Development and validation of the human *islet*OrganDOT™ 3D culture system for evaluation of insulin secretagogues

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ASTERAND BIOSCIENCE
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Abstract

Diabetes affects approximately 9%[1] of the world's population and >90% of those with the disease have type 2 diabetes. Each year over 1.5 million people die from complications associated with poor glycaemic control. Whilst a number of drugs with different mechanisms of action are currently available for the treatment of diabetes, new and efficacious drugs are urgently needed to combat the looming global health challenge of the disease.

Insulin secretagogues, such as sulphonylureas and incretin mimetics, are important drugs for combating type 2 diabetes, and extensive research into the identification and development of new insulin secretagogues continues. Evaluation of secretagogues for their effects on glucose- stimulated insulin secretion (GSIS) using freshly isolated human pancreatic islets has long been considered the 'gold standard' but freshly isolated islets present several challenges, not least the relatively rapid loss of functionality following isolation from the pancreas. Using a method based on that described by Sundstrom *et al*[2] for the generation of reconstituted tissues on an air-liquid interface, we have developed the human *islet*OrganDOT™ system; a 3D organotypic culture model for profiling new insulin secretagogues that has several advantages over freshly isolated human pancreatic islets. The human *islet*OrganDOT system maintains islet-like structures that contain the expected complement of islet α, β, and δ cells and express key drug target genes such as the GLP-1 receptor, GPR40 and GPR119. Furthermore, these organotypic cultures retain GSIS functionality for up to 4 weeks and represent a robust and reliable test system for compound profiling. Finally, the extended culture life of *islet*OrganDOTs cultures enables repeat GSIS assays and offers the potential for evaluation of compounds designed to protect β cell health and/or promote β cell proliferation.

Methods

Human isolated pancreatic islets used in this work were obtained with informed consent and ethical approval for research use.

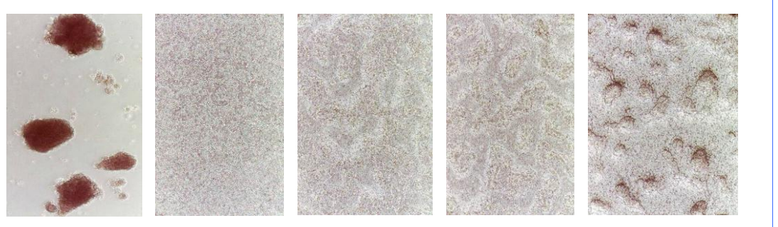
Preparation of *islet*OrganDOT cultures
Freshly isolated intact islets were dispersed enzymatically, washed by centrifugation and re-suspended at high density in M199 medium supplemented with serum and antibiotics/antimycotics. *Islet*OrganDOT cultures were created by 'spotting' 3μL of cell suspension into the centre of each membrane of a 24-well Millicell cell culture insert plate, with the feeder plate previously primed with culture medium sufficient to achieve basolateral feeding. The *islet*OrganDOT cultures were maintained at 30ºC at 5% CO_2, with medium changes every 3 or 4 days.

Glucose stimulated insulin secretion (GSIS) assays and insulin ELISA
GSIS assays were performed at 37ºC by incubating *islet*OrganDOT cultures with test solutions applied basolaterally only. Cultures were initially equilibrated to low glucose by incubating with HBSS containing 1.67mM glucose. Basal insulin secretion was then determined by further incubation for 30 minutes with 1.67mM glucose. Cultures were then shifted to a final test solution, typically 6.7mM or 16.7mM glucose ± test compound, for 30 minutes to yield stimulated insulin secretion. Basal and stimulated secretions were assayed for insulin by ELISA (Mercodia) according to the manufacturer's instructions. Data are plotted as net insulin secretion (stimulated minus basal) and Student's t-test was used to evaluate statistical differences between the various treatment conditions.

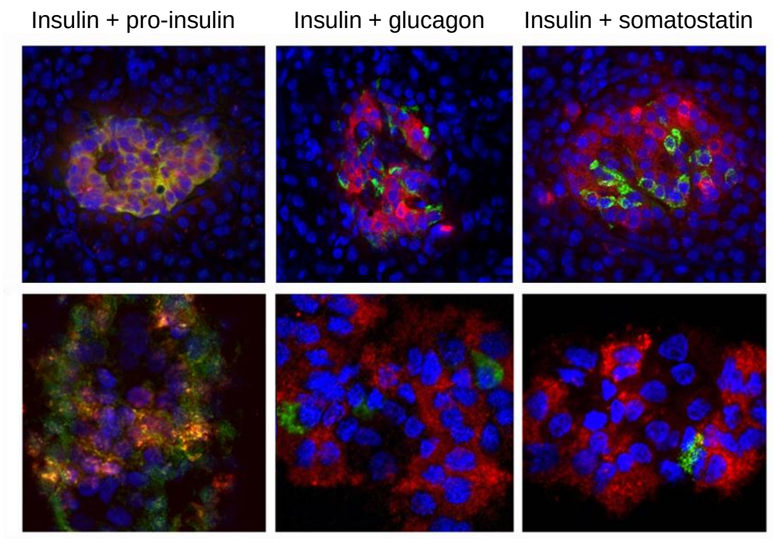
Immunofluorescence analysis of *islet*OrganDOT cultures
*Islet*OrganDOTs were mounted on SuperFrost*plus* slides and then fixed using ice-cold acetone:methanol. Slides were washed with Dako Envision FLEX buffer, incubated with primary antibodies for 60 minutes, washed again with FLEX buffer and then incubated for a further 60 minutes with appropriate Alexa-conjugated secondary antibodies. After a final wash with FLEX buffer, the slides were counterstained with DAPI, cover-slipped using Permafluor (Vector Laboratories), dried at 4ºC in the dark and viewed using confocal fluorescence microscopy (Zeiss LSM 510 META). For comparison with the *islet*OrganDOT cultures, frozen sections of human pancreas were stained for islet α, β, and δ cells as described above.

Expression of islet cell marker genes
RNA was extracted from freshly isolated pancreatic islets or *islet*OrganDOT cultures using an SV Total RNA Isolation System (Promega) and cDNA was created with Applied Biosystems' High Capacity cDNA Reverse Transcription Kit. The expression of eight key islet cell marker genes was measured using TaqMan® qRT-PCR (primer-probe sets from Applied Biosystems; PerfeCTa® qPCR Supermix from Quanta Biosciences). PCR conditions were 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. A suitable threshold value was set for the determination of C_T values from the PCR amplification curves.

Results



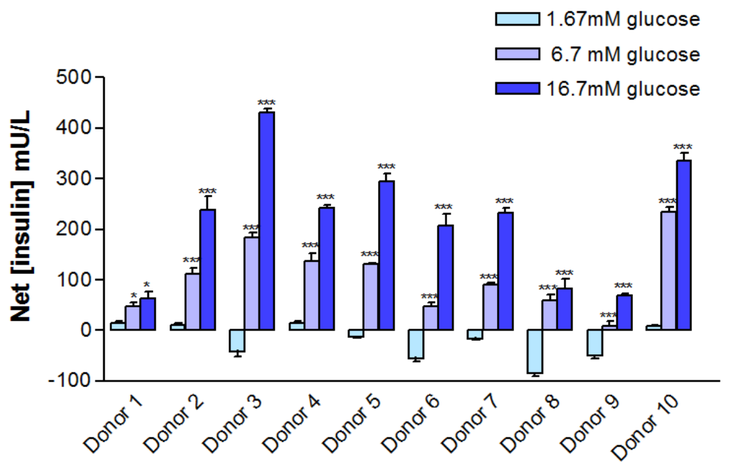
Dispersed human islet cells re-aggregate in OrganDOT culture.
Intact pancreatic islets were enzymatically dispersed and the isolated cells 'spotted' on to a Millicell culture membrane were maintained by feeding basolaterally only. These 'air-liquid' interface conditions cause capillary compaction and the phase contrast images show that the cells re-aggregate and form 3D structures after a few days in culture.



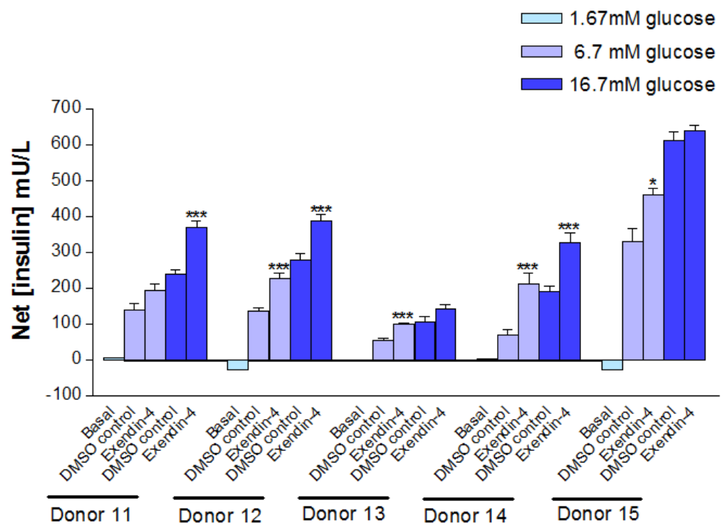
Islet α, β and δ cells are maintained in *islet*OrganDOT cultures
Dual labelling immunofluorescent detection of pancreatic islet marker proteins in frozen pancreas sections (top row) and *islet*OrganDOTs (bottom row). Insulin is visualised as red and other islet cell marker proteins as green fluorescence. Overlapping distribution appears as yellow fluorescence. These confocal images indicate that the α, β and δ cell phenotypes are retained in *islet*OrganDOT cultures.

	Genes and qPCR C_T values							
	Insulin	GLP-1 receptor	Glucagon	GPR40	GPR119	SLC2A2	PCSK1	GCK
Islet OrganDOT (Day 7)	14.8	24.5	18.9	24.9	29.0	28.8	23.7	29.1
Islet OrganDOT (Day 13)	15.0	23.9	18.2	24.4	28.9	28.4	24.0	29.0
Native islets (Donor 1)	17.0	29.0	21.4	29.2	30.6	30.9	27.4	32.4
Native islets (Donor 2)	17.0	27.8	20.0	28	29.5	28.5	26.2	33.2
Native islets (Donor 3)	17.8	29.4	19.8	29.1	31.2	32.8	26.8	32.5

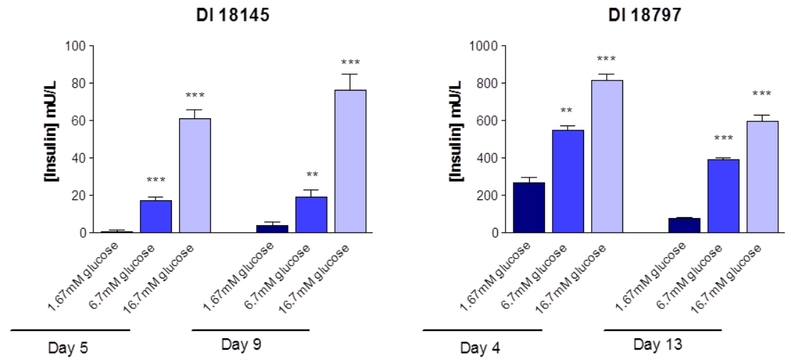
Expression and maintenance of key genes in *islet*OrganDOT cultures
Gene expression was measured using TaqMan qRT-PCR and C_T values for eight pancreatic islet cell genes are shown for *islet*OrganDOT cultures from a single donor after 7 and 13 days in culture and for native intact islets from three donors. The data show that expression of these key genes was higher (the lower the C_T value the higher expression) in the *islet*OrganDOT cultures compared with native islets and that the level of expression was maintained after at least 13 days in culture. (SLC2A2 = solute carrier family 2, member 2, PCSK1 = proprotein convertase subtilisin/kexin type 1, GCK = glucokinase)



Robust GSIS responses of *islet*OrganDOT cultures
*Islet*OrganDOT cultures were prepared with islets from 10 different donors and GSIS assays performed 4 – 7 days post seeding of the cells. Consistent and statistically significant (*p<0.05, ***p<0.001) increases in insulin secretion were measured at both 6.7mM and 16.7mM glucose when compared with insulin secretion in the presence of 1.67mM. Note that where the test solution is 1.67mM glucose, calculation of net insulin secretion yields a negative value for some donors. Data are mean+sem (n=4 replicates).



The GLP-1 receptor agonist exendin-4 potentiates GSIS in *islet*OrganDOT cultures
GSIS assays were performed with 6.7mM and 16.7mM glucose in the presence or absence of 25nM exendin-4 (Exenatide). When compared with the respective 'glucose only' controls, statistically significant (*p<0.05, *** p<0.001) potentiation of insulin secretion was apparent with exendin-4 with *islet*OrganDOTs from all five donors. Data are mean+sem (n=4 replicates).



***Islet*OrganDOT cultures exhibit robust GSIS responses in extended culture**
*Islet*OrganDOT cultures were prepared with islets from two donors and GSIS assays were performed 5 days (donor 18145) and 4 days (donor 18797) after seeding. Increased glucose concentration elicited statistically significant (** p<0.01, *** p<0.001) stimulation of insulin secretion from both sets of cultures. Repeat GSIS assays were performed with the same *islet*OrganDOT cultures 4 days (donor 18145) and 8 days (donor 18797) later and robust GSIS responses were again apparent. Data are mean+sem (n=4 replicates).

Conclusions

- The human *islet*OrganDOT system offers a robust platform for GSIS assays allowing in-depth profiling of insulin secretagogues under development for the treatment of diabetes.
- Consistent responses to glucose and insulin secretagogues, such as exendin-4, are key features of the system, and the demonstration of the expression of genes such as GPR40 and GPR119, for which agonists are currently under intensive investigation as insulin secretagogues[3,4] extends the value of this test system.
- *Islet*OrganDOTs have several advantages over intact human islets, particularly retention of functionality after extended culture, which allows re-testing of compounds using the same cultures.
- The ability of *islet*OrganDOT cultures to remain viable and functional after extended culture also offers the potential for the system to be used for investigation of compounds designed to promote β cell proliferation or maintain β cell numbers in diabetes, which are important new areas for diabetes drug discovery[5].

References

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