SUR1-mutant iPSC cell-derived islets recapitulate the pathophysiology of congenital hyperinsulinism

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Abstract

Aims/hypothesis Congenital hyperinsulinism caused by mutations in the K_{ATP} -channel-encoding genes ($K_{ATP}HI$) is a potentially life-threatening disorder of the pancreatic beta cells. No optimal medical treatment is available for patients with diazoxide-unresponsive diffuse $K_{ATP}HI$. Therefore, we aimed to create a model of $K_{ATP}HI$ using patient induced pluripotent stem cell (iPSC)-derived islets.

Methods We derived iPSCs from a patient carrying a homozygous $ABCC8^{V187D}$ mutation, which inactivates the sulfonylurea receptor 1 (SUR1) subunit of the K_{ATP} -channel. CRISPR-Cas9 mutation-corrected iPSCs were used as controls. Both were differentiated to stem cell-derived islet-like clusters (SC-islets) and implanted into NOD-SCID gamma mice.

Results SUR1-mutant and -corrected iPSC lines both differentiated towards the endocrine lineage, but SUR1-mutant stem cells generated 32% more beta-like cells (SC-beta cells) (64.6% vs 49.0%, $p = 0.02$) and 26% fewer alpha-like cells (16.1% vs 21.8% $p = 0.01$). SUR1-mutant SC-beta cells were 61% more proliferative (1.23% vs 0.76%, $p = 0.006$), and this phenotype could be induced in SUR1-corrected cells with pharmacological K_{ATP} -channel inactivation. The SUR1-mutant SC-islets secreted 3.2-fold more insulin in low glucose conditions (0.0174% vs 0.0054%/min, $p = 0.0021$) and did not respond to K_{ATP} -channel-acting drugs in vitro. Mice carrying grafts of SUR1-mutant SC-islets presented with 38% lower fasting blood glucose (4.8 vs 7.7 mmol/l, $p = 0.009$) and their grafts failed to efficiently shut down insulin secretion during induced hypoglycaemia. Explanted SUR1-mutant grafts displayed an increase in SC-beta cell proportion and SC-beta cell nucleomegaly, which was independent of proliferation.

Conclusions/interpretation We have created a model recapitulating the known pathophysiology of $K_{ATP}HI$ both in vitro and in vivo. We have also identified a novel role for K_{ATP} -channel activity during human islet development. This model will enable further studies for the improved understanding and clinical management of $K_{ATP}HI$ without the need for primary patient tissue.

Keywords Beta cells · Congenital hyperinsulinism · Disease modelling · Induced pluripotent stem cells · K_{ATP} -channel · Pancreatic islet development · Stem cell-derived islets

Abbreviations

CHI Congenital hyperinsulinism
CXCR4 C-X-C motif chemokine receptor 4
GBC Glibenclamide
iPSC Induced pluripotent stem cell

$K_{ATP}HI$ Congenital hyperinsulinism due to loss-of-function of the K_{ATP} channel
PDX1 Pancreatic and duodenal homeobox 1
RNP Ribonucleoprotein
SC-alpha cell Stem cell-derived alpha-like cell

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Research in context

What is already known about this subject?

- Loss of K_{ATP} -channel activity leads to constitutive insulin secretion from beta cells and is the leading genetic cause of congenital hyperinsulinism ($K_{ATP}HI$)
- Previous models using K_{ATP} -channel gene knockout mouse strains have demonstrated a milder phenotype than is observed in human patients
- In addition to constitutive insulin secretion, beta cells of $K_{ATP}HI$ patients display increased proliferation and a higher occurrence of nucleomegaly

What is the key question?

- Can these features of $K_{ATP}HI$ be replicated in a model based on stem cell-derived islets?

What are the new findings?

- Excessive secretion of insulin seen in $K_{ATP}HI$ can be recapitulated both in vitro and in vivo using patient induced pluripotent stem cell-derived islets
- More subtle features of the disease phenotype, such as increased proliferation and nucleomegaly, are also recapitulated in the model
- Loss of K_{ATP} -channel activity leads to increased beta cell mass in in vitro differentiation

How might this impact on clinical practice in the foreseeable future?

- This work establishes a platform that can be used to screen novel treatments for $K_{ATP}HI$

SC-beta cell	Stem cell-derived beta-like cell
SC-islet	Stem cell-derived islet-like cluster
SUR1	Sulfonylurea receptor 1

Introduction

Congenital hyperinsulinism (CHI) defines disorders of pancreatic beta cells characterised by hypoglycaemia caused by inappropriate insulin secretion. Pancreatic beta cells regulate blood glucose by linking their intracellular glucose metabolism with insulin secretion via the K_{ATP} -channel [1]. Inactivating mutations in the beta cell K_{ATP} -channel genes are the most common cause of CHI ($K_{ATP}HI$), comprising 36–61% of all cases [2, 3]. In $K_{ATP}HI$, the K_{ATP} -channels are either insensitive to the changes in intracellular ATP/ADP ratio, or they are missing from the plasma membrane. This leads to constant membrane depolarisation and constant influx of calcium through the voltage-gated calcium channels, resulting in constitutive insulin secretion from the beta cells. [4–6]. Thus, $K_{ATP}HI$ typically presents as persistent, severe neonatal hypoglycaemia. The major histological type of $K_{ATP}HI$ is recessively inherited diffuse $K_{ATP}HI$, in which all beta cells are affected. K_{ATP} -channel-activating diazoxide is used as the first-line CHI therapy. However, due to the lack of

the K_{ATP} -channel activity, patients with diffuse $K_{ATP}HI$ are in most cases resistant to this therapy [4, 7]. Therapy with non-specific second-line drugs, such as octreotide and nifedipine, or with frequent feeding is then attempted. However, up to 55% of the $K_{ATP}HI$ patients remain without adequate glycaemic control using these treatments, requiring near-total pancreatectomy [8]. Pancreatectomy acutely results in euglycaemia in only 31% of the patients [9] with 91–96% of the patients developing diabetes in long-term follow-up [10, 11]. The currently suboptimal management of diffuse $K_{ATP}HI$ highlights the need for novel therapies.

Islet material from CHI patients is extremely scarce and K_{ATP} -channel knockout mouse studies have not fully recapitulated the severity of the human phenotype [12–15], and both these issues have hindered the study of CHI pathophysiology and pharmaceutical development needed for improved patient care.

The advent of induced pluripotent stem cell (iPSC) technology has allowed the limitless expansion of stem cells carrying the patient mutation [16, 17]. CRISPR-Cas9 genome editing [18] has made it possible to efficiently correct specific mutations in order to generate isogenic cell lines that are discordant only for the mutation of interest [19, 20]. Differentiating human embryonic stem cells with a $K_{ATP}HI$ mutation in 2D towards beta-like cells has been previously shown to replicate the insulin hypersecretion phenotype in vitro [21].

Our objective was to create an improved model of $K_{ATP}HI$ by using a 2D-to-3D protocol [20] to differentiate sulfonylurea receptor 1 (SUR1)-mutant patient iPSCs into stem cell-derived islets (SC-islets) and implanting them into mice to encompass the disease phenotype both in vivo and in vitro. Additionally, we studied the effect of K_{ATP} -channel inactivation on islet differentiation.

Methods

Derivation of the iPSC lines The patient iPSC line HEL113.5 was derived from a $K_{ATP}HI$ patient carrying a homozygous c.560T>A (V187D) mutation in the exon 4 of the *ABCC8* gene encoding the SUR1 protein [6]. It was derived at the Biomedicum Stem Cell Center, University of Helsinki, from donated skin fibroblasts with a Sendai virus (SeVdp) vector carrying the reprogramming factors octamer-binding transcription factor (OCT)3/4, Krüppel-like factor 4 (KLF4), SRY (sex determining region Y)-box 2 (SOX2) and c-Myc. The healthy control cell line HEL46.11 [22] was derived similarly. The Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (no. 423/13/03/00/08) approved the patient informed consent for the derivation of the hiPSC lines used in this study.

Correction of the mutation in the patient iPSC line Guide RNA (TCGTGGAGGTCAATGTCATC-AGG) targeting the *ABCC8* gene V187D mutation site was designed using a web-based tool, Benchling (<https://benchling.com>, accessed 1 Feb 2018), with cut site 8 bp from the mutation. To perform the genome editing in the HEL113.5 cell line, guide RNA was used together with Cas9 in a complexed ribonucleoprotein (RNP).

The RNP complex was delivered by electroporation using the Neon Transfection System (ThermoFisher, USA) to 2 million Accutase (ThermoFisher, cat.no. A11105-01) dissociated HEL113.5 cells, together with 2 µl of a 100 µmol/l solution of electroporation enhancer (IDT, USA, cat.no. 1075915), 2 µg of pCXLE-mp53DD plasmid (Addgene no. 41859) and 2 µg of donor template, as described elsewhere [20]. Limited dilution cloned colonies were screened using PCR followed by HincII restriction. Recombinant colonies were validated using Sanger sequencing and checked for chromosomal abnormalities by G-banding karyotyping. Absence of pCXLE-mp53DD plasmid was determined using PCR for plasmid EBNA and ORIP backbone sequences. Full sequences for the PCR primers and correction template are available in the electronic supplementary material (ESM) Methods.

Cell culture Mycoplasma-free iPSCs were maintained on Matrigel-coated plates in Essential 8 (E8) medium (ThermoFisher, cat.no. A1517001). The differentiation was carried out

essentially as described previously [20] with a protocol modified from key publications [23–26]. The complete protocol is available in the ESM Methods. In brief, the iPSCs were dissociated and seeded on Matrigel-coated plates in E8 to create a confluent monolayer. Differentiation was initiated 24 h later by changing to the stage 1 medium. Differentiations that failed to achieve >90% positivity for C-X-C motif chemokine receptor 4 (CXCR4) at the end of stage 1 were aborted. After the pancreatic progenitor stage, stage 4, the cells were dissociated with TrypLE (ThermoFisher, cat.no. 12563029), and they were allowed to spontaneously aggregate in suspension on plates placed on a rotating platform.

In some experiments, 0.1 µmol/l glibenclamide (GBC) (Tocris, UK, cat. no. 0911) was added to inactivate the K_{ATP} -channels during stages 5, 6 and 7. An equal volume of DMSO was added to the control wells.

Flow cytometry A single cell suspension was created by dissociating the cells with TrypLE. For definitive endoderm (stage 1) flow cytometry, the cells were stained directly for 30 min in room temperature with CXCR4 antibody in 100 µl PBS + 5% FBS and analysed the same day. For pancreatic progenitor (stage 4) flow cytometry, the cells were fixed for 20 min with Cytofix/Cytoperm (BD Biosciences, USA, cat.no. 554714), and stained overnight in +4°C with NKX6.1 and pancreatic and duodenal homeobox 1 (PDX1) antibodies in 100 µl Perm/Wash (BD Biosciences, cat.no. 554714) + 5% FBS. The samples were run with FACSCalibur flow cytometer (BD Biosciences). The data were analysed with FlowJo software v10 (FlowJo, USA). After filtering the debris using FSC/SSC gating, the gating for the positive cells was done using isotype controls when the populations were not clearly separated and when they were, the gate was placed in the midpoint between the positive and negative peaks. Antibody details are available in ESM Table 1.

In vitro tests of insulin secretion One hundred SC-islets were hand-picked on stage 7 day 5–9, washed and preincubated in KRB with 3.3 mmol/l glucose for 1–2 h. Multi-step tests were carried out in sequence on the same SC-islets in KRB. Full test protocols are available in the ESM Methods.

SC-islet implantation Implantation of SC-islets under the kidney capsule of male and female NOD-SCID-gamma mice (Jackson laboratories strain no. 005557) was performed as previously described [19]. In brief, approximately 1000 stage 7 SC-islets were compacted into PE-50 tubing and implanted into the exposed left kidney with a syringe (Hamilton, USA, ref.no. 81301). Animals from each litter were randomised to receive a SUR1-mutant or a healthy graft (non-isogenic control or SUR1-corrected graft). Further details are available in the ESM Methods and ESM Table 2.

Insulin tolerance test and graft explantation Four months after the implantation, the mice were subjected to an insulin tolerance test. The mice were fasted for 5 h, a baseline blood glucose measurement (Lifescan, USA, OneTouch ultra) and a 75 µl blood sample from the saphenous vein was obtained. Then, 0.75 IU/kg of human insulin analogue (Novo Nordisk, Denmark Actrapid) was administered intraperitoneally. Blood glucose was measured at 20, 40 and 60 min and another 75 µl blood sample obtained at 40 min post injection. Human specific C-peptide was measured from the serum samples with ELISA assay (Mercodia, Sweden). After the test, the mice were euthanised in a CO₂ chamber, rapidly dissected and the kidneys containing the grafts fixed in 4% PFA for 2 days.

Immunohistochemistry and slide imaging The SC-islet samples were taken on stage 7 day 6–8, fixed for 2 h in 4% PFA, embedded in paraffin, cut into 5 µm sections, subjected to heat-induced epitope retrieval in 0.1 mol/l citrate buffer, immunostained and then imaged with the Zeiss (Germany) Axio Observer Z1 using the Apotome 2 as previously described [20]. Sections that would be later compared were imaged using the same settings and exported with the same intensity scaling. Antibody details are available in the ESM Table 1.

Morphometric analyses of stage 7 SC-islets and explanted 4-month grafts Immunostainings were quantified using a custom image analysis pipeline on CellProfiler 3.0 [27] (<https://cellprofiler.org>). First, nuclei were identified from the image displaying the DNA signal. The locations of the nuclei were then used as seeds to be propagated along the intensity gradient of the insulin or glucagon signal to identify the cytoplasm. Speckles of <50 px² were excluded. If >50% of insulin-positive cytoplasm was covered by glucagon-positive cytoplasm, the cells were considered to co-stain. To obtain accurate cell counts even if the cytoplasm was fragmented, the overlap of nuclear border on the surrounding cytoplasm was compared. If >15% of the nuclear border was surrounded by insulin, glucagon or co-staining cytoplasm the nucleus was counted positive for that marker. The final data are based on the counts of these nuclei and not fragments of cytoplasm. To identify Ki-67- and TUNEL-positive cells, the pipeline was configured to create images displaying borders of nuclei assigned to insulin-positive cells on the Ki-67 or TUNEL signal containing image. The co-localisation was manually verified from these images. Thresholds were set manually and the same pipeline settings were used in the comparable SUR1-mutant- and SUR1-corrected samples in effect making the analysis blinded.

Statistical analyses and data collection PRISM 8.0 (Graph Pad software, CA, USA) was used for statistical analyses. Statistical methods for each analysis are listed in figure

legends. SUR1-corrected and healthy donor iPSC-derived SC-islets were pooled together for analyses of in vitro and in vivo function, but displayed with separate symbols on graphs. Numbers in the main text are mean values unless otherwise indicated. All data points represent independent observations: differentiation experiments, animals, explanted grafts or individual cells. All data points are visible on the graphs, except in Fig. 5e. Researchers were not blinded to the genotype of the samples except when conducting the morphometric analyses. Littermate control animals were randomly assigned to receive grafts of the different genotypes used, but randomisation was not used in other stages of the study.

Results

SUR1 mutation correction with CRISPR-Cas9 in patient iPSC

The iPSC line HEL113 was derived from a K_{ATP}HI patient carrying a homozygous c.560 T > A (V187D) mutation in the exon 4 of the *ABCC8* gene encoding the SUR1 protein [6]. To allow reliable conclusions concerning the impact of the mutation, we corrected the V187D-mutation with CRISPR-Cas9 (Fig. 1b), yielding four homozygously corrected clones (Fig. 1c). These clones were validated using Sanger sequencing and karyotyping, presenting correction of the mutation, no chromosomal abnormalities, and no remnants of coelectroporated plasmid sequence (Fig. 1c, ESM Fig. 1a,b,c). Upon stem cell culture, clone number 1 displayed a higher rate of proliferation and abnormal morphology compared with the parental and sister clones and was excluded from further analyses.

SUR1 mutation leads to increased beta cell proliferation and mass in SC-islets

We differentiated the iPSCs towards beta cells using a previously published 7-stage protocol (Fig. 1a) [20]. The efficiency of each differentiation experiment was studied at the end of the definitive endoderm induction (stage 1 of the differentiation) and the pancreatic progenitor formation (stage 4) with flow cytometry. SUR1-mutant and SUR1-corrected cells had similar proportions of CXCR4⁺ cells at stage 1 (median 97.1% vs 94.4%, $p = 0.31$) (ESM Fig. 2a) and PDX1⁺NKX6.1⁺ cells (median 78.1% vs 76.9%, $p = 0.57$) (ESM Fig. 2b) at stage 4. Thus demonstrating that the mutation did not affect the development of endodermal or pancreatic progenitors.

To study how the SUR1 mutation affects islet endocrine cell mass, we quantified the populations of cells positive for insulin (SC-beta cells), glucagon (SC-alpha cells) or both of these hormones in immunohistochemical sections of SC-islets at the end of the differentiation protocol (stage 7), using a custom automated segmentation pipeline (Fig. 2a). SUR1-mutant SC-islets contained 32% more SC-beta cells (64.6% vs 49.0%, $p = 0.02$), an equal number of cells positive for both

insulin and glucagon (4.9% vs 5.5%, $p = 0.45$) and 26% fewer SC- α cells (16.1% vs 21.8% $p = 0.01$) (Fig. 2b) than the SUR1-corrected SC-islets. The sum of these three distinct endocrine populations was higher in the SUR1-mutant SC-islets (76.2% vs 65.2%, $p = 0.02$) (ESM Fig. 2c).

Proliferation has previously been found to be increased in diffuse K_{ATP} -HI patient beta cells [28–32], and could contribute to the increased number of insulin-positive cells we detected in the SC-islets. We therefore examined the expression of the proliferation marker Ki-67 using immunohistochemistry (Fig. 2c). The SUR1-mutant SC-beta cells co-stained more frequently with Ki-67 than the SUR1-corrected SC-beta cells (1.23% vs 0.76%, $p = 0.006$) (Fig. 2d, ESM Fig. 2e). To confirm that the increased proliferation was dependent on K_{ATP} -channel inactivation, we treated the cells with GBC, a drug which inactivates the K_{ATP} channel, throughout endocrine cell formation and maturation (stages 5–7). Upon addition of GBC, the proportion of proliferative SC-beta cells in SUR1-corrected SC-islets increased to 1.10% (from 0.76%, $p = 0.03$) (Fig. 2d, ESM Fig. 2d). The proliferation rate of non-SC-beta cells was higher than that of the SC-beta cells (around 1.6%), but neither genotype nor GBC treatment had an effect on the proliferation rate of these cells (ESM Fig. 2e).

SUR1-mutant SC-islets have higher insulin secretion in low glucose and fail to respond to K_{ATP} -channel acting pharmaceuticals Next, we studied the in vitro function of the SC-

islets using sequential static incubations, to assess whether our model replicates the hypersecretion of insulin seen in K_{ATP} -HI patient islets in vitro [4]. The SUR1-mutant SC-islets secreted 3.2-fold more of their insulin content in low glucose (3.3 mmol/l) compared with SUR1-corrected controls (0.0174%/min vs 0.0054%/min $p = 0.0021$) (Fig. 3a, ESM Fig. 3a,b). Treatment of SUR1-corrected SC-islets with GBC through stages 5, 6 and 7 rendered them hypersecretory as their secretion was increased 2.6-fold compared with DMSO treatment (0.0140%/min vs 0.0054%/min, $p = 0.0063$), despite GBC having been washed out 1 h prior to the test (Fig. 3a). Both SUR1-mutant and SUR1-corrected SC-islets (analysed together with healthy non-isogenic SC-islets) were unresponsive to high glucose (Fig. 3b) indicating SC-islets derived using this protocol lack mature stimulation-secretion coupling. These healthy SC-islets responded to the first-line CHI treatment, K_{ATP} -channel opener diazoxide by a 56% reduction and to the K_{ATP} -channel-closing sulfonylurea drug tolbutamide by a 54% increase of insulin secretion, while neither had effect on the SUR1-mutant SC-islets (Fig. 3b).

To study whether the SUR1-mutant SC-islets are responsive to non- K_{ATP} -channel-dependent modulators of insulin secretion, we exposed them to the glucagon-like peptide 1 (GLP1)-receptor agonist exendin-4, α_2 -adrenoceptor agonist clonidine and to sequestration of extracellular Ca^{2+}

Fig. 1 Creation of the K_{ATP} -HI model using SC-islets. **(a)** scheme of the overall experimental strategy. **(b)** Genome editing strategy for the patient iPSCs. The correction template was designed to change the encoded aspartate (GAC) amino acid causing the disease to the wild-type valine codon (GTC), as well as to include a HincII restriction enzyme cut site to facilitate corrected clone screening, and to block the PAM-site to prevent

Cas9 from cutting the correction template. **(c)** Sequencing of the parent clone with the homozygous (Homoz.) *ABCC8*^{V187D} SUR1 mutation and the four homozygously corrected clones. The mouse image in **(a)** is adapted from Gwiltz/Wikimedia Commons under a Creative Commons Attribution-Share Alike 4.0 International license; the baby image is from pixabay.com

Fig. 2 SUR1-mutant SC-islets contain more insulin-positive cells. **(a)** Characterisation of stage 7 SC-islets with insulin–glucagon–DNA immunohistochemistry, along with quality control output images produced by the automatic segmentation pipeline, displaying nuclear segmentation and identification of insulin (INS) and glucagon (GCG) positive cytoplasm (cytopl.) and assigning of nuclei to the corresponding cytoplasm. Scale bars, 100 μ m. Insets show 3 $\times$ magnification. **(b)** Quantification of insulin–glucagon immunohistochemistry. **(c)** Characterisation of stage 7 (S7) SC-islets with insulin–Ki-67–DNA immunohistochemistry, and quality control output images, displaying nuclear segmentation,

identification of insulin signal and identification of Ki-67 positive nuclei. Scale bars, 100 μ m. Insets show 3 $\times$ magnification. **(d)** Quantification of insulin–Ki-67 immunohistochemistry. GBC indicates GBC treatment 100 nmol/l during stages 5, 6 and 7; DMSO was used as the vehicle control. **(b, d)** Analysis by Welch's unequal variances *t* test: **p*<0.05; ***p*<0.01. Data presented as mean $\pm$ SEM, pooled data from 2 SUR1-mutant (red bars) and 3 SUR1-corrected clones (blue bars). Each data point represents population-wide characteristics of an independent differentiation experiment

by EGTA. SUR1-corrected SC-islets increased insulin secretion by 50% in response to exendin-4 in the presence of high glucose (Fig. 3d), but not in low glucose (Fig. 3c); SUR1-mutant SC-islets remained unresponsive to exendin-4 regardless of glucose concentration. Both clonidine and EGTA inhibited insulin secretion by 40–50% compared with the baseline secretion in low glucose, an effect equal in both SUR1-mutant and SUR1-corrected SC-islets (Fig. 3c).

The insulin content of the SC-islets was analysed after these tests. There were no differences in the insulin content between SUR1-mutant and SUR1-corrected SC-islets nor GBC- and DMSO-treated SC-islets (ESM Fig. 3c).

Grafts of SUR1-mutant SC-islets cause hyperinsulinaemic hypoglycaemia in vivo To create an in vivo model of $K_{ATP}HI$, we implanted SUR1-mutant, SUR1-corrected and healthy non-isogenic SC-islets into the kidney subcapsular space of immunocompromised mice. Four months after engraftment, we subjected

the mice to a 5 h fast and an immediate insulin tolerance test. The SUR1-mutant-grafted mice exhibited 38% lower fasting blood glucose (4.8 vs 7.7 mmol/l, *p* = 0.009), reaching hypoglycaemic (<3.3 mmol/l) levels in 3/9 of the SUR1-mutant-grafted mice (Fig. 4a); this was in conjunction with 6.7-fold higher levels of circulating human C-peptide, when compared with the pooled SUR1-corrected and healthy non-isogenic SC-islet grafted mice (901 vs 135 pmol/l, *p* < 0.0001) (Fig. 4b). We then challenged the mice with exogenous insulin to study the graft function in hypoglycaemic conditions. Critically, the circulating C-peptide levels in the SUR1-mutant-grafted mice remained high after the insulin injection, being approximately 16.4-fold higher than in the healthy controls (627 vs 38.2 pmol/l, at 40 min, *p* < 0.0001) (Fig. 4b), despite 7/9 of the mice being hypoglycaemic (<3.3 mmol/l blood glucose). C-peptide levels before and after the insulin injection showed that the healthy grafts reduced their C-peptide secretion by 67%, whereas the reduction was 30% in the SUR1-mutant grafts (*p* = 0.009) (Fig. 4c).

Fig. 3 SUR1-mutant SC-islets are hypersecretory and unresponsive to K_{ATP} -channel-acting drugs. **(a)** Insulin secretion during 1 h incubation in 3.3 mmol/l glucose (G3.3). Some SC-islets were treated with GBC (100 nmol/l) through stages 5 to 7. GBC was washed out 1 h before the test. DMSO was used as the vehicle control. Analysis by two-way ANOVA. **(b)** Sequential static incubation responses to 20 mmol/l glucose (G20), diazoxide (100 μ mol/l) and tolbutamide (Tolb., 100 μ mol/l). Analysis by two-way ANOVA. **(c)** Sequential static incubation responses to extendin-4 (Ex4, 50 ng/ml), clonidine (Clon., 2 μ mol/l) and EGTA (100 μ mol/l) in 3.3 mmol/l glucose. Analysis by one-sample *t* test vs a value of

1.0. **(d)** Fold increase in response to extendin-4 treatment in 20 mmol/l glucose vs 20 mmol/l alone. Analysis by Welch's unequal variances *t* test. Data presented as mean $\pm$ SEM, from SUR1-mutant clones (red bars), SUR1-corrected clones (blue bars, circles) and healthy donor iPSCs (blue bars, crosses). **(a)** 2 mutant and 3 corrected clones **(b)** 1 mutant, 1 corrected clone and 1 healthy donor iPSC clone. **(c, d)** 1 mutant and 1 corrected clone. Each data point represents an SC-islet sample from an independent differentiation experiment. **p*<0.05; ***p*<0.01; ****p*<0.001, as indicated, and by analysis as detailed above

Increased beta cell proportion in engrafted SUR1-mutant SC-islets is maintained in vivo After the insulin tolerance test at 4 months post engraftment, the grafts were explanted and analysed with immunohistochemistry to observe whether the changes in hormonal cell populations and proliferation marker expression were also present in vivo (Fig. 5a). Analysis of the insulin- and glucagon- immunoreactive cells revealed that a larger proportion of the hormone-positive cells in the SUR1-mutant grafts were insulin positive (71.5% vs 55% *p* = 0.06) and a smaller proportion glucagon positive (18.9% vs 36.9% *p* = 0.005) compared with the SUR1-corrected grafts (Fig. 5b). The proportion of cells co-expressing insulin and glucagon was equally low in SUR1-mutant and -corrected grafts (3.3% vs 2.7% *p* = 0.81)

(Fig. 5b). SUR1-mutant insulin-positive cells no longer expressed Ki-67 more frequently than the SUR1-corrected insulin-positive cells (Fig. 5c).

The disease progression in diffuse K_{ATP} HI patients is often self-limiting as insulin secretion reduces over time and many develop diabetes over long-term follow-up. Apoptosis of the hyperactive beta cells could contribute to this self-limitation and thus we examined it in our 4-month material using TUNEL as a marker. However, the overall rate of insulin-TUNEL co-expression was low and not significantly different between SUR1-corrected and SUR1-mutant grafts (4 out of 17,961 vs 8 out of 25,598 SC-beta cells, respectively) (ESM Fig. 4).

Fig. 4 Hyperfunctionality of SUR1-mutant SC-islet grafts at 4 months after engraftment. **(a)** Mouse blood glucose after a 5 h fast (0 min) and after an insulin injection (20, 40 and 60 min). Measurements <1 mmol/l are marked as 1 mmol/l. Horizontal line at 3.3 mmol/l to mark hypoglycaemic level of blood glucose. **(b)** Human specific C-peptide at fasted (0 min) and insulin-challenged conditions (40 min). **(c)** Human C-

peptide secretion at 40 min, relative to 0 min. **(a, c)** Analysis by Welch's unequal variances *t* test; **(b)** Two-way ANOVA: ***p*<0.01; ****p*<0.001. Data presented as mean $\pm$ SEM, from one SUR1-mutant clone (red bars), one SUR1-corrected clone (blue bars, circles) and one healthy donor iPSC clone (blue bars, crosses). Each data point represents measurements from an individual animal

Fig. 5 SC-islet grafts explanted at 4 months display a higher proportion of insulin-positive cells with sporadic nucleomegaly. **(a)** Characterisation of explanted SC-islet grafts, showing H&E staining; insulin (INS)–glucagon (GCG)–DNA immunohistochemistry and associated analysis images displaying the cytoplasm (cytopl.) and nuclei assigned to the cytoplasm; and insulin–Ki-67–DNA immunohistochemistry and associated analysis image displaying nuclei assigned to insulin-positive cytoplasm and Ki-67-positive nuclei (white arrows). Scale bars 100 μm . **(b)** Quantification of insulin–glucagon immunohistochemistry; each individual population (INS⁺GCG[−], INS⁺GCG⁺ and INS[−]GCG⁺) was compared with the sum of the populations. **(c)** Quantification of insulin–Ki-67 immunohistochemistry: % of insulin-positive cells that co-stain with Ki-67. **(d)** Example of abnormally large nuclei present in SUR1-mutant grafts, with nuclear

diameter displayed. **(e)** Quantification of the surface area of all nuclei assigned to insulin-positive cells ($n=8236$ –9011) and all nuclei assigned to insulin-positive cells that co-stain with Ki-67 ($n=59$ –98). **(f)** The percentage of nucleomegalic insulin-positive cells out of all insulin-positive cells in the individual grafts. Data presented as mean $\pm$ SEM (except in **e**) from SC-islet grafts derived from one SUR1-mutant clone (red bars) and one SUR1-corrected clone (blue bars). Each data point in **(b, c, f)** represents population-wide characteristics of independent graft explants. **(e)** Data pooled from four individual SUR1-corrected (Corr.) and SUR1-mutant (Mut.) grafts and presented as violin plot displaying range, median and interquartile range of the surface areas and approximate distribution. **(b, c, e, f)** Welch's unequal variances t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Additionally, we noticed a subpopulation of SC-beta cells with strikingly large nuclei (Fig. 5d) in the SUR1-mutant grafts. This nucleomegaly is observed in most islet specimens from K_{ATP}HI patients, and rarely in healthy islets [33]. To characterise this phenomenon further, we quantified the surface area of all SC-beta cell nuclei in our study material and also determined whether those SC-beta cells expressed Ki-67 as a marker of proliferation. The median SUR1-mutant SC-beta cell nucleus was 38% larger (47.6 vs 34.3 μm^2 , $p = 0.0005$) when the cell co-expressed Ki-67, and 14% larger when the cell did not co-express Ki-67 (41.7 vs 36.6 μm^2 , $p =$

0.0001) (Fig. 5e), compared with the median SUR1-corrected SC-beta cell nucleus. We next determined the rate of SC-beta cell nucleomegaly by using nuclear area of 100 μm^2 as a cut-off. The SUR1-mutant grafts had 2.9-fold higher rate of SC-beta cell nucleomegaly than the SUR1-corrected grafts (36 out of 9011 vs 11 out of 8236 SC-beta cells nucleomegalic). This rate in individual grafts was higher in the SUR1-mutants but not statistically significant (Fig. 5f). A single nucleomegalic SC-beta cell from the SUR1-mutant grafts co-expressed Ki-67, while none were observed in the SUR1-corrected SC-beta cells (ESM Fig. 5).

Discussion

We created a novel model for the study of $K_{ATP}HI$ by recapitulating the disease phenotype in vitro and in vivo with SC-islets. Our approach has several advantages: differentiation of stem cells into SC-islets mimics normal development and enables the study of developmental effects of mutations in a reliable isogenic comparison. The use of human cells circumvents the inherent issues associated with SUR1 knockout mouse models arising from species differences [34–37], while the subcapsular transplantation techniques still allow in vivo studies. Furthermore, the automated segmentation pipeline used for morphometric analysis reduces analysis bias.

The in vitro differentiation of iPSCs to SC-islets offers a unique window to the antenatal effects of K_{ATP} -channel inactivation, which would be near impossible to address in clinical studies. We discovered that SC-islets generated from SUR1-mutant iPSCs contained more SC-beta cells and fewer SC-alpha cells compared with SUR1-corrected SC-islets. Earlier morphometric studies have provided conflicting results regarding the presence of increased beta cell mass in diffuse $K_{ATP}HI$ islets, with one group reporting increased total insulin-positive area in sections [32], and others not detecting such a difference [30, 38]. The data obtained with our individual cell-based analysis gives weight to the argument that K_{ATP} -channel inactivation increases beta cell mass in humans.

In this study, SUR1-mutant SC-beta cells exhibited a higher proliferation rate, which could explain the increased final mass of SC-beta cells, with increased beta cell lineage allocation during differentiation possibly contributing. Proliferation of beta cells is known to be greatly increased in focal $K_{ATP}HI$ owing to inactivation of growth suppressing $p57^{KIP}$ [39], and slightly increased in diffuse $K_{ATP}HI$ because of an unknown mechanism [28–32]. Rodent studies have shown that sulfonylurea drug treatment increases the replicative potential of beta cells, also pointing to the role of the K_{ATP} -channel in this process [40, 41]. K_{ATP} -channel inactivation may lead to multiple intracellular perturbations that could explain the increased proliferation. These include at least the autocrine effect of secreted insulin through the insulin receptor [42], changes in signalling pathways due to altered metabolic flux as a consequence of increased energy demand [32, 43], activation of the nuclear factor of activated T-cells (NFAT)/calcineurin pathway because of the constitutively high intracellular Ca^{2+} [44], and elevation of endoplasmic reticulum stress to a level that promotes proliferation [45]. In future studies, our model could be used to elucidate the relevance of these pathways for increased beta cell proliferation in diffuse $K_{ATP}HI$.

The critical aspect of the $K_{ATP}HI$ patient phenotype on the beta cell level is the constant secretion of insulin. Our SC-islets demonstrate a 3.2-fold higher basal insulin secretion, in line with the 2.8-fold difference seen in a study using

$K_{ATP}HI$ patient islets [4]. We could also replicate the inability of SUR1-mutant cells to respond to diazoxide. Recapitulating the disease phenotype in vitro combined with the fact that the SC-islets can be generated in large quantities highlights the possibility of using this model for screening of novel anti-hypoglycaemic drugs for $K_{ATP}HI$.

On a systemic level, the patient phenotype is characterised by the failure to shut down insulin secretion when blood glucose falls below a threshold, which in a healthy person serves as the first-line safeguard against hypoglycaemia. This excess insulin secretion is sufficient to overcome the capacity of the counterregulatory machinery [46], particularly in a young infant, resulting in severe hypoglycaemia. The data from our in vivo model indicate that the SUR1-mutant SC-islet grafts were potent enough in secreting insulin that they could lower the fasting glucose levels of the recipient mice. After induced hypoglycaemia the SUR1-mutant grafts replicated the most devastating feature of the disease phenotype by continuing to secrete high levels of insulin.

The increase in insulin-positive cells persisted in grafts in vivo after 4 months, even when the rate of proliferation lowered, which fits with the established finding of a reduction in the proliferation rate of beta cells after birth [47]. This suggests that the abnormal hormonal populations are maintained without the need for constant proliferation of the SC-beta cells. Interestingly, the insulin-positive cells in SUR1-mutant grafts exhibited nucleomegaly, a classical histological diagnostic sign for diffuse $K_{ATP}HI$ [33, 38] in addition to a general shift in the size distribution towards larger nuclei [38]. The explanation for this phenomenon is unknown, but our data add evidence to the notion that nucleomegaly is not linked to proliferation but rather to the constitutive hyperfunctionality of the cells. Our finding that apoptosis is not increased in the SUR1-mutant SC-beta cells does not rule out the possibility that beta cell apoptosis would be responsible for the self-limitation of hyperinsulinaemia in patients; however, the lifespan of the grafted mice limits the usefulness of our model in answering questions regarding such long-term pathophysiology.

Another limitation of our model is that the differentiation protocol used in this study produces beta-like cells that lack full coupling of glucose metabolism and insulin secretion present in adult human beta cells. This is not a critical shortcoming as $K_{ATP}HI$ is primarily a neonatal condition and neonates lack this coupling as well [48]. Furthermore, the corrected SC-islets gain the ability to control their insulin secretion in vivo. Nevertheless, the model could be further refined by taking advantage of recent advances in the differentiation protocols to generate more mature SC-islets in vitro [49].

In conclusion, we show that patient-derived SUR1-mutant SC-islets recapitulate the cardinal feature of $K_{ATP}HI$, the dysregulated insulin secretion in vitro and in vivo, along with

increase in beta cell mass and proliferation. Our model constitutes a valuable tool to use in future endeavours to improve the management of CHI and in studying the basic biology of hypersecretory beta cells.

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Data availability Data collected for this manuscript are available upon reasonable request from the corresponding authors. The CellProfiler pipelines developed for this study are available upon request from VL.

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