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Exendin-4 Enhances GSIS By Upregulating Genes Related to Maturation, Glucose-Sensing Apparatus and Mitochondrial Oxidative Phosphorylation Machinery in hPSC-Derived Islets Without Increasing β-Cell Number

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Abstract

Emerging β-cell replacement with human pluripotent stem cell (hPSC)–derived β-cells could provide remedial cell therapy for diabetes. Most *in vitro* differentiation protocols generated hPSC-derived β-cells with immature phenotypes such as impaired or weakened glucose-stimulated insulin secretion (GSIS) relative to primary β-cells. Evidence has shown the effectiveness of exendin-4 in increasing β-cell mass and function *in vivo*. This study investigates the effect of exendin-4 on maturation and functionality of hPSC-derived β-cells. Differentiation of two hPSC cell lines (HUES8 and iPSC824) into islets was carried out using a 3D differentiation protocol. 50 nM Exendin-4 was added to the suspension culture during the last three days of differentiation. The gene expression patterns in both cell lines showed that expression of the pluripotent marker OCT4 was lost upon initiation of differentiation. The definitive endoderm marker SOX17 was transiently and significantly increased at Stage 1. PDX1, a marker of the pancreatic progenitor 1 started being significantly upregulated at Stage 3; while the β-cell specific markers including insulin, NKX6-1 were strongly induced at stage 5, and 6. In HUES8 there was no difference in c-peptide secretion between low (2.8mM) and high (20 mM) glucose; suggesting a lack of functional β-cells. While flow cytometry data showed no significant difference between control and exendin-4 treated groups in NKX6.1^{+}/insulin^{+} (β-cell markers), the addition of exendin-4 significantly enhanced GSIS in both cell lines. This was associated with increased expression of maturation (NeuroD1, Six2, MAFA) glucose-sensing (Glut2, GCK) and mitochondrial oxidative phosphorylation machinery (NDUSF1, NDUSF2) genes. In summary, we demonstrated for the first time in 3D differentiation of hPSC-derived β-cells that addition of exendin-4 enhances GSIS without increasing β-cell number.

Keywords: Exendin-4; 3D differentiation; diabetes; GSIS; hPSC-derived β-cells.

Introduction

Diabetes mellitus, which affects more than 537 million people worldwide (www.idf.org) is characterized by impaired glucose metabolism that results from a defect in insulin secretion, action, or both [1]. The American Diabetes Association (ADA) has classified diabetes into two main categories, Type 1 diabetes (T1DM) and Type 2 diabetes (T2DM) (American Diabetes Association 1997 Report). T1DM, also known as juvenile diabetes or insulin-dependent diabetes is a result of a selective immune-mediated pancreatic β-cell

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destruction leading to nearly complete deficiency of insulin production in the body and represents 5-10% of diabetic patients. T2DM, on the other hand, results from peripheral tissue insulin resistance and β-cell dysfunction and represents 80-90% of diabetic patients [2-4]. Now it is well established that T1DM and severe forms of T2DM commonly share an important feature, a marked reduction in the number of the pancreatic β-cells that negatively impacts insulin secretion [5], suggesting that β-cell replenishment would be of an ideal therapeutic option in both diseases in the future. Patients with T1DM predominantly rely on exogenous insulin injections to combat insulin deficiency [6]. Whilst the exogenous insulin injections are considered as a life-saving treatment, it remains unfortunately imperfect as it could result in acute/severe hypoglycemia, and weight gain for many patients [7]. Cadaveric islet transplantation using Edmonton protocol has demonstrated to be an effective treatment for T1DM, which could allow temporal exogeneous insulin independence [8]. However, this islet transplantation approach is significantly hindered by its high costs and healthy islet donor shortage as well as a potential risk of tissue rejection [9]. Therefore, transplantation option cannot be widely implemented in clinical practice to the diabetic population. One of the alternatives to resolve the cadaveric islet shortage is the generation of surrogate and transplantable pancreatic β-cells from pluripotent stem cells (PSC) due to their infinite self-renewal capacity and ability to differentiate into derivatives (cell types) of all three germ layers in the body.

In the context of diabetes therapy, patient specific PSC-derived β-cells also known as induced PSC (iPSC) or autologous PSC-derived β-cells would help to circumvent the inadequate islet supply and allogeneic immune rejection. Indeed, iPSC do not impose any ethical concerns as compared to human embryonic stem cells (hESC); making the appropriate choice for diabetes therapy. Moreover, iPSC-derived β-cells from patients with diabetes are also critical to gain a better understanding of the disease and its progression [10]. Over the last two decades, great effort has been concentrated by scientists on developing technologies and protocols to efficiently and reproducibly differentiate PSC into mono-hormonal insulin-expressing cells *in vitro* that show key features of a bona fide mature β-like cells capable of maintaining the physiological blood glucose setpoint following transplantation. Most *in vitro* protocols for differentiating PSC into insulin-expressing β-cells using specific soluble inducers or small molecules have produced hPSC-derived β-cells with an immature phenotype. This is characterised by impaired or weakened glucose-stimulated insulin secretion (GSIS) and low or absent expression of key mature transcription factors (e.g MAFA, NEUROD1, SIX2) as compared to primary β-cells [7, 11]. The reason and mechanisms behind the production of immature PSC-derived β-cells *in vitro* vary and are not fully understood. Evidence has shown that β-cell mitochondria play a central role in coupling glucose metabolism to insulin exocytosis. Therefore, the impairment of GSIS in hPSC-derived β-cells may be attributed to mitochondrial dysfunction. There is consensus that mitochondrial metabolism is a major determinant of insulin secretion from islet β-cells [12]. More specifically, in response to extracellular glucose, β-cells facilitate GSIS through increased mitochondrial oxidative ATP production [13-15], indicating that increased mitochondrial activity is a cellular component required for GSIS. Therefore, disruption of mitochondrial oxidative metabolism has been identified as an important contributor to impaired GSIS [16, 17]. Moreover, the paramount role of mitochondria in GSIS is demonstrated by the substantial positive correlation between mitochondrial membrane potential and GSIS [18] as well as by complete inhibition of GSIS when OXPHOS is suppressed [19]. Also, hPSC-derived β-cell clusters obtained *in vitro* are often characterized by low amplitude of GSIS as compared to native pancreatic β-cells despite equal amount of mitochondrial mass per cell [20], suggesting that differentiated hPSC-derived β-cells might have metabolically dysfunctional mitochondria. Interestingly, reduced anaplerotic cycling in the mitochondria has been identified as an underlying mechanism associated with reduced GSIS in hPSC-derived β-cells [20]. Therefore, proper mitochondrial function is a cornerstone of β-cell in coupling glucose metabolism to insulin exocytosis. In addition, besides intrinsic factors (genetic program), the efficiency of differentiation is modulated by external microenvironment. Interestingly, the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 [21] has been incorporated among the extrinsic factors to enhance PSC-derived β-cell differentiation using conventional two-dimensional (2D) cell culture systems [22-25]. Exendin-4 is a 39-amino-acid peptide which has been found to act as a long-acting GLP-1 receptor agonist and has been reported to stimulate both β-cell replication and neogenesis, resulting in increased β-cell mass and improved the insulin secretion function [26]. However, the effects of exendin-4 on 3D suspension culture throughout the entire differentiation protocol [27-30] specifically have not been studied adequately.

Materials and Methods

Stem cell culture and in vitro pancreatic beta cell differentiation

Both embryonic (HUES8) and induced (iPSC824) pluripotent stem cell lines were used in this study. iPSC824 line was generated by Qatar Biomedical Research Institute stem cell core from commercially available consented healthy (female) donors' dermal fibroblasts. Undifferentiated HUES8 and iPSC824 cells were cultured on Matrigel-coated (Cat #

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3542277.) 2D plates and maintained in mTeSR+ (Cat # 100-0276.) for expansion. After reaching ~90% confluency and undergoing at least three passages, cells were dissociated into a single-cell suspension, counted and 20 million cells were transferred into 30ml spinner flasks (ABLE Corporation, Tokyo, Japan) placed on a 6-position stir plate set at rotation rate of 65 rpm in a 37°C incubator, 5% CO2, and 100% humidity to form 3D clusters. Stem cells clusters were passaged every 3-4 days using Gentle Cell Dissociation Reagent (GCDR; Cat# 100-0485) for cluster dissociation, followed by passing the cells through a 37 uM strainer and seeded at 0.5 million cells/ml in mTeSR$^+$ + 10 µM Y27632 (ROCK inhibitor; Cat #72308). Two stem cell lines, one embryonic (HUES8) and one induced-pluripotent (iPSC824) stem cell lines were differentiated into pancreatic β-like cells using a modified 3D differentiation protocol [28]. The differentiation process was carried out in six stages, as detailed below: differentiation was initiated 48 hours after passage, when clusters reached a diameter of 200-250 µM, by removing mTeSR$^+$ medium and replacing with the stage-specific medium and growth factor/small molecules supplements. Media changes were as follows – Day 1: S1 +100ng/ml ActivinA + 3 µM Chir99021 + 10 µM Y27632. Day 2: S1 + 100 ng/ml ActivinA. Day 3: S1 + 100 ng/ml Activin A. Days 4, 6: S2 + 50 ng/ml KGF. Days 7, 8: S3 + 50 ng/ml KGF + 0.25µM Sant1 + 2µM RA + 200nM LDN193189 (only Day 7) + 500 nM PdBU + 10µM Y27632. Days 9, 11, 13: S3 + 50 ng/ml KGF + 0.25 µM Sant1 + 100nM RA + 10µM Y27632. Days 14, 16: S5 + 0.25 µM Sant1 + 100 nM RA+1 µM XXI + 10µM Alk5i II + 1µM T3 +20 ng/ml Betacellulin. Days 18, 20: S5 + 25 nM RA + 1µM XXI+ 10 µM Alk5i II + 1µM T3 + 20ng/ml Betacellulin. Days 21–35: S3 (changed media alternate days). For the Exendin-4 treatment study, S3 media supplemented with 50 nM of Exendin-4 (Cat # E7144-1MG) was added during the last 3 days of differentiation (Days 33-35). The 50nM were chosen as ideal concentration based on a dose-dependent effect of exendin-4 on cell viability (Supplementary figure 1).

Flow cytometry

At each stage of the differentiation, clusters from the suspension culture were collected and dissociated using TrypLE Express (Gibco; Cat#12604013) at 37 °C and mechanically disrupted to form single cells. Cells were fixed using 4% PFA (Cat # J61899.AP) for 20 min at 4 °C. Fixed cells were washed twice with stain buffer (Cat # 554656) and then incubated in permeabilizing/washing buffer (Cat # 51-2091KZ) with primary antibodies (30 min for conjugated antibodies or overnight at 4 °C for unconjugated antibodies). The cells were washed by centrifugation (300 rcf, 5 min) three times with permeabilizing/washing buffer and incubated with secondary antibodies in permeabilizing/ washing buffer for 2 hours at room temperature. Following three more washes, the cells were resuspended in stain buffer and analyzed using the , Accuri C6 flow cytometer (BD Biosciences). Flow cytometry data were then analyzed using FlowJo software. Results presented are representative of at least three independent differentiations.

Immunofluorescence staining

At each stage of the differentiation, clusters were collected, washed, and fixed in 4% PFA for 1 hour on ice. Fixed clusters were washed and incubated in sucrose overnight at 4 °C before being frozen in Optimal Cutting Temperature compound (O.C.T). The clusters were then sectioned (8 µm thickness) and placed on a glass slide. For staining, slices were washed with PBS, permeabilized for 30 minutes in permeabilization buffer (0.3% Triton-X in PBS) and blocked for two hours in blocking buffer (6% BSA, 2% donkey serum, 0.3% Triton-X in PBS). The clusters were incubated with primary antibodies overnight at 4 °C in blocking buffer, washed three times and incubated with secondary antibodies for 2 hours in blocking buffer at room temperature. The clusters were then washed, incubated with DAPI for 10 min and then washed twice before placing a coverslip over the slide using permount mounting media (Cat#SP15-100). The list of antibodies used, and their dilution are mentioned in supplementary Table 1. Images were acquired using at 20X/0.8 numerical aperture (NA) objective (LD LCI Plan-Apochromat; Carl Zeiss Inc, Oberkochen, Germany) using confocal microscopy on a laser scanning microscope (LSM 780; Carl Zeiss Inc, Oberkochen, Germany). Images were analyzed using ZEN imaging software (Carl Zeiss Inc.). Images shown are representative of at least three biologically separate differentiations.

Gene expression using real-time RT-PCR

At each stage of the differentiation, total RNA was extracted from clusters using RNeasy Mini kit (Cat # 74104) as instructed by the manufacturer, then converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Cat # 4374967). Gene expression was measured using RT-PCR (QuantStudio 6 Flex system) using SYBR Green (Cat # 4309155). Relative expression was calculated by the Livak comparative ΔΔCt method [31]. GAPDH and Actin genes were used as housekeeping genes. The primer sequences are listed in Table 1.

Whole cell lysates and western blot

Total proteins were extracted from exendin-4 treated and control cells using RIPA buffer (Cat # 89901). Protein concentration was determined by the BCA method using γ-globulin as a standard, and 20 µg of proteins were loaded on 10% SDS-PAGE gels and used to detect OXPHOS, MAFA, NeuroD1. Proteins were then transferred onto PVDF membranes, blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h, and then probed with the primary antibody for overnight

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Gene	Forward	Reverse
OCT4	5'-GAAACCCACACTGCAGCAGA-3'	5'-TCGCTTGCCCTTCTGGCG-3'
SOX17	5'GGGAAATGGGAGGGGTGCAAAAGAGG-3'	5'TTGCGTGAGTGTGGATGGGATTGGTG-3'
PDX1	5'-GATACTGGATTGGCGTTGTTTG-3'	5'-TCCCAAGGTGGAGTGCTGTAG-3'
NKX6.1	5'-GCCTGTACCCCTCATCAAGGA-3'	5'-AAGTGGGTCTCGTGTGTTTTCTC
Insulin	5'-CAATGC CACGCTTCTGC-3'	5'-TTCTACACACCCAAGACCCG-3'
Glucagon	5'-AGCTGCCTTGTACCAGCATT-3'	5'-TGCTCTCTCTTCACCTGCTCT-3'
MAFA	5'-GAGAGCGAGAAGTGCCAACT-3'	5'-TTCTCCTTGTACAGGTCCCG-3'
NeuroD1	5'-ATCAGCCCACTCTCGCTGTA-3'	5'-GCCCCAGGGTTATGAGACTAT-3'
Six2	5'-AAGGCACACTACATCGAGGC-3'	5'-CACGCTGCGACTCTTTTCC-3'
Glut2	5'-ACAGATGAACTGCCCACAAT-3'	5'-ATGCAGTCATTCCACCAACT-3'
GCK	5'-ATGCTGGACGACAGAGCC-3'	5'-CCTTCTTCAGGTCCTCCTCC-3'
G6PC2	5'-GGTCATCGACCTTACTGGTG-3'	5'-TTCCTGGACCTGTTTCACAT-3'
NDUSF1	5'-TGCCAAAGGATTGTTTCATT-3'	5'-TGCTGAGCTCTACCCTCAGT-3'
NDUSF2	5'-GTTCCTCCAGGAGCCACATA-3'	5'-CTTGTCCAAACCAGCCAGAT-3'
PGC1a	5'-GCCAAACCAACAACTTTATCTCTTC-3'	5'-CACACTTAAGGTGCGTTCAATAGTC-3'
TFAM	5'-TCCCCCTTCAGTTTTGTGTA-3'	5'-GTTTTTGCATCTGGGTTCTG-3'
Actin	5'-TCATGAAGTGTGACGTGGAC-3'	5'-GCAGTGATCTCCTTCTGCAT-3'
GAPDH	5'-CCACTCCTCCACCTTTGACG-3'	5'-ATGAGGTCCACCACCCTGTT-3'

Table 1: Primer list and sequences

at 4 °C. Actin and GAPDH were used as internal control. Antibodies recognizing OXPHOS, MAFA, NeuroD1, Actin, and GAPDH were used at dilutions of 1:1000, 1:1000, 1:1000, 1:1000, and 1:10,000, respectively. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000 for 2 h at room temperature. Protein bands were visualized by chemiluminescence, and the images were captured using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA). For densitometric analysis, the intensity of the bands was determined using ImageJ 1.52v software (NIH, Bethesda, MA, USA).

Glucose stimulated insulin secretion (GSIS) Assay

hPSC-derived islet clusters (35 days of differentiation) were collected for glucose-stimulated insulin secretion (GSIS) assay as previously described [32]. Krebs buffer (KB) [128 mM NaCl, 5 mM KCl, 2.7 mM CaCl2 Krebs buffer (KB) [128 mM NaCl, 5 mM KCl, 2.7 mM CaCl2, 1.2 mM MgSO4, 1 mM Na2HPO4, 1.2 mM KH2PO4, 5 mM NaHCO3, 10 mM HEPES (Life Technologies; 15630080), 0.1% BSA in deionized water] was freshly prepared on the day of the experiment. Clusters were washed twice with low-glucose (2.8 mM) KB and incubated for 1 hour in 37°C incubator. They were washed once in low-glucose KB to remove any residual insulin, incubated in low-glucose KB for 1 hour, and the supernatant was collected. To challenge the cells, the clusters were then incubated in high-glucose (20 mM) KB for 1 hour, and the supernatant was collected. Finally, clusters were incubated in low-glucose KB containing 30 mM KCl (forced depolarization challenge) for 1 hour, and then the supernatant was collected. Clusters were finally dispersed into single cells using TrypLE Express, and cell number was counted to normalize insulin level by the cell number. Supernatant samples containing secreted C-peptide (a proxy marker for insulin co-released at equivalent molar) were processed using the human ultrasensitive C-peptide ELISA kit (Mercodia, Uppsala, Sweden).

Statistical analysis

All assays were performed at least in triplicate and a minimum of three independent experiments. Results are presented as means ± SEM and were plotted using GraphPad (Prism v7, La Jolla, CA). We used one-way ANOVA for comparison of the groups with post-hoc Tukey's test or the student t-test and paired two-sided t-test, as appropriate. A p-value < 0.05 was considered statistically significant.

Results

Differentiation of human pluripotent stem cell lines into 3D human pancreatic islets

Using a 3D differentiation protocol [30] with slight modification, both HUES8 and iPSC824 stem cell lines were differentiated into insulin-expressing cells. In this study, we used a new induced-pluripotent stem cell line, iPSC824, which was established in our Stem Cell Core. iPSC824 expressed pluripotency markers SOX2, OCT4, SSEA4, and TRA1-60; and successfully differentiated into ectoderm, mesoderm, and endoderm germ layers; and had normal 46, XX karyotype (Supplementary Fig. 2). To determine the efficiency of the differentiation, stage-specific markers were assessed in cells collected from different stages of the differentiation using RT-qPCR, flow cytometry and immunofluorescence. Gene expression pattern of pancreatic development (or lineage cell differentiation) markers is outlined in Figure 1 and data showed that expression of the pluripotent marker OCT4 was lost upon initiation of differentiation. The definitive endoderm marker SOX17 was transiently upregulated at stage 1 (S1) (day 4) by around >5,000 times as compared to stem cell control cells (day 0). PDX1, a marker of pancreatic progenitors was upregulated significantly at stage 3 (S3) (day14); while β-cell markers including NKX6-1 and insulin were strongly induced at stages 5 (S5) (day 21), and 6 (S6) (day 35). Additionally, glucagon (GCG), a hormone secreted by alpha cells, was transiently upregulated at S5, followed by a reduction in expression at S6 in HUES8-derived pancreatic

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islets. To ensure the efficiency of the differentiation, stage-specific markers were measured at different stages using flow cytometry. The results showed 96.3% OCT4^{+} cells (pluripotency marker) at S0; 89.4% SOX17^{+} cells (definite endoderm marker) at S1 and 83.4% PDX1^{+} cells (pancreatic progenitor marker) induction at S3. Subsequently, β cell-specific markers were induced, as evidenced by the generation of over 40% NKX6.1/insulin double-positive cells (Figure 2). The expression of stage-specific markers was also confirmed by immunofluorescence (Figure 3). To assess reproducibility of the differentiation, the differentiation was conducted using the newly generated hiPSC line (iPSC824) and we observed consistent results in the expression of stage-specific markers and β cell-specific markers (Figures 1-3).

Exendin-4 enhances glucose-stimulated insulin secretion without increasing β-cell number

Several studies reported the effectiveness of Exendin-4 in increasing β-cell mass and function [33-35]; however, none of these studies examined the effect of Exendin-4 on the functionality and maturation of 3D hPSC-derived pancreatic islets. Therefore, we investigated whether addition of exendin-4 could improve the functionality of hPSC-derived islets by performing the static GSIS assay. Data displayed in Figure 4 indicated that, in control HUES8 non-treated group, the clusters failed to secrete more c-peptide in response to high glucose (20 mM) challenge. However, they were responsive to direct cellular depolarization-mediated c-peptide secretion by KCl, indicating non-functional islets. Interestingly, in

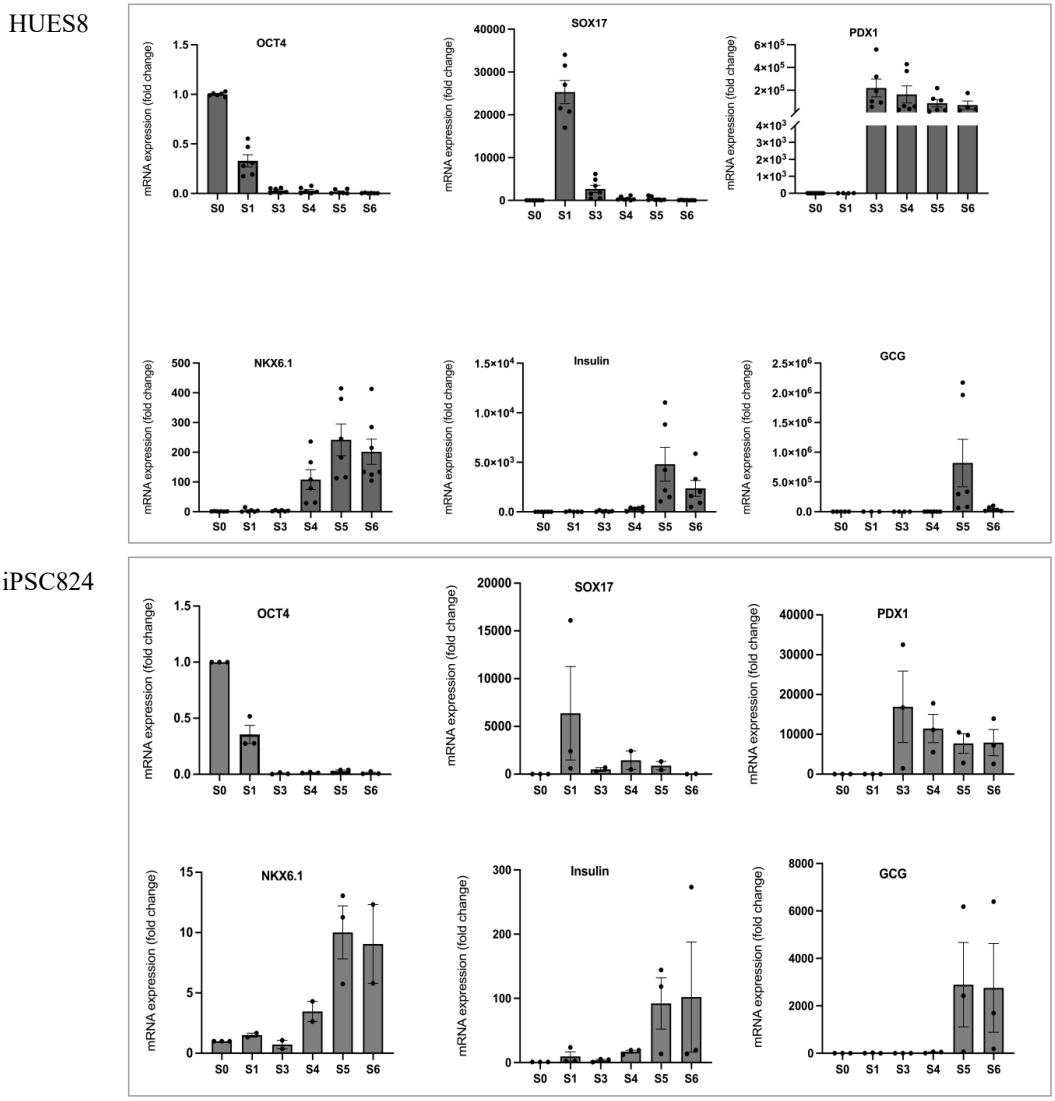

Figure 1: Gene expression profiling of stage-specific markers. Relative expression of pluripotent marker (OCT4), endoderm marker (SOX17) and pancreatic progenitor 1 marker (PDX1), endocrine β-cells markers (NKX6.1, Insulin, Glucagon) in HUES8 and iPSC824 at different stages. Relative expression was calculated by the comparative ΔΔCT method, and the fold change ($2^{-\Delta\Delta Ct}$) were calculated using stage 0 as control. The data are mean ± SEM for at least n=3 per cell line.

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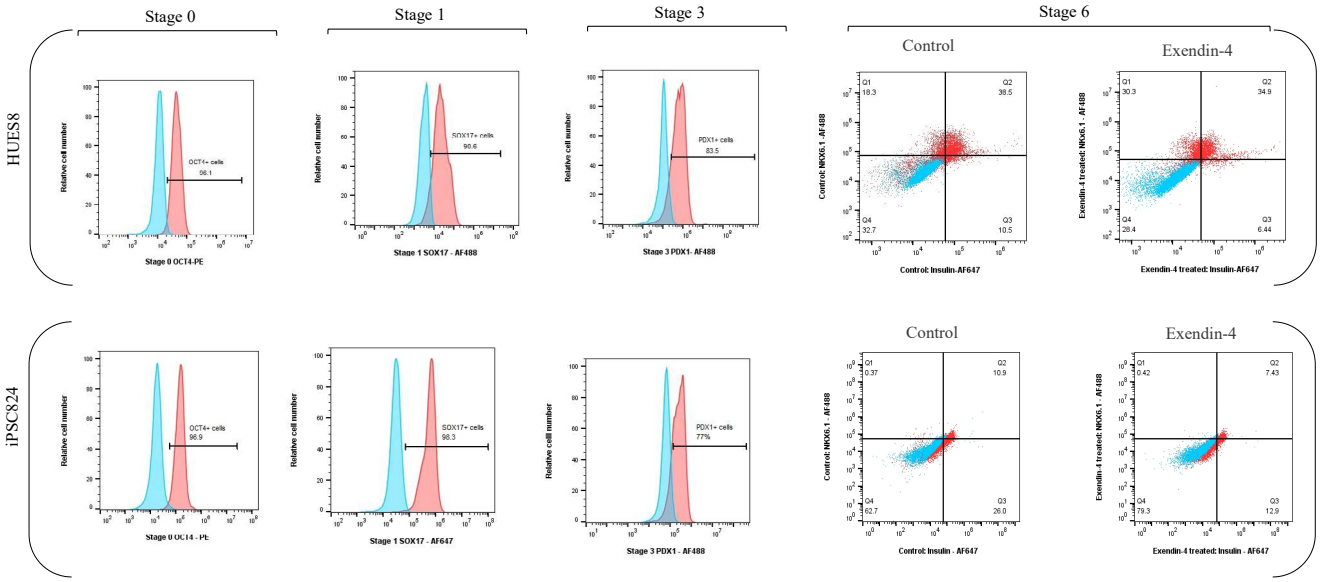

Figure 2: Representative flow cytometry plots of relevant stage-specific markers of HUES8 and iPSC824 differentiation into insulin-producing β-cells. Cell clusters were collected from different stages of the differentiation included stage 0 (OCT4 for pluripotency), stage 1 (SOX17 for definitive endoderm), stage 3 (PDX1 for pancreatic progenitor) and stage 6 (NKX6.1/insulin for β-like cells, following exendin-4 treatment). Data were analyzed using FlowJo.

Fig. 3a (HUES8):

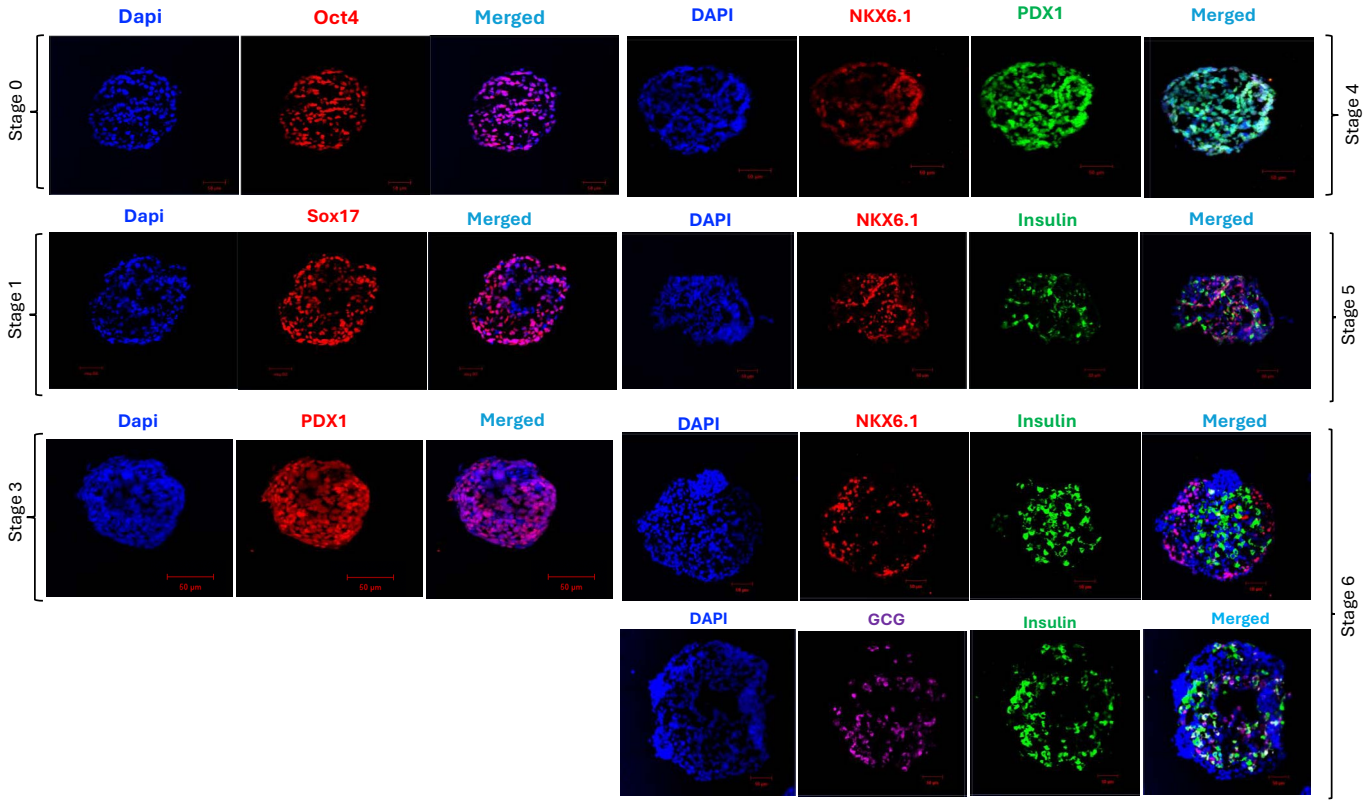

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Fig. 3b
(iPSC824):

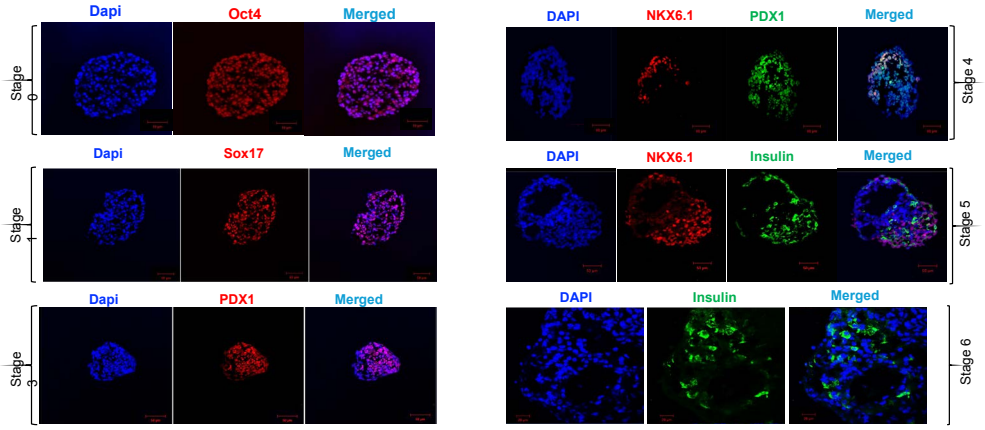

Figure 3: Representative immunofluorescence staining images at each stage of the 6-stage 3D differentiation protocol for HUES8 (Fig. 3a) and iPSC824 (Fig. 3b) to qualitatively assess differentiation status using key relevant stage-specific markers. The images were acquired using a 20X magnification with a Zeiss LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany). Negative staining images at each stage of the differentiation when only using the secondary antibodies (without primary antibodies) and negative control for NKX6.1 and insulin staining at stage 6 are shown in supplementary Figure 5.

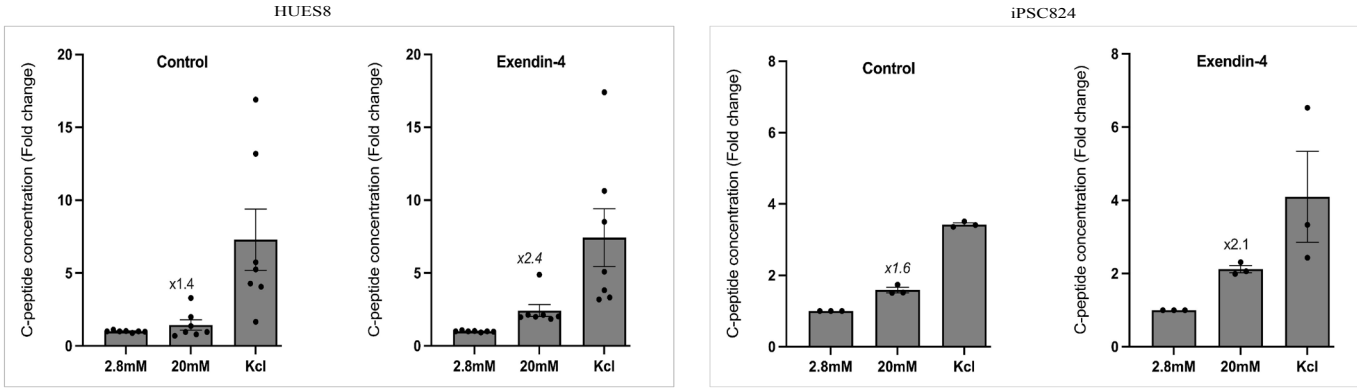

Figure 4: Static glucose-stimulated insulin secretion of stage 6 HUES8 and iPSC824 clusters, un-treated or treated with 50 nM Exendin-4 (paired two-sided t-test was used to compare low and high glucose in untreated and treated groups, n=7 for HUES8 and n=3 for iPSC824).

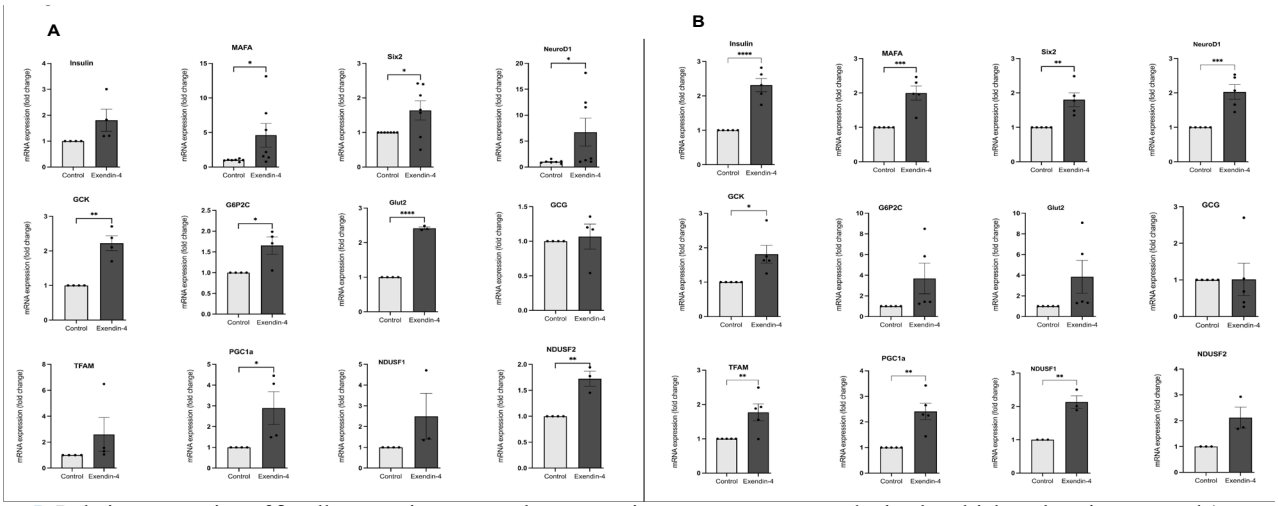

Figure 5: Relative expression of β-cell maturation genes, glucose sensing apparatus genes and mitochondrial markers in untreated (control) or treated with 50 nM Exendin-4 clusters differentiated from HUES8 (A) and iPSC824 (B). Relative expression was calculated by the comparative ΔΔCT method, and the fold change (2−ΔΔCt) were calculated. Data are mean ± *SEM* (n=7 for HUES8; n=5 for iPSC824).

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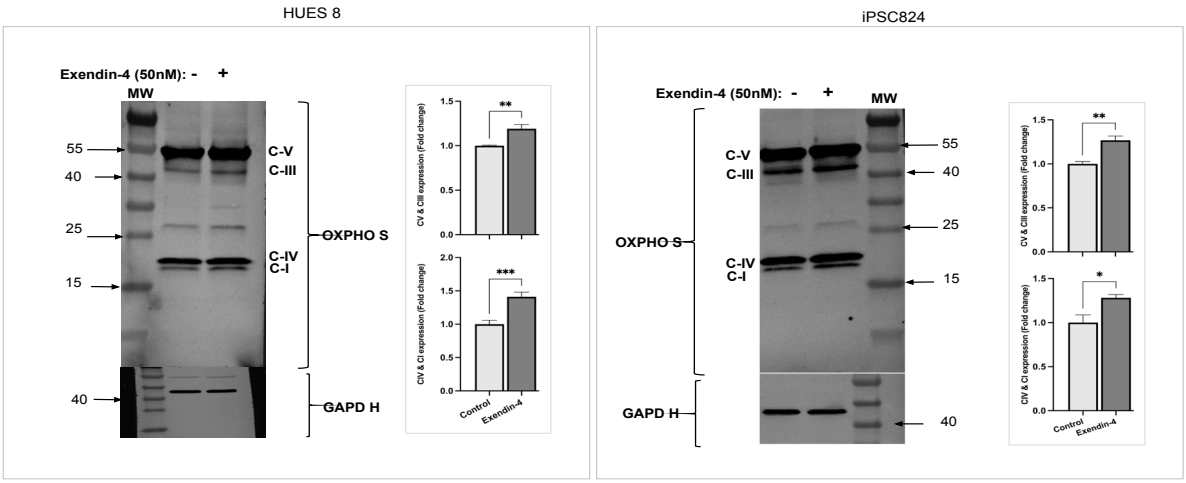

Figure 6: Representative western blot confirming the positive effect of exendin-4 on the expression of mitochondrial proteins involved in oxidative phosphorylation. As compared to control, 3-day treatment with 50 nM of exendin-4 resulted in increased expression of mitochondrial complexes (C-I, C-IV, C-III, C-V). Actin and GAPDH were used as internal controls to monitor for protein loading differences. Full-length blots are displayed in Supplementary figure 6.

exendin-4 treatment group, a 2.4-fold increase in c-peptide secretion was observed by clusters when challenged from low (2.8mM) to high (20 mM) glucose, (Figure 4) in spite no difference in β-cell number measured by NKX6.1/insulin double-positive cells as compared to control non-treated group, (Figure 2), suggesting a positive effect of exendin-4 in enhancing the functionality of hPSC-derived pancreatic β-cells rather than an increase in cells number. Moreover, exendin-4 had no effect on glucagon (α-cells) and somatostatin (δ-cells) expression (supplementary figure 3).

To determine whether the effect of exendin-4 on the functionality of stem cell-derived islets was cell line specific (i.e, HUES8 cell line) , we examined its effect on iPSC824-derived β-cells. Compared to HUES8-derived β-cells control clusters, iPSC824-derived β-cells control clusters showed a 1.6-fold increase in c-peptide release when challenged from low (2.8mM) to high (20 mM) glucose, indicating functional β-cells and treatment of exendin-4 further improved the functionality with a 2.1-fold increase in c-peptide secretion when challenged with high glucose (Figure. 4). Taken together, our data indicate that exendin-4 positively modulates GSIS without increasing β-cell number nor impacting glucagon (α-cells) and somatostatin (δ-cells) expression (supplementary figure 4)

Exendin-4 stimulates the expression of mitochondrial biogenesis and oxidative phosphorylation machinery genes

Dysfunction of mitochondria and/or its biogenesis has been linked to impaired GSIS [36-39]. Also, exendin-4 has been known to promote mitochondrial function [40]. We measured the expression of key representative mitochondrial marker genes following exendin-4 treatment. As shown in Figure 5, in both HUES8- and iPSC824-derived islets, addition of exendin-4 significantly increased the expression of mitochondrial biogenesis (PGC-1a, TFAM) and oxidative phosphorylation machinery (NDUSF1, NDUSF2) genes (Figure 5; $P<0.05$). Consistent with this finding, exendin-4 also triggered an increase in the expression of the protein levels of mitochondrial complexes (C-I, C-IV, C-III, C-V) involved in oxidative phosphorylation (Figure 6).

Exendin-4 stimulates the expression of β-cell mature markers and glucose-sensing apparatus genes.

The contribution of impaired maturation genes and glycolysis pathway to the immature phenotype of stem cell-derived islets is well established [7, 41]. We therefore investigated whether the GSIS-promoting effect of exendin-4 is associated with upregulation of genes known to be expressed in mature β-cells and involved in the glucose sensing apparatus. Data displayed in Figure 5 indicate that 3-day treatment of both HUES8- and iPSC824-derived clusters with 50 nM of exendin-4 led to a marked increase in the expression of NeuroD1, Six2, MAFA, GCK, Glut2, G6PC2 mRNA ($P<0.05$) as compared to untreated controls. Additionally, the expression of the protein levels of MAFA and NeuroD1 were upregulated following exendin-4 treatment (supplementary figure 4).

Discussion

Cellular replacement therapy is a promising treatment option for long-term blood glucose control in diabetes mellitus. While replenishment of malfunctioning pancreatic β-cells via human islet transplantation has an aptitude to restore normoglycemia and prevent hypoglycemia in

diabetic patients, its potential is hampered by healthy islet donor shortage. Stem cell-derived pancreatic 'pseudoislets' generated in vitro could potentially become an infinite surrogate source of insulin-secreting β-cells to overcome the limited donor supply as a potential therapy for diabetes. However, many protocols developed to differentiate stem cells into insulin-expressing β-cells in vitro have encountered a major challenge: the resulting β-cells often exhibit an immature phenotype with impaired glucose-stimulated insulin secretion (GSIS) associated with low or absence of expression of key maturation transcription factors (e.g MAFA, NEUROD1, SIX2) compared to primary islets [7]. This limitation highlights the need for further refinement of differentiation protocols to achieve the generation of more mature and functional hPSC-derived β-cells. GLP-1 agonists are thought to play an important role in improving β-cell morphology and function [42]. Exendin-4, a well characterized dipeptidyl peptidase IV (DPP-IV)-resistant GLP-1 analog, has been reported to stimulate both β-cell proliferation and insulin secretion *in vivo* or in isolated islets *in vitro* [43-45]. Consequently, exendin-4 has emerged as an important extrinsic factor to promote stem cell differentiation into insulin–producing β-cells. However, the exact mechanisms of action of exendin-4 during the differentiation process are not fully understood. In the present study, we added exendin-4 to investigate its role in the differentiation of hPSC cells into mature, functional β-cell using a more physiologically relevant 3D model that better mimics the in vivo cell niche compared to a 2D cell culture protocol [12]. Our results provide evidence that addition of 50 nM exendin-4 enhanced the functionality of hPSC-derived islets, as demonstrated by a greater than a 2-fold increase in c-peptide release when clusters were challenged with high (20 mM) glucose compared to low glucose in a static GSIS assay; replicating previous findings in other cellular systems [40, 46]. The question arising from this investigation is the mechanism underlying exendin-4-mediated enhancement of the functionality of hPSC-derived islets. The most likely mechanisms for GSIS induction involve activation of islet-enriched transcription factors that regulate maturation as well as stimulation of mitochondria; the master regulator that couples glucose metabolism to insulin exocytosis. Under our experimental conditions, we observed that exendin-4 supplementation increased the mRNA levels of genes involved in mitochondrial biogenesis (PGC-1a, TFAM) and oxidative phosphorylation machinery (NDUSF1, NDUSF2), suggesting that exendin-4 may enhance GSIS by promoting mitochondrial biogenesis and function. Glucose-sensing factors such as Glut2 and GCK, are essential for glycolysis, which subsequently coordinates with mitochondrial function to produce ATP in GSIS [12]. In pancreatic β-cells, glucose enters by facilitated diffusion through the glucose transporter (GLUT2) and is phosphorylated by glucokinase (GCK), thereby initiating glycolysis [47]. The main end product of glycolysis, pyruvate, is imported into mitochondria to fuel the tricarboxylic acid (TCA) cycle. Activation of TCA cycle lead to ATP production via oxidative phosphorylation (OXPHOS), which is required for the K_{ATP} channel-dependent pathway to trigger insulin exocytosis [48]. Furthermore, the critical role of GCK in GSIS is demonstrated by MODY2 (a form of monogenic diabetes) caused by mutation in the GCK gene [49]. Reduced GCK activity in β-cells has been reported as the primary contributor to hyperglycemia in MODY2. Therefore, our data indicate that the GSIS-promoting effect of exendin-4 may be associated with the upregulation of Glut2 and GCK mRNA, the principal sensors of glucose flux in β-cells, consistent with previous studies [50, 51]. It is known that multiple islet-enriched transcription factors are involved in pancreas development and the maturation and function of β-cells. Among these, MAFA, and NeuroD1 are known to be vital for maintaining the function of mature β-cells [52-55]. These transcription factors coordinate the stimulation of insulin synthesis by activating the insulin gene promoter in response to glucose challenge [56]. Six2 is reported to play an essential role in regulating the functional maturity and fate of human pancreatic β-cells, as the loss of Six2 markedly reduces insulin secretion and impairs the expression of genes governing β-cell insulin processing and output in primary human pseudoislets [53]. Similarly, studies on MafA mutant mice (global $MafA^{-/-}$, pancreas-specific $MafA^{-/-}$, and β cell–specific $MafA^{-/-}$) have established a key role for this transcription factor in promoting β-cell maturation and GSIS [57-59]. Moreover, NeuroD1 has been shown to be required for pancreatic β-cells to achieve and maintain functional maturity [55, 60, 61]. Thus, in the present study, the GSIS-enhancing effect of exendin-4 observed in the treated group may be driven by the upregulation of MAFA, Six2 and NeuroD1 following treatment.

Conclusion

This study reveals the beneficial enhancing effects of exendin-4 on GSIS in hPSC-derived insulin-producing β-cells. The principal novel finding of this study is that exendin-4 upregulates genes associated with maturation, glucose-sensing apparatus, and mitochondrial oxidative phosphorylation machinery, which underlie the molecular mechanisms to promote β-cell functions (GSIS). These findings also provide a unique approach to substantially improving the 3D differentiation protocol for generation mature-like and functional hPSC-derived β-cells, with potential applications in drug screening and β-cell replacement for T1DM and severe forms of T2DM in the future. However, despite the improvements in hPSC-derived β-cells function (GSIS) observed in this study; a limitation remains: further investigations, including in vivo testing in animal models of diabetes, are needed to validate whether exendin-4-treated,

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functional HUES8 and iPSC824-derived islets could lead to diabetes reversal.

Author Contributions: Study design: H.H.A, A.D, R.B.A.M; data collection: A.D, R.B.A.M, N.A.A, A.A, N.T.S, S.K.; data analysis and interpretation: A.D, R.B.A.M, H.H.A; manuscript writing: A.D.; manuscript reviewing and editing: H.H.A, R.B.A.M.; study supervision: H.H.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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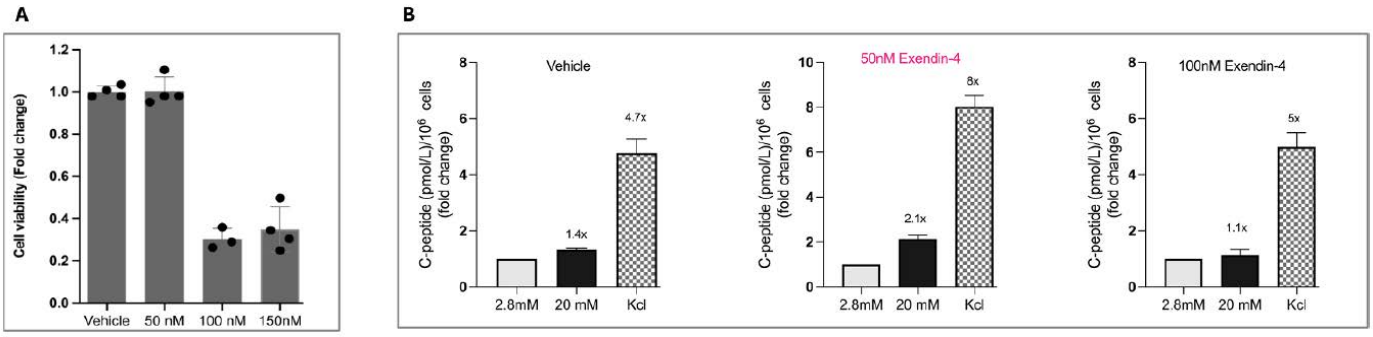
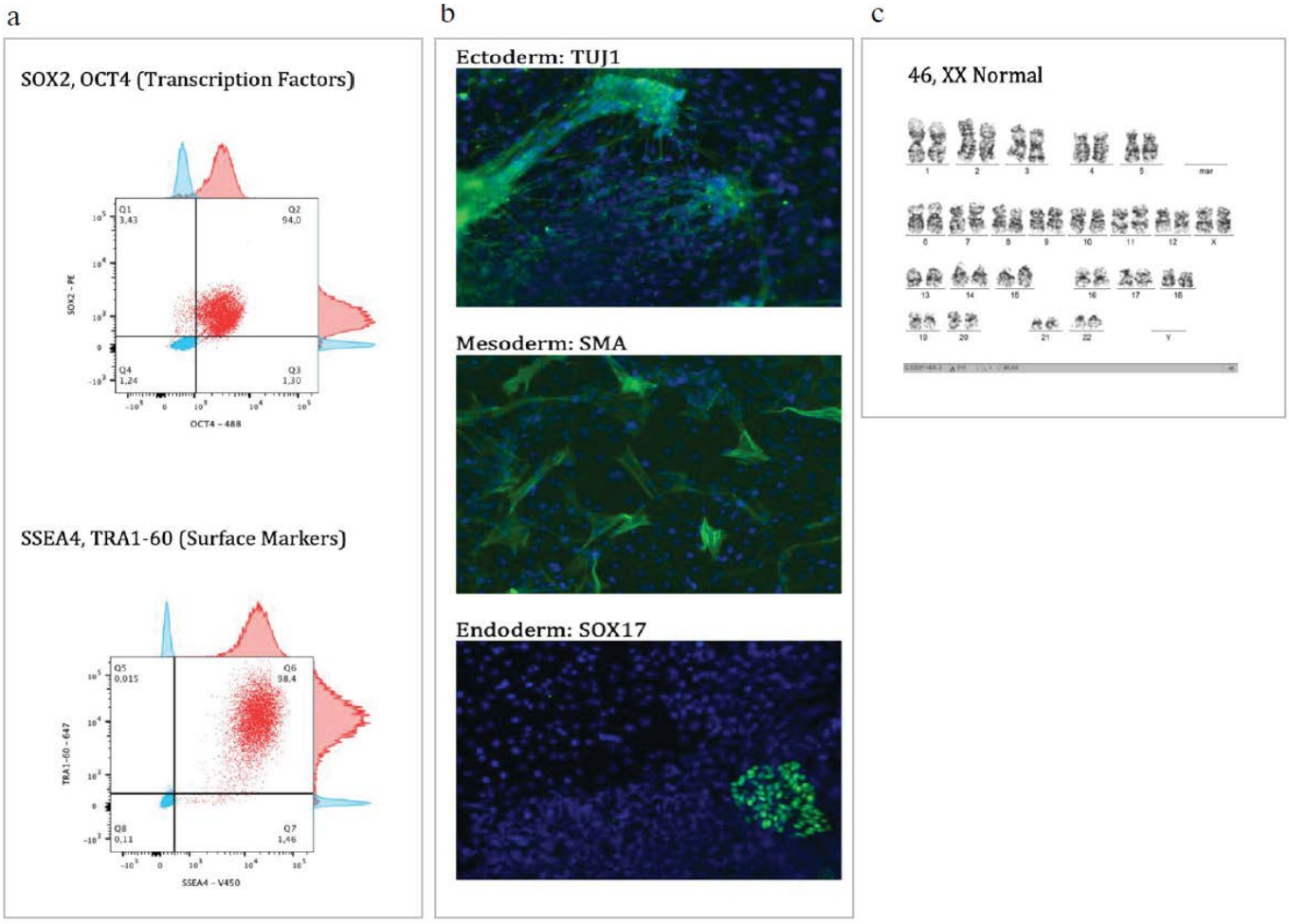


Supplementary figure 1: Dose-dependent effect of exendin-4 on cell viability (A) and GSIS (B). Cell viability significantly decreased after 3 consecutive days addition of 100ηM and 150ηM of exendin-4 ($p < 0.001$). The concentration of 50ηM showed comparable cell viability to the vehicle (control) along with increased GSIS in response to high glucose (20 mM).



Supplementary figure 2: iPSC824 line generated from commercially available consented healthy donors' dermal fibroblasts. **a**). The cells express high levels of the four tested Bonafide pluripotency markers (SOX2, OCT4, SSEA4, TRA1-60). **b**) spontaneous differentiation into various germ layers was performed in 10% FBS media culture. Immunostaining for Tuj1, smooth muscle actin (SMA) and SOX17 confirm ectodermal, mesodermal and endodermal lineages, respectively. **c**) G binding karyotyping showed normal karyotype of the 20 metaphase counted nuclei.

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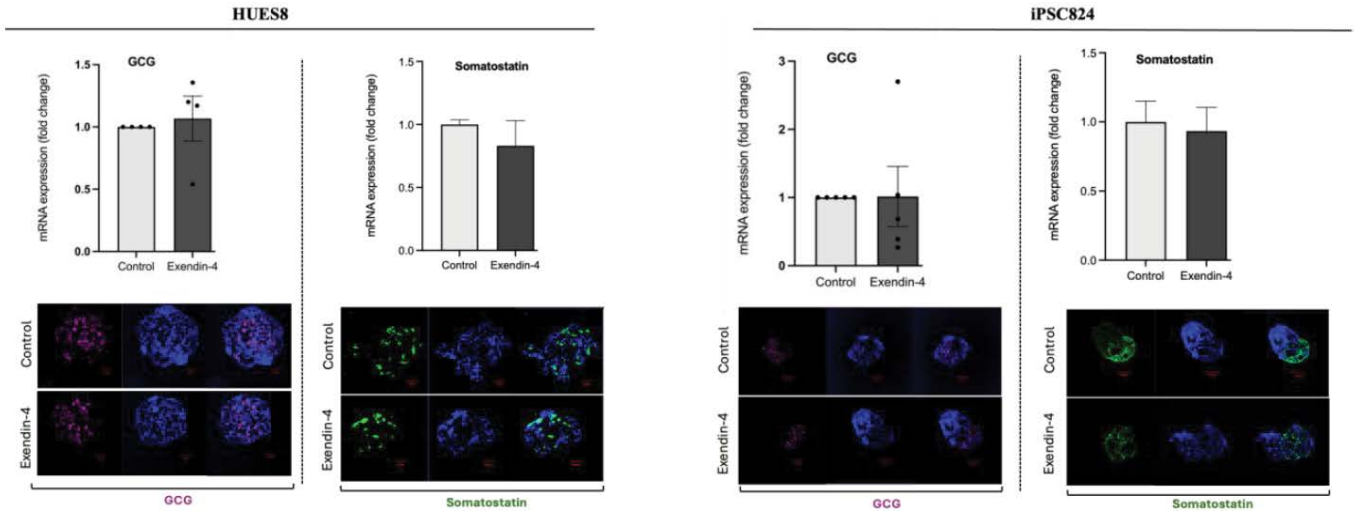
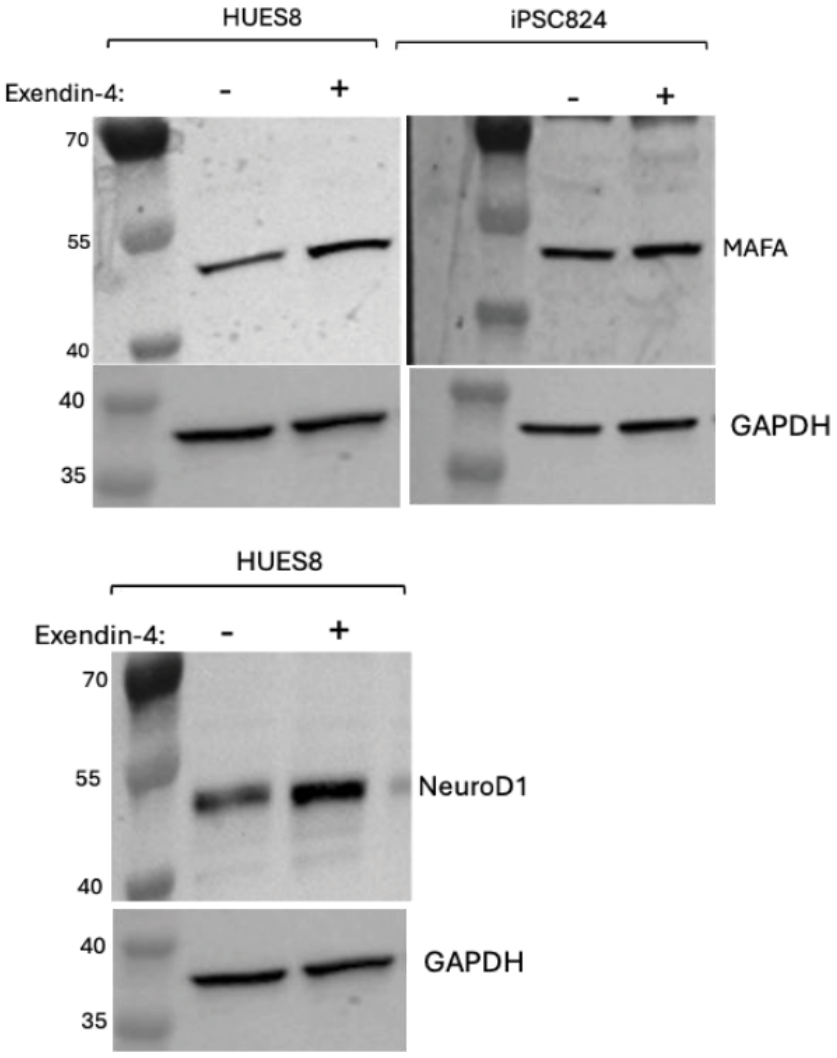


Supplementary figure 3: effect of exendin-4 on glucagon (α-cells) and somatostatin (δ-cells) expression using both gene expression with RT-PCR and immunofluorescence



Supplementary figure 4: effect of exendin-4 on MAFA and NeuroD1 protein expression.

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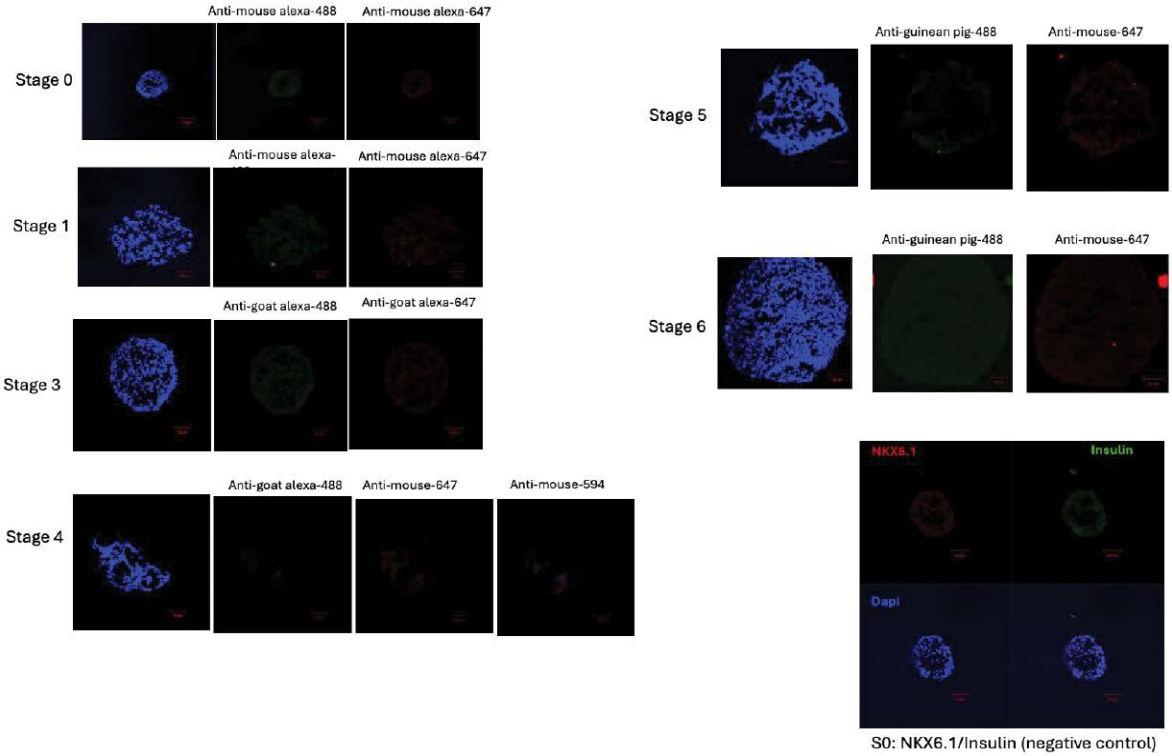
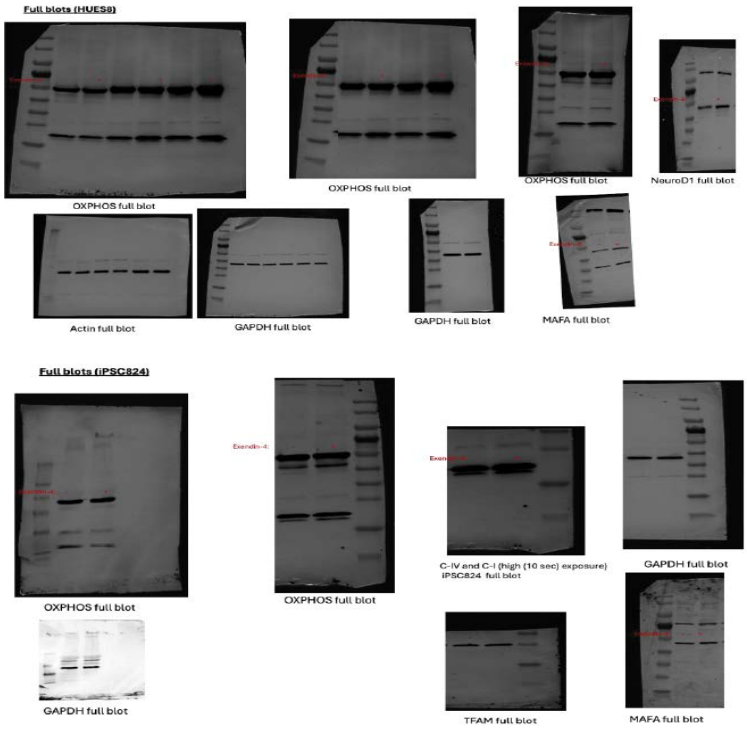


Supplementary figure 5: Fluorescence images demonstrating negative staining at each stage when omitting primary antibodies and only using the secondary antibodies. Stem cells clusters (stage 0) were also used as a negative control for NKX6.1 and insulin staining at stage 6.



Supplementary figure 6: Exendin-4 induces the expression of mitochondrial proteins involved in oxidative phosphorylation proteins in HUES8 and iPSC824 cells as revealed by western blots (full blots). Clusters were treated for 3 days with 50ηM of exendin-4. GAPDH was used as internal control.

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Supplementary Table 1: List of antibodies used

	Supplier	Catalogue number	Flow cytometry	Immunofluorescence staining	Western Blot
Primary antibodies					
Oct-04	Santa Cruz	sc-5279	1:300	1:300	
SOX17	BD Biosciences	561590	1:300	1:300	
PDX1	R&D systems	AF2419	1:300	1:400	
NKX6.1	DSHB	F55A12-c	1:100	1:300	
NKX6.1	DSHB	F55A12-s	1:100	1:100	
Somatostatin	ThermoFisher	PA5-82678		1:500	
Insulin	Dako	IR002	1:20	1:5	1:1000
OXPHOS	Abcam	ab110411			1:250
MafA	ThermoFisher	720262			1:1000
NeuroD1	Cell Signaling Techonology	4373			1:1000
GAPDH	Santa Cruz	sc-32233			1:5000
Secondary antibodies					
Donkey anti-goat 488	ThermoFisher	A11055	1:500	1:500	
Donkey anti-mouse 488	ThermoFisher	A21202	1:500	1:500	
Donkey anti-Guinea pig 488	ThermoFisher	A11073	1:500	1:500	
Donkey anti-mouse 594	ThermoFisher	A21203	1:500	1:500	
Donkey anti-goat 647	ThermoFisher	A21447	1:500	1:500	
Donkey anti-mouse 647	ThermoFisher	A31571	1:500	1:500	1:5000
Rabbit anti-mouse HRP	Abcam	ab6728			1:5000
Goat anti-rabbit HRP	Abcam	ab205718			1:5000

Citation: Abdoulaye Diane, Razik Bin Abdul Mu-U-Min, Asma Allouch, Noora Ali Al-Shukri, Nuha Taysir Swaidan, Shahryar Khattak and Heba Hussain Al-Siddiqi. Exendin-4 Enhances GSIS By Upregulating Genes Related to Maturation, Glucose-Sensing Apparatus and Mitochondrial Oxidative Phosphorylation Machinery in hPSC-Derived Islets Without Increasing β-Cell Number. Fortune Journal of Health Sciences. 8 (2025): 367-382.