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Original Article

MOLECULAR METABOLISM

EndoC-βH5 cells are storable and ready-to-use human pancreatic beta cells with physiological insulin secretion

Bruno Blanchi [1,], Marion Taurand [1], Claire Colace [2], Sofia Thomaidou [3], Charlotte Audeoud [4], Federica Fantuzzi [5,6], Toshiaki Sawatani [5], Sevda Gheibi [7], Joan Sabadell-Basallote [8,13], Fransje W.J. Boot [9], Thibault Chantier [4], Aline Piet [1], Charlotte Cavanihac [1], Marion Pilette [1], Adélie Balguerie [1], Hamza Olleik [1], Françoise Carlotti [9], Miriam Ejarque [8], Malin Fex [7], Hindrik Mulder [7], Miriam Cnop [5,10], Decio L. Eizirik [5], Ouardane Jouannot [4], Anne-Lise Gaffuri [4], Paul Czernichow [11], Arnaud Zaldumbide [3], Raphaël Scharfmann [12], Philippe Ravassard [2,*]**

ABSTRACT

Objectives: Readily accessible human pancreatic beta cells that are functionally close to primary adult beta cells are a crucial model to better understand human beta cell physiology and develop new treatments for diabetes. We here report the characterization of EndoC-βH5 cells, the latest in the EndoC-βH cell family.

Methods: EndoC-βH5 cells were generated by integrative gene transfer of immortalizing transgenes hTERT and SV40 large T along with Herpes Simplex Virus-1 thymidine kinase into human fetal pancreas. Immortalizing transgenes were removed after amplification using CRE activation and remaining non-excized cells eliminated using ganciclovir. Resulting cells were distributed as ready to use EndoC-βH5 cells. We performed transcriptome, immunological and extensive functional assays.

Results: Ready to use EndoC-βH5 cells display highly efficient glucose dependent insulin secretion. A robust 10-fold insulin secretion index was observed and reproduced in four independent laboratories across Europe. EndoC-βH5 cells secrete insulin in a dynamic manner in response to glucose and secretion is further potentiated by GIP and GLP-1 analogs. RNA-seq confirmed abundant expression of beta cell transcription factors and functional markers, including incretin receptors. Cytokines induce a gene expression signature of inflammatory pathways and antigen processing and presentation. Finally, modified HLA-A2 expressing EndoC-βH5 cells elicit specific A2-alloreactive CD8 T cell activation.

Conclusions: EndoC-βH5 cells represent a unique storable and ready to use human pancreatic beta cell model with highly robust and reproducible features. Such cells are thus relevant for the study of beta cell function, screening and validation of new drugs, and development of disease models.

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Keywords Human pancreatic beta cell line; Human beta cell function; Glucose and incretin stimulated insulin secretion; Type-I diabetes disease model

1. INTRODUCTION

More than 500 million adults currently live with diabetes worldwide, a number that is predicted to continue rising. The World Health Organization (WHO) and Global Burden of Disease (GBD) initiative report an increase in diabetes cases and diabetes related age-standardized mortality rates; the increase in diabetes prevalence is particularly significant in low and middle income countries [1] (IDF Diabetes Atlas). During the same period of time, the probability of dying from other major non transmissible diseases has globally decreased [1], ruling out improved diagnosis or deteriorated health care as plausible causes for the increased number of diabetes cases observed and legitimizing its description as a pandemic [2]. The need for physiologically relevant human cellular models to study pancreatic endocrine cell function, diabetes mechanisms and treatment strategies is thus greater than ever. Easily accessible and functionally validated human beta cells would

[1]Human Cell Design, Canceropole, Toulouse, France [2]Paris Brain Institute, Sorbonne Université, Inserm U1127, CNRS UMR 7225, Paris, France [3]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, Netherlands [4]Adocia, Lyon, France [5]ULB Center for Diabetes Research, Université Libre de Bruxelles, Brussels, Belgium [6]Department of Medicine and Surgery, University of Parma, Parma, Italy [7]Department of Clinical Sciences, Unit of Molecular Metabolism, Lund University Diabetes Centre, Malmö, Sweden [8]Unitat de Recerca, Hospital Universitari de Tarragona Joan XXIII, Institut d'Investigació Sanitària Pere Virgili, Tarragona, Spain [9]Department of Internal Medicine, Leiden University Medical Center, Leiden, Netherlands [10]Division of Endocrinology, Erasmus Hospital, Université Libre de Bruxelles, Brussels, Belgium [11]Animal Cell Therapy, Paris, France [12]Université Paris Cité, Institut Cochin, CNRS, INSERM U1016, Paris, 75014, France [13]Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

*Corresponding author. E-mail: philippe.ravassard@icm-institute.org (P. Ravassard).

**Corresponding author. E-mail: b.blanchi@humancelldesign.com (B. Blanchi).

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facilitate the development of novel and better, i.e. more efficient, easier to distribute and administer or less expensive, drugs as an essential complement to indispensable prevention programs and public health policies.

We previously developed immortalized human beta cell lines using targeted oncogenesis in human foetal pancreatic tissue in which two transgenes were integrated using lentiviral vectors. Both transgenes, SV40 large T antigen (SV40-LT) and human Telomerase Reverse Transcriptase (hTERT), were expressed under the control of the beta cell specific rat insulin promoter (RIP). The first generation of cells, named EndoC-βH1, expressed SV40LT and hTERT in a constitutive manner [3] whereas, in the second generation EndoC-βH2 and EndoC-βH3, these transgenes were excisable upon CRE recombination mediated by the presence of a loxP site located in the 3' LTR of the lentiviral backbone [4,5]. EndoC-βH cells have become a broadly used model giving rise to numerous innovative publications and discoveries [6].

Here, we developed and characterized an optimized EndoC-βH version with improved function and greatly enhanced practicality. Similar to previous EndoC-βH lines, EndoC-βH5 cells were obtained through targeted oncogenesis of a human foetal pancreas using lentiviral vectors expressing excisable SV40 LT and hTERT. In EndoC-βH5 cells, the immortalizing genes have been integrated along with Herpex simplex 1 (HSV1) thymidine kinase (TK) in the same excisable cassette. With such construct, the presence of TK allowed ganciclovir-dependent elimination of the potential population of non-excised cells that could expand and take over the culture over time as observed in EndoC-βH3 [4] Following massive amplification and excision of immortalizing transgenes, cells that resisted transgene excision were depleted upon ganciclovir treatment. The resulting cells were frozen and stored as ready-to-use EndoC-βH5 cells.

EndoC-βH5 cells secrete insulin in response to glucose in a robust and reproducible way when tested in independent laboratories. Furthermore, EndoC-βH5 cells display dynamic insulin secretion and robust and dose-dependent response to incretins. EndoC-βH5 cells are ready-to-use, distributed as functionally validated cells that are storable and highly reproducible. Overall, EndoC-βH5 cells are a novel human pancreatic beta cell solution to develop human diabetes models and drug screening and validation platforms.

2. METHODS

2.1. DNA constructs and lentiviral vector productions

The lentiviral constructs, pTRIP ΔU3loxP-RIP405-SV40-LT and pTRIP ΔU3loxP-RIP405-SV40-hTERT have been previously described [5]. Both vectors were digested with EcoRI in order to insert a synthetized EcoRI flanking DNA fragment that contained the 235 bp Polyoma virus enhancer (pY) upstream of the HSV1 TK coding sequence followed by the human beta actin polyA signal (Sigma Aldrich). Clones that integrated this fragment in reverse orientation relative to the transcribed proviral RNA were selected, resulting in two novel vectors pTRIP ΔU3loxP-pY-TK-RIP405-SV40-LT and pTRIP ΔU3loxP-pY-TK-RIP405-hTERT. The pTRIP ΔU3 RIP405-CRE-ERT2 was described elsewhere [7]. A dual expression cassette containing the coding sequence of HLA-A2, an Internal Ribosome Entry Site (IRES) and the puromycin-resistance gene was synthetized (Sigma Aldrich) and flanked with Gateway recombination sites attL1 and attL2. The HLA-A2 IRES PURO sequence was inserted using Gateway LR clonase (Invitrogen) according to manufacturer instructions into the pTRIP ΔU3 CMV RFA destination vector to create the lentiviral vector pTRIP ΔU3 CMV HLA-A2 IRES PURO that expressed both HLA-A2 and the puromycin-resistance gene under the control of the ubiquitous cytomegalovirus (CMV) promoter.

Lentiviral vector stocks were produced by transient transfection of HEK 293 T cells by encapsidation of the p8.9 plasmid (ΔVprΔVifΔVpuΔNef), pHCMV-G, that encodes the VSV glycoprotein-G, and the pTRIP ΔU3 recombinant vector, as previously described [8]. Supernatants were treated with DNAse I (Roche Diagnostic) prior to ultracentrifugation, and pellets were re-suspended in PBS, aliquoted, and frozen at −80 °C until use. The amount of p24 capsid protein was quantified by the HIV-1 p24 antigen ELISA (Beckman Coulter). All transductions were normalized relative to p24 capsid protein quantification.

2.2. Derivation of EndoC-βH5

EndoC-βH5 cells were derived from a human fetal pancreatic tissue with slight modifications of previously described methods [3,5]. Briefly, human fetal pancreatic explants were cotransduced with pTRIP ΔU3loxP-pY-TK-RIP405-SV40-LT and pTRIP ΔU3loxP-pY-TK-RIP405-hTERT lentiviral vectors. They were transplanted under the kidney capsule of SCID mice leading to the formation of an insulinoma eight months later. Two successive rounds of transplantation in SCID mice were used to amplify the insulinoma cells prior to establishment of the cell line as described elsewhere [3,5].

2.3. Cell culture and production of ready-to-use EndoC-βH5

EndoC-βH1 [3] and proliferating EndoC-βH5 cells were cultured on βCoat® coated plastic plates in Opti-β1 and Ulti-β1 culture media, respectively. Cells were propagated every 7 days using trypsin (0.05%, Thermofisher). HLA-A2 expressing EndoC-βH5-HLA-A2 line was produced by lentiviral transduction of EndoC-βH5 with pTRIP ΔU3 CMV HLA-A2 IRES PURO using 100 ng of p24 capsid protein per 10^5 cells. Cells were amplified for three weeks in presence of 1 μg/ml puromycin to select transduced cells. For complete maturation, EndoC-βH5 and EndoC-βH5—HLA-A2 cells were cultured for 3 additional weeks and treated with 5 μM tamoxifen and 0.5 μM ganciclovir for excision of immortalizing genes and selection of excised cells. The resulting cells were collected using trypsin (25300—054, ThermoFisher), frozen in Nutrifreez® (Biological Industries) and stored as working batches. When needed, frozen cells were rapidly thawed in a 37 °C water bath, resuspended in Ulti-β1® culture medium, centrifuged (500 g, 5min) in order to remove cryoprotectant, gently resuspended in Ulti-β1® culture medium and seeded on βCoat® coated plastic plates and cultured in Ulti-β1® culture medium. Unless specified, cells were maintained in culture for 7 days before undergoing experimental procedure.

Overall, cells used in this study were ready-to-use EndoC-βH5 and EndoC-βH5—HLA-A2 cells that had undergone CRE-mediated immortalizing transgene excision and the TK-dependent selection.

2.4. Flow cytometry

EndoC-βH5 cells were seeded onto βCoat® coated 12-well plates at 3.75×10^5 cells/well. Six days later, cells were collected using 0.05% trypsin and fixed for 15 min (Cytofix, BD Biosciences) and then stained (Perm III permeabilization buffer and FBS stain buffer, BD Biosciences). The following antibodies were used: mouse anti-insulin AF-647 and isotype (1 μl/10^5 cells, 565689 and 557783, BD Biosciences), mouse anti-PDX1 AF488 and isotype (1 μl/10^5 cells, 562274 and 557721, BD Biosciences) and mouse anti-Nkx6.1 PE and isotype (0.5 μl/10^5 cells, 563023 and 554680, BD Biosciences). Flow cytometry analyses were performed using MACSQuant 10 flow cytometer (Miltenyi).

2.5. Immunofluorescence

EndoC-βH5 cells were seeded onto βCoat® coated 8-well IBIDI chamber slides at $1-2 \times 10^5$ cells/well. Six days later, cells were fixed for 10 min in 4% paraformaldehyde. The following antibodies

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were used for immunostaining: guinea pig anti-insulin (1/500, A0564, Dako); rabbit anti-somatostatin (1/500, A0566, Dako); mouse anti-PDX1 (1/33, 685A5, BD Biosciences). Secondary antibodies were AF-594 anti-guinea pig and AF-488 anti-rabbit or anti-mouse (Thermo Fisher, 1/600 and 1/400, respectively). Digital images were captured using an Eclipse-Ti2 fluorescent microscope (Nikon).

2.6. Quantification of somatostatin expressing cells

Three independent vials of EndoC-βH5 cells were cultured on lab-tek chamber slides (Sigma Aldrich) and stained for SST along with DAPI. All slides were digitized using an AxioScan.Z1 (Zeiss). The resulting images were imported into QuPath for downstream processing [9]. Cellular segmentation was done using a pre-trained StarDist model. A simple threshold approach was used to quantify the percentage of cells expressing SST (n = 736, 554 total cells analyzed).

2.7. Insulin secretion and content

EndoC-βH5 cells were seeded onto βCoat® coated plates 12-well (3.75×10^5 cells/well) or 96-well plates (10^5 cells/well). Six days later, medium was replaced with Ulti-ST® starvation medium containing 0.5 mM glucose for 24 h. Medium was then replaced with βKrebs® glucose stimulated insulin secretion (GSIS) buffer supplemented with 0.1% fraction V fatty acid free BSA for 60 min. Cells were then incubated with βKrebs®/BSA supplemented with D-glucose (Sigma), L-glucose (Sigma), exendin-4 (Sigma), D-[Ala2]-GIP (Tocris), exendin-9 (Tocris) or diazoxide (Sigma) for 40 min. Incubation medium was collected, spun down and analysed by ELISA. For insulin content measurement, cells were lysed in Tris/Triton X-100 based lysis buffer for 5 min, collected and analysed by ELISA. Insulin was measured by ELISA using Human Insulin Kit (10–1113, Mercodia) following manufacturer's instructions.

2.8. Dynamic insulin secretion using perifusion

For perifusion experiments, EndoC-βH5 spheres (2,000 cells) were formed using Elplasia ultra-low attachment microwell plates (Corning). Spheres were starved overnight in Ulti-ST® starvation medium. Two hundred spheres were placed in a flow chamber (Biorep Technologies) that was perfused with a krebs based GSIS buffer containing different glucose concentrations. An initial 54 min habituation step was performed using 1.5 mM glucose, followed by 12 min of low glucose (LG, 1.5 mM), 32 min of high glucose (HG, 8.5 mM), 12 min wash-out (LG), 6 min of KCl (40 mM) and 10 min washout (LG). Output was collected in 4 °C cooled 96-well plates. Insulin secretion was measured by ELISA. Secretion index was calculated by dividing average insulin level during the 32-min HG phase by average insulin level during the 12-min LG phase.

2.9. RNA isolation and RNA-seq

Total RNA from 5 independent batches of EndoC-βH1 and EndoC-βH5 cells was isolated from frozen pellets (10^6 cells per sample) using the RNeasy Micro Kit 50 (74,004, Qiagen) according to the manufacturer's instructions. 300 ng of total RNA was used for library preparation following manufacturer's recommendations using KAPA mRNA hyperprep (Roche Diagnostic). Each final library preparation was quantified with fluorimeter from DENOVIX and qualified with 2200 Tapestation (Agilent). Final samples of pooled library preparation were sequenced on ILLUMINA Novaseq 6000 with S1-200 cartridge at 2×100 M reads/sample.

For evaluation of cytokine responses, total RNA isolated from EndoC-βH5 cells treated or not with a mixture of 1000 U/ml IFNγ and 2 ng/ml IL1β for 24 h (n = 3) was isolated using Nucleospin miRNA Kit (740,971, Bioke) according to manufacturer's guidelines. RNA quality was characterized using Experion RNA StdSens 1 K Analysis Kit (7007103, Bio-Rad) on Experion Automated Electrophoresis System (Bio-Rad) following the manufacturer's protocol. Samples were sequenced on Illumina NovaSeq 6000 (20 M reads/sample).

Raw sequence reads from RNA-seq are available from GEO under accession number GSE224732.

2.10. RNA-seq analysis

Quality of raw data was evaluated with FastQC [10]. Poor quality sequences and adapters were trimmed or removed with fastp tool, with default parameters, to retain good quality paired reads. Illumina DRAGEN bio-IT Platform (v3.8.4) was used for mapping on hg38 reference genome and quantification with gencode v37 annotation gtf file. Library orientation, composition and coverage along transcripts were checked with Picard tools. The following analyses were conducted with R software. Data were normalized with edgeR (v3.28.0) bioconductor package [11], prior to differential analysis with glm framework likelihood ratio test from edgeR, and/or DESeq2 workflow. Multiple hypothesis adjusted p-values were calculated with the Benjamini-Hochberg procedure to control FDR. Enrichment analysis was conducted with clusterProfiler R package (v3.14.3) with over-representation analysis, on gene ontology database, KEGG pathways and Descartes Cell Types and Tissue ontology [12].

2.11. HLA determination and T-cell activation assays

HLA-A2 expression was determined by FACS using FITC mouse anti-human HLA-A2 antibody (clone BB7.2, BD Bioscience) at 1/100 dilution. For T cell activation assays, EndoC-βH5 and EndoC-βH5—HLA-A2 cells were treated with 1000 U/ml IFNγ and 2 ng/ml IL1β in the presence or absence of the JAK inhibitor baricitinib (4 μM) (Selleckchem) for 24 h. Cells were harvested and cocultured with allo-A2 reactive cytotoxic T lymphocytes (CTLs) at increasing effector/target ratios (25,000/50,000/100,000 effectors to 50.000 target cells). Co-cultures were incubated at 37 °C for 16 h in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% human albumin and 40 U/ml IL-2 (Novartis). Supernatant was assayed for MIP-1β production by T-cells using MIP-1β ELISA kit (88-7034-22, Thermo Fisher Scientific) according to the manufacturer's protocol.

2.12. Study approval

Human fetal tissue was collected in compliance with French bioethic legislation. Approval was obtained from the French Agence de Biomedecine (Paris), under approval number PFS08-005. The mother gave written consent. Experiments using animals were reviewed and approved by the Direction Départementale de la Protection des Populations (Paris) under agreement number A75-13-19 in compliance with French legislation.

3. RESULTS

3.1. Ready-to-use EndoC-βH5 cells, the third generation of immortalized human beta cell lines

Here, a third generation of the EndoC-βH beta cell family was produced from an 8-week-old female human fetal pancreas using integration vectors containing SV40LT or hTERT and loxP sites similar to first and second generation lines, respectively [3,5], and with an additional transcription unit expressing HSV1 TK under the control of the ubiquitous polyomavirus enhancer pY (Figure 1A). The derived cell line was transduced with a lentiviral vector expression CRE-ERT2, the tamoxifen inducible form of CRE under the control of RIP. Next, cells were massively amplified, treated for 7 days with tamoxifen for transgene

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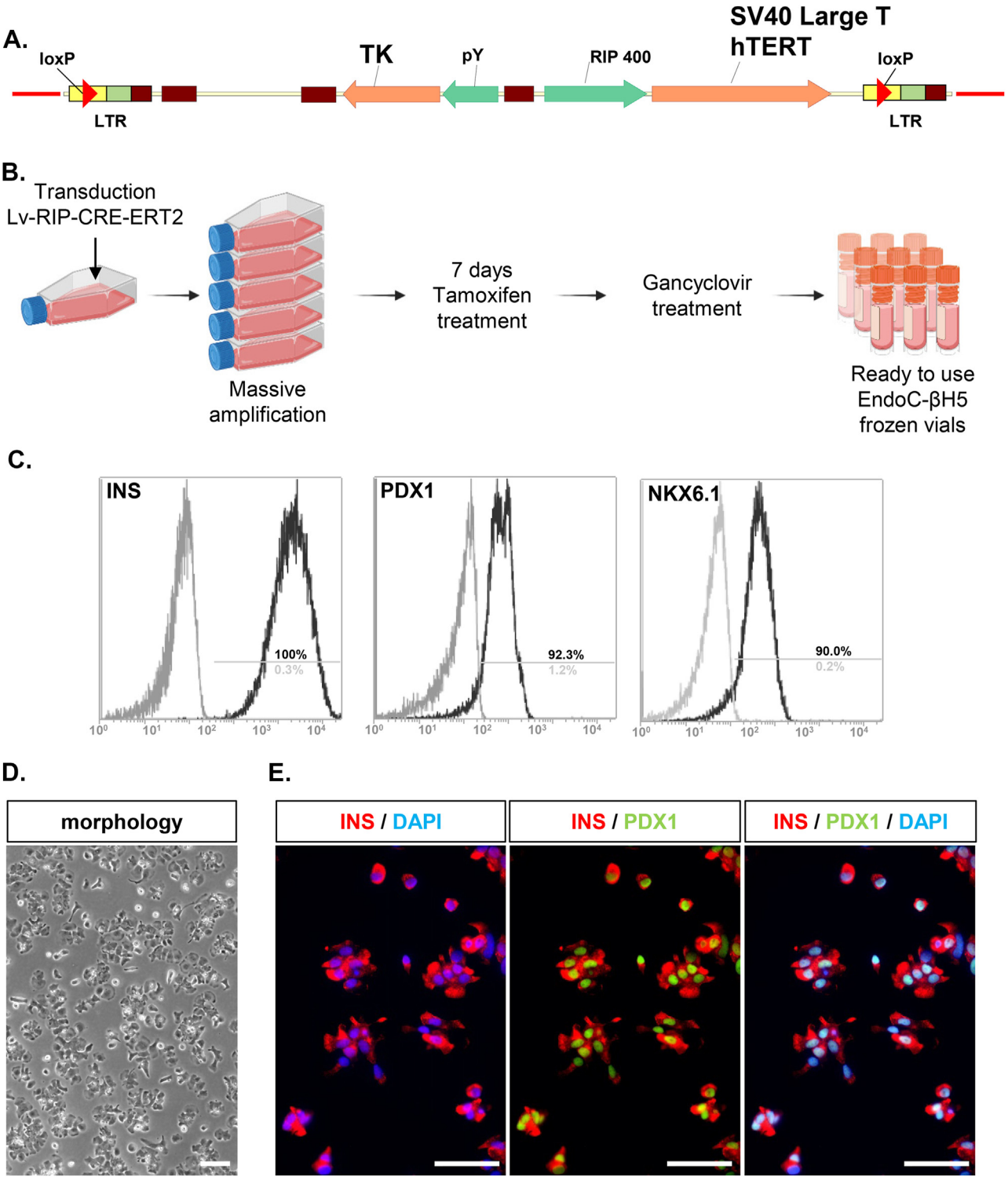

Figure 1: Production of ready to use EndoC-βH5 cells and characterization by immuno-labeling. (**A**) Schematic representation of integrated lentiviral vector used to produce EndoC-βH5 cells. Upon integration the loxP site located into the deleted U3 3′ LTR region is duplicated. The entire integrated sequences are flanked with loxP sites thus allowing CRE mediated excision. The integrated sequence contains a first transcription unit expressing SV40 LT or hTERT under the control of the rat insulin promoter (RIP) and a second unit expressing HSV1 TK under the control of the polyomavirus enhancer (pY). (**B**) The immortalized cell line is transduced with a lentiviral vector expressing a tamoxifen inducible form of CRE (CRE-ERT2) expressed under the control of RIP. After massive amplification, cells are treated for 7 days with 1 μM tamoxifen followed by 1 day of ganciclovir to allow SV40LT and hTERT excision and destruction of non-excised cells that expressed TK. The resulting EndoC-βH5 cells are frozen to produce ready to use vials. Panels A and B were created with BioRender.com. (**C**) FACS analysis profile (black curves) of insulin, PDX1 and NKX6.1 expression in EndoC-βH5 cells. The grey curve represents the isotype control. (**D**) Phase contrast photograph of ready to use EndoC-βH5 thawed cells. (**E**) Insulin (red), PDX1 (green) and nuclei (DAPI, blue) immunofluorescent staining of EndoC-βH5 cells. Scale bar = 50 μm.

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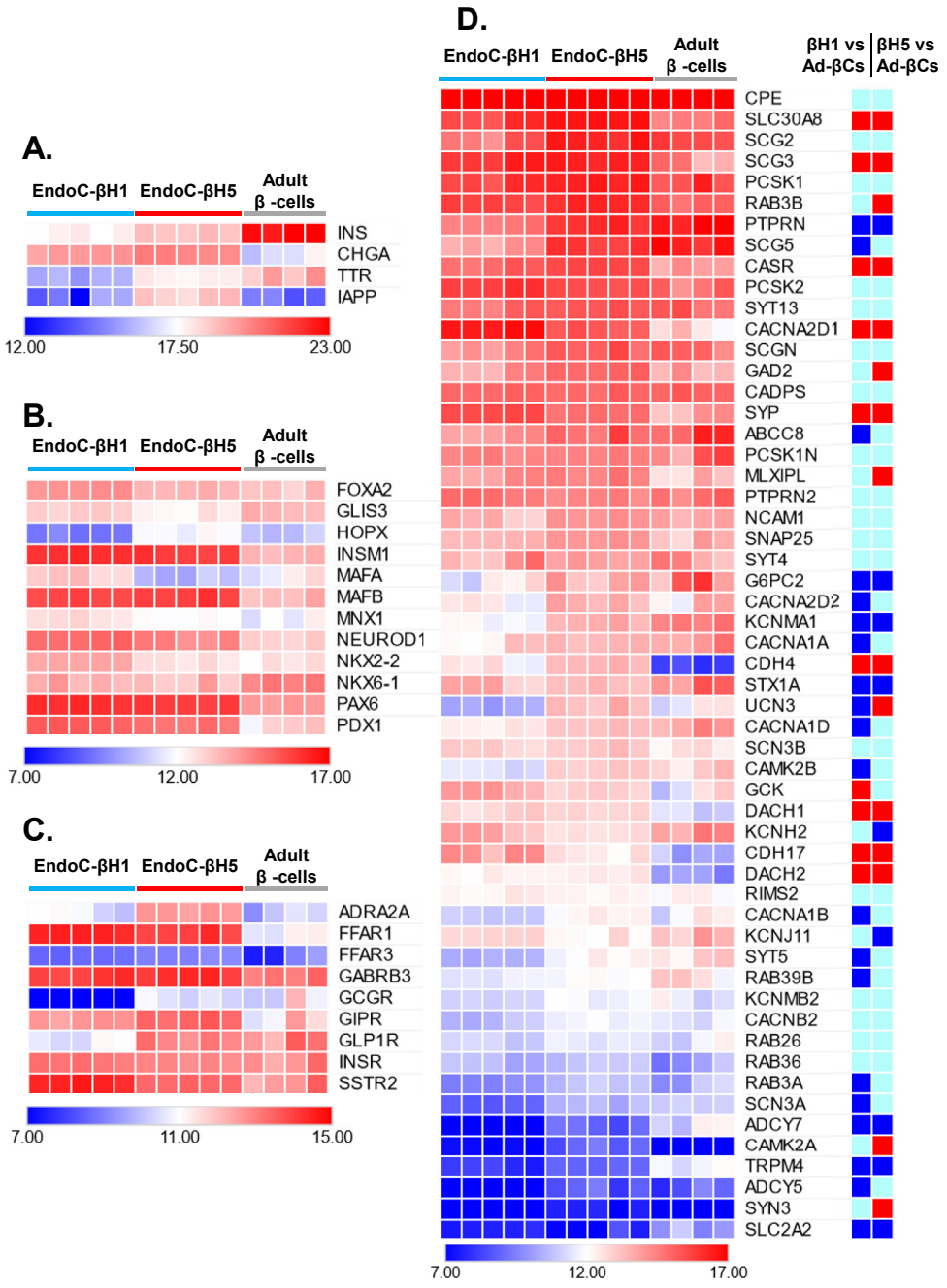

Figure 2: Heat map of gene expression profiling in EndoC-βH5, EndoC-βH1 and FACS purified human adult beta cells. The heat maps show the variance stabilizing transformation (VST) of the count data sets normalized to library size of 5 independent EndoC-βH5 and EndoC-βH1 cell preparations and 4 publicly available data sets of FACS purified human adult beta cells (adult β cells). (**A**) INS, CHGA, TTR and IAPP represent the top four non-mitochondrial transcripts expressed in EndoC-βH5 cells, ranking 1st, 7th, 14th and 18th respectively. (**B**) List of transcription factors essential for beta cell development and identity. (**C**) List of receptors regulating insulin secretion in beta cells. (**D**) Gene lists of transcripts important for beta cell function, generated using published transcriptomics data, literature data, and manual curation. Genes do not overlap with the 3 lists presented in **A**, **B** and **C**. Left panel, heat maps of expression (VST) in EndoC-βH1, EndoC-βH5 and adult β cells. Right panel, differential expression between EndoC-βH1 and adult β cells and EndoC-βH5 and adult β cells. Significant changes corresponding to a fold change >2 and an FDR <0.05 are represented in blue and red for reduced or increased expression respectively. Light green squares represent non-significant changes.

excision upon CRE-ERT2 activation. The cell population that escaped CRE dependent excision expressed TK and were then depleted form the culture following one day of ganciclovir treatment The resulting cells, named ready-to-use EndoC-βH5 cells, were frozen and stored for extensive period of time (Figure 1B).

3.2. Flow cytometry and immunofluorescence analyses

Flow cytometry analyses indicated that all EndoC-βH5 cells were positive for insulin. PDX1 and NK6.1 expression was detected in a vast majority of the cells (92.3% and 90% respectively) (Figure 1C). EndoC-βH5 cells formed small adherent clusters on coated plastic (Figure 1D).

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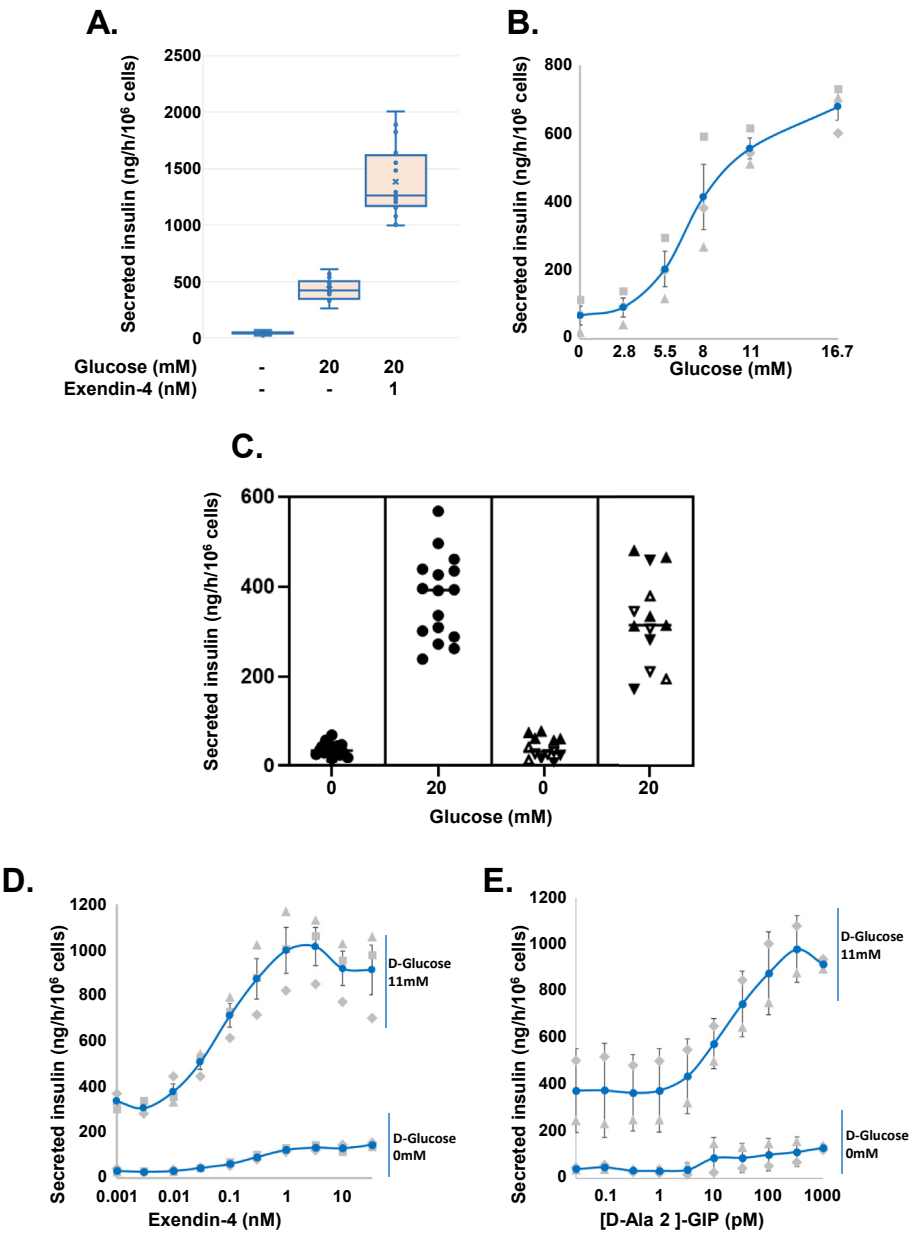

Figure 3: Robust and highly reproducible glucose and GLP-1/GIP receptor agonist stimulated insulin secretion by EndoC-βH5 cells, Box-plot (A) and graphs (B, C, D) showing static insulin secretion by EndoC-βH5 cells subjected to various stimuli and expressed as ng insulin that is secreted per h and per million cells (ng/h/10^6 cells). **(A)** Box-plot of GSIS results from 16 independent EndoC-βH5 batches. Cells were stimulated with D-Glucose (20 mM) or D-Glucose (20 mM) plus exendin-4 (1 nM). **(B)** D-glucose dose response (2.8—16.7 mM). Graph shows results of 3 independent experiments (grey symbols) and average (blue circles and curve). **(C)** Plot of individual GSIS results (LG, 0 mM; HG, 20 mM) obtained at HCD (left, circles, 16 independent experiments) and in four European laboratories (right, triangles, 13 independent experiments). Upward/downward and solid/empty triangles correspond each of the four separate laboratories. **(D)** Exendin-4 dose response (0.001—33 nM) in presence (top) or absence (bottom) of D-Glucose (11 mM). Graph shows results from 3 independent experiments (grey symbols) and average (blue circles and curve). Bottom experiment (no D-Glucose) was repeated twice. **(E)** [D-Ala^2]-GIP dose response (0.033 pM to 1 nM) in presence (top) or absence (bottom) of D-Glucose (11 mM). Graph shows results from 3 independent experiments (grey symbols) and average (blue circles and curve).

By immunofluorescence, all EndoC-βH5 cells expressed insulin and PDX1 (Figure 1E), in keeping with the flow cytometry analyses.

3.3. EndoC-βH5 transcriptome profile

PolyA + RNA from 5 independent batches of EndoC-βH5 and EndoC-βH1 cells were processed to generate RNA-seq data that were compared to publicly available data from FACS purified human primary adult beta cells (referred to as adult β cells, ArrayExpress Archive under accession number E-MTAB-1294) [13]. Insulin was the most abundant transcript in the three cell groups (Figure 2A). In addition to insulin, chromogranin-A (CHGA), transthyretin (TTR) and islet amyloid polypeptide (IAPP) were amongst the most expressed transcripts ranking 7th, 14th and 18th respectively in EndoC-βH5 cells. In EndoC-βH1 and -βH5 cells, markers of acinar lineages such as amylase 1 A and 1 B (AMY1A, AMY1B) and chymotrypsinogen B1 (CTRB1) as well as markers of ductal and mesenchymal cells such as cystic fibrosis

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transmembrane conductance regulator (CFTR) and vimentin (VIM) were not or very lowly expressed (tpm <0.1) (Supplementary Figure. 1A). In contrast, in adult β cells, CTRB1, CFTR and VIM were expressed at 1.5, 1.3 and 430 tpm respectively (Supplementary Figure. 1A). In EndoC-βH5 cells, expression of somatostatin (SST) was observed (Supplementary Figure. 1A). This was consistent with rare (0.89% +/− 0.06) SST positive cells detected by immunostaining (Supplementary Figure. 2). Only rare glucagon (GCG) transcripts were detected in EndoC-βH5 cells (Supplementary Figure. 1A), but no GCG protein was observed by immunostaining (not shown). Finally, no expression of pancreatic polypeptide (PP) was detected.

Expression of all transcription factors important for beta cell identity and function [14] was detected in EndoC-βH5 cells. Their expression levels were either similar (FOXA2, MNX1, NKX2.2) or higher (HOPX, MAFB, NEUROD1, PAX6, PDX1) in EndoC-βH5 cells compared to adult β cells (Figure 2B). Only GLIS3, NKX6.1 and MAFA were expressed at lower levels in EndoC-βH5 cells (17, 124 and 61 compared to 44, 230 and 119 tpm in adult β cells).

Receptors linked to insulin secretion were expressed in EndoC-βH5 cells, including receptors for adrenalin (ADRA2A), fatty acids (FFAR1, FFAR3), GABA (GABRB3), glucagon (GCGR), somatostatin (SSTR2) and incretins, such as glucagon like peptide-1 (GLP1R) and gastric inhibitory polypeptide (GIPR). Importantly, GLP1R, GIPR and GCGR were more abundant in EndoC-βH5 than in EndoC-βH1 cells (Figure 2C).

We next focused on 54 transcripts encoding proteins important for beta cell function (Figure 2D) [5,14,15]. Differential expression analysis, using a cut-off of fold change >2 and a corrected p-value (FDR) < 0.05, revealed that only 9 out of these 54 genes are down-regulated in EndoC-βH5 cells compared to adult β cells, whereas 30 and 15 are, respectively, not or up-regulated (Figure 2D). When performing the same comparison between EndoC-βH1 and adult β cells, 20 of the 54 genes were down-regulated in EndoC-βH1 compared to adult β cells and 24 and 10 were not or up-regulated (Figure 2D).

Differential expression analysis between EndoC-βH5 and EndoC-βH1 using the same cut-off as above indicated that down-regulated transcripts in EndoC-βH5 are enriched in KEGG pathway terms related to cell cycle and DNA replication (Supplementary Table 1).

Finally, we evaluated the expression of 8 genes that were recently reported to be islet and beta cell disallowed genes in an article describing putative roles, mechanisms of repression and evidence of increased expression in type 2 diabetes [16]. As expected for disallowed genes, IGFBP4, YAP1, MGLL, PDGFRA, HSD11B1 and SMOC2 were not or very lowly expressed (tpm<0.1) in EndoC-βH5 cells (Supplementary Figure. 1B). Only lactate dehydrogenase A (LDHA) and monocarboxylate transporter 1 (SLC16A1) were expressed in EndoC-βH5 (77 and 8.4 tpm respectively), but at levels that were 4.4-fold (FDR $= 3.2\ 10^{-17}$) and 16.4-fold (FDR $= 1.7\ 10^{-30}$) lower than in EndoC-βH1 cells (Supplementary Figure. 1B).

3.4. Insulin secretion in response to glucose

EndoC-βH5 cells from 16 independent cell preparations were thawed and seeded in order to estimate their capacity to secrete insulin upon

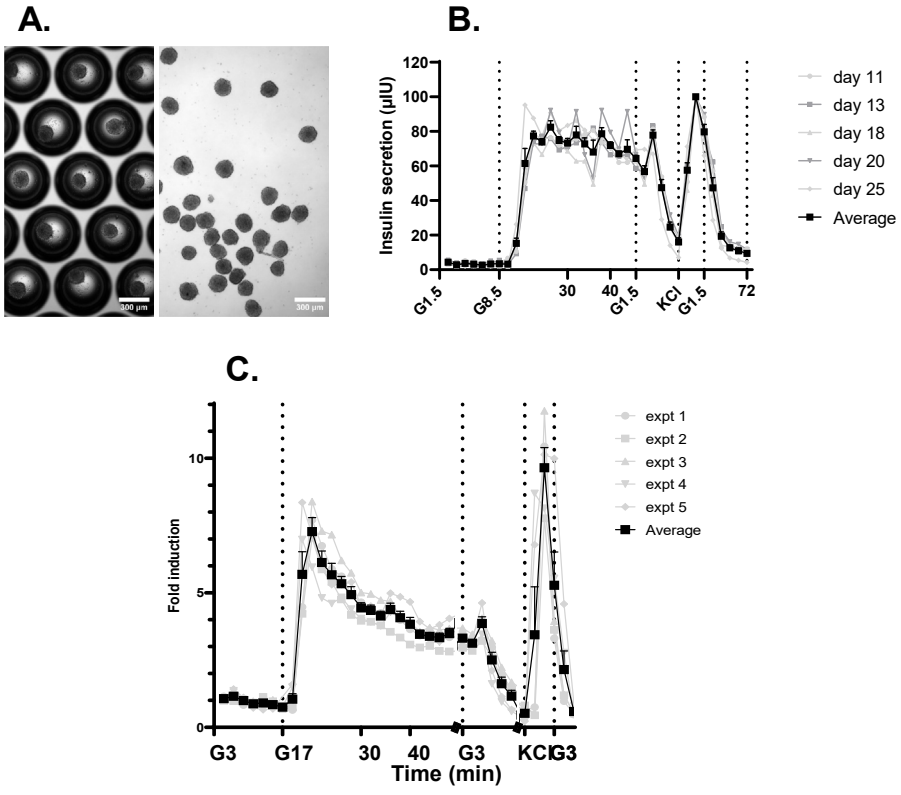

Figure 4: Dynamic insulin secretion by EndoC-βH5 spheres. (**A**) Morphology of EndoC-βH5 spheres. (**B**) Dynamic insulin secretion by EndoC-βH5 spheres in a perifusion setting in presence of 1.5 mM glucose (G1.5), 8.5 mM glucose (G8.5) or 40 mM KCl (KCl). Five experiments were performed, using two independent preparations of spheres at three (day 11, 18 and 25) and two (day 13 and 20) time points. Insulin secretion is expressed as ng insulin secreted per time-point (2-minute) and per million cells (ng/10^6 cells). (**C**) Dynamic insulin secretion by EndoC-βH5 spheres maintained in 0.5 mM glucose showing initial first-phase peak. Spheres were stimulated with 3 mM glucose (G3), 17 mM glucose (G17) or 40 mM KCl (KCl). In two of the experiments, G17 and second G3 incubation duration were longer. This additional time is not shown (broken X axis) Five experiments were performed, using two independent preparations of spheres. Insulin release is expressed as fold induction relative to the first G3 point.

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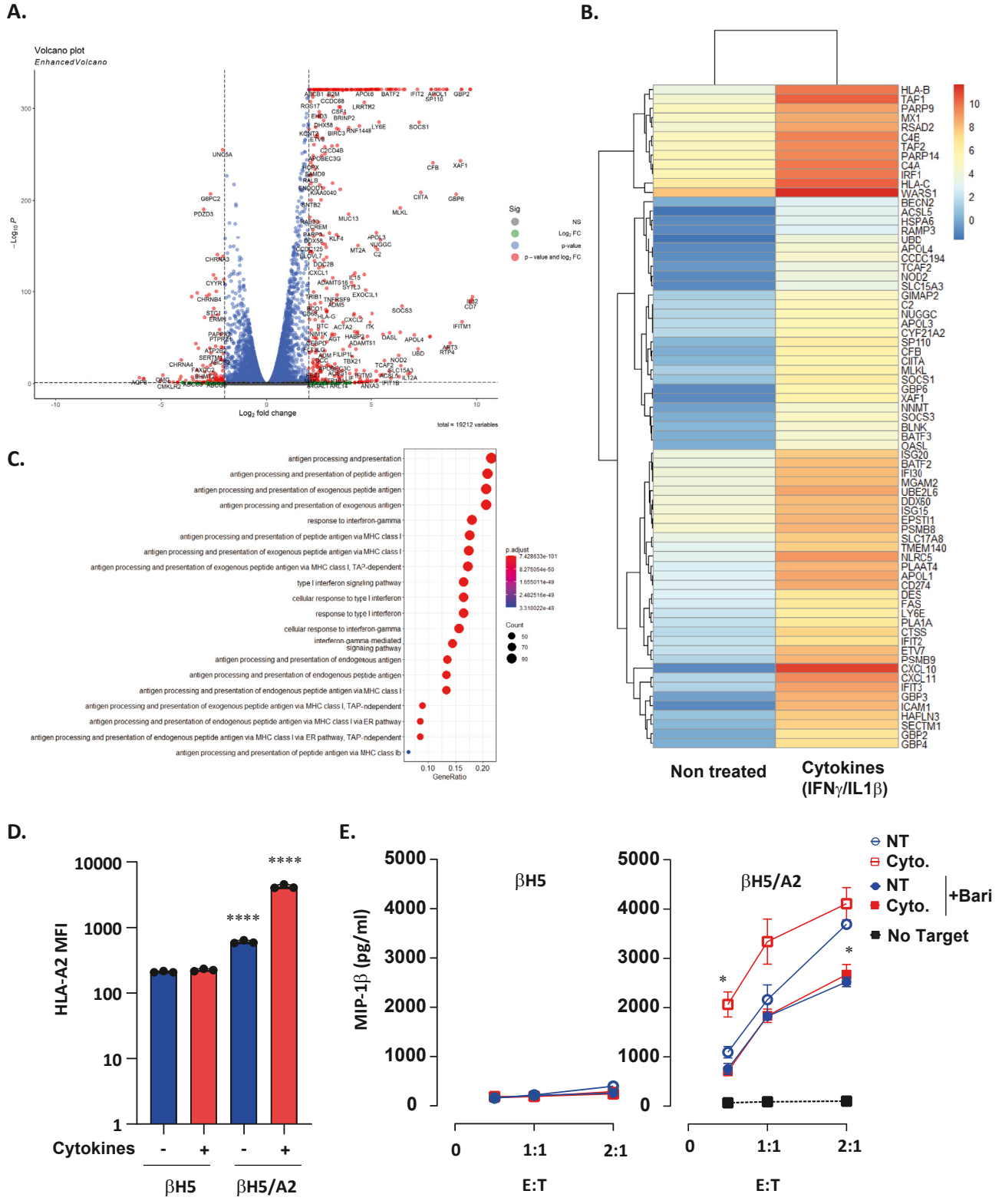

Figure 5: EndoC-βH5 are responsive to cytokines and EndoC-βH5/A2 is applicable in T cell reactivity assays. **(A)** Volcano plot of protein coding transcripts expression detected in RNA-seq of EndoC-βH5 cells treated with IFNγ/IL1β for 24 h (n = 3). The Volcano plot was generated using EnhancedVolcano. **(B)** Dot plot of enriched gene ontology terms within the group of transcripts upregulated by cytokine treatment (logFC>2, FDR<0.05). The dot plot was generated using clusterProfiler. **(C)** Heatmap of the most upregulated transcripts (logFC>5, FDR< 0.05). Read counts were averaged over the triplicates and only transcripts with an average > 3 are depicted. The heatmap was generated using Pheatmap. **(D)** HLA-A2 expression in EndoC-βH5 and EndoC-βH5/HLA-A2 cells in normal or IFNγ/IL1β treated condition. Data are presented as mean fluorescent intensity (MFI). **(E)** MIP-1β secretion of allo-HLA-A2 reactive CTLs after overnight coculture with EndoC-βH5 (left panel) or EndoC-βH5/HLA-A2 (right panel). Target cells were untreated or treated with IFNγ/IL1β with or without 4 μM baricitinib (Bari) for 24 h. N = 3 independent experiments. N.B. In this figure, cytokines means IFNγ and IL1β.

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glucose stimulation and the reproducibility of their response. Basal (0 mM D-glucose) insulin secretion was 42.8 ± 3.4 ng/h/10^6 cells (average secretion ± SEM) whereas 20 mM D-glucose stimulated insulin secretion was 431.7 ± 23.9 ng/h/10^6 cells (Figure 3A). Total insulin content was 4.5 ± 0.4 μg insulin per million cells (n = 16 experiments). When expressing secretion as percentage of insulin content, EndoC-βH5 cells secreted 0.7 ± 0.1% of insulin content in 40 min under basal condition and 6.8 ± 0.8% under 20 mM D-glucose (Supplementary Figure. 3). The stimulation index between basal and high D-glucose conditions was 11.6 ± 1.8. Specificity of insulin secretion in response to D-glucose was verified by applying L-glucose (20 mM) that did not elicit insulin secretion (Supplementary Figure. 4A) or the potassium channel opener diazoxide (100 μM) that completely blocked D-glucose induced insulin secretion (Supplementary Figure. 4B).

Insulin secretion increased in a D-glucose concentration dependent manner from 68.8 ± 27.3 at 0 mM D-glucose to 679.2 ± 39.5 ng/h/10^6 cells at 16.7 mM D-glucose (Figure 3B). The steepest increase in insulin secretion was between 5.5 and 8 mM D-glucose with 204.7 ± 51.3 and 414.9 ± 94.8 ng/h/10^6 cells respectively.

3.5. Reproducibility of glucose stimulated insulin secretion by EndoC-βH5 cells across laboratories

To address the question of reproducibility across laboratories, glucose stimulated insulin secretion experiments were performed by collaborating laboratories. Fourteen GSIS experiments were performed by four laboratories using various batches of EndoC-βH5 cells. Insulin secretion at 0 or 20 mM D-glucose was, respectively, 42.8 ± 3.4 and 431.7 ± 23.9 ng/h/10^6 cells at HCD and 33.2 ± 4.5 and 351.5 ± 41.8 ng/h/10^6 cells in the other laboratories with respective stimulation index of 11.6 ± 1.8 and 10.5 ± 1.6 (Figure 3C). There was no difference between HCD and the other laboratories in terms of basal and stimulated insulin secretion as well as fold induction.

3.6. Insulin secretion in response to GLP1R/GIPR agonists

GLP1R and GIPR mediated signalling potentiated glucose stimulated insulin secretion by EndoC-βH5 cells. In 16 independent experiments, stimulation index between 20 mM D-glucose and 20 mM D-glucose plus 1 nM GLP1R agonist exendin-4 was 3.2 ± 0.1. Insulin secretion increased from 431.7 ± 23.9 to 1382.8 ± 78.7 ng/h/10^6 cells (Figure 3A); the percentage of secreted insulin went from 6.8 ± 0.8% to 18.3 ± 1.6% (Supplementary Figure. 3).

In dose response experiments, insulin secretion increased from 338.6 ± 19.6 to 1014.6 ± 84.4 ng/h/10^6 cells in cells treated with 1 pM and 3.3 nM exendin-4, in presence of 11 mM D-glucose, respectively (Figure 3D). Insulin secretion also increased from 381.9 ± 38.1 to 996.0 ± 90.6 ng/h/10^6 cells in cells treated with 0.03 and 330 pM GIPR agonist D-[Ala2]-GIP, in presence of 11 mM D-glucose (Figure 3E). Stimulation index between low and high concentrations was 3.5 ± 0.3-fold for exendin-4 and 3.1 ± 0.4-fold for D-[Ala2]-GIP. Exendin-4 and D-[Ala2]-GIP elicited only minor insulin secretion (33.8 ± 12.0 to 146.7 ± 14.5 ng/h/10^6 cells and 38.1 ± 6.3 to 106.1 ± 46.1 ng/h/10^6 cells respectively) in the absence of D-glucose (0 mM, Figure 3D,E).

Importantly, in a set of experiments using three separate batches of EndoC-βH5 cells, insulin secretion in response to D-glucose and exendin-4 was maintained over a 4-week culture period. D-glucose (20 mM) stimulated insulin secretion was 514.6 ± 44.7 and 503.7 ± 68.7 ng/h/10^6 cells and D-glucose/exendin-4 (1 nM) stimulated secretion was 1365.7 ± 133.5 and 1556.1 ± 138.9 ng/h/10^6 cells, one and four weeks after seeding, respectively. The percentage of cells that survived was 86.3% ± 2.6% and 72.3% ± 4.7% after one and four weeks, respectively.

The specificity of GLP1R mediated potentiation of GSIS was verified using GLP1R antagonist exendin-9. Exendin-9 dose dependently blocked exendin-4 potentiation of GSIS (Supplementary Figure. 5).

3.7. Dynamic glucose stimulated insulin secretion of EndoC-βH5 cells

EndoC-βH5 cells were clustered and formed spheres that were homogenous in size and shape (Figure 4A). Insulin secretion experiments were then performed in a perifusion setting. Five groups of 11- to 25-day-old EndoC-βH5 spheres, originating from two independent sphere productions, gave rise to highly reproducible and dynamic insulin secretions in response to glucose. Mean basal low glucose (1.5 mM) secretion was 8.8 ± 0.5 ng/h/10^6 cells whereas mean high glucose (8.5 mM) secretion was 159.3 ± 8.4 ng/h/10^6 cells (Figure 4B). Stimulation index between low and high glucose concentrations was thus 18.1. Upon glucose washout, insulin secretion rapidly decreased to basal level whereas KCl further induced it.

One of the characteristics of healthy mature human pancreatic beta cells is the biphasic shape of their glucose induced insulin secretion, with marked initial peak of secretion [17,18]. In the experiments presented above, cells were cultured in 5.5 mM glucose and did not show such a biphasic response. In contrast, biphasic insulin secretion was observed in EndoC-bH5 cells maintained in 0.5 mM glucose (Figure 4C).

3.8. EndoC-βH5 cells expressing HLA.A2: a tool to study interaction between β cells and CD8+ T cells

Responsiveness of EndoC-βH5 cells to cytokines was examined by RNA-seq of cells treated with IFNγ and IL1β for 24 h. The treatment resulted in profound changes in their transcriptome and upregulation of genes involved in the inflammatory pathway (e.g. STAT1, IFIT1, IFIT3) and antigen processing and presentation (e.g. PSMB8, PSMB9, b2m, TAP1, TAP2, HLA-A/B/C) (Figure 5A–C), similar to previous studies on EndoC-βH1 cells and adult human islets [19–21]. EndoC-βH5 cells hence represent a powerful tool to investigate the dialogue between beta cells and the immune system. As an illustration, we performed co-culture assays with EndoC-βH5, or its HLA-A2 positive derivative, with allo-HLA-A2 reactive T-cells and determined T cell activation status by measuring MIP1β secretion in presence or absence of proinflammatory cytokines (e.g. IFNγ/IL1β). HLA-A2 expression in the EndoC-βH5-HLA-A2 cells was amplified upon exposure to cytokines (Figure 5D) and correlated with a strong HLA A2 specific T-cell response following coculture with A2 alloreactive CTLs (Figure 5E,F). In these assays, we tested the effect of the JAK/STAT inhibitor baricitinib [22] that is clinically approved for rheumatoid arthritis and has been shown to prevent IFN-induced HLA class I expression in adult human islets [23]. We found that baricitinib abrogates the deleterious effect of IFNγ/IL1β on T-cell activation by HLA-A2 expressing EndoC-βH5 cells (Figure 5E). Altogether, these results suggest that these cells can be used not only for testing T-cell reactivity, but also for investigating antigen presentation pathways and modulating agents.

4. DISCUSSION

4.1. Summary of main results

The number of diabetes patients is continuously growing, underlining the need for physiologically relevant and easily accessible human pancreatic beta cell models that can accelerate the understanding of disease mechanisms and development of new drugs. Here, we show that

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EndoC-βH5 cells, the most recent generation among the EndoC-βH human pancreatic beta cell family [3—5], bears great potential for diabetes research. EndoC-βH5 cells are directly available as frozen validated functional human beta cells that can thus be used at any time. They display strong standardization and batch to batch reproducibility allowing for confident data interpretation. EndoC-βH5 cells performed well in all functional assays tested to date. The cells secreted insulin in response to glucose in a dose dependent manner and glucose stimulated insulin secretion was further potentiated by incretins. Finally, modified HLA-A2 expressing EndoC-βH5 cells elicited alloreactive T lymphocyte activation recapitulating some of the auto-immune mechanisms involved in type 1 diabetes. Of note, EndoC-βH5 cells have also been used in recently published articles as models to study type 1 diabetes related sensitivity to pro-inflammatory cytokine and β cell senescence mechanisms as well as to identify modulators of insulin secretion in a genomic screen [24—26].

4.2. dose dependent glucose responsiveness

The main feature of pancreatic beta cells is their ability to secrete insulin in response to glucose, in a dose-dependent manner and following a sigmoidal shape. The role of this sigmoidal response to glucose is to efficiently detect increasing blood glucose levels and maintain it in a narrow range of concentrations (3—9 mM) [27]. Response to physiological glucose concentrations is made possible by rate-limiting glucokinase (GCK) activity, while upstream glucose transport, downstream ATP dependent potassium channel closure and alternative metabolic amplifying pathway are not limiting in the early response to increased glucose levels [28,29]. EndoC-βH5 cells show exclusive expression of GCK but no other HK genes (not shown), a landmark of human beta cells. The cells recapitulate sigmoidal dose response to glucose with an inflexion point between 3 and 4 mM glucose, similar to what is described for primary human beta cells, and maximum increase in insulin secretion between 5 and 8 mM glucose, in the range of physiological glucose concentrations [30].

Insulin content in EndoC-βH5 cells is 4.5 $\pm$ 0.4 μg/10^6 cells, which is two to three times below that of primary human islets [31,32]. Absolute values of insulin secretion in EndoC-βH5 cells at zero and high glucose and the stimulation index are similar to those described in primary human islets [30,33,34]. When expressed as percentage of insulin content, insulin secretion in EndoC-βH5 appears to be $>$ 2-fold higher (10% per h) when compared to published data on perifused human islets (2—4% per h) [32], possibly reflecting the absence of paracrine regulation of insulin secretion in EndoC-βH5 cells [35]. Perifusion experiments demonstrated that EndoC-βH5 cells very dynamically respond to glucose, and when maintained in low glucose, show a biphasic response with a prominent first phase peak, similar to what is expected from physiological human pancreatic beta cells. Overall, these results confirmed elevated insulin secretion stimulation index of EndoC-βH5 cells in an independent setting.

Importantly, similar 2D static GSIS responses were also obtained between different laboratories demonstrating the reproducibility of the model in terms of functional activity. In addition, stimulation index which varies a lot from poorly to highly responsive preparations in primary human islets [30,33,34], are very reproducible in EndoC-βH5, guarantying optimal and reproducible assay resolution.

Overall, EndoC-βH5 response to glucose appears to be physiological. They respond to physiological concentrations of glucose and present highly dynamic range of insulin secretion and marked initial peak of secretion. In order to further characterize EndoC-βH5 cell maturity, however, it would be interesting to investigate response to other stimuli. An interesting example is the ability of human pancreatic beta cells to respond to amino acids (AA). Even though several reports have shown that adult islet human respond to elevated AA concentrations [17,18], a recent report by Helman et al. suggests that low physiological AA concentrations may discriminate non-responsive adult mature from immature human pancreatic beta cells [36]. Also, culture in specific AA concentrations may control the emergence of this mature phenotype. It would thus be interesting to assess response of EndoC-βH5 cells to culture and stimulation with AA in future works. Finally, in comparison to EndoC-βH5 cells which present highly dynamic range and reproducible secretion between laboratories, broadly used EndoC-βH1 cells show more modest and variable insulin secretion responses with 2.8 $\pm$ 0.3 stimulation index when compiling results from six published articles that provide secretion values, and an estimated 4.1 $\pm$ 0.6 stimulation index when estimating graph results from 26 published articles, with stimulation index values ranging from 1.7 to 12.4 (Supplementary Table 2).

4.3. GLP1R and GIPR stimulated insulin secretion

Insulin secretion is modulated by various secretagogues among which the incretin hormones GLP-1 and GIP that are secreted by the gut in response to food intake. GLP-1 and GIP act as potentiators of glucose induced insulin secretion through their cognate G protein coupled receptors located at the beta cell surface, activation of Gα and adenylate cyclase and increased cAMP production [37,38]. There are currently no easily accessible human cell models to study insulin secretion modulation by incretin receptor agonists and recently developed GLP1R/GIPR dual agonists. In vitro, human islets stimulated with glucose increase insulin secretion 2 to 3 times upon incretin exposure [39,40]. Reproducibility is limited due to the variable quality of islet preparations [41,42]. EndoC-βH5 cells, which show increased expression of incretin receptors compared to previous EndoC-βH versions, display very reproducible responses to GLP1R and GIPR signalling. GLP-1 and GIP analogues exendin-4 and D-ALA2-GIP potentiate glucose stimulated insulin secretion in a dose dependent manner with 2 to 3-fold stimulatory indexes, similar to primary cells. The GIP analogue D-ALA2-GIP acts at lower concentrations than exendin-4 in Endoc-βH5 cells, similar to what has been reported in other models, in particular human receptor expressing rodent cells [37,43—45]. In comparison, there is, to our knowledge, no report of GIP receptor agonist response in EndoC-βH1 cells whereas GLP-1 receptor agonist response is very limited, in line with significantly reduced expression of GLP-1R in EndoC-βH1 cells compared to EndoC-βH5 cells.

Overall, EndoC-βH5 cells represent an easy-to-use and highly reproducible model for the study of incretin receptor signalling and insulin secretion modulation in human beta cells.

4.4. Expression of functional pancreatic beta cell markers in EndoC-βH5 cells

RNA-seq analysis showed elevated expression of major beta cell transcription factors and functional markers and absent or low expression of markers of non-beta exocrine as well as acinar, ductal and mesenchymal lineages, demonstrating the homogenous beta cell identity of EndoC-βH5 cells. Differential analyses of beta cell markers showed greater proximity of EndoC-βH5 cells to primary human beta cells than that of EndoC-βH1 cells. This is particularly true for incretin receptors and corroborates the functional response to incretins. On top of functional expression of GLP1R and GIPR, EndoC-βH5 cells express the glucagon receptor, recently recognized as an important regulator of insulin secretion [46,47]. Expression of GLP1R and GCGR in EndoC-βH5 cells is in the order of magnitude of that of primary human beta cells while they are less expressed (GLP1R) or barely detected (GCGR) in

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EndoC-βH1 cells. GIPR is also abundant in EndoC-βH5 cells. This identifies EndoC-βH5 cells as a unique human cellular model to study recently developed dual and triple GLP-1/GIP/GCG receptor agonists [48,49].

4.5. Type 1 diabetes modeling

Our data also demonstrated that HLA-A2 expressing EndoC-βH5 represent a valuable model for immunological studies and a potential screening platform to repurpose anti-inflammatory drugs or for discovery of inhibitors of class I antigen peptide processing and presentation with a cytokine response similar to primary human islets [20,21]. The coculture of HLA-A2 alloreactive T-cells with EndoC-βH5 cells showed a strong HLA-A2 specific response, amplified by IFNγ/IL1β stimulation. We observed that baricitinib prevented the IFNγ stimulation of T cell activation. This effect was also observed in non-IFNγ-treated conditions, possibly reflecting a suppression of IFNγ endogenously produced by activated T cells during coculture.

5. CONCLUSION

We have developed a fully functional, close to physiology human pancreatic beta cell model with highly dynamic glucose stimulated insulin secretion and modulation by incretins. We demonstrate high reproducibility of the model, both between batches and between laboratories. Finally, storability and ready to use format allow for anticipation and precise experimental planning and should result in significative gain of time when developing projects. Overall, EndoC-βH5 cells bear great potential to accelerate diabetes research.

AUTHOR CONTRIBUTIONS

MT, AP, CC, MP and AB performed cell culture, insulin secretion, immunofluorescence and flow cytometry experiments. CC performed EndoC-βH5 derivation experiments. ST performed cytokine treatment and T lymphocyte activation experiments under AZ supervision. CH and TC performed perifusion experiments under OJ and ALG supervision. FF, TS, SG, JSB and FWJB performed insulin secretion experiments under FC, MC, DLE, MF, HM and ME supervision. BB and PR designed the experiments, analysed the data and wrote the manuscript. RS contributed to the scientific discussion and reviewed the manuscript. PR and RS conceptualized the EndoC-βH5 cells. All authors contributed to the article and approved the submitted version.

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DECLARATION OF COMPETING INTEREST

BB, MT, AP, CC, MP, AB and HO are or were employees at Human Cell Design SA, France, the company that commercializes EndoC-βH1 and EndoC-βh5 cells and associated media. RS, PC and PR are shareholders at HCD.

DATA AVAILABILITY

RNAseq data have been deposited in GEO. Accession number GSE224732

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Ready to use EndoC-βH5 and HLA-A2 EndoC-βH5 vials are commercially available from Human Cell Design (https://www.humancelldesign.com/).

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101772.

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