The first unbiased human β cell line secretome

1 Graphical abstract

3 Highlights

- We validate EndoC- β H1 as a translational human β cell model using omics.
- We present the first unbiased proteomics composition of human β cell line secretome.
- The secretome of human β cells is more extensive than previously thought.
- Untreated cells secreted 671 proteins and stressed cells secreted 3,278 proteins.
- Secretion of exosomes and bioactive peptides constitute directions of future research.

The first unbiased human β cell line secretome

Characterization of the secretome, transcriptome and proteome of human β cell line EndoC- β H1

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37 **Abbreviations**

38 ACN – acetonitrile

39 DIA – Data Independent Acquisition

40 FA – formic acid

41 FACS – Fluorescence-activated cell sorting

42 FDR – False Discovery Rate

43 GLP1R – glucagon like peptide 1 receptor

44 GO – Gene Ontology

45 GPCR – G-protein coupled receptor

46 HCD – Higher-energy C-trap dissociation

47 HPRP Fractionation – High pH Reversed-Phase Fractionation

48 LC – liquid chromatography

49 MS/MS – tandem mass spectrometry

50 PBS – Phosphate Buffered Saline

51 SDB-RPS – styrene divinylbenzene reversed-phase

52 T2D – Type 2 Diabetes

53 TMT – Tandem Mass Tag

54 TPM – transcripts per million

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ABSTRACT

Early diabetes research is hampered by limited availability, variable quality and instability of human pancreatic islets in culture. Little is known about the human β cell secretome, and recent studies question translatability of rodent β cell secretory profiles. Here, we verify representativeness of EndoC- β H1, one of the most widely used human β cell lines, as a translational human β cell model based on omics and characterize the EndoC- β H1 secretome. We profiled EndoC- β H1 cells using RNA-seq, Data Independent Acquisition (DIA) and Tandem Mass Tag proteomics of cell lysate. Omics profiles of EndoC- β H1 cells were compared to human β cells and insulinomas. Secretome composition was assessed by DIA proteomics. Agreement between EndoC- β H1 cells and primary adult human β cells was ~90% for global omics profiles as well as for β cell markers, transcription factors and enzymes. Discrepancies in expression were due to elevated proliferation rate of EndoC- β H1 cells compared to adult β cells. Consistently, similarity was slightly higher with benign non-metastatic insulinomas. EndoC- β H1 secreted 671 proteins in untreated baseline state and 3,278 proteins when stressed with non-targeting control siRNA, including known β cell hormones INS, IAPP, and IGF2. Further, EndoC- β H1 secreted proteins known to generate bioactive peptides such as granins and enzymes required for production of bioactive peptides. Unexpectedly, exosomes appeared to be a major mode of secretion in EndoC- β H1 cells. We believe that secretion of exosomes and bioactive peptides warrant further investigation with specialized proteomics workflows in future studies.

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83 INTRODUCTION

84 Close to half a billion people are living with diabetes worldwide and this number is expected to increase
85 by 25% in 2030 (1). Despite diabetes being a global health burden, the mechanisms underlying normal
86 β cell function and failure are not yet fully elucidated. This is partly due to limited availability of high
87 quality human pancreatic islets for research purposes combined with variable quality and variation in
88 relative cell type composition of islet preparations (2, 3). Moreover, primary human islets rapidly loose
89 functional expression pattern in culture (4). To overcome these limitations, pancreatic islets from
90 rodents have been used to study β cell biology for decades leading to significant advances in our
91 understanding of the disrupted pathways causing impaired β cell function. However, the cellular
92 architecture and secretory profile of rodent islets is substantially different from that of human islets (5,
93 6), manifesting the need for human β cell lines, which mirror the expression and secretory pattern of
94 primary human β cells.

95 Several human β cell lines are currently available - 1.1B4, 1.4E7 and 1.1E7 (7), T6PNE (8), EndoC-
96 β H1 (9), EndoC- β H2 (10), EndoC- β H3 (11) and ECN90 (12). The corresponding full text searches for
97 these cells lines in Google Scholar retrieved 774 (22.5%), 993 (28.8%), 947 (27.5%), 13 (0.4%), 598
98 (17.4%), 67 (1.9%), 41(1.2%) and 11(0.3%) entries as of March 2021, respectively. Thus, EndoC- β H1
99 cells remain one of the most commonly used human β cell lines despite availability of next-generation
100 cell lines EndoC- β H2 (10) and EndoC- β H3 (11) with inducible growth-arrest. Taken together, these
101 three generations of EndoC- β H cells are used by over 150 research labs world-wide
102 (<https://www.humanbetacelllines.com/>)(13).

103 Validity of EndoC- β H1 cells as a human β cell model has been previously established based on
104 functional experiments such as glucose-stimulated insulin secretion (14-16) and marker expression
105 profiling by qPCR (9, 16). Recently, EndoC- β H1 open chromatin, transcriptomics and miRNA
106 landscapes were reported to be most similar to adult human β cells or islets compared to α cells,
107 adipocytes, muscle and peripheral blood cells (17). Two proteomics studies investigated the response
108 to interferons in EndoC- β H1 cells (18, 19), but did not validate EndoC- β H1 as a β cell model. It remains

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unclear what are the discrepancies of EndoC- β H1 with adult β cells and limitations (scope / applicability) of the model.

The aim of this study is to validate EndoC- β H1 as an *in vitro* human β cell model based on omics. We evaluate the EndoC- β H1 cell transcriptome and proteome against corresponding omics profiles of primary β cells and insulinoma samples. Due to their proliferative nature, EndoC- β H1 are more similar to human primary insulinomas than to adult human β cells which have an extremely low proliferation rate. We show that EndoC- β H1 cells belong to the β cell lineage and recapitulate features of adult primary β cells well. We observe that EndoC- β H1 exhibit ~90% similarity with reference adult human β cell transcriptome and proteome. Similarity exceeding 90% was noted for β cell markers, transcription factors and enzymes. We also extensively studied negative control markers of other cell and tissue lineages and confirm lack of major expression abnormalities.

Further, we address a major knowledge gap: β cells are a crucial endocrine cell type, yet little is known about the β cell secretome, especially in humans. Few studies reported secretome composition of rodent insulinoma cell lines (20-22), while the secretome of human immune-cell depleted islet tissue has been profiled using the SOMAscan 1,300 proteomics assay only this year (23). The SOMAscan study suggests that the human and murine secretomes may be quite distinct (23), making it unclear to what extent findings of secretome studies in murine β cells can be extrapolated to human. In this study, we present the first characterization of human β cell line EndoC- β H1 secretome by Data Independent Acquisition (DIA) proteomics. This novel data indicates that the secretome of β cells may be more extensive than previously thought, and highlights surprising abundance of exosome-associated proteins.

EXPERIMENTAL PROCEDURES

Experimental Design and Statistical Rationale

The experiment series was designed to investigate the effects of PCSK9 knockdown on β cell transcriptome, proteome and secretome. Non-targeting control siRNA samples were used to evaluate EndoC- β H1 as a translational β cell model and to characterize composition of the secretome, and are presented in this manuscript. Effects of PCSK9 knockdown are further explored in [reference]. For all

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experiments, the PCSK9 knockdown efficiency was analyzed at the mRNA level by RT-qPCR and at the protein level by Western blot in both cell extracts and conditioned media prior to conducting omics data acquisition. Pilot Tandem Mass Tag (TMT) proteomics cell lysate time course (PXD027898) contained untreated baseline and siRNA control samples and was used to verify lack of major impact of control siRNA treatment on proteome composition. Five other experiments included non-targeting control siRNA and siPCSK9 samples, had confirmed PCSK9 knockdown and supported both parts of the study. EndoC- β H1 cells treated with control siRNA and PCSK9 siRNA at 72h were profiled by RNA-seq (GSE182016), cell lysate DIA proteomics (PXD027911), cell lysate TMT proteomics (PXD027921) and secretome DIA proteomics (PXD027913). In addition, a time-course profiling of the secretome of PCSK9 siRNA vs control siRNA cells at 24, 48 and 72 hours was performed with DIA proteomics (PXD027920).

Sample size calculation: RNA-seq, cell lysate TMT proteomics and DIA secretome time course were unpowered pilot experiments with three replicates per condition. Sample size for cell lysate and secretome DIA proteomics at 72 hours (PXD027911 and PXD027913) was calculated based on the pilot cell lysate TMT experiment (PXD027921) as 12 non-targeting control siRNA and 12 siPCSK9 samples (power 80%, alpha 0.05, N proteins expected to be detected 9,000, alpha corrected for multiple testing $5.6e-6$, Cohen d for PCSK9 knockdown -2.93, distribution of normalized protein intensities assumed to be approximately normal, two-tailed t-test).

EndoC- β H1 cell culture

EndoC- β H1 cells were cultivated in low glucose DMEM medium (5,6 mmol/l; Sigma-Aldrich) supplemented with 2% BSA fraction V (Roche Diagnostics), 50 μ mol/L 2- β -mercaptoethanol (Sigma-Aldrich), 10 mmol/L nicotinamide (Calbiochem), 5.5 μ g/mL transferrin (Sigma-Aldrich), 6.7 ng/mL sodium selenite (Sigma-Aldrich), 100 units/mL penicillin, and 100 μ g/mL streptomycin (ThermoFisher Scientific). The cells were seeded on plates previously coated with 1.2% Matrigel containing-3 μ g/ml fibronectin (both from Sigma-Aldrich) and cultured at 37°C and 5% CO₂ as previously described (9).

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For siRNA transfection, EndoC- β H1 cells were passaged, seeded at 10^5 cells /cm². 24 hours later, cells were transfected in OptiMEM using Lipofectamine RNAiMAX (Life Technologies, Saint Aubin, France) with siRNA SMARTpools (Horizon Discovery LTD): non targeting Control (siCTRL, D-001810-01-20) or siRNA PCSK9 (M-005989-01-0005) at the final concentration of 80 nM as described (24). Medium was replaced 2.5 hours later with fresh EndoC- β H1 culture medium.

For proteome experiments, EndoC- β H1 cells were pre-incubated for 2 hours in BSA/phenol red-free culture medium before transfection. After transfection, the cells were grown in fresh BSA/phenol red-free culture medium for 3 days. Cell pellets were collected by trypsinization and washed 3 times in PBS. Conditioned media were collected, treated with protease and phosphatase inhibitors (Roche Diagnostics) and the debris eliminated by centrifugation at 1,500 rpm at 4 °C. All the samples were stored at -80°C.

Fluorescence-activated cell sorting

EndoC- β H1 cells were trypsinized and washed three times in PBS. Cell death was assessed by incubating the cells with annexin-V antibody (APC, #B187891, Biolegend) for 15 minutes at room temperature, in the dark in annexin-V buffer (#422201, Biolegend). Propidium iodide was added before FACS analysis. For proliferation studies, cells were fixed, and permeabilized with a transcription factor staining buffer set (#00-5523-00, Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were then incubated with Ki67 antibody (PerCP/Cy5.5, #B238642, Biolegend) for 30 minutes at room temperature in the dark in permeabilization buffer, and then with DAPI (BD Biosciences) for 10 minutes at room temperature in the dark. FACS analysis was carried out using a FACS Aria III (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo 10.7 software (RRID:SCR_008520).

RNA-sequencing

RNeasy Micro Kit (Qiagen) was used to extract total RNA from EndoC- β H1 cells. Genomic DNA was removed by DNase treatment following the RNeasy Micro Kit protocol. RNA quality (RNA integrity number) was determined by electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies,

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Palo Alto, CA, USA) as per the manufacturer's instructions. To construct the libraries, 600 ng of high quality total RNA sample (RIN >8.5) was processed using TruSeq Stranded mRNA kit (Illumina) according to manufacturer's instructions. Briefly, after purification of poly-A containing mRNA molecules, mRNA molecules were fragmented and reverse-transcribed using random primers. Replacement of dTTP by dUTP during the second strand synthesis enabled strand specificity. The addition of a single A base to the cDNA was followed by ligation of Illumina adapters. Then, the libraries were quantified with Qubit fluorometer DNA HS kit (Invitrogen). The library profiles were assessed using the DNA High Sensitivity LabChip kit on an Agilent Bioanalyzer. Libraries were sequenced on an Illumina Nextseq 500 instrument using 75 base-length reads V2.5 chemistry in a paired-end mode. After sequencing, a primary analysis based on AOZAN software (Ecole Normale Supérieure, Paris) was applied to demultiplex and control the quality of the raw data (based on FastQC modules / version 0.11.9). Raw reads were aligned to human genome GRCh38 release 96 using STAR version 2.7.1a (25). Transcript quantification was performed with RSEM (26). Quality control metrics were generated with Picard ["Picard Toolkit." 2019. Broad Institute, GitHub Repository. <http://broadinstitute.github.io/picard/>; Broad Institute] and STAR (25) and examined visually in addition to clustering and PCA. All samples passed quality control. TPM (transcript per million) normalization was applied for descriptive analysis in this study.

Cell Proteome Sample Preparation

Cell pellets were thawed on ice and subjected to sample preparation with the PreOmics iST NHS or iST kit (PreOmics, Planegg, Germany) according to manufacturer's protocols. Briefly, iST or iST-NHS lysis buffer was added directly to the cell pellet and incubated at 95°C for 10 min for cell lysis, reduction and alkylation of proteins. Cell lysate was normalized using BCA assay (Thermo Fisher Scientific) and 50 μ g from each condition was subjected to enzymatic cleavage for 3 hours by adding equal amounts of endoproteinase Lys-C and trypsin in a 1:50 (wt/wt) enzyme:protein ratio. For experiments utilizing Tandem Mass Tags™ (TMT™), TMT™ reagents were reconstituted in anhydrous acetonitrile (ACN) and added to peptides at a 4:1 reagent:peptide. De-salting and purification were performed according to the PreOmics iST or PreOmics iST-NHS protocol on a styrene divinylbenzene reversed-phase

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sulfonate (SDB-RPS) sorbent. IST Purified peptides were vacuum-centrifuged to dryness and reconstituted in double-distilled water with 2 vol% ACN and 0.1 vol% formic acid (FA) for single-run LC-MS analysis. IST-NHS TMT™ labeled peptides were reconstituted in pH 10.0, 20mM ammonia hydroxide and combined evenly to create a single sample.

HPRP Fractionation

TMT™ labeled peptides were fractionated into 96 fractions across a 96 well plate, using high pH reverse phase chromatography on an Agilent 1100 system and Phenomenex Gemini 5 μ m C18 250mm x 2mm column. The linear gradient profile consisted of 2% to 90% mobile phase B in 70 minutes at flow rate of 0.20mL/min. Mobile phase A consisted of pH 10.0, 20mM ammonia hydroxide in water and mobile phase B consisted of 20mM ammonia hydroxide in ACN. The 96 fractions were combined within each column orthogonally into a total of 12 concatenated analytical samples. Samples were vacuum-centrifuged to dryness and were then reconstituted with double-distilled water with 2 vol% ACN and 0.1 vol% FA for LC-MS analysis.

Secretome Sample Preparation

Cell conditioned media was thawed on ice and was concentrated down to ~100 μ L using Amicon® Ultra-15 3k MWCO centrifugal filter units (Millipore Sigma). Buffer exchange into PreOmics iST lysis buffer was performed, followed by incubation at 95°C for 10 min for reduction and alkylation of proteins. Protein amounts were normalized using BCA assay (Thermo Fisher Scientific) and equal amounts from each condition was subjected to enzymatic cleavage for 3 hours by adding equal amounts of endoproteinase Lys-C and trypsin in a 1:50 (wt/wt) enzyme:protein ratio. De-salting and purification were performed according to the PreOmics iST protocol on a SDB-RPS sorbent. Purified peptides were vacuum-centrifuged to dryness and reconstituted in double-distilled water with 2 vol% acetonitrile (ACN) and 0.1 vol% formic acid (FA) for single-run LC-MS analysis.

LC-MS/MS Measurement

Orbitrap Exploris 480 Mass Spectrometer (PXD027911 and PXD027920)

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Peptides were loaded onto a 25cm IonOpticks Aurora Series emitter Column (25cm x 75u ID, 1.6um C18; IonOpticks, Australia) performed by a Dionex Ultimate 3000 coupled online to an Exploris 480 Mass Spectrometer equipped with a Nanospray Flex Ion Source, integrated with a column oven (PRSO-V1, Sonation, Biberach, Germany) maintained at 50°C. Peptides were separated using a non-linear gradient. Mobile Phase A was 0.1 vol% FA, 3 vol% ACN in water, while Mobile Phase B was 90 vol% ACN, 0.1 vol % FA. The gradient was operated at 400nl/min flowing 3 vol% B for 25min, 5-17 vol% B over 72min, 17-24 vol % B over 18min, 24-30 vol % B over 10min, 30-85 vol % B over 3min, hold at 85 vol% B for 7min, 85-3 vol% B over 0.1min, and hold at 3 vol% B for 15min. Orbitrap Exploris 480 was operated in BoxCar Data Independent Acquisition (DIA) positive mode where spray voltage was set to 1600V, funnel RF at 40%, and heated capillary temperature at 275°C. Method Timeline experiment consisted of 1 MS1 Scan, 1 tSIM scan and 1 tMS2 scan. MS1 scan was operated at 120k resolution, 400-1200 m/z scan range, 40% RF lens, 300% AGC target, and 54ms IT. MS1 tSIM was operated with Multiplexed Ions enabled (12 ions), at 120k resolution, 300% AGC target, 20ms IT with a set loop control of 2N (number of spectra). BoxCar windows spanned 400-1200m/z space. A total of 48 MS2 DIA variable windows were operated at 15k resolution with normalized collision energy of 28%, 1000% AGC target with 22ms IT spanning 400-1200m/z range taken into account heavily dense peptide regions.

Orbitrap Fusion Lumos Mass Spectrometer (PXD027913)

Peptides were loaded onto a ReproSil-Pur 120 C18AQ 1.9um in-house packed to a 5um tip 75u ID x 360u x 50 cm column using a Thermo EASY-nLC1200 connected through a Nanospray Flex Ion Source, integrated with a Column Oven (PRSO-V1, Sonation, Biberach, Germany) maintained at 50°C. Mobile Phase A was 0.1 vol% FA, 3 vol% ACN in water, while Mobile Phase B was 90 vol% ACN, 0.1 vol% FA. The gradient which had a flow of 300nl/min consisted of a non-linear ramp of 3-24 vol% B in 85min, 24-30 vol% B for 15min, 30-95 vol% B in 5 min, 95 vol% B hold for 5min, and re-equilibration to 3 vol% B for 10min. The LC was connected to an Orbitrap Fusion Lumos Mass Spectrometer operated in DIA Positive mode. Briefly, spray voltage was set to 2500V, Ion transfer tube set to 300°C, MS1 resolution at 120k, MS2 resolution at 30k, scan range of 350-1650 m/z for MS1 and

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350-1650 m/z for MS2. RF lens was set to 30%, MS1 IT to 20ms, MS2 IT to 60ms. MS1 AGC target set to 3e6, MS2 AGC target set to 1.5e6 with 28% HCD collision energy. DIA variable windows covered 400-1650 m/z space taken into account heavily dense peptide regions.

Orbitrap Fusion Lumos Mass Spectrometer (PXD027898 and PXD027921)

Peptides were loaded onto a ReproSil-Pur 120 C18AQ 1.9 μ m in-house packed to a 5 μ m tip 75 μ ID x 360 μ x 50 cm bed volume column using a Thermo EASY-nLC1200 connected through a Nanospray Flex Ion Source, integrated with a Column Oven (PRSO-V1, Sonation, Biberach, Germany) maintained at 50°C. Mobile Phase A was 0.1 vol% FA, 3 vol% ACN in water, while Mobile Phase B was 90 vol% ACN, 0.1 vol% FA. The gradient which had a flow of 250nl/min consisted of non-linear ramp of 3-5 vol% B in 5min, 5-20 vol% B for 80min, 20-32 vol% B in 30 min, 32-95 vol% B in 1min, and hold at 95 vol% B for 14 min. The LC was connected to an Orbitrap Fusion Lumos Mass Spectrometer operated in Data Dependent Acquisition Positive mode. Briefly, spray voltage was set to 2200V, Ion transfer tube set to 275°C, MS1 resolution at 60k, MS2 resolution at 15k, scan range was set to 375-2000 m/z for MS1 and first mass of 100 m/z for MS2. MS1 IT to 50ms, MS2 IT to 22ms. MS1 AGC target set to 1e6, MS2 AGC target set to 1e6 with 38% HCD collision energy. Quadrupole isolation window was set to 1m/z with 30 second exclusion duration. MIPS filter was set to peptide, filter intensity threshold was activated with Maximum intensity set to 1E20, minimum intensity set to 4E4, intensity filter type set to intensity threshold.

Mass Spectrometry Analysis

DIA experiments were analyzed using Spectronaut™ V14.10 (Biognosys AG, Switzerland) direct DIA analysis using a Swiss-Prot human canonical database downloaded on 2/28/2020; the database contained 20,350 entries and utilized Pulsar as the search engine. Analysis settings were maintained to factory settings where identification was set to 1% FDR for precursor and protein level and quantification was conducted on MS2 level for specific digest type of Trypsin/P. Static modifications of Carbamidomethyl (+57.021 Da) and dynamic modification N-terminal acetylation (+42.011 Da).

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TMT experiments were analyzed using Thermo Proteome Discoverer 2.4. Briefly, the raw files were searched through Sequest HT using canonical database as above with the following parameters: Trypsin (full), max missed cleavage of 2, minimum peptide length of 6, max peptide length of 144, precursor mass tolerance of 10ppm, fragment mass tolerance of 0.02Da, dynamic modification: oxidation (+15.995 Da) of methionine, N-terminal acetylation (+42.011 Da), static modification: TMT (+229.163 Da) on peptide N-terminus and lysine, PreOmics cystine modification (+113.084 Da). Target/decoy was performed using Percolator: concatenated, q-value, with FDR on peptide and protein level of 1%. TMT reporter ion quantification was carried out using 50% co-isolation threshold, and average reporter S/N threshold of 10.

Down-stream analysis

TMT proteomics data were normalized using scaling factor normalization and log2 transformed. DIA proteomics data were normalized using quantile normalization as implemented in limma (27) and log2-transformed. DIA proteomics data contained small amount of missing values: 0.7 (0.2-1.8)% missing protein intensity values per sample in cell lysate PXD027911 and 1.5 (0.9-2.3)% in secretome PXD027913 experiments. Missing values were imputed using sequential imputation method (28, 29). Approximately normal distribution of normalized protein intensities were confirmed by visual examination of quantile-quantile plots. Ambiguously annotated proteins (e.g., tryptic peptides aligning to several immunoglobulin kappa chain proteins) were excluded prior to statistical analysis. Differential protein expression analysis of siRNA control vs untreated cells was performed with mixed-effect linear model with condition as fixed effect term and TMT plex as random intercept. Functional classification of the proteins (30) was evaluated using AmiGO2 (31) and PANTHER (32). Proteins may have more than one functional annotation (e.g., be involved in several pathways), therefore the sum of percentages of proteins with different annotations may exceed 100%. Figures were made in R version 4.0.2(33) with ggplot2 (34) and ggVennDiagram (35).

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RESULTS

Quality control of the biological material

One of the biggest challenges in secretome studies is determining whether the proteins are truly secreted by viable cells or are observed as a result of cell death or leakage. Therefore, we verified that treatment with non-targeting control siRNA did not drastically affect cell viability, cell type identity or functional state. We confirmed that treatment with control siRNA did not affect cell proliferation (**Fig 1A**) or viability (**Fig 1B** and **C**), which are important for secretome assessment as these factors could affect membrane permeability. We assessed the differences in protein expression between untreated baseline and control non-targeting siRNA treated EndoC- β H1 cells. Treatment with control siRNA altered expression of 3.3% of proteins. The cells treated with control siRNA displayed signs of altered lipid metabolism and stress. However, the proteins quantified at baseline were a complete subset of the proteins quantified at 72h after transfection with control siRNA (**Fig 1D**). Thus, treatment with control siRNA did not change the repertoire of proteins expressed by the cells and cell type identity.

Validation of EndoC- β H1 as a translational human β cell model

We compared the transcriptome and proteome of EndoC- β H1 both globally and across selected positive control and negative control marker categories to published human primary β cells and insulinomas. The reference human β cell preparations were selected based on purity of the tissue: RNA-seq of adult primary human β cells from 7 non-diabetic donors, isolated by FACS sorting with at least 97% β cell purity in GSE67543 (36) and proteomics of 4 non-diabetic human islet cultures on nanostructured zirconia with $80\% \pm 10\%$ purity of β cells in PXD007569 (37). The reference insulinoma samples were selected from tumors with typical presentation and without metastases: RNA-seq samples WT_MK27 and WT_MK8 in GSE118014 (38) and proteomics by Song and colleagues (39). The reference transcriptome and proteome contained less molecular species than the EndoC- β H1 transcriptome and proteome acquired in this study. Hence, we calculated percentage of the reference transcriptome/proteome that was recapitulated by EndoC- β H1 cells.

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First, we compared EndoC- β H1 cells to primary human adult β cells. Overall, transcriptome and proteome of EndoC- β H1 cells resembled adult human β cells (**Fig 2A** and **2B**). EndoC- β H1 recapitulated 90.7% of β cell protein-coding transcriptome and 87.7% of the β cell – enriched islet proteome. The reference human β cell proteomics data originated from an older study and contained limited number of protein species. Therefore, the agreement between marker gene sets was calculated based on transcriptomics. Good agreement between 69% and 91% vs adult β cells was observed across individual gene/protein categories such as β cell markers, genes associated with diabetes, transcription factors, enzymes, ion channels and lncRNA (**Table S1, Fig S1-S6, Fig S8**). The lowest agreement was obtained for G-protein coupled receptors (GPCRs) with 52% of β cell GPCRs detected on mRNA level and only a few species confirmed by proteomics (**Fig S7**). In particular, median GLP1R expression in control siRNA treated EndoC- β H1 cells was 0.8 TPM whereas median expression in primary adult β cells was 61.7 TPM. We also verified low GLP1R expression with median 5 TPM in an external experiment with untransfected EndoC- β H1 cells sequenced at 200 million reads per sample in GSE133218 (18).

Next, we investigated possible ectopic expression patterns. EndoC- β H1 expressed β cell hormones INS and IAPP at gene and protein levels and ADCYAP1 (also known as PACAP) at mRNA level. (**Fig 2C-F**). EndoC- β H1 expressed mRNA of other islet endocrine hormones (**Fig 2D**) which can also be observed in primary β cells (**Fig 2C, Fig S9**). However, detection of proglucagon and somatostatin on protein level (**Fig 2E, Fig 2F**) is atypical for adult human β cells. Somatostatin expression in EndoC- β H1 cells has been noted previously (16) and is observed in insulinomas (39). Proglucagon protein was detected based on a single peptide DFINWLIQTK aligning to amino acid positions 166-175 and corresponding to glucagon-like peptide 2. Proglucagon co-expression with insulin is a feature of insulinomas (39, 40). No ectopic expression was observed for other α cell markers (**Fig S10**). EndoC- β H1 were derived from fetal islet cells at 9 to 11 weeks of gestation (9). Interestingly, expression of endocrine progenitor markers was considerably reduced in EndoC- β H1 cells compared to human fetal pancreas at 9 weeks gestation (**Fig S11**). Expression of NEUROG3 was lost. Markers of fetal β cell at 12 to 18 weeks gestation were not expressed (**Fig S12**). We also observed no ectopic expression of

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markers of exocrine pancreas acinar (**Fig S13**) and ductal cells (**Fig S14**) and markers of other tissues (**Fig S15**).

Overall, the genes and proteins quantified in EndoC- β H1 cells but not in primary β cells were annotated with cell cycle, DNA repair, proliferation, ATP generation and mitochondrial function Gene Ontology (GO) terms. This ectopic expression pattern was consistent with proliferative phenotype. EndoC- β H1 cells double in number every 174 hours (14) whereas proliferation of adult human β cells is an extremely rare event (41). Accordingly, the EndoC- β H1 transcriptome and proteome were more similar to insulinomas than to primary human β cells (**Fig 2G-H**). EndoC- β H1 cells recapitulated 96% of the reference insulinoma transcriptome and 90.7% of the reference insulinoma proteome. Good agreement was observed across individual protein categories (**Fig 3**).

The secretome of EndoC- β H1 cells

In order to quality-control the secretome, we cross-referenced coordinates of peptides detected in the secretome experiments and mapped to membrane proteins with topology of proteins in the human surfaceome (42). If a secretome preparation is high-quality, peptides should map to extracellular regions that can be cleaved-off or shed. By contrast, contamination with peptides that map to transmembrane or intracellular regions indicates lysis or mechanical cell damage. The ratio of combined lengths of extracellular vs intracellular regions in the reference database was 2.4:1. This expected ratio was preserved in the cell lysate DIA experiment PXD027911 with 65.6% peptides mapped to extracellular regions and 30.9% peptides mapped to intracellular regions. By contrast, 94.4% peptides mapped to extracellular regions in PXD027913 and 93.2% in PXD027920 secretome experiments. Cell lysate proteome in PXD027911 and secretome in PXD027913 were generated based on the same EndoC- β H1 cell cultures. Expression abundances of proteins with cytoplasmic subcellular location according to UniProt (43) did not correlate between cell lysate and the corresponding secretome samples - median IQR Spearman rho 0.02 (-0.12 to 0.16), - indicating that leakage was unlikely. Proglucagon was not detected in the secretome experiments. Somatostatin was secreted by cells treated with control siRNA but not by untransfected EndoC- β H1 cells.

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Higher number of proteins were detected in the secretome of EndoC- β H1 cells treated with control non-targeting siRNA than in untreated cells at baseline (**Fig 4A**).

EndoC- β H1 cells secreted known β cell hormones INS and IAPP and autocrine regulator IGF2 (44) (**Fig 4B**). The secretome contained proteins known to generate bioactive peptides such as all 8 proteins of the granin family (**Fig 4B**), among which chromogranins A and B, secretogranins 2 and 3 and VGF have been previously reported in insulinomas (45). Secretome proteomics indicated potential presence of several bioactive peptides (**Fig S16-S50**) including chromogranin A peptides pancreastatin and vasostatins, chromogranin B peptides GAWK and CCB, secretogranin-2 peptide manserin and VGF peptides. Some peptides were established β cell secretion products: IGF2 peptide preptin is co-secreted with insulin (46) while VGF peptides neuroendocrine regulatory peptide-2 (47) and TLQP-62 (48) regulate glucose-induced-insulin-secretion. The secretome also contained CPE, PAM and proprotein convertases PCSK1 and PCSK2 – the enzymes involved in generation of bioactive peptides from prohormones. Interestingly, EndoC- β H1 cells also secreted PCSK9, a member of proprotein convertase family without known enzymatic function but involved in LDL receptor clearance in hepatocytes. We further investigated the function of PCSK9 in EndoC- β H1 cells in a separate follow-up study [**reference**].

We quantified exosome markers (49) in the secretome samples - CD63, CD81, heat shock 70kDa proteins and TSG101 (**Fig 4B**) and members of the Rab family (**Fig 4D**). Exosomal proteins were a major component of the secretome (**Fig 4C**). Secretion of exosomes is increased in stressed β cells (50). Therefore, higher number of protein species quantified in EndoC- β H1 cells treated with control siRNA than in untreated cells was consistent with exosomes being the major “mode of secretion”.

The EndoC- β H1 cell secretome after 72 hours of incubation with control siRNA contained proteins implicated in β cell proliferation: NR3C1 (glucocorticoid receptor), GSK3B and cell cycle-regulating kinases CDK1, CDK2, CDK4 and CDK6. These proteins can be detected in exosomes based on ExoCarta database (51), although most of the referenced research works were conducted in cancer tissues or cell lines rather than non-cancerous tissues. Hence, it might be theoretically possible to infer

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β cell state based on exosome markers that are normally intracellular and inaccessible for measurement in plasma or serum.

The second most common category of proteins in the secretome were classically secreted proteins containing signal peptide (**Fig 4C**). For example, both untreated and control siRNA treated EndoC- β H1 cells secreted factors regulating cell differentiation and survival NENF, MANF, MYDGF and CREG1. EndoC- β H1 cells also secreted proteins that are measurable in circulation (52) such as annexins, apolipoproteins, serpins, IGF-binding proteins and VEGFA.

In addition, we captured known proteins with shed extracellular domain in islets or rodent cell lines (21, 53, 54) in the EndoC- β H1 cell secretome (**Fig 4B**). Sheddases ADAM10, ADAM17, ADAM22 and ADAM9 were quantified in the EndoC- β H1 secretome. Sheddases BACE1 and BACE2 were not quantified in the secretome samples, but were quantified in the DIA and TMT cell lysate experiments.

Finally, we investigated the most abundantly secreted proteins in the untreated (**Table S2**) and control siRNA treated samples (**Table 1**). β cell hormones were among the most abundant secreted proteins confirming β cell line identity.

DISCUSSION

EndoC- β H1 cells are among the most commonly used human β cell lines for early-stage diabetes research, yet EndoC- β H1 cells have not been validated as an *in vitro* human β cell model based on omics. Here, we confirm that EndoC- β H1 are cells of β cell lineage using transcriptomics and proteomics data. The observed discrepancies with adult human β cells and increased similarity with insulinomas were due to elevated proliferation rate (14). Therefore, inducible growth arrest will likely ameliorate the discrepancies in gene and protein expression pattern. In fact, next generation EndoC- β H2 and EndoC- β H3 cells display dramatic increase in insulin content and secretion capacity after the immortalization cassette is excised (10, 11). Increased expression of β cell markers post-excision has also been characterized in second-generation EndoC cells (10).

Limited detection of GPCRs in the proteomics experiments might be of technical nature. In general, membrane proteins are hydrophobic. Much harsher lysis buffers than the buffers were used in these

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proteomics experiments are required for solubilizing GPCRs. In addition, the hydrophobic nature of GPCRS leads to low frequency of polar aminoacids (i.e., very few lysin and arginine residues), which affects tryptic digestion and subsequent detection by mass-spectrometry (55, 56). By contrast, low GLP1R expression appeared to be a true biological finding. GLP1R expression may also be remedied by using next-generation cell lines. For example, dose-dependent response to GLP-1 mimetics has been reported by vendors in Endoc- β H5 cells (<https://www.humancelldesign.com/human-beta-cells-endoc-bh5/>).

In summary, growth-arrested next-generation EndoC cells may be more suitable for some research questions such as studies of factors influencing proliferation of adult β cells, GLP1R-mediated drug delivery or effects GLP-1 mimetics.

As EndoC- β H1 cells proved to be a reasonable translational human β cell model, we used EndoC- β H1 cells to uncover the β cell secretome. EndoC- β H1 cells secreted β cell hormones INS and IAPP. Both adult human β cells and insulinoma cells secrete low amounts of insulin under basal conditions and not only after glucose or KCl challenge (57, 58). Hence, presence of insulin and co-secreted products IAPP and IGF2/preptin in the secretome of unstimulated EndoC- β H1 is not abnormal. Albumin was not quantified in the EndoC- β H1 RNA-seq samples. Therefore, albumin in the secretome samples likely represents the traces of albumin derived from the culture medium where the cells had been previously grown.

Our secretome proteomics workflow was aimed at identification of secreted proteins and contained a tryptic digestion step. Thus, we could not establish full-length sequences of endogenously cleaved peptides. However, we could make inference based on the spectra and prior knowledge of protein families generating bioactive peptides. Our secretome data indicated that β cells may be capable of producing and secreting bioactive peptides. In fact, we confirmed the presence of preptin, neuroendocrine regulatory peptide-2 and TLQP-62 (46-48) in conditioned medium from EndoC- β H1 cells. We also found that EndoC- β H1 cells secrete proteins of the granin family and possess the enzymatic machinery necessary to produce bioactive peptides, which suggests that the endogenous bioactive peptide repertoire of β cells might be richer than previously anticipated. Bioactive peptides

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may play an important role in both autocrine and endocrine signaling as well as cross-talk between β cells and other islets cell types. We believe that presence of full-length endogenous peptides and their secretion dynamics and function warrant further investigation in subsequent studies with specialized proteomics methods.

The secretome of EndoC- β H1 encompassed classically secreted proteins with signal peptide. However, we speculate that they may play a limited role as biomedically relevant biomarkers. Only a few proteins in the secretome had enriched expression in β cells (INS, IAPP) or nervous system and β cells (e.g. PTPRN, VGF) (59). In addition, the relative contribution of β cells to the plasma pool of broadly expressed proteins in human is unknown, and deconvoluting tissue-of-origin for such blood-borne biomarkers can be extremely challenging. By contrast, classically secreted proteins may play an autocrine or paracrine function. For example, the functions of NENF, MANF, MYDGF and CREG1 as regulators of cell survival and differentiation were described in a different tissue context (43) but they may play similar roles in islets. In fact, MANF has been recently characterized as a β cell protecting factor (60).

Abundance of exosome-associated proteins in untreated and control siRNA treated cells was an unexpected and potentially biomedically relevant finding. Exosomes may offer additional opportunities to discover and non-invasively monitor biomarkers reflecting β cell state. Another important observation was quantification of CD63 and CD81 in the EndoC- β H1 cell secretome. Antibodies recognizing these exosome surface markers are used in exosome isolation protocols, and our findings indicate that standard protocols could be applicable to isolate β cell derived exosomes in *in vitro* studies. Future studies need to further elucidate composition of β cell exosomes and their secretion dynamics, and to establish if it is possible to enrich β cell derived exosomes from plasma, serum or fecal samples based on additional biomarkers.

Data availability

Data sets will be made public upon publication of the manuscript in a peer-reviewed journal.

RNA-seq data have been deposited to NCBI GEO (61) with accession GSE182016.

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (62) partner repository with the following identifiers: PXD027898, PXD027921, PXD027920, PXD027911 and PXD027913. Each data set contains raw files, annotated spectra files in .sne or .msf format, files with parameters for Proteome Discoverer or Spectronaut software run and a processed_data.zip. Each processed_data.zip files contain raw protein quantification table, log2 normalized and imputed protein quantification table, sample description and analysis log file. Peptide quantification tables and the corresponding log files are included in PXD027911, PXD027913 and PXD027920.

Gene/protein lists corresponding to Figure 2 and Figure 4 are provided in the supplementary excel file.

The previously published data sets used as reference primary human biological material were: GTEx (59), Human Protein Atlas (HPA) (52), GSE67543 (36), GSE57973 (63), GSE118014 (38), GSE84133 (64), GSE81608 (65), E-MTAB-5061 (66), GSE86469 (67), PXD007569 (37). Marker lists were obtained from HGNC (68), OMIM (69), GENECODE v36 (70) and supplementary data sets in (39, 71-74).

Author contribution statements

R.S., S.A., K.Se., G.H. – design and planning of the study. K.S. – cell culture, RNA-seq and FACS experiments. C.B. – FACS experiments. G.H., A.J. – mass-spectrometry data acquisition, pre-processing. M.R. – down-stream analysis, first draft. All authors – data interpretation, scientific feedback, revisions of the manuscript and approval of the submitted version.

Conflict of interest

S.A., K.Se., M.R., A.J., C.R.U and G.H. are employed by AstraZeneca. R.S. is a shareholder in and a consultant for Univercell-Biosolutions.

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Fig. 1. Non-targeting control siRNA did not affect EndoC- β H1 cells proliferation, viability and composition of the proteome. Ki67/Dapi staining for cell proliferation (A) and Annexin-V/PI staining for apoptosis (B) and live cells (C) assessed by FACS in untransfected cells compared to 72 hours post transfection with control siRNA or siPCSK9. (D) Proteome composition of untransfected cells and cells transfected with control siRNA at 72 hours in pilot TMT cell lysate experiment. Expression cut-off for proteins: quantified in 3 samples and unambiguously annotated.

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Fig. 2. Transcriptome and proteome of EndoC- β H1 cells compared to adult human β cells and insulinomas. (A) Venn diagram showing the number of quantified protein-coding genes and proteins in EndoC- β H1 cells vs adult non-diabetic β cells (GSE67543). (B) Comparison to human β cell-enriched islet proteomics (PXD007569). (C-F) Hormone expression. (G) Comparison to protein-coding genes with expression > 1 TPM in human low-grade insulinomas without lymph node and distant metastases (GSE118014). (H) Comparison to proteome of human insulinomas with typical tumor presentation and without lymph node and distant metastases (39). EndoC- β H1 cells were treated with control non-targeting siRNA and harvested at 72 h. Expression cut-off for genes: median expression $\geq$ 1 TPM. Expression cut-off for proteins: quantified in $\geq$ 3 samples and unambiguously annotated. Sample sizes in EndoC- β H1 experiments: RNA-seq N = 3, TMT proteomics = 3, cell lysate DIA proteomics = 12 replicates.

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Fig. 3. PANTHER classification of EndoC- β H1 cell proteome compared to insulinoma proteome.

(A) Top 100 pathway by the number of annotated proteins. (B) Protein-containing complexes. (C) Protein classes. Percentages indicate the number of proteins annotated with a given functional term vs the total number of proteins quantified in the experiment. EndoC- β H1 cells were treated with control non-targeting siRNA and harvested at 72 h. Insulinoma samples from (39). Expression cut-off for proteins: quantified in ≥ 3 samples and unambiguously annotated.

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Fig. 4. Composition of the EndoC- β H1 cell secretome. (A) Secretome of untreated cells compared to secretome of cells treated with control non-targeting siRNA for 72 hours. **(B)** Known positive control proteins that were quantified in EndoC cell secretome. **(C)** Number of proteins by mode of secretion. Proteins can belong to more than one category (e.g., secreted based on both UniProt and HPA annotation). **(D)** Top 50 protein families. Expression cut-off for proteins: quantified in min 2 out of 3 baseline samples, quantified in min 3 out of 12 samples at 72 hours.

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Table 1. Top 150 secreted proteins. Proteins are ordered by decreasing median normalized expression abundance in EndoC- β H1 cells treated with control non-targeting siRNA at 72 hours in the DIA secretome experiment (PXD027913). N=12.

rank	UniProt	Symbol	rank	UniProt	Symbol	rank	UniProt	Symbol
1	P02787	TF	51	P06703	S100A6	101	P55327	TPD52
2	P10645	CHGA	52	P05060	CHGB	102	P40926	MDH2
3	P62805	H4C1	53	P35241	RDX	103	P30048	PRDX3
4	P02768	ALB	54	P11021	HSPA5	104	P14625	HSP90B1
5	P01308	INS	55	P01034	CST3	105	P00390	GSR
6	P62937	PPIA	56	P08758	ANXA5	106	P14543	NID1
7	P02790	HPX	57	Q15084	PDIA6	107	Q13822	ENPP2
8	P68133	ACTA1	58	O75347	TBCA	108	P60660	MYL6
9	Q9BUJ0	ABHD14A	59	P00492	HPRT1	109	P07942	LAMB1
10	P00352	ALDH1A1	60	O75367	MACROH2A1	110	P19338	NCL
11	P23528	CFL1	61	P07900	HSP90AA1	111	P37837	TALDO1
12	P40925	MDH1	62	P61088	UBE2N	112	P26641	EEF1G
13	P63261	ACTG1	63	P61978	HNRNPK	113	Q92820	GGH
14	P02766	TTR	64	P10599	TXN	114	P50395	GDI2
15	P22626	HNRNPA2B1	65	Q96JB3	HIC2	115	Q14624	ITIH4
16	P31946	YWHAB	66	P29401	TKT	116	P42167	TMPO
17	P07951	TPM2	67	P48637	GSS	117	P17174	GOT1
18	P30101	PDIA3	68	P08238	HSP90AB1	118	P00558	PGK1
19	Q92616	GCN1	69	P14618	PKM	119	P15311	EZR
20	P13521	SCG2	70	P07108	DBI	120	P11142	HSPA8
21	Q9UHG2	PCSK1N	71	P67936	TPM4	121	P62942	FKBP1A
22	P09936	UCHL1	72	Q8WXD2	SCG3	122	Q15293	RCN1
23	P32119	PRDX2	73	P16403	H1-2	123	P13639	EEF2
24	Q13404	UBE2V1	74	P04075	ALDOA	124	P20962	PTMS
25	P61769	B2M	75	P23526	AHCY	125	Q9BWD1	ACAT2
26	P60174	TP11	76	P25391	LAMA1	126	P35527	KRT9
27	P62258	YWHAE	77	P16401	H1-5	127	O75874	IDH1
28	P35908	KRT2	78	P25398	RPS12	128	O94760	DDAH1
29	P16870	CPE	79	Q15631	TSN	129	P28066	PSMA5
30	Q71U36	TUBA1A	80	Q86YB8	ERO1B	130	P25205	MCM3
31	Q9Y6V0	PCLO	81	Q99497	PARK7	131	P26885	FKBP2
32	P11047	LAMC1	82	P29966	MARCKS	132	P27348	YWHAQ
33	P23284	PPIB	83	P06733	ENO1	133	Q01469	FABP5
34	Q562R1	ACTBL2	84	Q12906	ILF3	134	P09493	TPM1
35	Q05519	SRSF11	85	O43707	ACTN4	135	P24534	EEF1B2
36	P09651	HNRNPA1	86	P13645	KRT10	136	P04264	KRT1
37	Q06830	PRDX1	87	P63279	UBE2I	137	P06744	GPI
38	P06753	TPM3	88	P30041	PRDX6	138	P26599	PTBP1
39	P54652	HSPA2	89	P62318	SNRPD3	139	P27797	CALR
40	P01023	A2M	90	P52209	PGD	140	Q02818	NUCB1
41	P00441	SOD1	91	O94985	CLSTN1	141	P04080	CSTB
42	P07237	P4HB	92	Q04760	GLO1	142	P38159	RBMX
43	P61457	PCBD1	93	Q96AE4	FUBP1	143	Q9POL0	VAPA
44	Q16555	DPYSL2	94	Q99574	SERPINI1	144	Q86V81	ALYREF
45	P10909	CLU	95	Q13867	BLMH	145	P05387	RPLP2
46	P07195	LDHB	96	P49321	NASP	146	P50990	CCT8
47	P02533	KRT14	97	O76038	SCGN	147	P30040	ERP29
48	P62857	RPS28	98	Q14194	CRMP1	148	P10997	IAPP
49	Q14103	HNRNPD	99	Q14011	CIRBP	149	P09874	PARP1
50	Q6ZNA1	ZNF836	100	P55145	MANF	150	P60900	PSMA6