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Research paper

Abnormal regulation of glucagon secretion by human islet alpha cells in the absence of beta cells

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ABSTRACT

Background: The understanding of the regulation of glucagon secretion by pancreatic islet α-cells remains elusive. We aimed to develop an in vitro model for investigating the function of human α-cells under direct influence of glucose and other potential regulators.
Methods: Highly purified human α-cells from islets of deceased donors were re-aggregated in the presence or absence of β-cells in culture, evaluated for glucagon secretion under various treatment conditions, and compared to that of intact human islets and non-sorted islet cell aggregates.
Findings: The pure human α-cell aggregates maintained proper glucagon secretion capability at low concentrations of glucose, but failed to respond to changes in ambient glucose concentration. Addition of purified β-cells, but not the secreted factors from β-cells at low or high concentrations of glucose, partly restored the responsiveness of α-cells to glucose with regulated glucagon secretion. The EphA stimulator ephrinA5-fc failed to mimic the inhibitory effect of β-cells on glucagon secretion. Glibenclamide inhibited glucagon secretion from islets and the α- and β-mixed cell-aggregates, but not from the α-cell-only aggregates, at 2.0 mM glucose.
Interpretation: This study validated the use of isolated and then re-aggregated human islet cells for investigating α-cell function and paracrine regulation, and demonstrated the importance of cell-to-cell contact between α- and β-cells on glucagon secretion. Loss of proper β- and α-cell physical interaction in islets likely contributes to the dysregulated glucagon secretion in diabetic patients. Re-aggregated select combinations of human islet cells provide unique platforms for studying islet cell function and regulation.

1. Introduction

Pancreatic islet cell function is of critical importance in glucose homeostasis. It is now understood that both deficient insulin release by islet β-cells and dysregulated glucagon secretion by islet α-cells contribute to hyperglycemia in patients with diabetes [1–6]. Persistently elevated circulating glucagon levels under fed states [2,7–12] and abnormally reduced secretion of glucagon in response to hypoglycemia [12–19] have been observed paradoxically in diabetic patients. In addition, islet α-cells of diabetic donors lack the appropriate increase of glucagon secretion under low concentrations of glucose [20,21] and demonstrate a dedifferentiated phenotype [21,22]. Despite the fact that the involvement of abnormal glucagon secretion in the etiology of diabetes was first highlighted more than 40 years ago [13,23,24] and supported by recent studies [2,25], understanding of glucagon secretion under normal physiological as well as diabetic conditions is limited. The regulation of glucagon secretion which is induced by hypoglycemia and suppressed by hyperglycemia, involves extremely complex and only partially understood mechanisms including direct regulation by glucose, intracellular ion flux, energy availability (changes in ATP/ADP ratio), autonomic nervous system and paracrine effects of insulin and somatostatain [26–29]. The causes of α-cell abnormalities in diabetes also remain unclear. Local inflammation, immunological insults, diminished paracrine β-cell influence (including reduction of intra-islet insulin) under diabetic conditions, or the diabetic milieu of hyperglycemia

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Research in context

Evidence before this study

Glucagon secretion from pancreatic α-cells and its regulation have been studied using rodent α-cells, transgenic mouse models, intact human islets and in human subjects. The secretion is suggested to be modulated by factors such as blood glucose, signals from the nervous system, and islet paracrine factors including insulin, somatostatin, Zn^{++}, GABA, glutamate, and serotonin. A human α-cell model that allows the direct examination of influences of various treatments on glucagon release has not been reported.

Added value of this study

By utilizing purified and then re-aggregated human islet α-cells, this study ascertained that fluctuations in glucose concentration (ranging from 2.0 mM to 28.0 mM), insulin at the physiological level, the supernatants containing all the secreted factors of β-cell at either low or high concentrations of glucose, somatostatin or glibenclamide had insignificant direct effects on human α-cell glucagon secretion, in the absence of β-cells. Addition of β-cells to the α-cell aggregates partially restored their functional responsiveness to glucose and glibenclamide. On the other hand, the pure β-cell-aggregates were capable of responding to glucose stimulation with increased insulin secretion. The increased secretion was further potentiated when re-aggregated together with the sorted α-cells. Activation of the juxtacrine EphA-ephrin signaling pathway by adding the Fc fragment of ephrin5A to the human α-cell aggregates, however, significantly stimulated, rather than inhibited, glucagon release. In addition, somatostatin was not found to be essential for mediating the inhibitory effect of β-cells on glucagon secretion in the mixed α- and β-cell aggregates at high glucose.

Implications of all the available evidence

Taken together, this work establishes aggregates of human islet α-cells, β-cells and mixed α- and β-cells as models for investigating the regulation of islet hormone secretion. This study reveals an essential role for the presence of β-cells for proper regulation of glucagon secretion by glucose, islet paracrine factors and sulfonylureas, and the mechanisms involved are likely different than that of rodent islet cells. The fact that insulin at doses much higher than physiological level are required to achieve sufficient inhibition of glucagon release, in the absence of β-cells, provides further evidence demonstrating that β-cell replacement is likely a more efficient therapy than insulin treatment is in controlling α-cell dysfunction in diabetes. Aggregates of various combinations of sorted human islet cells provide unique and important tools for investigating islet cell function, metabolism, gene expression, cell-to-cell interactions, and drug screening for the treatment of diabetes.

can all potentially contribute to α-cell dysfunction [21]. Further characterization of the function of α-cells in the presence or absence of proper input from β-cells in the pathophysiological environment of diabetes is needed for better understanding of diabetes development and for identifying better treatment options for diabetes. In addition, it is of clinical importance to investigate whether the current diabetic treatments which target mostly islet β-cells, such as sulfonylureas [30], have any direct effects on human α-cell glucagon secretion.

Several model systems including intact islets isolated from deceased humans and wild-type or genetically modified rodents [31,32], pancreas tissue fragments [33,34], re-aggregated islet cells [35], purified rodent α-cells [36,37] and rodent α-cell lines [38,39] have been used to investigate α-cell function in vitro. While important information regarding α-cell physiology can be generated utilizing these models, it is challenging to monitor the direct effects of any potential regulators on α-cells or to examine the complex paracrine interactions between the multiple islet cell types that are known to modulate α-cell function within intact islets or tissue fragments. In addition, findings generated from genetically modified rodent islets, purified rodent α-cells or immortalized rodent α-cell lines need to be verified using their human counterparts.

We hypothesized that the purified and then re-aggregated human α-cells, with or without other islet cell types, represent a clinically relevant model for the investigation of intrinsic mechanisms underlying the direct glucose- or paracrine factor-induced changes in cellular metabolism, membrane potential, gene expression, cellular stress level, and glucagon secretion. As an initial attempt, we aimed to establish an in vitro model using purified and then re-aggregated human islet α-cells isolated from deceased donors to investigate the regulation of human α-cell glucagon secretion at various glucose levels, the paracrine interactions between islet α- and other endocrine cells or factors, and the potential direct effects of diabetic drugs or compounds such as glibenclamide on α-cell glucagon secretion. This study, for the first time, provided clear evidence demonstrating that exogenous insulin or the entire β-cell secreted factors, molecules, and granules collected at either low or high concentrations of glucose, IGF-1, somatostatin, or glibenclamide had no significant direct influence on purified human α-cell glucagon secretion. Addition of purified β-cells to the α-cell aggregates restored the responsiveness, at least in part, to glucose and glibenclamide.

2. Materials and methods

2.1. Human islet culture and single cell preparation

Islets isolated from 32 non-diabetic deceased human donors were used in the study. Among the 32 donors, islets from 28 donors were generously provided by Dr. Tatsuya Kin at the Clinical Islet Laboratory at the University of Alberta Hospital, 2 were isolated by the Islet Core at Columbia University, and 2 were obtained from the Integrated Islet Distribution Program (IIDP). Donor information was summarized in Table 1. To ensure quality, islets were sorted and used in experiments within 2–4 days post-isolation. Islets and the subsequently dispersed cells were cultured at 37 °C with 5% CO_2 in a CMRL-1066 supplemented CIT medium (Cellgro, Cat. #98-304-CV) with 10 IU/ml Heparin (Sagent Pharmaceuticals, Cat. #25021-400-10), 1 μg/ml IGF-1 (Cell Sciences, Cat. #CRI500), and 10% fetal bovine serum (GE Life Sciences, Cat. #SH30088.03HI). For single cell preparations, islets were incubated with 0.025% trypsin (Millipore Sigma, Cat. #SM-2004-C) for approximately 6 min at 37 °C. Undispersed material was excluded using a 40 μm cell strainer. The dispersed cells were re-suspended in CMRL-1066 supplemented CIT medium with 2% FBS (Hyclone, Cat. #SH30084.03 HI).

2.2. Fluorescence-activated cell sorting (FACS)

The dispersed cells were incubated with two monoclonal antibodies, HIC1-2B4 and HIC3-2D12 [40,41], which were provided by our collaborator Dr. Craig Dorrell at the Oregon Health and Science University. After incubation on ice for 30–40 min, the cells were washed with cold DPBS (Gibco, Cat. #70011-044), then stained with secondary antibodies DyLight 405-conjugated anti-mouse IgG and Alexa Fluor 647-conjugated anti-mouse IgM (Jackson ImmunoResearch, Cat. #115-475-164 and #115-606-075) on ice for 30 min. The cells were washed with cold DPBS, re-suspended in CMRL with 2% FBS, and sorted using the BD Influx sorter at the Flow Cytometry Core of

Table 1
Donor information.

Donor	Age (years)	Gender	BMI	Diabetes (+/−)	Culture time (days)
1	55	F	26.0	–	3
2	54	M	33.4	–	3
3	53	F	37	–	2
4	20	M	21.2	–	2
5	45	M	25	–	3
6	43	M	29.1	–	4
7	64	M	24	–	3
8	33	F	36.5	–	3
9	22	M	24.3	–	3
10	78	F	31.5	–	3
11	62	M	28.7	–	3
12	65	M	25.3	–	4
13	35	M	30.9	–	2
14	21	M	23.25	–	3
15	73	F	27.4	–	4
16	21	M	24.5	–	3
17	38	M	37.9	–	3
18	51	M	37.5	–	3
19	45	M	29.8	–	3
20	62	F	22	–	2
21	55	F	35.7	–	3
22	50	M	28.1	–	3
23	47	F	32.9	–	3
24	61	M	23.4	–	3
25	32	M	35.9	–	4
26	36	F	31.6	–	4
27	50	F	24.6	–	2
28	19	M	23.2	–	4
29	43	F	37.0	–	4
30	51	M	27.3	–	3
31	38	M	26.9	–	3
32	39	F	21.2	–	3

Columbia Center for Translational Immunology (CCTI). A two-step sorting process, using the enrich mode followed by the pure sort mode, was performed to eliminate contamination from other pancreatic cell types.

2.3. Sorted human islet cell purity and culture

Sorted α- or β-cells were counted and cultured in 500 μL of CMRL-1066 with 10% FBS and 5.6 mM glucose for a period of 3 days in Corning Ultra-Low Attachment plates (Corning/Costar, Ithaca, NY). Each well contained either 20k sorted α-cells, mixed α- (20k) and β-cells (20k), or approximately 50k dispersed but non-sorted cells containing approximately 20k α-cells, calculated based on the α-cell percentage determined by flow cytometry. The cells were given 72 h to aggregate in culture. Intact human islets were included as controls.

Samples of the sorted cells were concentrated on glass slides by cytocentrifugation, fixed in 2.5% paraformaldehyde for 10 min, and then immunofluorescence stained for human glucagon (Sigma, Cat. #G2654), insulin (Dako, Cat. #A0564), Somatostatin (SST, ZYMED, Cat. #18-0078) and ghrelin (Abcam, Cat. #ab209790) to confirm purity. Secondary antibodies conjugated with Dylight-dyes (Jackson ImmunoResearch) were used for fluorescence detection. Digital images of fluorescence-labeled cells were acquired using a Zeiss fluorescence axial microscope attached with a digital camera or with a Zeiss LSM410 confocal laser-scanning microscope.

2.4. Treatment conditions and glucose challenge

The experimental groups include: (1) Intact islets (15 islets with diameters of 200–300 μM, containing an estimated 20,000 α-cells per well); (2) dissociated but non-sorted islet cell re-aggregates that were estimated to contain ~20,000 α-cells per well based on the percentage of α-cells analyzed by flow cytometry; (3) α-cells (20,000 per well); (4) β-cells (20,000 per well); (5) mixed α- and β-cells (20,000 each per well); and/or (6) islets, mixed cells, or α-cells treated with either 0.6–10,000 μg/L insulin (Sigma, Cat. #11376497001), 10 nM IGF-1 (Cell Sciences, Cat. #CRI500), 10 nM somatostatin (Sigma, Cat. #S1763), 10 nM recombinant human ephrin-A5 Fc chimera protein (Fisher Scientific, Cat. #374-EA-200), or 0.1 μM K_{ATP}-channel inhibitor Glibenclamide (Sigma, Cat. #G0639) during the second phase of the two-step static glucose challenge procedure. Duplicate or triplicate samples from each donor were tested for each condition. Cells from each donor were used as their own controls when testing and comparing function under the various treatment conditions.

After the 3-day culture, the cell aggregates and control intact islets were subjected to glucose challenge using a static incubation approach described for intact islets [42] with modifications. The cell aggregates or islets were carefully transferred into individual cell culture inserts with a pore size of 3 μm (EDM Millipore, Cat. #PITP01250) in 24-well plates (Non-tissue culture treated, Corning). The cells were first equilibrated in 2.0 mM glucose-containing HEPES-balanced Krebs-Ringer bicarbonate buffer (KRB) with 2 mg/ml Bovine serum albumin (Sigma-Aldrich, Cat. #S6003) at 37 °C, 5% CO_2 for 1 h. Following this, the inserts were removed from the wells, drained of any residual media and transferred to new wells containing KRB with 2.0 mM glucose and incubated at 37 °C for one hour. Afterwards, the inserts were transferred to new wells containing KRB supplemented with various concentrations of glucose and growth factors or compounds (e.g., Glibenclamide) for another hour. The cells used in the high-to-low glucose challenge were first incubated in KRB with high concentration glucose for one hour and then transferred to KRB containing 2.0 mM glucose for another hour. The supernatants from each of the one hour-incubations of the two step-glucose challenge assay were collected for islet hormone measurements. The cells were lysed for cell number quantification at the end of the experiment using the PicoGreen DNA assay kits (Invitrogen, Cat. #P7589) according to the manufacturer's protocol. In addition, subgroups of cells were also evaluated for viability using FAD (Sigma, Cat. #F7378) and PI (Sigma, Cat. #P4170) staining, and for glucagon content assessment by acid ethanol extraction (1.5% HCL in 75% ethanol) [43] followed by freezing and neutralization steps prior to glucagon concentration measurement.

2.5. Islet endocrine hormone measurements

The supernatant collected were analyzed for insulin and glucagon concentrations using the human insulin and glucagon ELISA kits (Mercodia, Cat. #10-1113-01 and #10-1271-01) and somatostatin EIA kits (Phoenix Pharmaceuticals, Cat. #EK06003) according to the manufacturers' instructions. The hormone released by each group of cells was expressed as the ratio of the hormone released during the second step incubation divided by that released in the first step incubation of the two-step glucose challenge assay, as described above. The ratio represents the function of cells in each sample in a cell-number independent manner. In addition, in order to compare the absolute amount of hormone secretion by a given number of cells under different experimental conditions, glucagon or insulin release was standardized to cellular DNA content measured as described above.

2.6. Statistical analyses

Data were reported as means ± SE. For comparing the difference between different conditions within the same group, paired *t-test* was used to determine the statistical significance. For two-group comparisons, unpaired *t-test* was used to determine the statistical significance. For multiple group comparisons, differences were analyzed by two-way analysis of variance (ANOVA) for repeated measures and by Tukey post-hoc test. All tests were performed using the Prism-Graphpad software. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Purity and re-aggregation of sorted human islet α-cells

When used in combination, HIC1-2B4 and HIC3-2D12 antibodies permit identification and efficient FACS-based isolation of major subtypes of human islet cells [40,41,44], including α- and β-cells. The purity levels of the sorted α- and β-cell populations were routinely examined by flow cytometric analysis (data not shown) and by immunofluorescence staining (Fig. 1). At least 200,000 α-cells can be expected to be recovered from a sample of 10,000 human islet equivalents (IEQs) with an average purity level of 96.6 ± 1.8% and as high as 99% with no insulin-positivity detected (Fig. 1(B)). The antibody-defined FACS thus allows for preparation of human α-cells, and β-cells (Fig. 1(C)) purified close to homogeneity. It has been reported that each IEQ (volume of 150 μm diameter sphere) has 1560 ± 20 cells, and among which 73.6 ± 1.7% of the islets cells are β-cells [45]. Assuming α-cells constitute ~20% of the human islet cells [46,47], 10,000 IEQs (15.60×10^6 cells) contains approximately 3.12×10^6 α-cell. Based on these numbers, the efficiency of α-cell recovery by our approach was less than 10%. In the non-collected cell populations that were theoretically negative for the endocrine markers recognized by the two antibodies used, we detected significant numbers of glucagon-positive or insulin-positive cells by immunofluorescence staining (data not shown), suggesting differential expression or possible enzymatic damage of the cellular surface protein epitopes needed for the antibody recognition. Digestion methods that are less harsh may improve the recovery rates of the islet endocrine cells by FACS. In addition, cell number loss introduced by the double sorting strategy applied in the experiments likely contributed to the poor yield. Nevertheless, the purity levels of the cells sorted were high and the functions of the cells were preserved as described below when used in the experiments 3–4 days later in culture.

After verification of sample purity, the α-cells were cultured in ultra-low attachment dishes for 3–4 days to allow re-aggregation [48,49] and formation of 3D structures (Fig. 1(D)), in the absence or presence of the sorted human β-cells. The size of the formed aggregates varied, and was dependent on the number of cells cultured in each well. Within a reasonable cell number range that allows healthy cell survival, the higher the number of cells seeded in each well, the bigger and tighter the aggregates formed. The sorted pure islet α-cells did not survive well if they were cultured at a density lower than 10,000 per well in the 24 well-plates (data not shown). Sorted pure β-cells and mixed α- and β-cells tended to form aggregates faster than pure α-cells. Due to the limited number of human islet cells available for our research, we routinely cultured only 20,000 viable sorted α- or β-cells in each well in order to make sure there were enough cells to be divided between the control and the experimental groups. We found that a 3-day culture was the minimum time required for the human islet cells to aggregate with baseline hormone secretion similar to that of intact islets or re-aggregated dispersed islet cells as shown below. No significant cell death was observed with any of the treatments in these experiments as verified by cellular DNA concentration measurement and FDA/PI staining (data not shown).

3.2. Pure human islet α-cell aggregates maintained normal levels of glucagon secretion under low glucose conditions, but responded poorly to changes in ambient glucose concentration

It is generally believed that islet α-cells sense low blood glucose levels and secrete glucagon accordingly to prevent hypoglycemia. It has been shown that the secretion by intact islets reaches maximal level in the absence of glucose, and is inhibited with rising glucose level [50]. In order to test whether the sorted and then re-aggregated human α-cells can secrete glucagon normally, they were subjected to

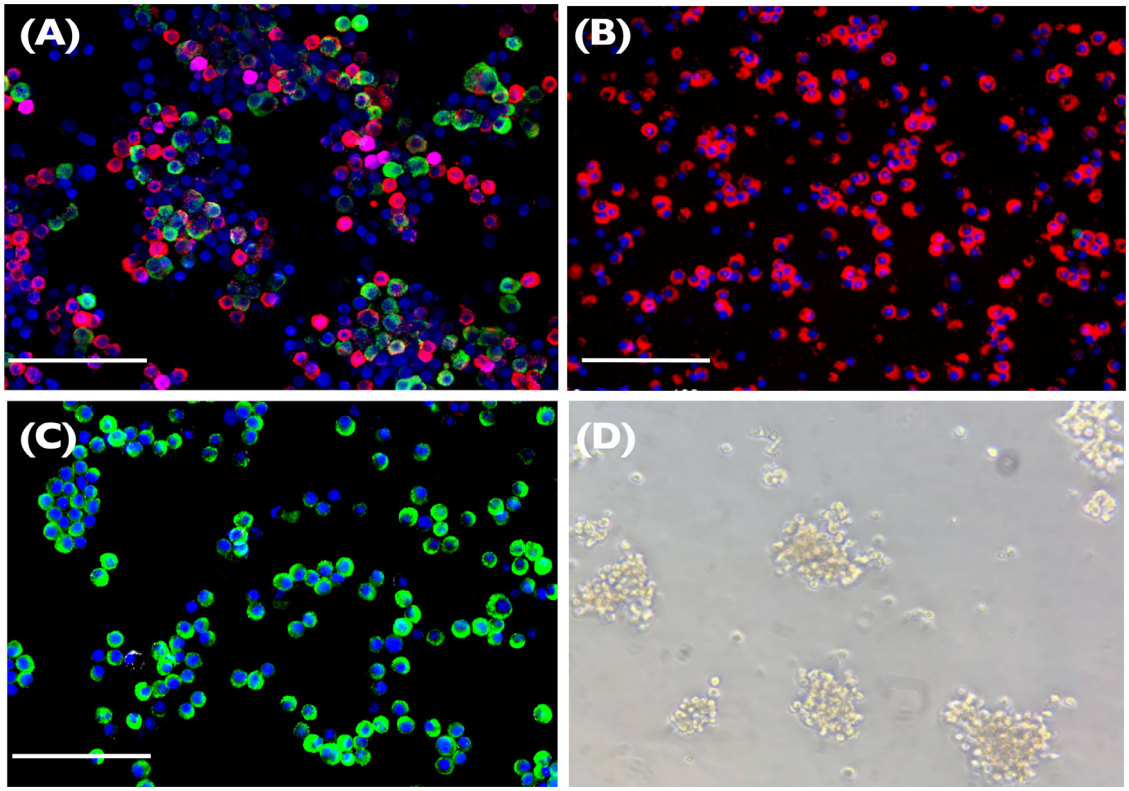

Fig. 1. Purity and re-aggregation of purified human islet cells. Representative Images of Immunofluorescence staining of islet cells for glucagon (red), insulin (green) and SST/Ghrelin (pink). (A) Dissociated but non-sorted human islet cells; (B) Sorted islet α-cell population; and (C) Sorted β-cell population. No insulin or glucagon positive cells were detected in the sorted α- or β-cell populations, respectively. The size of the scale bars was 100 μm. (D) Pure α-cell aggregates (100 fold-magnification).

glucose challenge in vitro. As shown in Fig. 2(A), the human α-cell aggregates incubated in the KRB solution containing 2.0 mM glucose maintained glucagon secretion capacity at levels similar to that of α-cells in the mixed cell aggregates or intact human islets under the same conditions, with no significant difference detected.

The intact islets and the non-sorted islet cell aggregates (both estimated to contain approximately 20,000 α-cells), the mixed cell aggregates of sorted 20,000 α- and 20,000 β-cells, as well as aggregates of 20,000 sorted human α-cells showed a robust glucagon secretion of 230.70 ± 23.70 pmol/L and 262.47 ± 9.70 pmol/L, 196.18 ± 12.37 pmol/L, and 231.09 ± 20.67 pmol/L, respectively, at 2.0 mM glucose (Fig. 2(A)). When the same groups of cells were subsequently exposed to KRB's solutions containing 16.8 mM glucose for another hour, the secretion of glucagon by all of the samples, except the α-cell-only group, were significantly decreased ($p < 0.05$) in comparison to that detected at 2.0 mM glucose. The glucose-inhibition index for each sample (Fig. 2(B)), calculated as the ratio of glucagon released at 16.8 mM glucose divided by that at 2.0 mM glucose of the same sample, was 0.62 ± 0.08, 0.58 ± 0.02, and 0.62 ± 0.04 for the intact human islets, the non-sorted islet cell aggregates, and the mixed cell aggregates, respectively, all significantly different ($p < 0.05$) than that of the α-cell-only aggregates (0.99 ± 0.11; Fig. 2 (B)). The glucagon content of the intact islets, the mixed cell aggregates, and the α-cell aggregates in each sample was measured to be 22,674 ± 3437 pmol/L (11.34 ± 1.72 pmol/15 islets, $n = 3$), 21,901 ± 1693 pmol/L (10.95 ± 0.85 pmols/20,000 cells, $n = 5$), and 18,182 ± 1487 pmol/L (9.09 ± 0.74 pmol/20,000 cells, $n = 5$), respectively, with no significant differences detected among the samples. The fractional glucagon secretion rates (percentage of glucagon content per hour) for the islets, the mixed cells, or the α-cell aggregates at 2.0 mM glucose were 1.0%, 0.9% or 1.2%, respectively. The glucagon release data also remained comparable between the pure α-cell and the mixed cell samples after they were standardized to cellular DNA concentration (data not shown). These data strongly suggested that the presence of other islet cell types or glucose are not required for human α-cell glucagon secretion at low glucose; whereas β-cell and likely other cell types in the islets or their released paracrine factors are critical for glucose inhibition of glucagon secretion.

The lack of glucose responsiveness of the α-cell-only aggregates was also verified by exposing the aggregates to additional concentrations of glucose in two-step glucose challenge assays (from 2.0 mM to 2.0 mM, 5.5 mM, 8.5 mM, 16.8 mM, or 28.0 mM) and compared to that of the mixed α- and β-cell aggregates under the same conditions. As shown in Fig. 2(C), the mixed-cell aggregates responded to the increased concentrations of glucose (5.5 mM, 8.5 mM, 16.8 mM, or 28.0 mM) with a significant inhibition of glucagon release of 52.18 ± 10.73%, 54.39 ± 13.54%, 71.47 ± 6.18% or 70.09 ± 10.40%, respectively, all significantly different than 120.84 ± 8.79% of the 2.0 mM-2.0 mM control. However, the α-cell-only groups responded to all the increased concentrations of glucose with insignificant changes in glucagon secretion of 76.78 ± 10.32%, 94.57 ± 16.97%, 103.56 ± 10.87% or 81.20 ± 7.73%, respectively (Fig. 2(D)), compared to 119.8 ± 13.69% for the 2.0 mM-2.0 mM control. These data further confirmed that the sorted and then re-aggregated α-cells were only capable of sensing increases in the ambient glucose concentration with corresponding changes in glucagon secretion when β-cells were present.

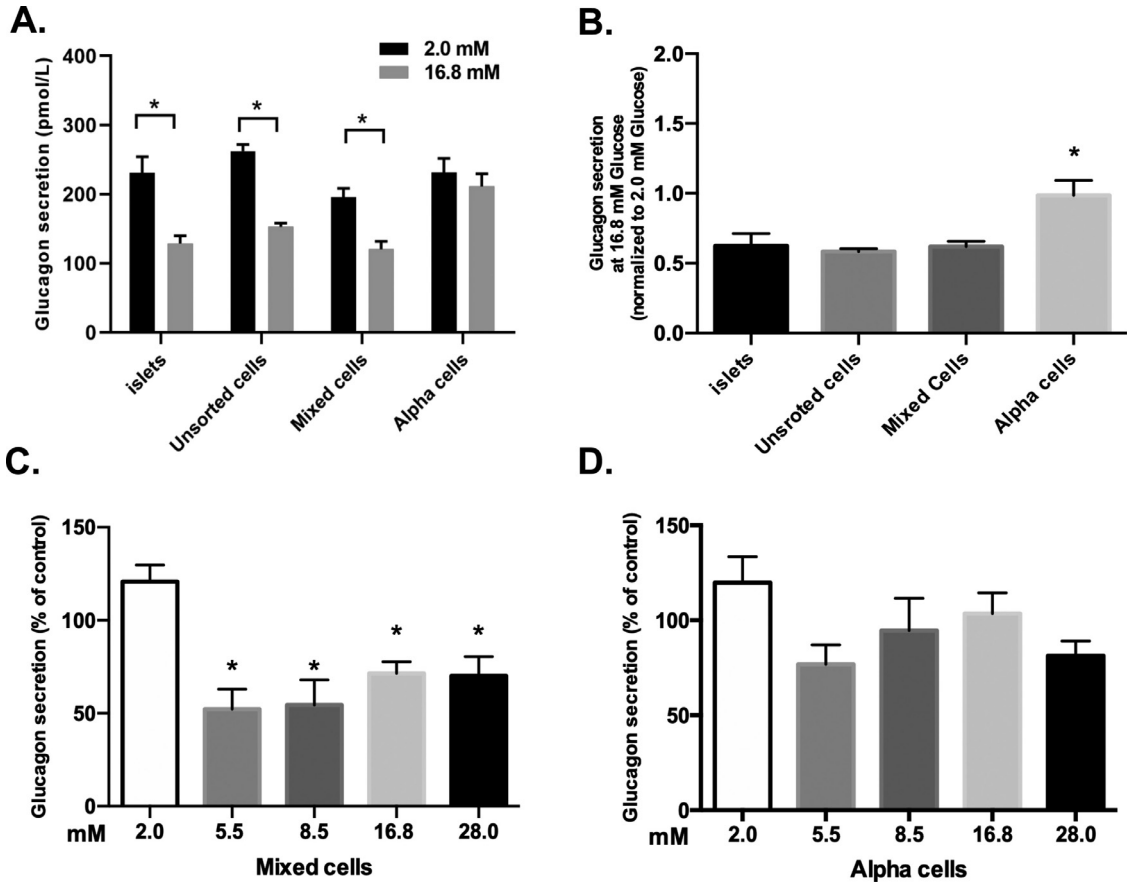

Fig. 2. Glucagon secretion in response to static incubation glucose challenge. (A) Glucagon secreted by intact human islets and aggregates of unsorted islet cells, sorted human α- and β-cells (mixed cells) or sorted pure α-cells ($n = 6$ for each group) in response to glucose challenge (2.0 mM to 16.8 mM). *p-value <0.05 (paired t-test). B. Ratio of glucagon released by the various groups of cells at 16.8 mM glucose versus that at 2.0 mM glucose. *p-value <0.05 (one-way ANOVA, Tukey post-hoc test). (C and D) Percent glucagon secretion by the mixed cell- or the α-cell aggregates, respectively, at 2.0 mM ($n = 6$), 5.5 mM ($n = 4$), 8.5 mM ($n = 5$), 16.8 mM ($n = 7$), or 28.0 mM ($n = 5$) glucose versus baseline of individual samples at 2.0 mM glucose. * denotes a p-value <0.05 versus the control 2.0 mM group (one-way ANOVA, Tukey post-hoc test).

3.3. Insulin dose response

Islet derived insulin has been suggested to be one of the β-cell mediators which play a role in glucagon secretion inhibition [32,51]. The insulin secreted by the β-cells in the mixed-cell aggregates at 16.8 mM glucose was measured to be 16.91 ± 1.71 μg/L; whereas the amount of insulin detected in the α-cell-only group was 0.39 ± 0.05 μg/L during the one-hour incubation, probably due to minute contamination of β-cells during FACS. In order to identify whether insulin was directly responsible for the inhibitory effect of β-cells on glucagon secretion, we performed a dose-response study by adding various concentrations of insulin (0.06 μg/L, 0.60 μg/L, 6.00 μg/L, 60.00 μg/L, 600 μg/L or 10,000 μg/L) to the α-cell-only aggregates at 16.8 mM or 2.0 mM glucose, for one hour. As shown in Fig. 3(A), only the 10,000 μg/L (~1.7 μM) dose but not the other tested doses of insulin, induced a significant inhibition on glucagon secretion in a 2.0 mM to 16.8 mM glucose challenge assay (Fig. 3(A)). The inhibition ratio (Fig. 3(B)) was 0.56 ± 0.05 for the 10,000 μg/L insulin, differing significantly from that of no insulin added control sample (1.0 ± 0.04) or the sample with 0.6 μg/L insulin (1.09 ± 0.09). Additionally, when 60 μg/L or 600 μg/L exogenous insulin was added to the second step of a 2.0 mM to 2.0 mM glucose challenge assay, no significant inhibition of glucagon secretion was observed comparing the ratios of glucagon secretion to that of the no-insulin control sample which showed a slight increase (1.32 ± 0.17-fold) in glucagon secretion during the second phase of glucose challenge at 2.0 mM (Fig. 3(C)). Since the 10,000 μg/L insulin dose was much higher than the physiological insulin level, it might act via an insulin-receptor-independent but rather IGF-receptor-dependent pathway. To test the possible involvement of the IGF-1 receptor signaling pathway in regulating glucagon secretion, we applied recombinant human IGF-1 directly to the pure α-cell aggregates in the second step of the 2.0 mM to 2.0 mM glucose challenge assay, but observed no significant changes in glucagon secretion under this treatment condition (Fig. 3(C), Left panel). These data provided clear evidence suggesting either very high concentrations of intra-islet insulin is needed or β-cell factors other than insulin are involved in the inhibition of glucagon secretion by neighboring α-cells, since the mixed α- and β-cell aggregates, although having only 16.91 μg/L insulin released at 16.8 mM glucose, were able to significantly inhibit glucagon secretion as shown in Fig. 2. Serotonin has also been shown to be an important mediator of the inhibitory effect of β-cells on glucagon secretion from both rodent and human islets [52]. However, addition of serotonin to the α-cell aggregates also did not inhibit glucagon secretion at either 2.0 mM or 16.8 mM glucose (data not shown).

3.4. Somatostatin

Islet δ-cell secreted somatostatin has also been suggested to influence the regulation of glucagon secretion under different physiological conditions, although its role has been controversial [53–55]. We tested but found no direct effect of somatostatin on glucagon secretion when 10 nM exogenous somatostatin was added to the purified α-cell aggregates at 2.0 mM glucose (Fig. 3(C), Left panel), suggesting that

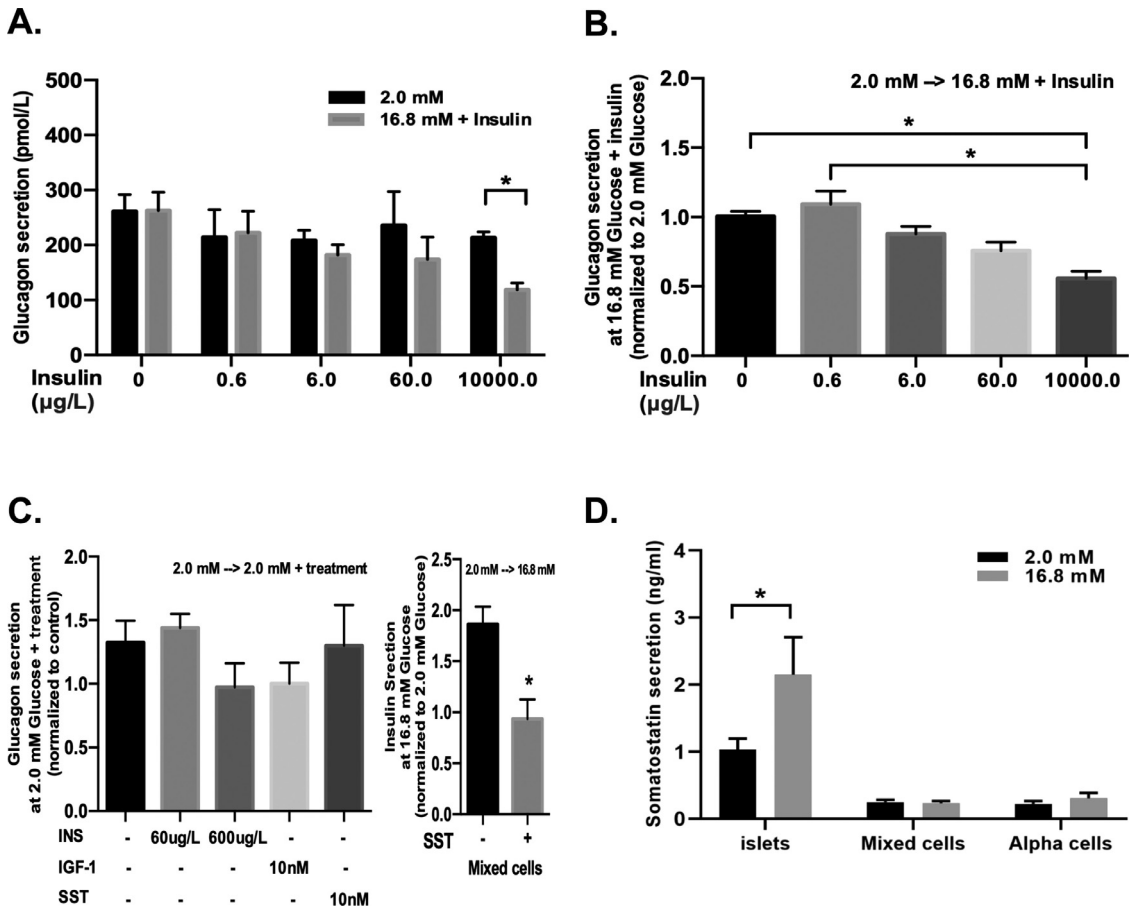

Fig. 3. The effect of insulin, IGF-1 or somatostatin on α-cell glucagon release. (A and B) Glucagon released by α-cells or the ratio of glucagon released during glucose challenge (2.0 mM to 16.8 mM ± insulin, n = 3 for each group), respectively. *p-value <0.05 ((A). paired t-test; (B) One-way ANOVA, Tukey post-hoc test). (C) The ratio of glucagon released at 2.0 mM glucose plus insulin (60 μg/L or 600 μg/L, n = 4 per group), IGF-1 (10.0 nM, n = 2) or somatostatin (10.0 nM, n = 2) divided by that at 2.0 mM glucose (Left panel). The ratio of insulin secretion at 16.8 mM glucose, in the absence or presence of somatostatin, divided by that at 2.0 mM glucose (Right panel). (D). Somatostatin released by human islets, mixed cell aggregates, or α-cell aggregates during glucose challenge assays (2.0 mM to 16.8 mM, n = 4 for each group). *p-value <0.05 (paired t-test).

somatostatin by itself was not sufficient to influence glucagon release by the pure α-cell aggregates at low glucose. Addition of the same dose somatostatin to the mixed α- and β-cell aggregates significantly inhibited glucose-stimulated insulin secretion (Fig. 3(C), Right panel), but slightly stimulated glucagon secretion at 16.8 mM glucose (data not shown) compared to that of non-somatostatin treated mixed cell aggregates. In addition, as shown in Fig. 3(D), while the intact human islets showed a 2.17 ± 0.36-fold increase in somatostatin secretion at 16.8 mM glucose (2.15 ± 0.56 ng/ml) compared to that of 2.0 mM glucose (1.03 ± 0.16 ng/ml), the mixed-cell aggregates which responded to glucose challenge with suppressed glucagon secretion, showed only minor levels of somatostatin (0.25 ± 0.03 ng/ml at 2.0 mM glucose and 0.23 ± 0.03 ng/ml at 16.8 mM glucose) which were not influenced significantly by changes of glucose concentration. Similarly, the supernatant of the α-cell-only samples contained 0.22 ± 0.04 or 0.30 ± 0.08 ng/ml at 2.0 mM or 16.8 mM glucose, respectively (Fig. 3 (D)). These data suggested that somatostatin was not essential for mediating the inhibitory effect of β-cells on glucagon secretion observed in the mixed α- and β-cell aggregates at 16.8 mM glucose (Fig. 2).

3.5. Cell-to-cell contact between α- and β-cells is essential for the regulated glucagon secretion

In an effort to identify the nature of the β-cell mediator(s) that were responsible for the inhibition of glucagon secretion occurring in the mixed cell aggregates, we conducted a novel glucose challenge experiment by first performing glucose-stimulated insulin secretion (GSIS, 2.8 mM to 16.8 mM glucose) on pure β-cell aggregates and then applying the supernatants collected from each phase of the GSIS to the pure α-cell aggregates sequentially (Fig. 4(A)). As expected, significant increases in insulin secretion were detected in the supernatants of mixed cells and β-cells at 16.8 mM glucose, compared to that at 2.0 mM glucose (Fig. 4(B)); however, no significant changes in the amount of glucagon released were observed between α-cells incubated in the β-cell supernatant (Beta KRB) of 2.0 mM glucose and that subsequently incubated in the Beta KRB of 16.8 mM glucose (Fig. 4(C)). While the mixed cell aggregates demonstrated a glucose inhibition ratio of 0.63 ± 0.07 in glucagon secretion, the α-cell aggregates showed ratios of 1.21 ± 0.13 or 1.18 ± 0.15 when challenged in regular KRB (2.0 mM to 16.8 mM glucose) or Beta KRB (2.0 mM to 16.8 mM glucose), respectively. These results point to a fact that, in the experimental setting, factors released by human β-cells as a result of glucose stimulation have no effect on α-cell glucagon secretion in the absence of physical interaction with β-cells.

The importance of α- and β- cell-to-cell contact has been demonstrated in rodent cells where juxtacrine signals between EphA4/7 on α-cells and ephrins on β-cells are believed to regulate α-cell F-actin concentration and glucagon secretion [35]. In order to test the potential importance of EphA-ephrin interaction and confirm the essential requirement of cell-to-cell contact between human islet α- and β- cells, we treated the pure α-cell aggregates with ephrinA5-Fc, the EphA stimulator. However, as shown in Fig. 4(C), addition of EphrinA5-Fc to the 16.8 mM glucose-containing KRB (in a 2.0 mM to 16.8 mM glucose

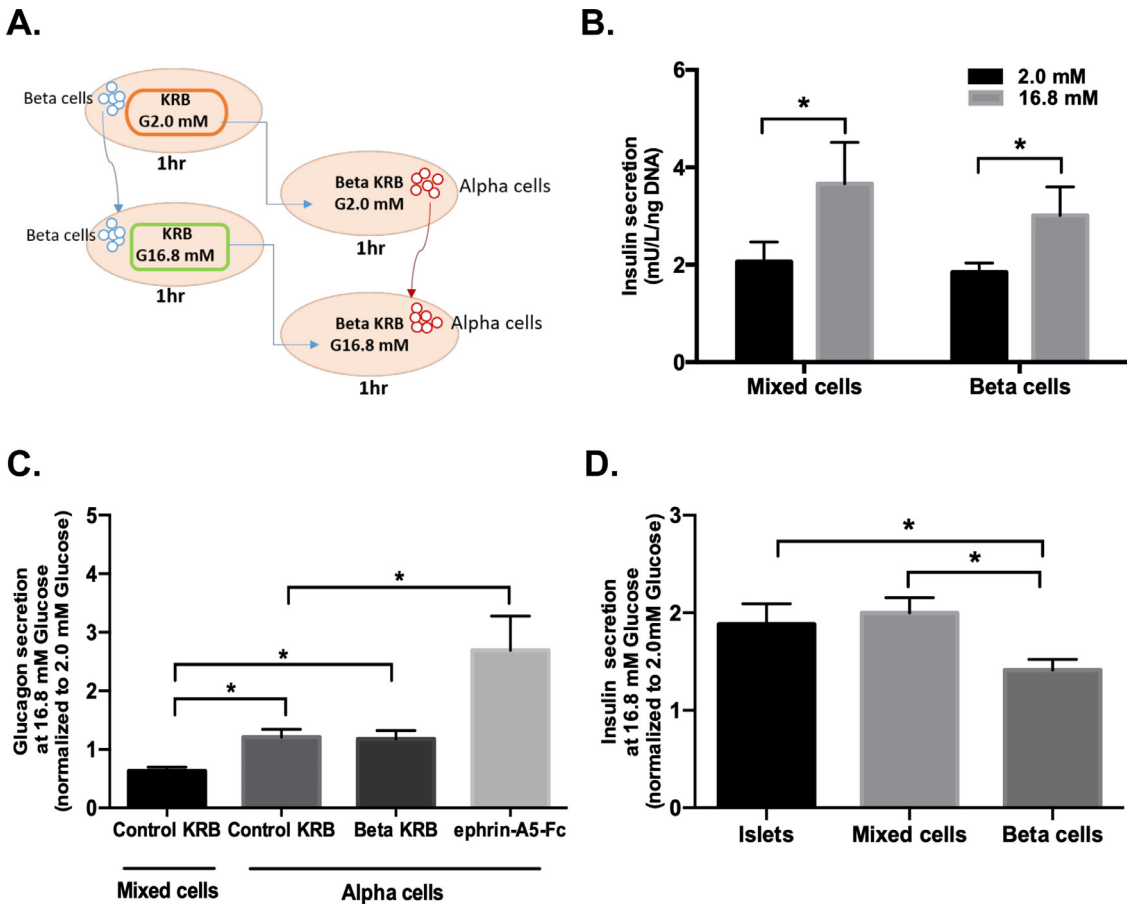

Fig. 4. Insulin or glucagon secretion from mixed or pure islet cell aggregates in control- or beta-KRB solutions. (A) An illustration of a unique glucose challenge approach where the supernatant collected from the β-cell glucose challenge at both 2.0 mM and 16.8 mM glucose were used sequentially in the α-cell glucose challenge performed next. (B) Insulin secretion by the mixed- or the pure β-cell aggregates. *p-value <0.05 (paired t-test, n = 4). C. Ratio of glucagon secretion in different types of KRB solutions (n = 7) or in the presence of ephrin-A5-Fc (n = 3). *p-value < 0.05 (one-way ANOVA, Tukey post-hoc test). (D) Ratio of insulin secretion. *p-value < 0.05 (one-way ANOVA, Tukey post-hoc test, n = 7).

challenge experiment) did not inhibit, but rather significantly stimulated, glucagon secretion with a near 3-fold induction by the α-cell aggregates, suggesting the EphA-ephrin signaling pathway is not sufficient to suppress glucagon release by human α-cells. Such stimulatory effect was not detected in intact islets. In fact, EphrinA5-Fc showed an inhibitory effect on glucagon secretion by intact human islets (not shown), consistent with what others have reported [35].

3.6. Human islet α-cells potentiated glucose-stimulated insulin release from β-cells

As shown in Fig. 4(D), the intact human islets and the mixed α- and β-cell aggregates responded to glucose challenge with an average of 1.88 ± 0.21 and 2.0 ± 0.16-fold increases of insulin secretion, respectively, with no significant difference observed between the two groups of samples. The pure β-cell aggregates also responded to glucose challenge with a significant induction (1.41 ± 0.11-fold) of insulin secretion, although the increase was significantly less than that of the islets or the mixed cell aggregates ($p < 0.05$), as shown in Fig. 4(D). These data suggested that the purified β-cell aggregates preserved the function of regulated insulin secretion in response to changes of ambient glucose levels, in contrast to the lack of responsiveness of the α-cell aggregates to glucose in the absence of β-cells. The data also suggested that the presence of α-cells in the mixed cell aggregates and intact islets potentiated GSIS, consistent with what others have reported [33,34,56–60].

3.7. Glibenclamide does not influence glucagon secretion by human α-cells in the absence of β-cells

Glibenclamide, a sulfonylurea, stimulates insulin secretion by inhibiting K_{ATP} channels on islet β-cells. One of the major side effects of sulfonylureas in diabetes treatment is hypoglycemia [30]. Given the importance of K_{ATP} channel-based medicines in the treatment of type 2 diabetes, it is essential to fully understand the actions of sulfonylureas in not only β-cells, but also other islet cell types including α-cells which also express the ATP-dependent K^+-channels [61–63]. To do this, we exposed islets, mixed cell aggregates, and pure α-cell aggregates to Glibenclamide (0.1 μM) in the presence of 2.0 mM glucose. As expected, Glibenclamide evoked a significant induction in islet insulin secretion (data not shown) and a suppression of islet glucagon secretion to 52.00 ± 8.70% of the baseline level (Fig. 5). Glibenclamide also significantly inhibited glucagon secretion by the mixed-cell aggregates, albeit to a lesser degree, to 88.78 ± 7.90% of the baseline level, confirming an added role of other islet cells for the glucagonostatic effect of K_{ATP} channel blockers as shown in rodent islet cells [64]. No significant changes in glucagon secretion from the α-cell aggregates, however, were observed in the absence or presence of Glibenclamide (Fig. 5).

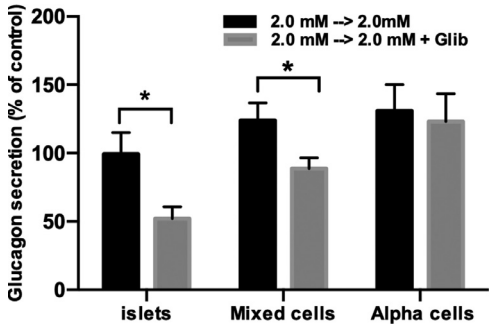

Fig. 5. The effect of glibenclamide on α-cell glucagon release. Glucagon secretion by intact human islets ($n = 4$), aggregates of mixed islet α- and β-cells ($n = 5$) or pure α-cells ($n = 3$).

*p-value <0.05 (paired t-test).

4. Discussion

Pseudo-islets formed by re-aggregation of all or selected combinations of islet cells have been used as model systems for in vitro studies of islet cell physiology and for transplantation in animal models of diabetes [35,65–72]. This work focuses on establishing re-aggregated pure human α-cells or α- and β-mixed cells to model the physiology of human pancreatic islet α-cells. The α-cells are relatively abundant in human islets, allowing for isolation of, albeit inefficient, sufficient numbers of the cells by the antibody-assisted FACS approach for clinically relevant research. We demonstrate that pure, viable and functional α- and β-cells from human islets can be isolated and re-aggregated either alone or as mixed populations to form 3D structures in culture which can be used for investigation of human glucagon secretion in vitro.

The human α-cells, devoid of or with only an insignificant amount of potential influence from other islet cell types or factors, are capable of secreting glucagon at levels similar to that secreted by the mixed α- and β-cell aggregates, unsorted islet cell aggregates, and intact islets, at no or low concentrations of glucose, although the dynamics of the secretion, which could potentially be different than those of α-cells in association with other types of islet cells, have not been measured. Less energy is likely available to fuel α-cell secretion at low glucose; however, glucagon and the co-released glutamate can potentially amplify glucagon secretion by raising α-cell cAMP [73,74] or calcium [75]. The re-aggregated purified human α-cells as described here can serve as a relatively clean model to test this positive feedback hypothesis.

It has been reported that α-cells, like β-cells, have glucose-sensing machinery including glucokinase [76] which is required for mouse α-cell glucose-dependent increase in intracellular ATP/ADP ratio, the closure of K_{ATP}-channel, and the suppression of glucagon secretion at euglycemic and hyperglycemic levels [76]. However, both inhibitory [77] and stimulatory [36,37] effects of glucose on glucagon secretion from the purified rodent α-cells have been reported, and the underlying mechanisms are still disputed. Our study shows that the human α-cell aggregates do not respond to changes in glucose level with significant changes in glucagon secretion in the absence of proper β-cell interaction, which may suggest that changes in blood glucose concentration ranging from hypoglycemia to hyperglycemia may only have a minute effect on islet glucagon secretion in patients with severe type 1 diabetes where most of the islet β-cells are lost as a result of autoimmune destruction [78]. This is consistent with clinical studies which show that reciprocal β-cell-mediated signaling predominates over potential direct regulation of glucagon exocytosis after mixed meal stimulation [79,80]. The presence of β-cells may change the functional properties of α-cells via cell-to-cell contacts and paracrine factors.

Insulin released from the β-cells is considered as a putative mediator of glucose-inhibited glucagon secretion [32,51]. Our study shows that exogenous insulin exerts a significant impact on glucagon secretion from α-cells only at doses much higher than the physiological level. Nevertheless, the highest insulin dose (~1.7 μM) tested in this study is not too different than the estimated 1.0 μM intra-islet insulin concentration [81], rendering the results physiologically relevant. Such insulin dosing studies need to be repeated using the mixed α- and β-cell aggregates at low glucose levels when minimal insulin is secreted to reveal whether the presence of β-cells alters the responsiveness of α-cells to the treatment. Data from the glucose challenge studies employing Beta KRBs clearly point to the potential important involvement of cell-to-cell contact between α- and β-cells, in addition to the potentially higher concentrations of paracrine factors released from neighboring β-cells, in the regulation of glucagon secretion. Cellular contact-mediated signals from neighboring cells constitute a major part of cellular microenvironment. Since the pulsatile secretion of both insulin and glucagon in islets are tightly

synchronized and the various islet cell types are electrically coupled, inversely or not, it is not surprising that the physical contact between the two types of cells through gap junctions and ligand-receptor signaling is critical for proper regulation of islet hormone secretion. It has been shown that the ephrinA5-Ac/Eph4/7 signaling pathway is capable of stimulating the formation of F-actin density in rodent α-cells which is critical for providing a secretion barrier for glucagon release [35]. Our study on purified human α-cells, however, did not confirm the interaction between ephrinA5-Ac and EphA4/7 as the important mediator of β-cell inhibition of glucagon secretion. In fact, such interaction stimulated glucagon secretion when exogenous ephrinA5 was added to the α-cells. The mechanisms involved in this stimulatory effect as well as the other potential cell-cell contact signaling between human α- and β-cells need to be explored in the future.

The α-cell aggregates from a few donors studied responded to increasing concentrations of glucose with increases in glucagon secretion, similar to that of sorted mouse α-cells when ample glucose concentration increased from 1.0 mM to 12.0 mM [37]. Averaging the responses of islet α-cells from multiple donors, however, did not reveal any significant effect of glucose on glucagon release. Donor-to donor variation in cellular function always remains to be a challenge when studying primary human cells. Larger sample populations and careful examination of the relationships between donor criteria and experimental data are required for studying human samples. However, no correlation between donor age, gender, BMI, or disease history and the responsiveness of the α-cells to glucose were clearly identified in the study.

Our study demonstrates that somatostatin and δ-cells are not essential for the inhibitory influence of glucose and β-cells on glucagon secretion. This is consistent with the finding that the specific antagonist of somatostatin-receptor subtype 2 expressed on human α-cells [82] does not affect the ability of glucose to inhibit glucagon secretion from human islets in vitro [83]. We cannot, however, rule out the possibility that δ-cells exert additional inhibition on glucagon secretion, since islets and the non-sorted islet cell aggregates containing δ-cells from some of the donors responded to high glucose with a stronger inhibition on glucagon secretion than that of the mixed cell aggregates without δ-cells from the same donors. In the presence of functional δ-cells where somatostatin can be induced by insulin [55], insulin at 100 nM is sufficient to inhibit glucagon secretion from human islets in vitro, much less than the effective dose (~1.7 μM) identified for the purified α-cells in this study. Addition of exogenous somatostatin in our study, however, did not result in an inhibition of glucagon secretion by the α-cells, consistent with the finding from the co-cultured mouse α- and δ-cells where no inhibition of glucagon secretion was induced by glucose [35]. All these results strongly suggest that multiple independent mechanisms including paracrine factors such as insulin and somatostatin and cell-to-cell contact between α- and β-cells need to act together to fully restore the responsiveness of α-cells toward glucose inhibition. The established aggregates of pure human α-cells provides a clean model for future investigation of the potential changes in signaling pathways, gene expression and cellular metabolism upon somatostatin treatment to further understand the role of somatostatin and δ-cells in regulating human glucagon secretion.

Our study also shows the lack of effect of Glibenclamide on glucagon secretion from the human α-cell aggregates, while it inhibits glucagon secretion from the islets and the mixed cell aggregates. This result is consistent with the finding that T1D patients lacking a detectable c-peptide response do not respond to sulfonylureas with hypoglycemia [84]. However, we cannot exclude the possibility that the sorted pure α-cells do not maintain the same membrane properties and channel expression as that of α-cells in intact islets or the mixed cells, resulting in the lack of direct impact of Glibenclamide on glucagon secretion. It is important to take into consideration the fact that the results generated from the purified cells may not completely reflect the responses of the cells in intact islets, due to the lack of proper cell-to-cell interactions [85], paracrine effects [86], and proper microenvironment cues [87]. Nevertheless, the pure α-cell model might represent, to a certain degree, the α-cell situation in T1D where interaction with β-cells is deficient, although the latter likely maintains most of the non-β-cell islet components. This study reveals an essential role for the presence of β-cells for proper regulation of glucagon secretion by glucose, islet paracrine factors and sulfonylureas, and that the mechanisms involved are likely different than that of rodent islet cells. The fact that insulin at doses much higher than physiological level are required to achieve sufficient inhibition of glucagon release, in the absence of β-cells, provides further evidence demonstrating that β-cell replacement is likely a more efficient therapy than insulin treatment is in managing α-cell dysfunction and glycemia in diabetes. Aggregates of various combinations of sorted human islet cells provide unique and important tools for investigating islet cell function, metabolism, gene expression, cell-to-cell interaction, and drug screening for the treatment of diabetes.

Declaration of Competing Interest

OHSU has commercially licensed HIC1-2B4/HPi2, of which C.D. is an inventor. This potential conflict of interest has been reviewed and managed by OHSU. All other authors declare no conflict of interest.

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