D Systems MAB1249) at 0.5 μg/ml for 2 h at RT. Following extensive washing, cells were filtered, visualized and quantified on Amnis Imaging Flow Cytometer ImageStreamX Mk II (Millipore). Secondary antibodies were purchased from Abcam: Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150077) were applied for 15 min in the dark room at RT. Acquisition of events were collected at medium speed in the positive stained population gate until 10,000 events were counted for each sample. The cells were passed through X40 magnification. The imaging cytometer and the software were set up as channel 01 (bright field), channel 12 (scattering channel) and channel 02 for Alexa Fluor® 488 conjugated. The lasers 488 and 745 were ignited to monitor fluorescence and side-scatter, and we set the flow rate to medium speed. The Alexa Fluor® 488 gate was plotted against side scatter intensity. This excluded the internal calibrator beads which the machine image capture system used to determine the flow speed [26].

2.4. Real time quantitative PCR

Total RNA was extracted using the Qiagen RNAeasy kit and reverse transcribed into cDNAs using SuperScript™ III Reverse Transcriptase (Invitrogen). NanoDrop SPectrophotometer was used to quantify the cDNAs. Gene-specific primers were ordered according to [9,27,28,] and then used to perform qPCR using SYBR Green PCR Master mix (Thermo Fisher) and an QuantStudio 3 (Thermo Fisher). Normalization of qPCR values was with values from qPCR of *GAPDH* values in the same samples.

2.5. Liraglutide treatment

Human islets were incubated with 1 μmol/·L liraglutide and 40 nM non-targeting control or 40 nM *FOXO1* Silencer Select siRNA s5259 (Thermo Fisher Scientific) using RNAiMax (Thermo Fisher Scientific) or 40 nM non-targeting control without liraglutide from day 3 to day 7 of *in vitro* culture of 16.7mM glucose. The media was
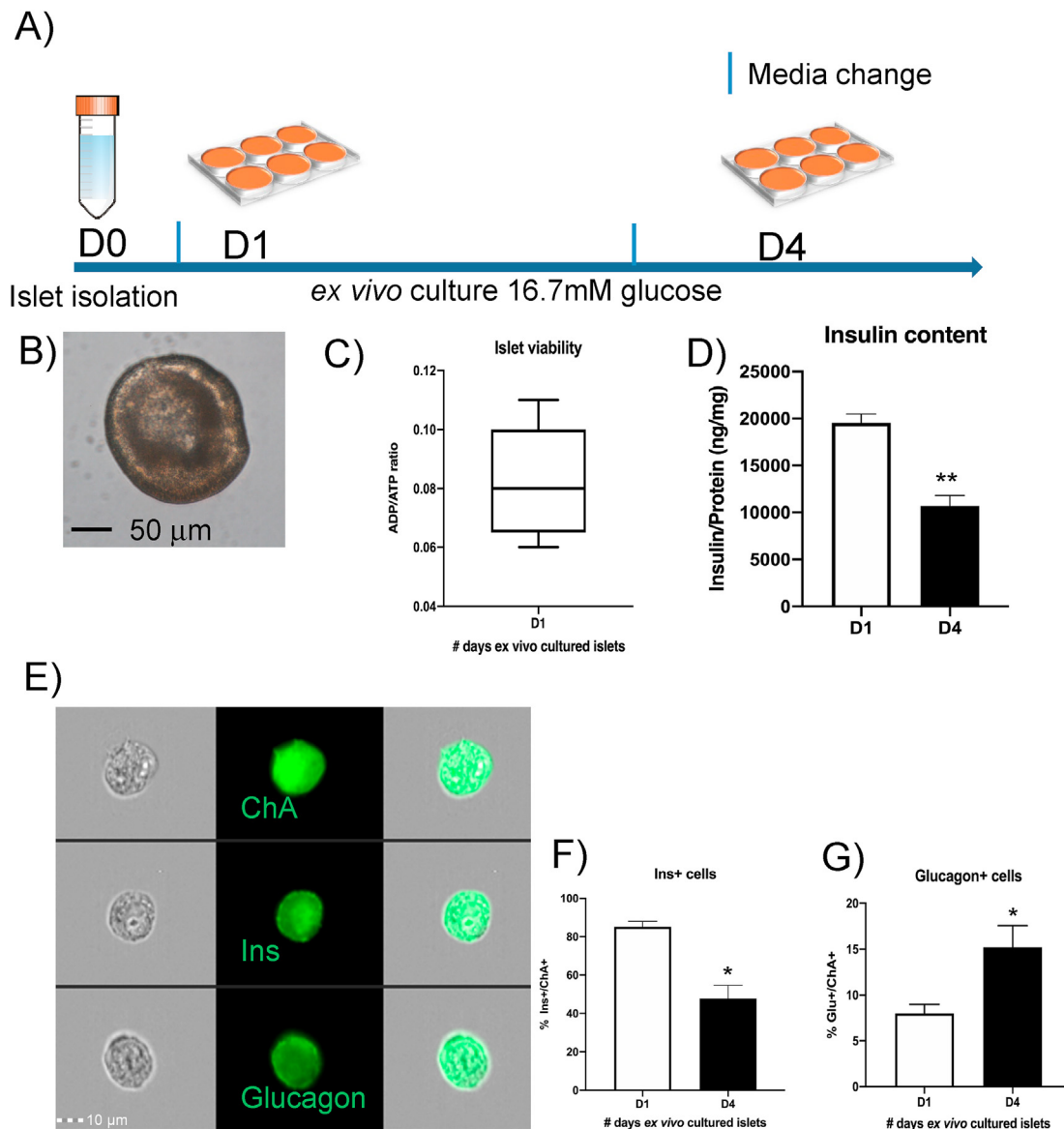
Figure 1. Isolated human islets when cultured *ex vivo* for 4 days showed the key features of dedifferentiated islets A) schematic experimental design from which islets were isolated from human pancreata on Day 0, cultured and media were changed at D1 and D3. B) a representative image of healthy islets isolated from human donors. C) ADP/ATP ratios of quality islets that were proceeded to dedifferentiation experiments. D) Isolated islets were measured their insulin contents relative to total protein contents. E) immunostaining of single cells positive to either CHROMAGRANIN-A, INSULIN, or GLUCAGON, dissociated from islets cultured from Day 1 or Day 4. F) &G) Quantification of insulin or glucagon positive cells as relative to total hormone positive (CHORMOGRANIN-A cells) measured by imaging flow cytometry. 10,000 cells were counted per read, and each sample was assayed in triplicates. E) Percentage of INSULIN+ cells. F) Percentage of GLUCAGON+ cells as relative to total CHROMAGRANIN-A cells from islets cultured until Day 1 or Day 4. Data show means ± SEM. * = p < 0.05 and ** = p < 0.01 by Student's t -test (n = 5).

changed at 24 h and 72 h after incubation. *FOXO1* siRNA was performed according to the manual protocol and the knockdown was evaluated using qPCR after 24 h post siRNA treated [31]. Islet viability functionality was assessed. We examined the β−cell dedifferentiation properties of liraglutide were evaluated by measuring insulin contents, hormone positive cells, gene expressions of markers of β−cell identities, β−cell machinery, progenitor markers, and mesenchymal markers. The GLP-1 related effect of liraglutide was evaluated by counting islet GLP-1 positive cells.

2.6. Statistical analyses

Data are presented as means ± SEM. R statistical package (http://www.R-project.org/) and Prism 8 GraphPad software were used to analyze 2-tailed Student's t-test. Differences were considered significant for *P < 0.05, and **P < 0.01.

3. Results

3.1. Ex vivo isolated human islets underwent dedifferentiation by day 4

Previous studies culturing isolated islets from animal and human conclusively showed that culturing islets *ex vivo* caused β−cells to engage in dedifferentiation process [7, 8], and not cell death. But, it remained unclear how *ex vivo* β−cell dedifferentiation was similar to pathological *in vivo* β−cell dysfunction. Therefore, we explored *ex vivo* islet culturing as a potential model to investigate mechanistic changes in human islets during dedifferentiation. To mimic hyperglycemic conditions, we set up

the dedifferentiation process by comparing isolated islet properties from islets that were cultured for 24 h or Day 1 and Day 4 (Figure 1A) under high glucose conditions (16.7mM glucose or the equivalent of 3 times physiological blood glucose levels). The process of islet purification involved non-enzymatic method [23, 24] from non-diabetic donors to minimize mechanical and cellular stresses from enzyme digestion and high temperature treatment. Figure 1B represents a healthy isolated islet. We further proceeded the experiments only when the purified islets illustrated ADP/ATP ratios of the human isolated islets within the range of 0.05–0.11 (Figure 1B), indicative of quality such that the isolated islets were at par of transplantation grade predicted to reverse diabetes *in vivo* [25]. Other conventional indicators reflect viability without information regarding *in vivo* performance [25]. In addition, the purity of islets was confirmed to contain no significant amounts of mRNAs of acinar and ductal markers. Next, we verified whether *ex vivo* dedifferentiated

pancreatic β−cells exhibited characteristics as identified *in vivo* diabetic β−cell dedifferentiated islets. First, we found 40–50% reduced insulin contents when isolated islets had been cultured to day 4 (Figure 1D) similar to our previous *in vivo* observation in diabetic islets [4]. To further quantify the extent of which changes occurred in hormone positive populations, we dissociated the islets to single cells and counted the immunoreactive insulin (INS), glucagon (GCG), and chromagranin A (CHGA) positive cells, using imaging flow cytometry. As frequently observed in diabetic islets, the results showed the reduced percentage of insulin$^+$ cells from 80-90% to approximately 40–60% of total chroma-granin A positive cells (Figure 1E-F). Meanwhile, the percentage of glucagon$^+$ cells was markedly increased (Figure 1G), suggesting that *ex vivo* dedifferentiated islets shared an important key feature of diabetic dedifferentiated pancreatic islets. Of note, we did not observe different percentages cell death between day 1 and day 4 (Figure 2A-B), but the
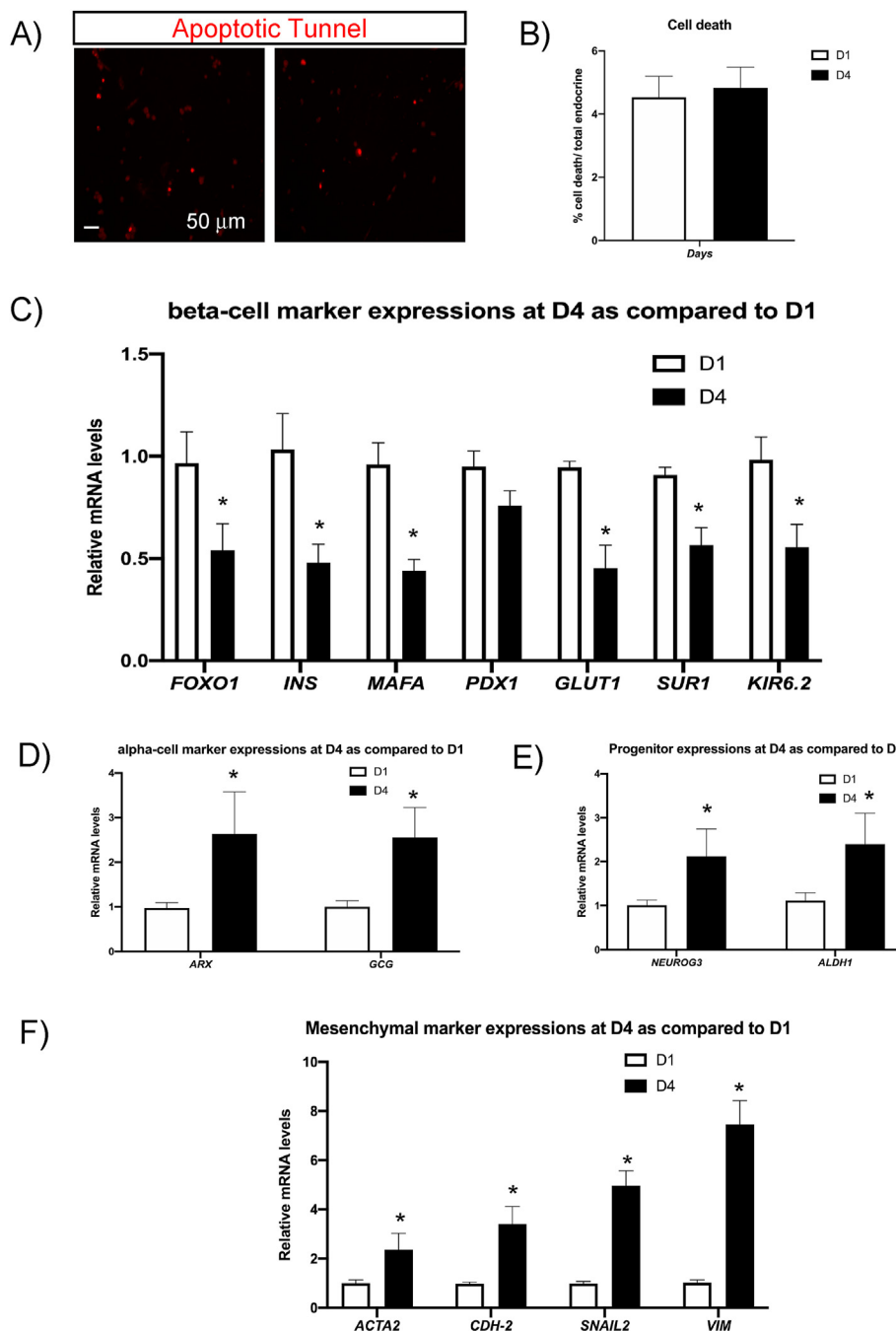

Figure 2. Ex vivo dedifferentiated human islets showed reduced expression of beta cells marker genes, while transcripts of progenitor genes, and alpha-cell genes were upregulated. A) Representative images of *ex vivo* islet cell death by TUNEL assay immonor-eactive to dissociated islet cells. B) Quantification of cell death in samples from Day 1 and Day 4 calculated as percentage relative to total CHROMAGRANIN positive populations. C) quantitative RT-PCR analysis of beta-cell marker genes. D) qRT-PCR analysis of alpha-cell genes, and E) qRT-PCR analysis of progenitor genes, and F) qRT-PCR analysis of mesenchymal markers. Data were normalized against the level of *GAPDH*. Data show means ± SEM. * = p < 0.05 and ** = p < 0.01 by Student's t -test (n = 5) 50 islets were sampled from each individual.

change in the number of insulin[+] staining as relative to chromogranin[+] staining as described in β−cell dedifferentiation [3, 4, 8]. Thus, these data showed that after 4 days, cultured islets lost insulin contents but exhibited expanded numbers of glucagon staining. This finding shared similar patterns to in vivo progressive diabetic endocrine pancreas [2].

3.2. Ex vivo dedifferentiated islets shared genetic markers of in vivo diabetic islets

In disease-causing β−cell dedifferentiation, FoxO1 was a linpinch transcription factor that maintained β−cell properties amidst physiological stresses [4]. Loss of FoxO1 induced subsequent loss of β−cell identity markers: MafA, Pdx1, Ins, depleted β−cell function indicators: Glut2, Sur1, and Kir6.2, and importantly increased progenitor markers: Neurog3, and Aldh1A3 [4,5]. To assess the mechanistic changes of ex vivo

dedifferentiated islets, we performed gene expression analysis of these islet properties. In human islets, GLUT1 is a primary glucose transporter [28]. The results revealed reduced levels of FOXO1, INS, MAFA, GLUT1, SUR1 and KIR6.2 from ex vivo cultured human islets at day 4 as compared to day 1 (Figure 2C). Coincided with the rises of glucagon^{+} cells observed in Figure 1G, ex vivo dedifferentiated islets exhibited increased transcripts of ARX, a glucagon regulator, and the proglucagon GCG (Figure 2D). Interestingly, transcripts of progenitor markers: NEUROG3, and ALDH1A3 in ex vivo dedifferentiated islets were upregulated in day 4 (Figure 2E) similar to the findings in diabetic islets of murine models. During islet dysfunctional process, epithelial-mesenchymal transition genes such as Nestin and Vimentin were observed during in vivo β−cell dedifferentiation [4], and expanded cultured human isolated islets [7, 9]. Similarly, we found the rises of mesenchymal gene expressions: ACTA2, CDH-2, SNAIL2, and VIM, in the day 4 high glucose ex vivo cultured islets
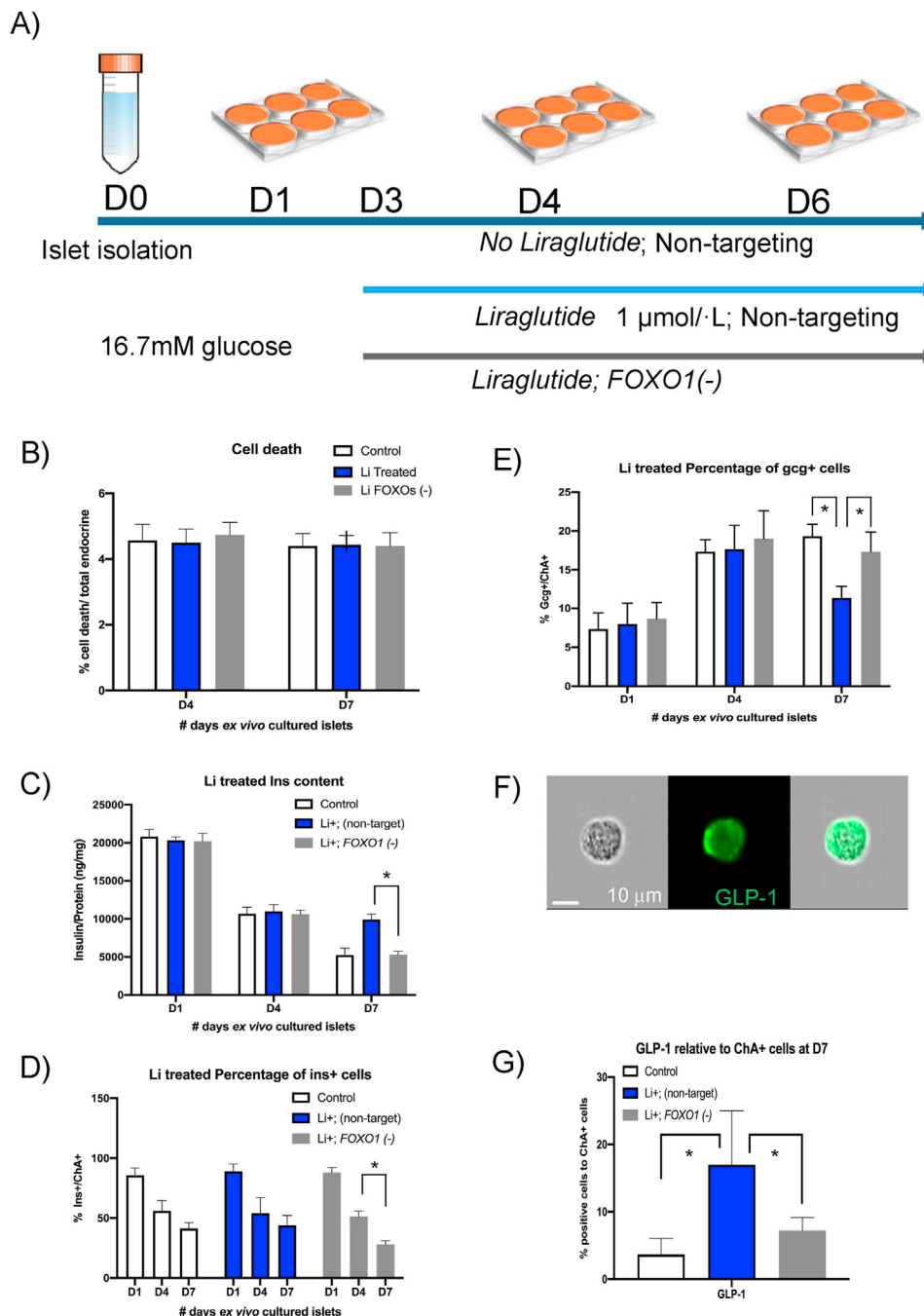

Figure 3. Liraglutide effects on dedifferentiated islets are FOXO1 depedent A) schematic experimental design from which islets were isolated from healthy human pancreata. Islets were called controls when there was no liraglutide treatment, but on Day 3 were added non-targeting siRNAs and dubbed non-targeting or FOXO1 (-) siRNA along with liraglutide treatment on Day 3, and samples were taken at Day 4 and Day 7 for analysis. B) Percent cell death in dissociated islets as calculated from immunostained TUNEL assay relative to CHROMOGRANIN-A positive cells. C) Insulin contents relative to total protein contents of isolated islets. Samples were evaluated at Day 1, Day 4, or Day 7. D) Percentage of INSULIN^{+} cells and E) Percentage of GLUCAGON^{+} cells as relative to total CHROMAGRANIN-A cells. F) Immunostaining of single cells positive to either CHROMAGRANIN-A, GLP-1from dissociated from islets cultured from Day 7. G) Quantification of GLP-1 positive cells as relative to total hormone positive (CHORMOGRANIN-A cells) measured by imaging flow cytometry. 10,000 cells were counted per read, and each sample was assayed in triplicates. Data show means ± SEM. * = p < 0.05 by Student's t -test (n = 5).

(Figure 2F). These gene expression data of *ex vivo* dedifferentiated islets further support the shared dysfunctional nature to *in vivo* diabetic islets [4].

3.3. Liraglutide treatment suppressed the increased glucagon+ cells but expanded islet GLP-1 in FOXOs-dependent manner

Studies of pancreatic diabetic islets have described increased glucagon+ cells and glucagon contents along with progressive diabetes [1, 2, 3]. Hyperglucagonemia in diabetic patients worsens their blood glucose due to glucagon action on liver glucose production. We observed the rises of percent glucagon+ cells in hormone+ populations at day 4 in *ex vivo* islet dedifferentiation (Figure 1G). A long-term treatment of ZDF diabetic rats with liraglutide study reported reduced glucagon+ cells based on immunostaining analysis of pancreatic diabetic islets as compared to non-diabetic rats [17]. However, it was unclear if the results were due to liraglutide effects on intestinal L-cells or direct impact of liraglutide on pancreatic islet properties. To distinguish between these two alternatives, we hypothesized that treatment of liraglutide directly on incubated *ex vivo* islets would affect islet properties while indirect effects of liraglutide would show no changes in the cultured islets. *Ex vivo* islet cells were incubated with or without liraglutide treatment from day 3 to day 7 while both were mixed with non-targeting RNAs (Figure 3A).

With or without liraglutide treatment, non-targeting nor *FOXO1* siRNAs, our findings reported no significant differences of cell death (as measured by TUNEL assay) among these different conditions at day 4 and day 7 (Figure 3B). Interestingly, these data further indicated that liraglutide treatment prevented insulin contents from further loss on day 7 as compared to no liraglutide treated controls. In islet cells treated with *FOXO1* siRNA and liraglutide, liraglutide was unable to prevent islets from progressively lost insulin contents indicating that FOXO pathway was involved in liraglutide action (Figure 3C-D).

Both with and without liraglutide incubation, the percentages of glucagon+ cells were increased on day 4. Only with liraglutide treatment, the percentage of glucagon+ cells were significantly down from the ranges of 20%–10% and this effect was reverted when *FOXO1* was further depleted (Figure 3E), suggesting that FOXO1 was essential in liraglutide effects on expanded glucagon+ cells. GLP-1 usually is processed in intestinal endocrine cells. To examine if islet GLP-1 was involved in liraglutide effects on *ex vivo* dedifferentiated islets, we analyzed the number of local GLP-1 positive cells relative to endocrine populations. Day 7 liraglutide treated islets showed significantly increased percentages of GLP-1 stained cells to the ranges of 15–30% of total islet endocrine cells (Figure 3F-G). The data distinguished direct impact of liraglutide treatment on islets as opposed to secondary effects due action of intestinal GLP-1 cells. These results suggest that early
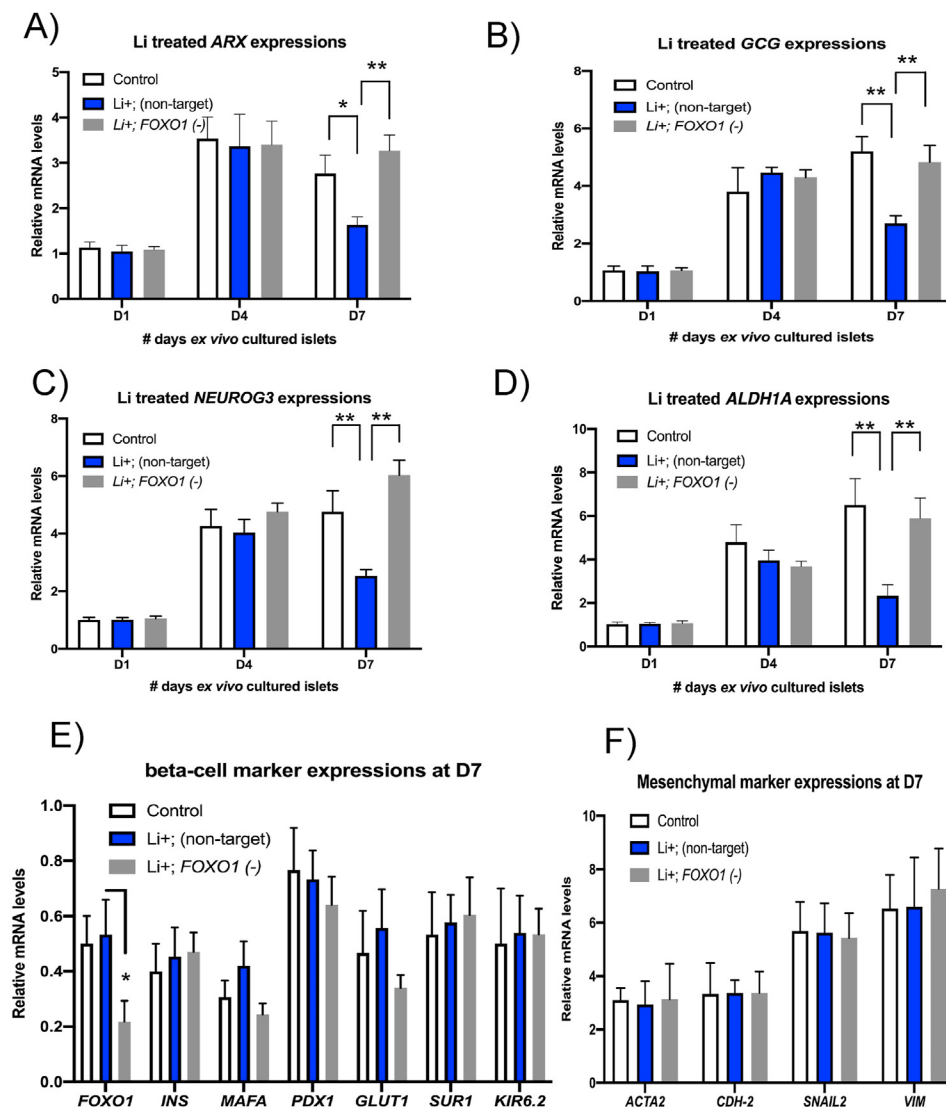

Figure 4. Suppression of alpha-cell relate genes and progenitor markers by liraglutide treatment. qRT-PCR analysis of alpha-cell genes: A) *ARX,* B) *GLUCAGON*, C-D) progenitor genes: C) *NEUROG3*, and D) *ALDH1A*, evaluated at D1, D4, and D7. E) quantitative RT-PCR analysis of beta-cell marker genes, and F) of mesenchymal marker genes: *ACTA2, CDH-2, SNAIL2, and VIM* measured at D7. Data were normalized against the level of *GAPDH* and expressed as relative folds to the transcript levels of isolated islets cultured until Day 1. Islets were dubbed as controls when there were no liraglutide treatment, but on Day 3 were added non-targeting siRNAs, and were called *non-targeting* or *FOXO1 (-)* siRNA along with liraglutide treatment on Day 3, and samples were taken at Day 4 and Day 7 for analysis. Data show means ± SEM. * = p < 0.05 and ** = p < 0.01 by Student's t -test (n = 5) 50 islets were sampled from each individual's replicate.

liraglutide treatment could affect development of diabetes by contributing to inhibition of expanded glucagon^{+} cells and activation of local GLP-1 during progressive islet endocrine dysfunction.

3.4. Direct suppression of progenitor expressions by liraglutide treatment

We asked if liraglutide treatment on *ex vivo* culture of human islets would alter islet gene expressions, and immune reactivities of islet endocrine cells. Clinical studies of liraglutide, GLP-1 analogue have shown improved glycemic outcomes [29]. In humanized mouse models, liraglutide compromised long-term islet health [30]. Therefore, the mechanistic understanding of liraglutide during *ex vivo* β−cell dysfunction would help elucidate the dilemma of liraglutide *in vivo* effects. In Figure 4A and B), we treated *ex vivo* human islets from day 3, and examined the results at day 4 and 7 as compared to non-targeting siRNA untreated liraglutide controls and we found that treated islets showed that *ARX* and *GCG* levels remained elevated. The increased percentage of GLP-1 positive cells seen in Figure 3E corresponded to higher *ARX* and *GCG* expressions. On the other hand, treatment of liraglutide on *ex vivo* cultured islets from day 3 to day 7 did not improve transcript markers of β−cell identities: *FOXO1, INS, MAFA, PDX1,* nor markers of β−cell function: *GLUT1, SUR1* and *KIR6.2*. Figure 4E the *FOXO1* mRNA level validated the degree of the knockdown to 70–80% of D1 controls. This incomplete knockdown could be due to islet structures, and the degrading nature of *FOXO1* in this hyperglycemic context. On the other hand, progenitor markers of β−cell [4], *NEUROG3* and *ALDH*[+] [5] signified diabetic progression via islet pathology. Surprisingly, Figure 4C and D showed that liraglutide treatment could rescue the elevated levels of these two markers during *ex vivo* β−cell dedifferentiation and these effects were blunted when *FOXO1* were further depleted. However, mesenchymal marker expressions: *ACTA2, CDH-2, SNAIL2, and VIM* sustained their elevation at day 7 in all sampled islets despite liraglutide treatment and knockdown of *FOXO1* in islet cells (Figure 4F). Our data indicate a role of GLP-1 pathway in beta-cell plasticity, but in the meantime, also exclude the involvement of liraglutide action from the ability to regulate β−cell mechanistic genes nor mesenchymal transition genes during islet dysfunction.

4. Discussion

β−cell dedifferentiation occurred *in vivo* and responsible for diabetic beta cell dysfunction in animal diabetic models. Dedifferentiated β−cells have been commonly observed in *ex vivo* conditions [7, 8]. But it was perceived as the difficulties of maintaining β−cell specific gene expressions [7, 8] and did not connect to the pathophysiology of T2DM. Our findings showed that *ex vivo* β−cell dedifferentiation shared some of mechanistic properties as *in vivo* diabetic β−cell dedifferentiation such as the loss of β−cell markers, and the rise of glucagon[+] populations, and the overexpression of progenitor markers: *NEUROG3* and *ALDH1A*, as well as elevated expressions of mesenchymal markers: *ACTA2, CDH-2, SNAIL2, and VIM,* in *ex vivo* dedifferentiated human islets. Our study reported percentages changes of immunoreactive GCG, GLP-1 and INS populations relative to total endocrine cells marked by CHG-A staining. The data presented percentages of insulin and glucagon immunostained cells did not fall within the average 60% insulin or 40% glucagon observed in human pancreatic islets by former surveys [32]. However, the percentages found in our samples were within ranges of human islet heterogeneity [33] and consistent with previously described in literatures [34, 35]. The discrepancies could be due to a) our islet isolation methods that might have biased toward smaller islet sizes, and b) these numbers could reflect Asian islet structures as the pancreatic analysis in [32] did not specifically report samples from Asian genetic backgrounds. Our analysis was bound within this limitation. It is important to note that we are interested in the dynamic changes of gene expressions as a result of *ex vivo* islet dedifferentiation as opposed to static numbers of hormone positive cells in islets. The results elucidated different aspects of

mechanistic understanding of liraglutide direct effects on islets that could alter hormone[+] populations, markers of progenitors, markers of GCG related genes, but not markers of β−cell function nor mesenchymal marker genes. This mechanistic understanding is clarified under *ex vivo* model as opposed to *in vivo* model. These detailed data could be useful for future antidiabetic drug targeting and design.

As we determined that liraglutide treatment partially protected dedifferentiated human islets from expanding GLUCAGON[+] cell numbers, liraglutide suppressed the elevated expression levels of *ALDH1A and NEUROG3 in FOXO1* dependent manner. Furthermore, liraglutide treatment of dedifferentiated human islets show increased GLP-1 positive cells but had little effects on expressions of β−cell function markers nor gene expressions that were elevated during epithelial mesenchymal transition such as *ACTA2, CDH-2, SNAIL2, and VIM*. However, mechanisms of local GLP-1 action and organ target sites were not well understood in human [36]. Our single staining study could not distinguish whether increased glucagon^{+} cells was due to beta-to-alpha conversion that was observed in other lineage-tracing studies [4, 20, 37]. When treated with liraglutide, dedifferentiated β−cell showed reduced number of glucagon positive cells similar to liraglutide treatment to diabetic mice *in vivo* [30]. Similar to *in vivo* treatment of liraglutide in STZ-induced diabetic models [21], and long-term treatment of liraglutide [30], we observed a decrease in glucagon^{+} cells and further solidified by reduced expressions of *GCG* related genes. Unlike Zhang et al, 2019, we did not see upregulated mRNA levels of *FOXO1, PDX1, and MAFA*, possibly because our system did not involve excessive oxidative stress from STZ-treatment. Our data coincided to the *in vivo* study of diabetic models that liraglutide was not sufficient to restore insulin content [18, 30]. In this work, we also did not find liraglutide to reverse insulin contents in *ex vivo* dedifferentiated human islets. Early treatment of liraglutide could potentially affect diabetic development by preventing uprising of progenitor genes and glucagon^{+} cells. Taken together, our data suggest that this system of high glucose induced *ex vivo* dedifferentiated β−cell cells could be a useful model for drug screening and testing for potential antidiabetic actions on human islets.

Declarations

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