Open
Functional, metabolic and transcriptional maturation of human pancreatic islets derived from stem cells

Diego Balboa (1,2,3,11), Tom Barsby (1,11), Väinö Lithovius (1,11), Jonna Saarimäki-Vire (1), Muhmmad Omar-Hmeadi (4), Oleg Dyachok (4), Hossam Montaser (4), Per-Eric Lund (4), Mingyu Yang (4), Hazem Ibrahim (1), Anna Näätänen (1), Vikash Chandra (1), Helena Vihinen (5), Eija Jokitalo (5), Jouni Kvist (1), Jarkko Ustinov (1), Anni I. Nieminen (6), Emilia Kuuluvainen (7), Ville Hietakangas (7,8), Pekka Katajisto (7,9), Joey Lau (4), Per-Ola Carlsson (4), Sebastian Barg (4), Anders Tengholm (4) and Timo Otonkoski (1,10)

Transplantation of pancreatic islet cells derived from human pluripotent stem cells is a promising treatment for diabetes. Despite progress in the generation of stem-cell-derived islets (SC-islets), no detailed characterization of their functional properties has been conducted. Here, we generated functionally mature SC-islets using an optimized protocol and benchmarked them comprehensively against primary adult islets. Biphasic glucose-stimulated insulin secretion developed during in vitro maturation, associated with cytoarchitectural reorganization and the increasing presence of alpha cells. Electrophysiology, signaling and exocytosis machinery of functional SC-islets have been lacking. Noteworthy differences to the most widespread protocols (8,9) include: (1) differentiation of hPSCs in adherent conditions until the pancreatic progenitor population in uniformly sized clusters (Supplementary Fig. 1a); and (4) improved final

The generation of functional pancreatic beta cells from human pluripotent stem cells (hPSCs) is a main goal of stem cell research, aiming to provide a renewable and consistent source of cells for the treatment of diabetes. Stem-cell-derived beta cells could solve the limitations of using cadaveric donor islets for transplantation and serve as a model system to understand the pathogenetic mechanisms leading to various forms of diabetes (1). Several studies have differentiated hPSCs to cell clusters that closely resemble primary islets (SC-islets) using multistage in vitro differentiation protocols that mimic the sequential inductive signals controlling pancreatic islet development in vivo (2,3). Individual studies have reported specific transcriptomic (2,3), functional (4-11) and metabolic (12) aspects of SC-islets. However, studies integrating these aspects with detailed analyses of stimulus-secretion coupling and exocytosis machinery of functional SC-islets have been lacking.

Here, we developed an optimized protocol for the generation of functional SC-islets. We compared SC-islets and primary human adult islets comprehensively to quantify systematically their similarities and differences. During the final, extended maturation stage, the cytoarchitecture of SC-islets was profoundly reorganized, and glucose-stimulated insulin secretion matured to a level similar to that of primary adult islets. We conducted detailed functional and physiological characterization of the SC-islets, supported by targeted metabolite tracing studies together with single-cell transcriptomic profiling of differentiating endocrine cell populations. This multipronged approach was conducted both during the timecourse of SC-islet maturation in vitro and after in vivo engraftment. Our integrated analyses show that a high level of beta cell functionality is achieved in vitro even if specific metabolic and transcriptomic differences persist between SC-islet beta cells and primary beta cells.

Results
SC-islets present organotypic cytoarchitecture and function. We devised an optimized differentiation protocol by combining previous advances in the generation of SC-islets (8,13,14) (Fig. 1a). Noteworthy differences to the most widespread protocols (8,9) include: (1) differentiation of hPSCs in adherent conditions until the pancreatic progenitor stage (S4); (2) optimized S4 step including nicotinamide, epidermal growth factor, Activin A and a ROCK inhibitor; (3) a microwell aggregation step that results in ≈ 80% PDX1+nKX6-1+ pancreatic progenitor population in uniformly sized clusters (Supplementary Fig. 1a); and (4) improved final
To determine the impact of the S7 maturation step on SC-islet function, we systematically characterized hPSC-derived SC-islet maturation from the beginning of S7 (S7w0) to the end of its sixth week (S7w6), using a series of morphometric, functional, metabolomic and transcriptomic analyses. At S7w0, SC-islets contained ≈40% insulin-positive mononuclear cells (INS+)—a proportion that remained relatively stable until S7w6 (Fig. 1b,c) and Supplementary Fig. 1b), consistent with previous studies demonstrating polyhormonal to alpha cell differentiation in vitro and in vivo20–22,23,24. Somatostatin (SST) positive cells were present at S7w0 around 4% and the proportion remained unchanged until S7w6 (Fig. 1c).

As functional maturation of beta cells is linked to reduced proliferation24–26, we examined markers of cell proliferation during the S7 and observed an 80% reduction (from 2.1% to 0.46%) in Ki-67+ INS+ cells (Fig. 1b,d). Critically, reduced proliferation was dependent on the use of ZM, NAC and T3 in S7 medium (Fig. 1e), while the proportions of INS+ and GCG+ cells increased from ≈5% to about 40–50% (Fig. 1f and Supplementary Fig. 1b), consistent with previous studies demonstrating polyhormonal to alpha cell differentiation in vitro and in vivo20–22,23,24. Somatostatin (SST) positive cells were present at S7w0 around 4% and the proportion remained unchanged until S7w6 (Fig. 1c).

SC-islet cytoarchitecture changed profoundly during S7 maturation. A high proportion of INS+ cells localized to the SC-islet periphery at S7w−1, but, by S7w3, SC-islets were polarized, with GCG+ and INS+ cells clustered separately (Fig. 1b). However, by S7w6, the cytoarchitecture varied from core-mantle organization (Supplementary Fig. 1d) to intermingled clusters of GCG+ and INS+ cells (Fig. 1b). Quantitatively, this reorganization resulted in an increased number of cell–cell contacts between GCG+ and INS+ cells from S7w0 to S7w6 (Supplementary Fig. 1e). Similar cytoarchitectural rearrangements have also been described during human fetal pancreatic islet development25,26.

While beta cell numbers remained unchanged during the first 3 weeks of S7, the insulin content of SC-islets increased fourfold (Supplementary Fig. 1f). Concurrently, SC-islet beta (SC-beta) cells progressively acquired dense core insulin granules with ultrastructural morphology resembling those of primary beta cells (Fig. 1i).

Adult primary islets are characterized by a tightly controlled, biphasic insulin secretion response to increases in glucose27. This is controlled through a metabolic response to glucose through KATP-channel activity (the triggering pathway), and modulated through neurohormonal and metabolic amplifying pathways28. At S7w0, high glucose concentrations alone (16.7 mM) failed to trigger insulin secretion. However, treatment with the GLP-1 analog exendin-4 and membrane depolarization with high K+ both triggered pronounced secretory responses. From S7w2 onwards, SC-islets displayed biphasic glucose-stimulated insulin secretion (GSIS) responses similar to primary islets, with gradual increases in the magnitude of the response until S7w6 (Fig. 1h). Of note, the primary islets in this study had secretory responses representing the lower end of responses recorded in previous studies29 and in publicly available databases30. The SC-islets sustained their second phase response for >70 min (Supplementary Fig. 1g). The acquisition of SC-islet function was replicated in two additional human iPSC-lines (Supplementary Fig. 1h) demonstrating the robustness of the maturation protocol. Omission of either ZM or NAC from S7 medium attenuated GSIS responses, while omitting all additives nearly abolished it (Fig. 1k)—an effect mostly explained by higher insulin release in low glucose (Supplementary Fig. 1i).

Immature fetal and infantile primary beta cells are unable to suppress their insulin secretion in low glucose31. Beta cell functional maturity is thus reflected by the glucose concentration threshold that triggers insulin secretion32. Immature S7w0 SC-islets released higher levels of insulin in low glucose (Supplementary Fig. 1j). This basal release could be reduced with the KATP-channel opener diazoxide (Supplementary Fig. 1k), suggesting inappropriate KATP-channel closing in basal conditions at S7w0. We assessed insulin secretion thresholds also by gradually increasing glucose concentration in perfusion assays. SC-islets at S7w0 showed no glucose-induced insulin release, whereas at S7w2 they responded at unphysiologically low glucose concentrations of ≈3 mM. However, at S7w3 and S7w6 they reached the adult threshold of ≈5 mM glucose (Fig. 1l). This shift was also reflected in the glucose concentration eliciting the half-maximal secretory response, (5.6–6.7 and 8.1 mM, at S7w2, S7w3 and S7w6, respectively) (Supplementary Fig. 1l).

These results demonstrate the generation of SC-islets in vitro, with biphasic glucose-dependent insulin secretion similar to that of adult islets. Functional maturation correlated with changes in SC-islet architecture and cell composition, but not with an increase in beta cell mass.

**Functional insulin secretion machinery in SC-beta cells.** To better understand the mechanisms of SC-islet glucose sensitivity, we dissected the stimulus-secretion coupling machinery of SC-beta cells with measurements of ion channel conductance, cytoplasmic Ca2+ and cAMP concentrations, as well as exocytosis. Patch-clamp...
recordings showed that S7w3 SC-beta cells fired action potentials (Fig. 2a), with 11 of 16 cells active in 3 mM glucose. In S7w6 cells, 1 of 17 cells fired action potentials in 3 mM glucose, which increased to 4 active cells in 16 mM glucose. SC-beta cells had Ca\textsuperscript{2+} - and Na\textsuperscript{+}-currents with voltage dependences similar to those in primary human beta cells (Fig. 2b,c). Ca\textsuperscript{2+}-current amplitude was similar in both cell types, while Na\textsuperscript{+}-currents were about twofold larger in SC-beta cells (Fig. 2c). K\textsubscript{\text{ATP}}-channel dependent K\textsuperscript{+}-conductance of S7w3 SC-beta cells was quantified using symmetric voltage-steps (Fig. 2d) or ramps (Fig. 2e). In 3 mM glucose, the membrane conductance was, on average, $53 \pm 4$ pS/pF ($n = 50$ cells) and increased in the presence of diazoxide in 49/50 cells to $273 \pm 30$ pS/pF ($n = 50$ cells). Both values are similar to those previously reported for human beta cells\textsuperscript{41}.

The cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) was recorded from individual cells in SC- and primary islets loaded with a fluorescent Ca\textsuperscript{2+} indicator. In S7w3 SC-islets, a subset of cells showed [Ca\textsuperscript{2+}] oscillations in low glucose with little change in response to high glucose (Fig. 2f). Other cells showed low and stable [Ca\textsuperscript{2+}], at low glucose, with increased, and often oscillatory, [Ca\textsuperscript{2+}], in high glucose (Fig. 2f). Primary islet cells also behaved heterogeneously but a higher proportion responded to elevated glucose (Fig. 2f,g). K\textsubscript{ATP}-channel opening with diazoxide reduced, and closure with tolbutamide increased, [Ca\textsuperscript{2+}], in both SC-islets and primary islets with more pronounced responses in the latter (Fig. 2f,g). Depolarization with 30 mM K\textsuperscript{+} increased [Ca\textsuperscript{2+}], in all cells with similar magnitude in SC- and primary islets (Fig. 2f,g). The overall [Ca\textsuperscript{2+}] responses or fraction of glucose-responsive cells in SC-islets did not change consistently during prolonged S7 maturation (range 42–74%; Supplementary Fig. 2a,b). However, among the glucose-responsive cells, basal [Ca\textsuperscript{2+}] decreased from 47 ± 0.4 to 24 ± 0.2% of the K\textsuperscript{+}-stimulated level and the increase induced by glucose stimulation improved from 5.2 ± 0.1% at S7w0 (n = 1,091 responsive cells) to 20.8 ± 0.4% at S7w7 (n = 1,659 responsive cells), consistent with the observed reduction of basal secretion and improved stimulation index. Since these unbiased analyses of indicator-loaded cells, in SC-beta cells identified by R-GECO1 under insulin promoter control. Recordings from SC-beta cells identified by R-GECO1 expression confirmed the response heterogeneity while also highlighting that at S7w7, 79% of the beta cells showed glucose-induced [Ca\textsuperscript{2+}], increases dependent on K\textsubscript{ATP}-channel closure ($n = 130$ cells; Fig. 2h and Supplementary Fig. 2c).

In the presence of low glucose, tolbutamide increased [Ca\textsuperscript{2+}], in both primary and SC-islets, but again to a higher degree in primary islet cells (Supplementary Fig. 2d,e). High glucose in the continued presence of tolbutamide caused a slight [Ca\textsuperscript{2+}], increase in SC-islets and a decrease in primary islet cells. Despite this lowered [Ca\textsuperscript{2+}], glucose amplified secretion under these conditions (Fig. 3g). Treatment with exendin-4 did not alter [Ca\textsuperscript{2+}], in primary islets and had only a weak tendency to increase [Ca\textsuperscript{2+}], in SC-islets (Supplementary Fig. 2d,e).

The concentration of submembrane cAMP ([cAMP]$_\text{m}$)—a modulator of insulin exocytosis—was recorded in single cells in intact SC- and primary islets. High glucose induced a small, and exendin-4 a much more pronounced, increase in [cAMP]$_\text{m}$ in both preparations (Fig. 2i–k) and in SC-beta cells identified by insulin-promoter-driven expression of R-GECO1 (Supplementary Fig. 2f), indicating that cAMP signaling in SC-islets closely resembles that in primary human islets.

Single-cell exocytosis was measured as membrane capacitance changes using patch clamp. A train of depolarizations (14×200 ms) resulted in identical capacitance increases (ΔC) of 0.087 ± 0.012 fF/pF ($n = 80$) in S7w3 beta cells and 0.084 ± 0.013 fF/pF ($n = 39$) in primary beta cells (Fig. 2l–m). Cell size, as assessed by the initial cell capacitance (Cm0), was slightly larger for SC-cells than for primary beta cells (by 27%, P = 0.0001, paired t-test) (Supplementary Fig. 2f).

Docking and exocytosis of insulin granules at the plasma membrane were studied by total internal reflection (TIRF) microscopy (Fig. 2n). Depolarization of S7w0 cells with elevated K\textsuperscript{+} (in the presence of diazoxide to prevent spontaneous depolarization) released 0.063 ± 0.008 granules (gr) μm$^{-2}$ ($n = 68$ cells; Fig. 2o). Exocytosis proceeded initially with a burst (4×10$^{-2}$ gr μm$^{-2}$ s$^{-1}$; <10s) and later decreased (to <0.7×10$^{-2}$ gr μm$^{-2}$ s$^{-1}$). We consistently observed more than a doubling of K\textsuperscript{+}-stimulated exocytosis following exendin-4 treatment (0.14 ± 0.01 gr μm$^{-2}$, $n = 71$ cells; Fig. 2o,p). All exocytosis values are similar to those reported in primary beta cells\textsuperscript{32}.

Notably, in S7w0, spontaneous exocytosis (no diazoxide) was similar in 3 mM and 10 mM glucose (0.045 ± 0.004 gr μm$^{-2}$, $n = 13$ versus 0.041 ± 0.002 gr μm$^{-2}$, $n = 12$) (Fig. 2q). In contrast, at S7w6,
basal exocytosis was lower (0.025 ± 0.002 gr μm⁻², n = 40) and doubled when glucose was raised to 10 mM (0.051 ± 0.003 gr μm⁻², n = 41; Fig. 2g).

The density of docked granules was ~ 0.6 gr μm⁻² in S7w3 cells (Supplementary Fig. 2g–k), which is identical to values reported for primary beta cells. Treatment with exendin-4 slightly increased docked granules when exocytosis was prevented with diazoxide (Supplementary Fig. 2g).

In summary, these analyses showed that SC-beta cells are equipped with the necessary ion channels, exocytosis components and intracellular signaling machinery required for fine-tuned regulation of insulin secretion.
SC-islets exhibit immature mitochondrial glucose coupling. As SC-islets display functionally mature exocytotic machinery, we next sought to uncover the extent of metabolic coupling to insulin release. Glucose-induced mitochondrial respiration is another characteristic feature of functional adult islets, which correlates with GSIS2,33–35. We assayed oxygen consumption rate (OCR) during glucose stimulation (Fig. 3a) and observed that glucose increased mitochondrial respiration in primary islets but not in SC-islets, despite similar insulin secretion dynamics (Fig. 1j). This lack of respiratory response to glucose was not explained by aberrantly low or high basal respiratory rates in SC-islets (Fig. 3b). In contrast, SC-islets responded with increased respiratory rates and insulin secretion at high concentrations of pyruvate, while primary islets remained unresponsive (Fig. 3c,d). This is indicative of a retention of immature metabolic characteristics in SC-islets as genes responsible for pyruvate sensitivity are ‘disallowed’ in adult islets46. Direct stimulation of mitochondrial metabolism using glutamine and leucine triggered similar insulin release in both primary islets and SC-islets, while the increase in respiratory rates was slightly higher for primary islets (Fig. 3e,f).

Since oxidative glucose metabolism is considered essential for the activation of the triggering pathway of insulin secretion, we next sought to clarify if a compensatory metabolic amplifying pathway might help explain SC-islet function despite the low oxidative metabolic response. We therefore exposed SC-islets and adult islets to high glucose under tolbutamide stimulation to determine the degree of insulin secretion occurring independently from KATP-channel closure. S7w3 and w6 SC-islets demonstrated a stronger initial insulin secretion response to tolbutamide than adult islets. Subsequent glucose-dependent metabolic amplification was detected in S7w3 SC-islets, but it was transient and lower compared with the initial KATP-channel dependent secretion (Fig. 3g). Conversely, adult islets displayed a sustained KATP-channel independent glucose-responsive amplification more similar in magnitude to that of KATP-channel dependent secretion, as has been reported in previous studies26.

Taken together, glucose processing seems aberrant or immature in SC-islets, resulting in undetectable mitochondrial respiratory responses. However, mitochondrial activity seems intact since respiration increases and dynamic insulin release can be elicited with other direct mitochondrial substrates, suggesting this discrepancy is not due simply to a low proportion of beta cells in SC-islets. Glucose-dependent insulin secretion independent of the KATP-channel is weakly present in SC-islets, suggesting metabolic amplification is a minor factor in explaining the discrepancy between robust insulin secretion and weak glucose-responsive respiration.

SC-islets demonstrate an immature glucose metabolism. To further probe the discrepancy between SC-islet functionality and low respiratory coupling we investigated how glucose metabolism differed between primary islets and SC-islets. We performed metabolite tracing analyses using uniformly labeled [U-13C6]-glucose comparing S7w0, w3 and w6 SC-islets together with primary adult islets, under low (3 mM) and high labeled glucose (17 mM) conditions (Fig. 4a). Beta cell glucose-sensing is mediated in part by the hexokinase step of glycolysis47. Over the course of SC-islet maturation, we detected a reduction in the ratio of labeled glucose-6-phosphate (G6P) to labeled glucose under both low and high glucose
conditions (Fig. 4b), suggesting a tighter control of glucose uptake and phosphorylation. This pattern is also evident from the relative abundances of both labeled G6P and residual labeled glucose under low glucose conditions (Supplementary Fig. 3a). Only primary islets displayed a trend for glucose-concentration-dependent increases in the G6P/glucose ratio, which may indicate a more complete degree of regulation of the hexokinase step. We also observed reduced labeled levels of 3-phosphoglycerate (3-PG) and phosphoenolpyruvate (PEP) in SC-islets compared with primary islets, despite similar levels of labeled dihydroxyacetone phosphate (DHAP) (Fig. 4c). These results are consistent with a proposed glycolytic 'bottleneck' due to reduced GAPDH activity. A significant decrease in the production of labeled lactate was a strong characteristic of SC-islet maturation during S7 (Fig. 4d) and is in agreement with studies that link lactate overproduction to reduced GSIS. The diversion of 3-PG into de novo serine and glycine biosynthesis, which is low in primary islets but significantly higher in less mature SC-islets, is another possible avenue of aberrant glucose metabolism (Fig. 4e).

SC-islets showed increased labeled glucose incorporation into the core tricarboxylic acid (TCA) cycle metabolites citrate, alpha-ketoglutarate (αKG), fumarate and malate upon stimulation with high glucose, but the response was clearly lower than in primary islets (Fig. 4f). Ratiometric analyses of labeled lactate/pepyruvate and labeled cis-aconitate-3-phosphoglycerate further demonstrated that metabolic trafficking of pyruvate is biased towards lactate production in SC-islets, and citrate/isocitrate formation in primary islets (Fig. 4g). We inferred flux through the TCA cycle by tracing the degree of 13C-glucose-derived carbon incorporation into each TCA metabolite. Primary islets showed enhanced oxidative TCA cycling for citrate, αKG, fumarate and malate compared with SC-islets (Supplementary Fig. 3b–e), as well as enhanced flux through the anaplerotic pyruvate carboxylase reaction, which resulted in a higher proportion of M+3 malate and fumarate isotopologues (Supplementary Fig. 3b–e). A ratiometric analysis of M+2/M+3 malate isotopologues indicated an increase in the bias towards the anaplerotic pyruvate carboxylase reaction during SC-islet maturation, towards the level seen in primary adult islets (Supplementary Fig. 3f).

Aspartate and glutamate are components of the malate–aspartate redox shuttle, a key constituent of beta cell metabolism that supports glucose-stimulated insulin secretion. Primary islets used a significantly higher proportion of glucose-derived carbons to generate these amino acids than SC-islets (Fig. 4h), as well as a significantly higher amount of labeled glutathione (GSH) (Fig. 4i). In contrast, the synthesis of labeled glycine (another GSH component) was barely detectable in primary islets (Fig. 4c). Of note, primary islets maintained higher total levels of reduced and oxidized forms of glutathione and the electron carriers NAD and NADP (Supplementary Fig. 3h). This is also reflected in the glucose concentration-dependent shifts in NAD+/NADH ratio, which were significantly more responsive in primary islets than in SC-islets (Supplementary Fig. 3g).

We next determined the extent of glucose-dependent changes in ATP/ADP ratio, an important determinant of the KATP-channel-dependent triggering pathway. SC-islets displayed a low, but nonsignificant, increase in the ATP/ADP ratio following glucose stimulation, as determined by metabolomic data and enzymatic assay (Supplementary Fig. 3i–j). In contrast, primary islets displayed a significant degree of glucose coupling to ATP/ADP ratio shifts.

Other metabolic pathways have been proposed to work in concert with the canonical oxidative phosphorylation-coupled insulin secretion model. The PEP cycle is one such model that has been suggested to function through anaplerotic regeneration of PEP from mitochondrial oxaloacetate. A hallmark of such cycling is the presence of M+2 labeled PEP and pyruvate, as oxidative generation of M+2 oxaloacetate would also be used in PEP regeneration. However, we were unable to detect M+2 PEP in SC-islet or primary islet samples (Supplementary Fig. 3k), and only a low percentage of M+2 pyruvate (<5%), which is in agreement with another recent study.

Reductive carboxylation of αKG to isocitrate and citrate via the IDH2 enzyme to fuel cytosolic redox reactions has been proposed recently as another mechanism of modulating insulin release in beta cells. Using 13C5-glutamine labeling, we observed that such reactions do occur in SC-islets, demonstrated by the high degree of M+5 cis-aconitate enrichment, an isotopologue that could only be generated by such a reductive carboxylation reaction (Supplementary Fig. 3l). By tracking the isotopolog profile of M+3 malate, we could infer the export of citrate (or isocitrate) from the mitochondria as a component of the pyruvate–citrate, pyruvate–malate and/or glycerolipid/FFA cycle (Supplementary Fig. 3l). We detected the generation of labeled pyruvate following labeled glutamine treatment, demonstrating some degree of pyruvate regeneration from TCA metabolites (Supplementary Fig. 3l).

Thus, primary human islets and SC-islets differ not only in their core TCA cycle turnover and respiratory rates under glucose stimulation, but also in the production of TCA-derived metabolites and redox pathway components. Despite the differences in both glycolytic and mitochondrial glucose metabolism, SC-islets do display dynamic glucose-sensitive insulin secretion responses.

**SC-islets control the glycemia of mice in vivo.** To investigate the in vivo functional potential of immature (S7w0) and more mature (S7w3) SC-islets, we implanted them under the kidney capsule of nondiabetic mice (Supplementary Fig. 4a). Circulating human C-peptide was detectable at 1 month postengraftment in all engrafted mice. However, mice engrafted with S7w3 SC-islets demonstrated twofold higher human C-peptide levels at 2 and 3 months than S7w0 engrafted mice (Supplementary Fig. 4b).
Overview of glucose trafficking

Adult islets
SC-islets

SC-Islets S7w0
SC-Islets S7w3
SC-Islets S7w6
Adult islets

Low glucose
High glucose

Glucose
DHAP
3-PG
PEP
Pyruvate

Serine/glycine

Aspartate
Glutamate

GSH

TCA Cycle

Malate
Fumarate
Citrate

Glucose
DHAP
3-PG
PEP
Pyruvate

Serine/glycine

Aspartate
Glutamate

GSH

TCA Cycle

Malate
Fumarate
Citrate
Fig. 5 | Single-cell transcriptomic profiling of stem cell derived islet cells. a, Experimental outline for scRNAseq transcriptomic profiling of SC-islets at the end of in vitro culture stages 5 (S5) and 6 (S7w0) and at week 3 (S7w3) and week 6 of S7 culture (S7w6), together with grafts retrieved after 1 (M1), 3 (M3) and 6 months (M6) postimplantation. b, UMAP-base embedding projection of an integrated dataset of 46,261 SC-derived endocrine cells and adult human islet cells\cite{1050}, colored by time and sample of origin. c, Clustering of the dataset in b into different cell types. d, Relative expression of marker genes for pancreatic progenitor cells (PDX1, NKX6-1, \textit{NEUROG3}) and alpha- (\textit{GCG}), delta- (\textit{SIX3}) and mature beta cell markers (\textit{INS}, \textit{MAFA}, \textit{SNAP25}). e, UMAP projection of the beta cell cluster indicating the relative expression of insulin (\textit{INS}) and alpha- (\textit{NEUROG3}), \textit{GCG}, SST) and alpha- (\textit{MAFA}, \textit{SNAP25}) genes for pancreatic progenitor cells (\textit{ESRRG}, \textit{INSR}, \textit{RIPK4}, \textit{GSK3B}, \textit{MTHFD2}, \textit{ITGB1}) cells. Dashed line indicates the beta cell origin of the trajectory. f, Pseudotime color scale. Earlier pseudotemporal points, the origin of the trajectory, are indicated in blue, and later pseudotemporal points in yellow on the pseudotime scale. g, PCA of the beta cell populations (Fig. 1e) coming from each independent sample with different time of origin (S5 to Adult islets) is represented. h, Clustering of beta cells according to their transcriptional similarity into early, late and adult beta cluster. i, Fractional contribution to each early, late and adult beta clusters of beta cells from different times of origin. j, UMAP projection of the beta cell cluster with RNA velocity vectors overlaid. Cells are annotated by latent-time dynamics. Earlier latent timepoints, the origin of the trajectory, are indicated in blue, and later pseudotemporal points in yellow on the pseudotime color scale. k, l, m, n, Relative expression levels of example genes that are upregulated (m) or downregulated (n) along the pseudotime trajectory inferred in l.
Correspondingly, blood glucose levels at 3 months were lower in S7w3 SC-islet engrafted animals (Supplementary Fig. 4c) and reached the human glycemic set point (4.5 mM) by 3 months postengraftment, as reported in primary islet engraftment studies. Glucose tolerance tests showed regulated insulin secretion in response to glucose injection in mice carrying both types of grafts (Supplementary Fig. 4d), but the glucose clearance was more rapid in S7w3 engrafted mice (Supplementary Fig. 4e,f). Next, we tested whether the S7w3 SC-islet grafts could sustain normoglycemia after streptozotocin (STZ)-induced loss of endogenous mouse beta cells. Glucose tolerance tests before and after STZ treatment (after 4 months of engraftment, with assays at 5 months postengraftment) showed that both control and STZ-treated animals presented robust glucose-regulated C-peptide secretion (Supplementary Fig. 4g). Despite C-peptide levels being lower in the STZ group, the glucose levels were similarly controlled in both groups (Supplementary Fig. 4h). The proportions of INS+ and GCG+ cells in the graft were not affected by the STZ treatment (Supplementary Fig. 4i,j). After removal of the engrafted kidney, the blood glucose levels increased sharply (Supplementary Fig. 4k), demonstrating that the engrafted SC-islets were actively controlling the glycemia of the diabetic mice. Extended in vitro culture in S7 conditions thus confers SC-islets a degree of maturation that results in improved functionality upon engraftment in vivo.

**SC-islets transcriptionally mature in vitro and in vivo.** To investigate the transcriptional changes associated with in vitro and in vivo SC-islet maturation, we performed single-cell RNA (scRNA) sequencing on SC-islets during in vitro differentiation (S5, S7w0, S7w3 and S7w6), as well as SC-islet grafts retrieved at 1, 3 and 6 months postengraftment (Fig. 5a). We obtained a dataset comprising 38,978 cells, which we integrated with previously published datasets from S5 hPSC-derived cells (4,458 cells) and human adult islets (19,435 cells). The full integrated dataset had a total of 62,871 cells, including 46,261 endocrine cells that were selected for further study (Supplementary Fig. 5a–d and Supplementary Table 1; Methods).

The endocrine cell dataset was clustered according to time of origin and cell identity (Fig. 5b–c, Supplementary Fig. 5e–g and Supplementary Table 1). These populations reconstructed a differentiation continuum, from multipotent pancreatic progenitors, through intermediate differentiating stages and finally into beta (44%), alpha (33%), delta and gamma cells (Fig. 5c–d, Supplementary Tables 1 and 2 and Supplementary Fig. 5f–i). We also detected a cell population expressing FEV (a beta cell developmental transcription factor) that could represent SC-EC (Supplementary Fig. 5i).

To cross-reference this and other cell types, we integrated our dataset with those described by Veres et al. Most of the cell types identified in our dataset clustered with the equivalent cell populations from the Veres et al. dataset (Supplementary Fig. 7). The cells identified as SC-EC by Veres et al. also clustered together with our FEV+ SC-EC cells, suggesting that they are the same cell type. However, the proportion of SC-EC cells identified in our dataset declined to undetectable levels during SC-islet maturation (Supplementary Fig. 7a).

We then focused on SC-beta cell subpopulations to determine the transcriptional changes associated with functional maturation (Fig. 5d–e and Supplementary Fig. 5j). Principal component and correlation analyses indicated that S7w3 and S7w6 SC-beta cells were transcriptionally more similar to grafted and adult beta cells than to S7w0 SC-beta cells (Fig. 5g and Supplementary Fig. 5k). We next examined the average expression of known mature beta cell marker genes across each individual sample (Fig. 5f). INS, G6PC2 and SIX2 gene expression increased early in vitro culture, whereas other mature beta cell markers such as HOPX, UCN3, IAPP, CPE and FXYD2 were upregulated only upon engraftment. CHGB and MAFA expression was sharply upregulated at 6 months postengraftment, suggesting the need of extended in vivo maturation for the upregulation of these genes. Interestingly RBP4 and SIX3 were detected primarily only in adult beta cells (Fig. 5f).

Beta cell differentiation is not a synchronous and homogeneous process, as evidenced by the coclustering of beta cells from different stages of maturation (Fig. 5b). To reduce the interference introduced by heterogeneous populations, SC-beta cells were unbiasedly clustered by transcriptional similarity, rather than by time of origin, into ‘SC-early’, ‘SC-late’ and ‘Adult beta’ categories (Fig. 5i). As expected, SC-beta cell proportions in the SC-late and Adult beta categories increased with the progression of time in vitro and in vivo (Fig. 5). Cells in the Adult beta category presented higher expression of genes related to insulin secretion (PCSK1, CPE, CHGB, ABCG8, FXYD2), beta cell maturation (MAFA, GDF15) and oxidative phosphorylation (OXPHOS) (Supplementary Table 3).

RNA velocity estimation (Fig. 5k) and pseudotemporal ordering (Fig. 5l) of beta cell subpopulations enabled us to infer a differentiation trajectory to investigate the genes differentially regulated upon beta cell maturation (Supplementary Fig. 5l–m and Supplementary Table 4). The expression of genes associated with pancreatic beta cell maturation and insulin secretion increased with pseudotime, together with ribosomal and HLA genes (Fig. 5m). Glutathione metabolism genes were also upregulated with pseudotime (Fig. 5m and Supplementary Fig. 5n), consistent with our metabolomic findings (Fig. 4i). Conversely, the expression of genes related to miTORC1 and MAPK signaling, cholesterol homeostasis, mitosis and MYC targets decreased with pseudotime (Fig. 5n). Genes associated with axon guidance (ROBO1, ROBO2) and adherens junctions were downregulated with pseudotime, indicating changes in cell migration, adhesion and cytoskeletal properties consistent with the observed cytoarchitectural changes upon maturation (Fig. 5n, Supplementary Fig. 5l–m and Supplementary Table 4). We calculated average expression levels for the genes in these processes to understand their dynamics across our dataset, showing that these mature beta program scores increased with time in vitro and in vivo, while miTORC1 and mitosis programs decreased (Fig. 6a and Supplementary Fig. 5o).

During SC-islet maturation following engraftment, SC-beta cells further upregulated genes related to beta cell maturation (G6PC2, UCN3, MAFA), insulin secretion (CHGB, GABRA2), cAMP signaling, OXPHOS and fatty acid metabolism. Whereas genes associated with miTORC1 signaling, cholesterol homeostasis and beta cell developmental transcription factors (FEV, ISL1) were downregulated (Fig. 6b–d and Supplementary Table 5). We performed an integrative analysis to understand the transcriptional differences between primary adult beta cells and the endpoints of SC-beta maturation in vitro (S7w6) and in vivo (M6) (Supplementary Fig. 5l–m and Supplementary Table 4). Glycolysis, OXPHOS and miTORC1 signaling-related genes were upregulated in more mature cells, while genes related to MAPK-, WNT- and estrogen-signaling pathways were downregulated (Supplementary Table 6 and Supplementary Fig. 6a–e). The expression of voltage-gated Na+ channel subunits was also downregulated upon extended maturation (Supplementary Fig. 6f), consistent with our electrophysiology findings (Fig. 2c).

**Transcriptional markers of in vitro SC-beta maturation.** We then investigated the transcriptional changes specifically occurring during in vitro maturation (S7w0 to S7w6) to better understand the acquisition of in vitro function. We found that beta cell maturation markers (IAPP, G6PC2, GLI3), together with oxycocytosis-related genes (KCNJ11, RAB27A, VAMP8), unfolded protein response pathway genes (TRIB3, DDIT3, HSPA5) and immediate-early transcription factors (FOS, JUN) were all significantly upregulated (Fig. 6e–f and Supplementary Table 7). Transcription factors involved in beta cell differentiation (SIX2, HOPX, ZBTB20) were also upregulated.

**In vitro SC-beta maturation.** We then investigated the transcriptional changes specifically occurring during in vitro maturation (S7w0 to S7w6) to better understand the acquisition of in vitro function. We found that beta cell maturation markers (IAPP, G6PC2, GLI3), together with oxycocytosis-related genes (KCNJ11, RAB27A, VAMP8), unfolded protein response pathway genes (TRIB3, DDIT3, HSPA5) and immediate-early transcription factors (FOS, JUN) were all significantly upregulated (Fig. 6e–f and Supplementary Table 7). Transcription factors involved in beta cell differentiation (SIX2, HOPX, ZBTB20) were also upregulated.

**In vitro SC-beta maturation.** We then investigated the transcriptional changes specifically occurring during in vitro maturation (S7w0 to S7w6) to better understand the acquisition of in vitro function. We found that beta cell maturation markers (IAPP, G6PC2, GLI3), together with oxycocytosis-related genes (KCNJ11, RAB27A, VAMP8), unfolded protein response pathway genes (TRIB3, DDIT3, HSPA5) and immediate-early transcription factors (FOS, JUN) were all significantly upregulated (Fig. 6e–f and Supplementary Table 7). Transcription factors involved in beta cell differentiation (SIX2, HOPX, ZBTB20) were also upregulated.
**Fig. 6 | Transcriptional maturation of stem-cell-derived beta cells.**

**a.** Mature beta cell signature of SC-beta and adult beta cells from different times of origin. **b.** Gene sets enriched in the in vivo implanted SC-beta cells upregulated and downregulated genes compared with in vitro SC-beta cells. **c.** Expression of selected marker genes upregulated in the in vivo SC-beta cells from S7w0, S7w3 and S7w6 times of origin. **d.** Expression of selected marker genes downregulated in the in vivo SC-beta cells from S7w0, S7w3 and S7w6 times of origin. **e.** Gene sets enriched in the in vivo implanted SC-beta cells upregulated and downregulated genes compared with in vitro SC-beta cells from different times of origin. **f.** Average expression of genes associated with mature beta cell hallmark processes in individual SC-beta cell in vitro samples from different times of origin. **g.** Average expression of glucose metabolism, noncanonical coupling factors and disallowed genes in individual SC-beta cell in vitro samples from different times of origin. **h.** Immunostaining for disallowed gene LDHA protein and insulin (INS) of in vitro SC-islets from S7w0, S7w3 and S7w6 timepoints. Scale bar, 100 μm. **i.** Quantification of LDHA positive cells out of all INS positive cells in SC-islets from S7w0, S7w3 and S7w6. Data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 One-way ANOVA with Welch’s correction; n = 3. **j.** Expression of genes associated with insulin secretion and oxidative phosphorylation in SC-beta cells with a high or low mature beta signature. **k.** Summary of functional and transcriptomic features of SC-islet maturation in vitro and in vivo.
and cell cycle inhibition (CEBP transcription factor family57, and SCRTT58,59), were also upregulated in S7w6 SC-beta cells, together with ligands (WNT4, TFF3)60,61 and receptors associated with beta cell function (GGCGR, Gabra2, FFAR1)62 (Fig. 6f). In line with the functional results, these transcriptional changes overall suggest that S7w6 beta cells present improved insulin production and exocytosis (Fig. 1j and Fig. 2g), which are associated with increased endoplasmic reticulum-stress levels and reduced proliferation (Fig. 1d)—all important hallmarks of mature beta cells22,63.

Glycolysis- and TCA cycle-related genes were upregulated during in vitro maturation, concomitantly with the increased expression of genes involved in noncanonical coupling processes that may act to trigger insulin exocytosis (PC, PCK2, SLC25A1, IDH1) (Fig. 6g). Contrastingly, the expression of disallowed genes such as HK1, monocarboxylate transporter SLC16A1 (MCT1; Fig. 6g). Given the heterogeneity observed in Ca2+ signaling, we investigated to what extent SC-islet functionality could be driven by a subpopulation of SC-beta cells. Mature beta cell marker expression was indeed heterogeneous across SC-beta cells (Supplementary Fig. 6g). We therefore calculated a gene expression score to classify them into high and low ‘mature beta signature’ (Supplementary Table 8). Consistent with our previous analyses, genes associated with insulin secretion and OXPHOS were upregulated in the mature beta signature high cells, suggesting that this subpopulation could represent beta cells better suited for improved functionality (Fig. 6j).

We have made our single-cell datasets available via an interactive single-cell portal to facilitate the access and exploration of this resource (https://singlecell.broadinstitute.org/single_cell/study/SCP1526/).

Discussion

Here, we describe an optimized protocol to generate human SC-islets that display glucose-sensitive insulin release and endocrine cell composition similar to that of primary islets. Moreover, through ind-depth functional assays, cell physiology analyses, metabolic tracing experiments and scRNAseq transcriptomic data throughout 6 weeks of in vitro maturation and 6 months of mouse engraftment, we show the temporal acquisition of metabolic programs and gene regulatory changes that contribute to beta cell functional maturation (Fig. 6k).

The acquisition of function observed throughout the final 6-week maturation step of in vitro differentiation correlated with a drop in markers of cell proliferation, a progressive rearrangement of SC-islet cytoarchitecture, a decrease in polyhormonal and SC-EC cell populations and a marked increase in alpha cell differentiation, all of which have been individually implicated in fetal islet development and the enhancement of mature islet function7,12,13,15,16,21,22,23,24.

Despite the stable number of monohormonal beta cells throughout S7 maturation, a clear physiological and transcriptomic heterogeneity is present. Only about two-thirds of SC-beta cells possess glucose-induced Ca2+ responses, in agreement with the scRNAsseq analyses, which demonstrate that beta cell subpopulations shift in proportion throughout in vitro differentiation and in vivo engraftment. Thus, beta cell maturation does not occur synchronously at a given time, but rather consists of progressive heterogeneous changes. Targeted beta cell enrichment approaches to exploit this finding, have been used successfully to obtain more functional SC-islets25. We envision that strategies to increase the proportion of functional beta cells, on the basis of knowledge gathered from single-cell analyses such as those presented here, will pave the way to improved differentiation protocols without the need for enrichment procedures.

Perhaps the most intriguing implication of this heterogeneity is the ability of SC-islets to display islet-like functional properties in vitro, despite the large differences between adult beta cell transcriptomic profiles and metabolic coupling pathways. In contrast to primary islets, SC-islets present significantly lower mitochondrial TCA metabolite enrichment, minimal ATP/ADP and NAD+/NADH ratio shifts, and absent respiration spikes during glucose stimulation, all of which are key aspects of the canonical triggering pathway in functional beta cells25. Modulation of the KATP-channel in SC-islets does show that glucose-induced Ca2+ influx and insulin release largely depend on KATP-channel closure. Therefore, while many elements of the stimulus-secretion coupling process are functional in at least a subset of SC-beta cells, the SC-islets as a whole are not robustly coupled metabolically to the canonical triggering pathway.

To further investigate this discrepancy in in vitro SC-islet function, we used metabolite tracing to assay other proposed metabolic coupling factors leading to insulin release. Pathways, such as the PEP cycle24,64, the pyruvate-isocitrate cycle65, the pyruvate–malate cycle66 and the malate–aspartate redox shuttle67 have all been reported to contribute to the coupling of glucose metabolism to insulin secretion7. Our results suggest that the PEP cycle and cytosolic redox pathways may contribute, at least partly, to the robust glucose-dependent secretion response seen in SC-islets.

The tightened regulation of the hexokinase step of glycolysis, a key control point in glucose-sensing metabolism in primary beta cells, also develops over the course of in vitro SC-islet maturation, correlating with an increasing glucose concentration threshold, and lowered basal exocytosis kinetics. We also see this across metabolic and transcriptomic data, with a more stringent production of glucose-6-phosphate relative to glucose abundance, a decrease in disallowed HK1 expression, and an increase in expression of the glucose transporter SLC2A2 and the G6PC2 phosphatase genes.

Despite positive functional outcomes, the maturation of beta cells is not complete in vitro. We see that, following mouse engraftment over a period of 6 months, a plethora of transcriptomic changes occur indicating continued maturation. Beta cell maturation markers such as G6PC2 and SIX2 are upregulated in the first few weeks of in vitro maturation. HOPX and UCN3 are only upregulated briefly after implantation, whereas MAFA and RBP4 display upregulation only after 3–6 months of engraftment, correlating with the humanization of mouse blood glucose levels and dynamic in vivo function. We also detect transcriptional modulation of energy-sensing mTORC1 signaling-related genes, particularly after engraftment, consistent with its reported role in postnatal mouse beta cell maturation and islet development68,69. This pattern is also reflected in the upregulation of mitochondrially encoded electron transport chain genes, which may indicate a stronger coupling of mitochondrial oxidative metabolism to glucose following implantation.

In summary, we present a protocol for the reliable generation of physiologically relevant SC-islets with cytoarchitecture and functionality similar to those of adult primary islets. Moreover, the multifaceted analysis that we present here constitutes a comprehensive effort to thoroughly benchmark maturing SC-islets against human primary adult islets, considered the ‘gold standard’ in the field. The combination of such integrated analyses with refined differentiation protocols will guide the generation of further improved SC-islets for the modeling of beta cell dysfunction, drug-screening purposes and cell replacement approaches, expanding both our understanding of the disease mechanisms and therapeutic possibilities to treat diabetes.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of
References

1. Balboa, D., Saarimäki-Vire, J. & Otonkoski, T. Concise review: human pluripotent stem cells for the modeling of pancreatic β-cell pathology. Stem Cells 37, 33–41 (2019).

2. Nair, G. G. et al. Recapitulating endocrine cell clustering in culture augments recovery of glucose-induced insulin secretion in normal human islets. Diabetologia 55, 358–373 (2012).

3. Rezania, A. et al. A human β-cell line with drug inducible excision of immortalizing transgenes. Mol. Metab. 7, e10851 (2018).

4. Obior, B. T. & Newton, T. C. β-cell proliferation in response to glucose-glucose pathway. Diabetologia 57, 169–179 (2014).

5. Basford, C. L. et al. The functional and molecular characterisation of human embryonic stem cell-derived insulin-positive cells compared with adult pancreatic beta cells. Diabetologia 55, 358–373 (2012).

6. Davis, J. C. et al. Glucose response by stem cell-derived β-cells in vitro is inhibited by a bottleneck in glycolysis. Cell Rep. 31, 107623 (2020).

7. Nourouzi, M. et al. Efficient generation of Nkx6.1+ pancreatic progenitors from multiple human pluripotent stem cells. Stem Cell Reports 4, 591–604 (2015).

8. Toyoda, T. et al. Rho-associated kinases and non-muscle myosin IIIs inhibit the differentiation of human iPSCs to pancreatic endoderm. Stem Cell Reports 9, 419–428 (2017).

9. Ryoo, P. et al. Aurora kinase A is critical for the Nkx6.1 mediated β-cell proliferation pathway. Islets 7, e1027854 (2015).

10. Rezania, A. et al. Production of functional glucagon-secreting α-cells from human embryonic stem cells. Diabetes 60, 239–247 (2011).

11. Rezania, A. et al. Enrichment of human embryonic stem cell-derived Nkx6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells in vivo. Stem Cells 31, 2432–2442 (2013).

12. Kelly, O. G. et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. Nat. Biotechnol. 29, 750–756 (2011).

13. Ramond, C. et al. Understanding human fetal pancreas development using subpopulation sorting, RNA sequencing and single-cell profiling. Development 145, dev165480 (2018).

14. Riopel, M., Li, J., Fellows, G. F., Goodyer, C. G. & Wang, R. Ultrastructural morphology of mammary gland stem cells. J. Histochem. Cytochem. 57, 811–824 (2009).

15. Gregg, B. E. et al. Formation of a human β-cell population within pancreatic islets is set early in life. J. Clin. Endocrinol. Metab. 97, 3197–3206 (2012).

16. Henquin, J.-C., Dufrane, D., Kerr-Conte, J. & Nenquin, M. Dynamics of glucose-induced insulin secretion in normal human islets. Ann. J. Physiol. Endocrinol. Metab. 309, E640–E650 (2015).

17. Henquin, J.-C. Regulation of insulin secretion: a matter of phase control and amplitude modulation. Diabetologia 52, 739–751 (2009).

18. Henquin, J., Dufrane, D. & Nenquin, M. Nutrient control of insulin secretion in isolated normal human islets. Diabetes 55, 3470–3477 (2006).

19. Lyon, J. et al. Research-Focused isolation of human islets from donors with and without diabetes at the Alberta Diabetes Institute IsletCore. Endocrinology 157, 560–569 (2016).

20. Henquin, J.-C. & Nenquin, M. Immaturity of insulin secretion by pancreatic islets isolated from one human neonate. J. Diabetes Investig. 9, 270–273 (2018).

21. Rorsman, P. & Braun, M. Regulation of insulin secretion in human pancreatic islets. Annu. Rev. Physiol. 75, 155–179 (2013).

22. Gandasi, N. R. et al. Glucose-dependent granule docking limits insulin secretion and is decreased in human type 2 diabetes. Cell Metab. 27, 470–478.e4 (2018).

23. Wikstrom, J. D. et al. A novel high-throughput assay for islet respiration reveals uncoupling of rodent and human islets. PLoS ONE 7, e33023 (2012).

24. Nicholls, D. G. The pancreatic β-cell: a bioenergetic perspective. Physiol. Rev. 96, 1385–1447 (2016).

25. Andruen kindness, L. E. et al. Characterization of stimulus-secretion coupling in the human pancreatic EndoC−βH1 cell beta line. PLoS ONE 10, e0120879 (2015).

26. Otonkoski, T. et al. Physical exercise-induced hypoglycemia caused by failed silencing of monocarboxylate transporter 1 in pancreatic β-cells. Am. J. Hum. Genet. 81, 467–474 (2007).

27. Ainscow, E. K., Zhao, C. & Rutter, G. A. Coacervation of lactate dehydrogenase-A perturbs beta-cell mitochondrial metabolism and insulin secretion. Diabetes 49, 1149–1155 (2000).

28. Sasaki, M. et al. Reduction of reactive oxygen species ameliorates metabolism-secretion coupling in islets of diabetic GK rats by suppressing lactate overproduction. Diabetes 62, 1996–2003 (2013).

29. Odegard, M. L. & Newton, T. C. Mechanisms controlling pancreatic islet cell function in insulin secretion. Nat. Rev. Mol. Cell Biol. 22, 142–158 (2021).

30. Lewandowski, S. L. et al. Pyruvate kinase controls signal strength in the insulin secretory pathway. Cell Metab. 32, 736–750.e5 (2020).

31. Ferdaoussi, M. et al. Isocitrate-to-SENP1 signaling amplifies insulin secretion and rescues dysfunctional β-cells. J. Clin. Invest. 125, 3847–3860 (2015).

32. Henquin, J.-C. & Nenquin, M. Immaturity of insulin secretion by pancreatic β-cells from CD177+ human in vitro islets. Cell Rep. 7, 107623 (2020).

33. Sasaki, M. et al. Reduction of reactive oxygen species ameliorates metabolism-secretion coupling in islets of diabetic GK rats by suppressing lactate overproduction. Diabetes 62, 1996–2003 (2013).

34. Odegard, M. L. et al. The multipotential 2-coupled rationally is part of a metabolic pathway that mediates glucose- and glutamine-stimulated insulin secretion. J. Biol. Chem. 285, 16530–16537 (2010).

35. Zhang, G.-F. et al. Reductive TCA cycle metabolism fuels glutamine- and glucose-stimulated insulin secretion. Cell Metab. 33, 804–817.e5 (2021).

36. Balboa, D. et al. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. eLife 7, e35519 (2018).

37. Rezania, A. et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 61, 2016–2029 (2012).

38. Kroon, E. et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat. Biotechnology 26, 443–452 (2008).

39. Rodriguez-Diaz, R. et al. Paracrine interactions within the pancreatic islet determine the glycemic set point. Cell Metab. 27, 549–558.e4 (2018).

40. Krentz, N. A. et al. Single-cell transcriptomic profiling of mouse and hESC-derived pancreatic progenitors. Stem Cell Reports 11, 1531–1564 (2018).

41. Xie, Y. et al. Pseudotime ordering of single human β-cells reveals states of insulin production and unfolded protein response. Diabetes 67, 1783–1798 (2018).

42. Arda, H. E. et al. Age-dependent pancreatic gene regulation reveals mechanisms governing human β-cell function. Cell Metab. 23, 909–920 (2016).

43. Haninlin, S. et al. Differentiation of human stem cells resembles fetal, not adult, β cells. Proc. Natl Acad. Sci. USA 111, 3038–3043 (2014).

44. Segerstolpe, Å. et al. Single-cell transcriptomic profiling of human pancreatic islets and without diabetes at the Alberta Diabetes Institute IsletCore.
62. Adams, M. T., Gilbert, J. M., Hinojosa Paiz, J., Bowman, F. M. & Blum, B. Endocrine cell type sorting and mature architecture in the islets of Langerhans require expression of Roundabout receptors in β cells. Sci. Rep. 8, 10876 (2018).
63. Moede, T., Leibiger, I. B. & Berggren, P.-O. Alpha cell regulation of beta cell function. Diabetologia 63, 2064–2075 (2020).
64. Wortham, M. et al. Integrated in vivo quantitative proteomics and nutrient tracing reveals age-related metabolic rewiring of pancreatic β cell function. Cell Rep. 25, 2904–2918.e8 (2018).
65. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J. & Maechler, P. The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. J. Biol. Chem. 279, 55659–55666 (2004).
66. Sinagoga, K. L. et al. Distinct roles for the mTOR pathway in postnatal morphogenesis, maturation and function of pancreatic islets. Development 144, 2402–2414 (2017).
67. Helman, A. et al. A nutrient-sensing transition at birth triggers glucose-responsive insulin secretion. Cell Metab. 31, 1004–1016.e5 (2020).
68. Jaafar, R. et al. mTORC1-to-AMPK switching underlies β cell metabolic plasticity during maturation and diabetes. J. Clin. Invest. 129, 4124–4137 (2019).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022
Methods

In vitro culture and differentiation of hPSCs. Human embryonic stem cell line H1 (WA01, WiCell) was used for most of this study. In Supplementary Fig. 1h, iPSC-lines HEL24.3 (ref. 1) and HEL1.13.5-correlated were used as well. The hPSCs were cultured in Matrigel (Corning, catalog no. 354277) coated plates in Essential 8 (EB) medium (Thermo Fisher, catalog no. A1517001) and passaged using EDTA. To prepare the differentiation experiments, near-confluent plates of stem cells were dissociated using EDTA and seeded on new Matrigel coated plates in EB supplemented with 5–10 mM Rho-Associated kinase inhibitor (ROCKi, catalog no. Y-27632; Selleckchem catalog no. S1049) at a density of 0.02 million cells cm−2 to achieve confluent plates. To start the differentiation, the medium was changed to D0 medium 24 h postseeding. The differentiation was carried out using a seven-stage protocol combined from key publications40,41 and the patent WO2017222879A1. Complete media formulations are available in the Supplementary Table 9. On the third day of D6 culture, the SC-islets were transferred from the microwells to suspension culture on ultralow attachment plates (Corning, catalog no. CLS3471) placed on rotor spinning at 95 rpm. Media changes were performed daily until the first day of D6 culture and every 2–3 days thereafter.

In vitro culture of primary adult islets. Primary islets were provided by the Nordic Network for Islet Transplantation (Uppsala University) and University of Alberta IsletCore (Canada). They were maintained in CMRL1066 supplemented with 10% FBS, 20 mM HEPES (Gibco, catalog no. 15630-056), 2 mM Glutmax and 100 µM penicillin and 100 µg/ml streptomycin on ultralow attachment plates. Islet donor characteristics are listed in Supplementary Table 10.

Flow cytometry. Stage 4 cells and stage 7 SC-islets were dissociated with TrypLE for 5–10 min in a 37 °C water bath and resuspended in 5% FBS-containing PBS. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, catalog no. 554714) for 20 min. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 30 min in RT in Perm/Wash buffer (BD Biosciences, catalog no. 554714) containing 5% FBS. The cells were run on FACSCalibur cytometer (BD Biosciences); data were collected with CellQuest Pro v.4.0.2 (BD Biosciences) and analyzed with FlowJo v.10 (BD Biosciences). Antibodies are listed in Supplementary Table 11.

Immunohistochemistry and image analysis. Samples of S7 SC-islets were fixed for 2 h and samples of explanted SC-islet grafts fixed overnight in 4% PFA and embedded in paraffin. Sections (5 µm) were deparaffinized and subjected to HIER in 0.1% citrate buffer. The slides were blocked with UV-block (Thermo Scientific, catalog no. TA-125-PBQ), and incubated with primary antibodies diluted in 0.1% Tween-20 overnight in +4 °C. Secondary antibodies for 30 min in RT in Perm/Wash buffer (BD Biosciences, catalog no. 554714) containing 5% FBS. The cells were run on FACSCalibur cytometer (BD Biosciences); data were collected with CellQuest Pro v.4.0.2 (BD Biosciences) and analyzed with FlowJo v.10 (BD Biosciences). Antibodies are listed in Supplementary Table 11.

Electrophysiology. SC-islets were dispersed into single cells in cell dissociation buffer (Thermo Fisher Scientific) supplemented with trypsin (0.005%, Life Technologies), washed and plated in serum-containing medium on 22 mm polylysine-coated coverslips, allowed to settle overnight, and then transduced with adenosine for enhanced green fluorescent protein under control of the RIP2 promoter to identify beta cells. Patch-clamp recordings were performed using an EPC-9 patch amplifier with PatchMaster v.2.90 software (HEKA Electronics). electrodes (resistance 2–4 MΩ) were pulled from borosilicate glass capillaries, coated with SiTygard and fire-polished. Cells were superfused with an extracellular solution containing 138 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM d-glucose, and 5 mM HEPES, pH 7.4 adjusted with NaOH at a rate of 0.4 ml min⁻¹ at 32 °C. Voltage-dependent currents and exocytosis were measured in whole-cell voltage-clamp mode with an intracellular solution containing 125 mM Cs-glutamate, 10 mM CaCl₂, 10 mM MgCl₂, 0.05 mM EGTA, 3 mM Mg-ATP, 0.1 mM CAMP and 5 mM HEPES, pH 7.2 adjusted using NaOH. For current-voltage (IV) relationships, the membrane was depolarized from −70 mV to +80 mV (in 10 mV steps) lasting 50 ms each. Currents were compensated for capacitive transients and linear leak using a P/4 protocol. Na⁺ and Ca²⁺ current contributions were separated by quantifying the initial peak current (0–5 ms; Na⁺) and average sustained current (5–45 ms; Ca²⁺).

Exocytosis was quantified using the lock-in module of Patchmaster (30 mV peak-to-peak; 1 kHz); with a train of 14 x 200 ms depolarizations to 0 mV at 1.4 Hz. KATP currents were measured in whole-cell mode using a pipette solution containing 140 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 3 mM Mg-ATP and 10 mM HEPES, pH 7.2 adjusted using KOH. The cell was held at −70 mV, and ±10 mV pulses (10 ms duration) were applied alternately at a rate of 15 Hz before and after the application of 200 µM diazoxide.

Membrane potential was measured in perforated whole-cell configuration, using a pipette solution containing 76 mM K₂SO₄, 10 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.3 adjusted with KOH, access was established with amphotericin (0.25 mg/ml). Glucose was varied as indicated in the text.

Exocytosis imaging. To visualize granule exocytosis, cells treated as described for electrophysiology were additionally injected with adNPY-taOrange2 (a well-established marker for granule exocytosis in pancreatic β-cells) and imaged after 30–60 min using a custom-built lens-type TIRF microscope based on an AxioObserver Z1 with a x100/1.45 objective (Zeiss). Excitation was from two DPSS lasers at 491 and 561 nm (Cobolt) passed through a cleanup filter (catalog no. ZT405/488/561/640rpc; Chroma) and controlled with an acousto-optical tunable filter (Opton). Emitted light was collected using a sCMOS camera (Hamamatsu) and emission light was separated chromatically onto separate areas of an EMCCD camera (Koper QuantumEM 512SC) using an image splitter (Optical Insights) with a cutoff at 565 nm (catalog no. 565dxcr; Chroma) and emission filters (catalog nos. ET525/50m and 600/50m, Chroma). Scaling was 160 nm per pixel.

Electrophysiology and exocytosis imaging for each condition were contained in one movie with a total duration of 138 s, and analyzed using custom-written Matlab software. Movies are available as Supplementary Movies 1 and 2.
Anaspec) or the KCl, channel opener diazoxide (200µM, Sigma-Aldrich; to prevent spontaneous depolarizations) was also present. Where stated, exocytosis was evoked by rapidly depolarizing cells with elevated K+ (75 mM KCl equimolarly replacing NaCl in the standard solution, by computer-controlled local pressure ejection). Spontaneous glucose-dependent exocytosis was recorded for 3 min per cell after equilibration >20 min in the stated conditions (no diazoxide).

**[Ca2+]i imaging.** SC- and primary islets were loaded with the fluorescent indicator Fura-2 LR (ion Biosciences) by 1h incubation with 1µM of its acetoxymethyl ester at 37 °C in experimental buffer containing 138 mM NaCl, 4.8 mM KCl, 340/26 nm (center wavelength/half-bandwidth) and 386/23 nm interference filter (Semrock, Omicron Laserage Laserprodukte) equipped with 340 and 385 nm diodes and 340/26 nm (center wavelength/half-bandwidth) and 386/23 nm interference filters (Semrock, IDEX Health & Science, LLC) provided excitation light that was led to the microscope via a liquid light guide. Emission was measured at 510/40 nm using a 400 nm dichroic beam splitter and an Evolve 512 EMCCD camera (Photometrics). Image pairs at 340/386 nm were acquired every 2 s with the MetaFluor v.7.7 software (Molecular Devices). [Ca2+]i was calculated from the background-corrected Fura-2 LR 340/380 nm fluorescence excitation ratio from manually defined cell-sized regions of interest. The data are presented as example traces or heatmaps from individual islets and cells and as histograms of the [Ca2+]i change under different conditions. For the expression of ATP/ADP ratios, the apparent [Ca2+]i values determined after in vitro calibration with the salt form of Fura-2 LR in Ca2+-deficient and -saturated buffers were systematically lower in SC than adult islet cells. This discrepancy could not be explained by a real difference in [Ca2+]i.

To enable comparison between the preparations, the response to each treatment was calculated as difference in time-averaged [Ca2+]i from the prescored response normalized to [Ca2+]i at 3 mM glucose. The histograms show the percentage of cells at different normalized [Ca2+]i, responses using bin widths of 0.018, 0.013 and 0.06 for glucose, diazoxide, tolbutamid and KCl, respectively, in Fig. 2g, and 0.015, 0.01 and 0.03 for tolbutamide, tolbutamide with high glucose, exendin-4 and KCl in Supplementary Fig. 2f. For comparison of the basal [Ca2+]i, the signal was normalized and adjusted at high KCl. All calculations were made using built-in functions of the Igor Pro 8 software (WaveMetrics).

We performed a series of experiments with SC-islets transfected with adenosine vector expressing the Ca2+ reporter protein R-GECO1 under control of the rat insulin promoter RIP2. The islets were allowed to express the protein for 48 h before microscopy analysis. Infected islets were reincubated for 1 h in experimental buffer. We performed imaging as described above but using a 505–600 nm LED (Omicron Laserage Laserprodukte) and a 561/2 nm filter for excitation (Semrock), a z/050/488/561/640rpc-UF2 dichroic mirror (Chroma Technology Group) and a 609/62 nm filter (Semrock) for emission. The R-GECO1 data is presented as the fluorescence intensity normalized to the basal level of fluorescence before microscopy analysis. Infected islets were preincubated for 1 h in experimental buffer. We performed a series of experiments with SC-islets transduced with adenovirus expressing the FRET-based ATP reporter Epac-SH188 (ref. 74) and cultured in an open 50 µl chamber superfused with buffer at a rate of 160 µl min–1. The chamber holder and 50:1, 1.5 NA objective were maintained at 37 °C. A modified version of the manufacturer’s protocol. Briefly, from three to five SC-islets were added to 40 µl of either low (3 mM) or high (17 mM) glucose in KRB in each well of a 96-well plate (white-walled, clear bottom) (Thermo Fisher Scientific). After 15 min of incubation at 37 °C, 90 µl of ATP reaction mix was added per well and incubated for 1 min (Measurement A). Luminous measurements were taken using a Fluostar eAspiti (3,400/545). After 20 min, a second measurement was taken (Measurement B), the addition of 3 µl of ATP reaction mix per well. A third and final measurement was taken after another 1-min incubation (Measurement C). ATP/ADP ratio was calculated as Measurement A/Measurement C (Treatment A – Measurement B).

**ATP/ADP ratio assay.** Glucose-responsive ATP/ADP ratios were measured using the Bioluminescent ATP/ADP Ratio Assay Kit (Merck MAK135) following a modified version of the manufacturer’s protocol. Briefly, from three to five SC-islets were added to 40 µl of either low (3 mM) or high (17 mM) glucose in KRB in each well of a 96-well plate (white-walled, clear bottom) (Thermo Fisher Scientific). After 15 min of incubation at 37 °C, 90 µl of ATP reaction mix was added per well and incubated for 1 min (Measurement A). Luminous measurements were taken using a Fluostar eAspiti (3,400/545). After 20 min, a second measurement was taken (Measurement B), the addition of 3 µl of ATP reaction mix per well. A third and final measurement was taken after another 1-min incubation (Measurement C). ATP/ADP ratio was calculated as Measurement A/Measurement C (Treatment A – Measurement B).

**[U-13C5] glutamine.** (Cambridge Isotope Laboratories, catalog no. CLM 1822). The high glucose condition was also supplemented with 5 mM unlabeled leucine (Sigma). Islets were then incubated for 1 h at 37 °C and 5% CO2. After incubation, islets were washed in cold PBS before cell lysis and metabolite extraction in 75 µl of lysis buffer (80% acetoneitrile in dH2O). Islets were lysed with mild tituration before centrifugation at 10,000g for 10 min at 4 °C. Supernatant was transferred into Chromosol (03-FIV5) MS vials with a 300 µl glass insert (Thermo Fisher) and sealed with Chromacol caps with white presplit septa (Thermo Fisher), and then analyzed with LC-MS (Quadrupole Orbitrap, Thermo Fisher Scientific). The HPLC was equipped with a photonic ZIC-pHILIC column (150×2.1 mm, 5 µm) with a ZIC-pHILIC guard column (20×2.1 mm, 5 µm, Merck Scientific). A 5 µl aliquot of each sample was used for each assay. Metabolite separation was achieved by applying a linear gradient of organic solvent (80–35% acetoneitrile, 20 mM ammonium bicarbonate) at 0.105 ml min–1 for 16 min at 45 °C. Metabolites were analyzed using heated electrospray ionization (H-ESI) with polarity switching (3,400 V for positive, 3,000 V for negative) at 280 °C, with ion transfer at 300 °C. Xcalibur v.1.31.9 software (Thermo Scientific) was used for LC-MS control. Confirmation of metabolite peak specificity was achieved via comparison to available standards (Merck, Cambridge Isotope Laboratories and Santa Cruz Biotechnology). LC-MS data quality was monitored throughout the run with running standard mixes, ion abundance quality controls and blanks for detecting carry over. Peak integration and metabolite isotopologue identification was accomplished using ionquant. Under SF2 software, an 0.1 A (Thermo Scientific)-width of labeled peak and isotopologues were confirmed using cell line controls, blank control samples and nonlabeled islet samples pre- and postincubation. Natural abundance was assayed using nonlabeled cell samples, and confirmed with correction calculations using IsoCor software on a subset of data. To avoid any possible confounding effect of naturally occurring M+1 labeling, M+1 isotopologues were omitted from the final analysis of the relevant metabolite. The liver area was normalized to the cell lysate DNA content or as a percentage of the total M+0 (nonlabeled) and M+1 (labeled) metabolite present in the sample. DNA normalization was also in strong agreement with levels of essential amino acids (that could not be metabolized from glucose) in each sample (data not shown). Relative abundance values are presented relative to the normalized metabolite level in primary adult islet samples in low (3 mM) ([U-13C5] glucose). For NAD+/NADH and ATP/ADP ratio data, values were calculated from total normalized abundances in low and high glucose conditions.

**Animal experiments.** Care and experiments were approved by a National Animal Experiment Board in Finland (EASAVI14852/2018). NOD-SCID-Gamma (NSG Jackson Laboratories, catalog no. 0555577) mice were housed in the Biomedical Helsinki conventional facility in 12 h light/dark cycle and fed standard chow. For SC-islet implantations, 250–750 SC-islets (diameter 100–200 µm), were aspirated into PE-50 tubing and compacted by centrifuging. Mice were anesthetized with isoflurane and the kidney exposed. A small opening to the kidney capsule was made and the capsule separated with a glass rod. The tubing was inserted in the opening and the SC-islets implanted using a Hamilton syringe. The kidney capsule was then closed by cautery before wound closure. Subcutaneous carprofen 5 mg kg–1 (Zoetics) and buprenorphine 0.05–0.1 mg kg–1 (RB Pharmaceuticals) were used as analgesics. Nonfasted blood samples were collected from the saphenous vein of mice. Some mice were subjected to a glucose-stimulation test (STZ, catalog no. 50310, Sigma-Aldrich) injection (130 mg kg–1) 4 months after engraftment to eliminate the mouse beta cells. To verify the functionality of the SC-islet graft, the engrafted kidney was removed 1 month after the STZ injection after ligation of the renal vein and artery and the ureter removing the entire kidney. To test the functionality of the SC-islet graft, mice were subjected to a glucose-stimulation test. Mice were fasted 6–8 h before the test. The mice were weighed, and blood glucose was measured before the test. Glucose (3 g kg–1) was injected intraperitoneally, and blood samples (30 µl) were taken from the saphenous vein after 15 min, 30 min, 60 min and 90 min to measure blood glucose and circulating human C-peptide levels by ELISA (Mercodia). scRNA sequencing sample preparation. SC-islets were incubated with 2 ml of a 1:1 mixture of TrypLE Select (Thermo catalog no. 12563-029) and Trypsin-EDTA.
scRNA sequencing analysis. Single-cell gene expression profiles were generated with 10x Genomics Chromium Single-Cell 3' RNAseq platform using the Chromium Next Gem Single-Cell 3’ Gene Expression v3.1.3 chemistry. This resulted in 220 M read pairs on average per sample (with 26–28 bp read 1, 8 bp i7′, 8 bp i5). SC-islets were encapsulated using the 10x Genomics Chromium platform. Single cells as described above. Single cells from in vitro and graft-recovered SC-islets were encapsulated using the 10x Genomics Chromium platform.

Data collection and statistical methods. Morphological data represents population-wide observations from independent differentiation experiments. Insulin secretion, respirometry and metabolomics data represents samples of independent SC-islet differentiation experiments or islet donors. Electrophysiology and measurements of Cα+, CAMP and exocytosis represent recordings from individual cells in independent experiments, which are pooled depending on the datasets described and perinur, or represented as individual measurements. In vivo data is derived from independent animals. Transcriptomics data represents data on the level of single cells, which are pooled from two to three independent differentiation experiments per timepoint. Statistical methods used are represented in figure legend.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All scRNA sequencing data are deposited in the Gene Expression Omnibus database under accession code GSE167880, and additionally on an interactive single-cell portal (https://singlecell.broadinstitute.org/single_cell/study/SCP1526). All other data are available upon reasonable request from the corresponding author.

References
69. Trokovic, R., Welter, J. & Otonkoski, T. Generation of iPSC line HEL24.3 from human neonatal foreskin fibroblasts. Stem Cell Res. 15, 266–268 (2015).
70. Lithovius, V. et al. SUR1-mutant iPS cell-derived islets recapitulate human pancreatic islets. Diabetes 64, 630–640 (2021).
71. Stirling, D. R. et al. CellProfiler 4: improvements in speed, utility and usability. BMC Bioinform. 22, 433 (2021).
72. Guéck, A. et al. Fusion pore regulation by CAMP/Epac2 controls cargo release during insulin exocytosis. Nat. Chem. 11, e41711 (2019).
73. Dyachok, O. & Gylfe, E. Store-operated influx of Cα+ in pancreatic beta-cells exhibits graded dependence on the filling of the endoplasmic reticulum. J. Cell Sci. 114, 2179–2186 (2001).
74. Klarenbeek, J., Goedhart, J., van Batenburg, A., Groenewald, D. & Jalink, K. Fourth-generation epac-based FRET sensors for CAMP feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for FLIM, for ratiometry and with high affinity. PLoS ONE 10, e0122513 (2015).
75. Midani, F. S., Wynn, M. L. & Schnell, S. The importance of accurately correcting for the natural abundance of stable isotopes. Anal. Biochem. 520, 27–43 (2017).
76. Lun, A. T. L. et al. EmptyDrops: distinguishing cells from empty droplets in droplet-based single-cell RNA sequencing data. Genome Biol. 20, 63 (2019).
77. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. 36, 411–420 (2018).
78. Korsunsky, I. et al. Fast, sensitive and accurate integration of single-cell data with Harmony. Nat. Methods 16, 1289–1296 (2019).
79. Klarenbeek, J., Goedhart, J., van Batenburg, A., Groenewald, D. & Jalink, K. Fourth-generation epac-based FRET sensors for CAMP feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for FLIM, for ratiometry and with high affinity. PLoS ONE 10, e0122513 (2015).
80. van Dijk, D. et al. Recovering gene interactions from single-cell data using cell diffusion. Cell 174, 716–729.e27 (2018).
81. Finak, G. et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. Genome Biol. 16, 278 (2015).
82. La Manno, G. et al. RNA velocity of single cells. Nature 560, 494–498 (2018).
83. Bergen, V., Lange, M., Peidli, S., Wolf, E. A. & Theis, F. J. Generalizing RNA velocity to cell transit times through dynamical modeling. Nat. Biotechnol. 38, 1408–1414 (2020).
84. Trapnell, C. et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat. Biotechnol.* **32**, 381–386 (2014).

85. Kuleshov, M. V. et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* **44**, W90–W97 (2016).

86. Zhou, Y. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* **10**, 1523 (2019).

87. Zhang, X. et al. Comparative analysis of droplet-based ultra-high-throughput single-cell RNA-seq systems. *Mol. Cell* **73**, 130–142.e5 (2019).

88. Fu, R. et al. clustifyr: an R package for automated single-cell RNA sequencing cluster classification. *F1000Res.* **9**, 223 (2020).

**Acknowledgements**

We thank C. Wollheim for invaluable feedback on the manuscript. H. Grym, A. Laitinen, S. Eurola and V. Parekh are thanked for expert technical support, and J. Jautila, S. Andersson and J. Morikka for the processing and acquisition of metabolite tracing data. We thank FIMM Single-Cell Analytics unit (supported by HiLIFE and Biocenter Finland) for scRNA sequencing services. We are grateful to the Nordic Network for Islet Transplantation (supported by the strategic grant consortium Excellence of Diabetes Research in Sweden, EXODIAB), and the IsletCore, University of Alberta, Canada, for providing human islets and P. MacDonald for providing IsletCore summary statistics. This study was supported by the Academy of Finland grant 297466 and MetaStem Center of Excellence grant 312437 to (T.O., V.H., P.K.), the Sigrid Jusélius Foundation Grant (to T.O.), the Novo Nordisk Foundation (to T.O., S.B., A.T., J.L.), Diabetes Wellness Finland (D.B., J.S.-V.) and the Helsinki University Hospital Research Funds (to T.O.) and an EMBO Long-Term Fellowship ALT295-2019 (to D.B.). Additional funding was provided by The Swedish Research Council (S.B., A.T., P.-O.C.), Barndiabetesfonden (S.B., A.T., P.-O.C., J.L.), Swedish Diabetes Foundation (S.B., A.T.), Family Erling Persson Foundation (P.-O.C., T.O.), Familjens Ernörs Foundation (A.T., J.L.), Diabetes Wellness Sweden (A.T.) the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement 115797 (INNODIA) and 945268 (INNODIA HARVEST). This Joint Undertaking receives support from the Union's Horizon 2020 research and innovation program and the European Federation of Pharmaceutical Industries and Associations, JDRF, and the Leona M. and Harry B. Helmsley Charitable Trust (to T.O., A.T.).

**Author contributions**

D.B. conceived and conceptualized the study, developed differentiation protocols, differentiated SC-islets, analyzed the single-cell transcriptomic data and wrote the manuscript. T.B. conceptualized and performed the metabolomic analyses, differentiated SC-islets and wrote the manuscript. V.L. developed differentiation protocols, performed and analyzed SC-islet differentiation-, insulin secretion- and IHC experiments and wrote the manuscript. J.S.-V. developed differentiation protocols, performed and analyzed differentiation and animal experiments and participated in the writing of the manuscript. M.O.-H., O.D., P.E.L. and M.Y. performed and analyzed cell physiology experiments. H.M. performed and analyzed differentiation and animal experiments, H.I., V.C. and J.U. participated in the differentiation experiment analysis. A.N. assisted in the single-cell transcriptomic experiments and J.K. in the data analysis. A.I.N. helped in the metabolomic analysis pipeline. H.V. and E.J. performed electron microscopical analyses. E.K., V.H. and P.K. helped in the analysis of metabolic data and participated in the manuscript writing. J.L. and P.-O.C. acquired funding and participated in the differentiation and animal experiments. S.B. and A.T. supervised the cell physiology experiments, acquired funding, and participated in manuscript writing. T.O. conceived and supervised the study, provided resources, acquired funding and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41587-022-01219-z.

Correspondence and requests for materials should be addressed to Timo Otonkoski.

Peer review information Nature Biotechnology thanks Maike Sander and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a Confirmed
- □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- □ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- □ The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- □ A description of all covariates tested
- □ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- □ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- □ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  BD Cellquest Pro v4.0.2, Axio Scan Blue Zeiss software Zen2 Blue Edition v2.2, TraceFinder v4.1

Data analysis  GraphPad Prism 8.4.2, CellProfiler 4.0, FlowJo v10, TraceFinder 4.1, 10x Genomics Cell Ranger v3.1, DropletUtils v1.6.1, Seurat v3.2.3, Harmony v1.0, Monocle2 v2.14.0, Souax v1.4.8, Rmagic v2.0.3, velocyto v0.17.17, scVelo v0.2.2, scanpy v1.5.0, Enrichr, CellPhoneDB v2.0.0, Patchmaster v2x90, MetaFluor 7.7, Igor Pro 8, isoCor plugin for Python, Agilent Wave v2.6

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Single cell RNA-seq data were deposited to GEO under accession number GSE167888, which is publicly accessible. All other data are available from the authors.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: No statistical method was used to determine sample size. Instead, biological repeats were collected based on availability of primary tissue. Stem cell derived tissue was tested with adequate repeats to confirm consistent data between parallel experimental setups. The range of biological repeats was therefore between n=3 to n=18 depending on availability of material and inherent variability within each assay.

Data exclusions: Low functioning primary islets (through batch variation or low cell viability) were excluded from the GSIS data sets. Metabolite tracing excluded peak data for low detection/non-specific metabolite measurements during data analysis. scRNAseq datasets excluded cell data with low reads or markers of high mitochondrial stress.

Replication: SC-islet differentiations were carried out by multiple researchers utilising the same protocol, and all produced SC-islet batches with similar levels of functionality and measurements between the multiple assays within the study. The number of independent SC-islet experiments used for each assay is detailed in the figure legends and up to 18 independent experiments with similar results were used in some of the functional tests. No SC-islet experiments were excluded from analysis, unless a technical issue with data collection occurred.

Randomization: No interventions requiring randomization were used for the study. When choosing SC-islets for various analyses, the samples were collected randomly from the whole batch.

Blinding: IHC immunostaining and quantification was blinded through processing batches simultaneously in the CellProfiler pipeline. Due to the experimental nature of comparing stem cell derived tissue and sporadically available primary tissue samples, no blinding could be achieved during experimental data collection. Data analysis was performed in a simultaneous and unbiased manner for all collected samples, where no blinding was necessary.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

Methods

| n/a | Involved in the study | n/a | Involved in the study |
|-----|-----------------------|-----|-----------------------|
| ☑   | Antibodies            | ☑   | ChIP-seq              |
| ☑   | Eukaryotic cell lines | ☑   | Flow cytometry        |
| ☑   | Palaeontology and archaeology | ☑   | MRI-based neuroimaging |
| ☑   | Animals and other organisms | ☑   |                       |
| ☑   | Human research participants | ☑   |                       |
| ☑   | Clinical data         | ☑   |                       |
| ☑   | Dual use research of concern | ☑   |                       |

Antibodies

Antibodies used

- Anti-insulin, Alexa 647 conjugate (FC), Rabbit, 1:160, Cell signaling technology [Cat#9008]
- Anti-insulin (IHC), Guinea pig, 1:500, DAKO A0564
- Anti-glucagon (FC/IHC), Mouse, 1:160 (FC) / 1:500 (IHC), Sigma-Aldrich #G2654
- Anti-somatostatin (IHC), Rabbit, 1:500, DAKO A21206
- Anti-Ki-67 (IHC), Rabbit, 1:500, Leica Microsystems #NCL-Ki67p
- Anti-SC18A1 (IHC), Rabbit, 1:500, Merck HPA063797
- Anti-LDHA (IHC), Rabbit, 1:250, Cell Signaling Technology #3582
- Anti-Guinea pig Alexa 594 conjugate (IHC), Goat, 1:500, Thermo-Fisher #A-11076
- Anti-Mouse Alexa 488 conjugate (FC/IHC), Donkey, 1:500, Thermo-Fisher #A-21202
- Anti-Rabbit Alexa 488 conjugate (IHC), Donkey, 1:500, Thermo-Fisher #A-21206

Validation

All antibodies used were validated using primary islet tissue or in-house tissue samples. The anti-insulin-AF647 antibody has flow cytometry validation data ("tested in-house for direct immunofluorescent analysis in rat cells and flow cytometry in mouse cells") and relevant citations on the Cell Signaling Technology website. The anti-insulin antibody (DAKO) has numerous citations of use for...
Eukaryotic cell lines

Cell line source(s)  H1 hESCs were purchased from WiCell. HEL24.3 and HEL113.5 iPSC lines were derived in Biomedicum Helsinki Stem Cell Core facility.

Authentication  De novo iPSC lines pluripotency and genomic stability were validated by the BSCC core facility with methods including G-band karyotyping, immunohistochemistry and quantitative PCR for pluripotency markers (OCT4, NANOG, TRA1-60, SOX2, SSEA3), teratoma assays and Promega StemElite STR ID system. HEL24.3 authenticated in Trokovic et. al 2015 and HEL113.5 in Lithovius et al. 2021.

Mycoplasma contamination  The iPSCs were tested routinely for Mycoplasma and they tested negative.

Commonly misidentified lines  Not used.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research.

Laboratory animals  Mus Musculus, NOD-Scid-Gamma, Male only, Age 2-10 months at SC-islet implantation. Mice maintained at the Biomedicum Helsinki animal facility on a 12-h light/dark cycle with ad libitum food. The temperature was kept at 23°C with 24 relative humidity (RH).

Wild animals  No wild animals were used in this study.

Field-collected samples  No field collected samples were used in this study.

Ethics oversight  Animal care and experiments were approved by National Animal Experiment Board in Finland [ESAVI/14852/2018].

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation  SC-islets were dissociated with TrypLE for 8-10 minutes before single-cell filtering and fixation/permeabilisation in BD Cytofix/Cytoperm solution for 20 minutes at room temperature. Primary antibodies were incubated overnight at 4°C in a 5% FBS PBS solution, secondary antibodies were incubated at room temperature for 30 minutes.

Instrument  BD FACSCalibur (Becton Dickinson)

Software  BD CellQuest Pro v4.0.2 [Acquisition] 

FlowJo v10 [Analysis]

Cell population abundance  The major endocrine cell populations were in high prevalence in this study. Cell population abundances ranged from 5% to >50% depending on the time of maturation within the experiment.

Gating strategy  Cells were gated with FSC and SSC to remove small interfering cellular debris. Positive and negative gating was determined through negatively stained cells within the population and non-stained controls.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.