Over-expression of Slc30a8/ZnT8 selectively in the mouse α cell impairs glucagon release and responses to hypoglycemia

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Abstract

Background: The human SLC30A8 gene encodes the secretory granule-localised zinc transporter ZnT8 whose expression is chiefly restricted to the endocrine pancreas. Single nucleotide polymorphisms (SNPs) in the human SLC30A8 gene have been associated, through genome-wide studies, with altered type 2 diabetes risk. In addition to a role in the control of insulin release, recent studies involving targeted gene ablation from the pancreatic α cell (Solomou et al., J Biol Chem 290(35):21432-42) have also implicated ZnT8 in the control of glucagon release. Up to now, however, the possibility that increased levels of the transporter in these cells may impact glucagon secretion has not been explored.

Methods: Here, we use a recently-developed reverse tetracycline transactivator promoter-regulated ZnT8 transgene to drive the over-expression of human ZnT8 selectively in the α cell in adult mice. Glucose homeostasis and glucagon secretion were subsequently assessed both in vivo during hypoglycemic clamps and from isolated islets in vitro.

Results: Doxycyclin-dependent human ZnT8 mRNA expression was apparent in both isolated islets and in fluorescence-activated cell sorting- (FACS) purified α cells. Examined at 12 weeks of age, intraperitoneal glucose (1 g/kg) tolerance was unchanged in transgenic mice versus wild-type littermates (n = 8-10 mice/genotype, p > 0.05) and sensitivity to intraperitoneal insulin (0.75U/kg) was similarly unaltered in transgenic animals. In contrast, under hyperinsulinemic-hypoglycemic clamp, a ~45 % (p < 0.001) reduction in glucose infusion rate was apparent, and glucagon release was significantly (~40 %, p < 0.01) impaired, in transgenic mice. Correspondingly, examined in vitro, glucagon secretion was significantly reduced (~30 %, p < 0.05) from transgenic versus control islets at low, stimulatory glucose concentrations (1 mM, p < 0.05) but not at high glucose (17 mM) glucose (p > 0.05). Over-expression of ZnT8 in glucagonoma-derived αTC1-9 cells increased granule free Zn²⁺ concentrations consistent with a role for Zn²⁺ in this compartment in the action of ZnT8 on glucagon secretion.

Conclusions: Increased ZnT8 expression, and a likely increase in intragranular free Zn²⁺ concentration, is deleterious in pancreatic α cells for stimulated glucagon release. These data provide further evidence that type 2 diabetes-associated polymorphisms in the SLC30A8/ZnT8 gene may act in part via alterations in glucagon release and suggest that ZnT8 activation may restrict glucagon release in some settings.
Background
Type 2 diabetes mellitus (T2D) is a complex polygenic disease which affects ~1 in 12 of the adult population and consumes ~10% of the health care budgets of most westernized societies [1]. Although genome-wide association (GWA) and other studies have in recent years identified multiple loci as affecting the risk T2D [2, 3], functional work in model systems remains important if we are fully to understand the physiological role(s), and potential as pharmacotherapeutic targets, of the implicated genes. Most such studies up to now have used gene deletion in mice, inactivating candidates either globally or in disease-relevant tissues [4, 5]. In the case of the endocrine pancreas-restricted secretory granule zinc transporter SLC30A8/ZnT8, identified in GWA studies for T2D [6], this approach has been pivotal in highlighting the role of the transporter in the control of secretory granule formation and Zn\textsuperscript{2+} storage [7, 8] insulin secretion [9] and hepatic clearance of the hormone [10]. Nevertheless, the impact of SLC30A8 risk variants [2] on ZnT8 activity and T2D risk are still debated. The common risk variant rs13266634 in the SLC30A8 gene encodes an amino acid exchange (R325W) which is believed to lower transporter activity [7, 11]. On the other hand, rare truncating variants of ZnT8 are protective [12]. The reasons for this complex relationship between ZnT8 levels and disease risk are not fully understood [13, 14]. Whilst the role of the transporter in the control of insulin secretion has been the chief focus of interest in recent years, the observation that ZnT8 is also expressed in the α cell in both rodents [7] and humans [15] leads to the possibility that an action via glucagon release may also affect diabetes risk. Indeed, Zn\textsuperscript{2+} ions have been shown by autometallography [16] to be present in the secretory granule of α as well as β cells. Correspondingly, we have recently shown, by α cell-selective deletion of ZnT8 in mice [17], an important role for this transporter in the control of glucagon secretion.

Importantly, and as well as providing insights into the aetiology of T2D, changes in the normal release of glucagon may also have consequences for glycemic control in Type 1 diabetes (T1D). In the latter disease, inadequate responses to hypoglycaemia constitute a substantial risk and limit the use of insulin treatment to achieve good glycemic control and minimize disease complications [18].

Although investigating the impact of the absence of a gene is usually highly informative, its overexpression may also provide important insights, particularly with respect to the possible impact of pharmacological approaches which activate the gene or its product. Inducible expression systems are consequently often used in mice to achieve both temporal and spatial (i.e. tissue-specific) control of the expression of a given gene. Components of the Tet Switches [19] originate from the tetracycline (Tet) resistance operon in E.coli and belong to one of the most evolved gene regulation systems. “Tet-Off” and “Tet-On” systems are used in the majority of the studies involving inducible expression. The Tet-Off system was initially developed in 1992 and in the presence of the antibiotic tetracycline the expression from a Tet-inducible promoter is decreased [19].

In order to utilize tetracycline as a regulator of transcription, a tetracycline-controlled transactivator (rtTA) is controlled by fusion of the tetracycline repressor with a transcriptional activation domain from Herpes Simplex Virus (HSV). Thus, in the absence of tetracycline, the fusion protein can bind tet operator sequences and promote transcription while in the presence of the antibiotic, its binding to the protein makes it unable to bind DNA leading to a decrease in gene expression. The “Tet-On” system was later developed by mutation of the repressor portion of the rtTA to create a reverse tetracycline controlled transactivator (rtTA) that relies on tetracycline for induction of gene expression rather than repression [20]. The system was first used in the pancreatic β-cell by Efrat and colleagues [21] and about ten years later in the α-cell [22].

Recently, our laboratory used this approach to determine the effects of ZnT8 over-expression in the pancreatic β-cell in mice, driving rtTA expression with the rat insulin 2 promoter [23]. In the present study, the rtTA sequence was placed under the control of the preproglucagon promoter in Glu-rtTA mice [22] allowing us to drive the expression of ZnT8 selectively in the α-cell in the adult mouse.

Using this approach we have investigated the effect of ZnT8 overexpression on glucagon secretion. Glu-rtTA mice were therefore crossed to mice bearing a human ZnT8 transgene whose expression was driven by the tet operator sequence. In contrast to the recently described effect of α cell-selective deletion of ZnT8 to enhance glucagon secretion at low glucose [17], we demonstrate that ZnT8 over-expression results in the suppression of glucagon release during hypoglycaemia, consequently enhancing glucose clearance.

Methods
Materials
Chemicals and biochemical were purchased from Sigma-Aldrich (Poole, Dorset, U.K.) unless otherwise indicated.

Generation and genotyping of αZnT8Tg mice
Glu-rtTA mice, which possess a 1.1 kb region of the preproglucagon promoter upstream of rtTA [22], were crossed to animals bearing a human ZnT8-overexpressing transgene (ZnT8Tg) as described in [23]. Heterozygous Glu-
rTA mice were crossed to homozygous ZnT8Tg animals. Two ZnT8Tg founders were used, corresponding to lines #31 and #23 in [23]. The litters comprised Glu-rTA offspring, which expressed the human ZnT8 transgene after induction with doxycycline (as described below), while littersmates bearing the ZnT8Tg allele alone, and born at Mendelian frequency (50 %), were used as controls. Doxycyclin, a tetracycline derivative, was given to all experimental mice in the drinking water (2 g/L) from the age of six weeks in order to induce ZnT8 expression in Glu-rTA *::ZnT8Tg* animals [22]. Animals were kept in a pathogen-free facility under a 12 h light-dark cycle with access to water and a standard mouse diet (Lillico Biotechnology). After weaning at 3–4 weeks of age mice were housed two to five per cage. All strains were maintained on a C57/BL6/J background.

Genotyping for Glu-rTA was performed using standard PCR on DNA extracted from ear biopsies (Fig. 1a) using the primers indicated in Table 1. For the ZnT8 transgene, qPCR was used to detect the presence in the genome of the firefly luciferase gene, included in the transgene in the antisense direction with respect to ZnT8 cDNA [23].

**Islet isolation and culture**

Mice were sacrificed by cervical dislocation and islets isolated essentially as described [24]. In brief, pancreata were inflated with collagenase solution at 1ug/ml (Serva) and placed in a water bath at 37 °C for 10 min. After centrifugation and washing islets were purified on a Histopaque 119 (Sigma) gradient, by centrifugation at 2500 rpm for 20 min. The islet layer was recovered and islets cultured in complete RPMI 1640 medium (Gibco; 11 mM glucose) for 2–4 h at 37 °C islets before hand-picking into fresh media.

**Islet dissociation, fluorescence-activated cell sorting (FACS) and FACS analysis**

FACS analysis was performed essentially as described [17]. After overnight incubation, 250–300 islets of the same genotype were handpicketed and dissociated into single cells by repeated pipetting in 150 μl of Hank’s based cell dissociation buffer (Invitrogen) containing 0.1 % BSA and 0.1 % trypsin. The reaction was stopped with the addition of FBS (20 μl; Seralab). Cells were incubated in near-IR dead cell stain (Life technologies) for 20 min. at 4 °C, washed with PBA (PBS, 1 % BSA, 0.1 % azide) and fixed in 2 % (w/v) PFA for 10 min. and then washed twice with PBA and once with Saponin (0.025 % in PBA) before 10 min incubation with Saponin at room temperature. Cells were incubated with primary antibodies against insulin or glucagon (see Table 2) before incubation with secondary antibodies (anti-mouse AF 405, anti-guinea pig AF 488, anti-rabbit AF 640) for 20 min and resuspension in PBA. The samples were run on a BD Fortessa Flow Cytometer (BD Bioscience).

**RNA extraction and cDNA synthesis**

Islets were washed once in phosphate-buffered saline (PBS) followed by addition of TRizol (ThermoFisher). Chloroform (200 μl per ml of TRizol) was added and after centrifugation the upper aqueous phase was removed and RNA was precipitated by adding 400 μl isopropanol per ml of TRizol. After re-centrifugation and washing the dried pellet was finally resuspended in nuclease-free water. The same quantity of RNA was used from each sample to perform a reverse transcription polymerase chain reaction (RT-PCR) and cDNA synthesis using random primers. cDNA was generated from RNA (up to 1 μg) using a High Capacity Reverse Transcription kit (Applied Biosystems) according the manufacturer’s instructions using the following thermocycler protocol: 10 min 25 °C, 2 h 37 °C, 5 min. 85 °C, hold 4 °C.

**Quantitative PCR (qPCR)**

For gene expression measurements cDNA (2 μl) from the RT-PCR reaction above were used as template for quantitative Real Time PCR (qPCR) using a Fast SYBR Green Master Mix (Invitrogen) according to the manufacturer’s instructions. The reaction was initiated at 50 °C for 2 min. followed by the activation and pre-denaturation step at 95 °C for 10 min. The run was made up of 40 cycles of 15 s at 95 °C and 1 min. at 60 °C.

**αTCT1.9 cell transfection**

αTCT1.9 cells were transfected with a human ZnT8 (W325 variant) expression vector or with the corresponding empty vector (EV), using Lipofectamine2000 (LifeTechnologies) as previously described [25, 26] and according to the manufacturer’s instruction. For cytosolic Zn²⁺ measurement, cells were co-transfected with an eCALWY-4 [27] expression vector. Imaging experiments were performed the day after transfection.

**Cytosolic Zn²⁺ measurement with eCALWY-4**

Cells on coverslips were washed twice in Krebs-HEPES-bicarbonate (KHB) buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.2 mM MgSO₄, 1.5 mM CaCl₂, 10 mM HEPES, 25 mM NaHCO₃), which was warmed, bubbled with 95:5 O₂/CO₂, set to pH 7.4, and contained 11 mM glucose. Imaging of [Zn²⁺] using eCALWY sensors [27] was carried out as optimized before [25, 26]. Briefly, cells were maintained at 37 °C throughout with a heating stage (MC60, LIMKAM, Scientific Instruments), and KHB was perfused (1.5 to 2 mL/min) with additions...
Fig. 1 (See legend on next page.)
as stated in the figures. Images were captured at 433 nm monochromatic excitation wavelength (Polychrome IV, Till photonics) using an Olympus IX-70 wide-field microscope with a 40×/1.35NA oil immersion objective and a zyla sCMOS camera (Andor Technology) controlled by Micromanager software. Acquisition rate was 20 images/min. Emitted light was splitted and filtered by a Dual-View beam splitter (Photometrics) equipped with a 505dcxn dichroic mirror and two emission filters (Chroma Technology, D470/24 for cerulean and D535/30 for citrine). Image analysis was performed with ImageJ software using a homemade macro and the fluorescence emission ratios were derived after subtracting background. Steady-state fluorescence intensity ratio citrine/cerulean (R) was measured, then maximum and minimum ratios were determined to calculate free Zn$^{2+}$ concentration using the following formula: $[\text{Zn}^{2+}] = \frac{K_d (R_{\text{max}} - R)}{(R - R_{\text{min}})}$. The maximum ratio ($R_{\text{max}}$) was obtained upon intracellular zinc chelation with 50 μM TPEN and the minimum ratio ($R_{\text{min}}$) was obtained upon zinc saturation with 100 μM ZnCl$_2$ in the presence of the Zn$^{2+}$ ionophore, pyrithione (5 μM).

**Granular Zn$^{2+}$ Imaging with Zinpyr-4**

αTC1.9 cells were incubated for 20 min. before imaging in 1 μM Zinpyr-4 (Santa Cruz Biotechnology), and washed twice in imaging buffer. Acquisitions were performed as described above with additions as stated on the figures. Zinpyr-4 was excited at 488 nm wavelength and signal was collected through a 535/50 nm emission filter. Differences in Zinpyr4 mean intensity between EV and ZnT8-expressing cells were measured using ImageJ.

**Table 1** PCR primers

| Gene       | Sequence                  |
|------------|---------------------------|
| β Actin    | FOR: CGAGTCGCGTCCACCC     |
|            | REV: CATCCA4TGGCAACTGTG   |
| Glucagon   | FOR: CCAGAGGAAACGGGAAACAAC|
|            | REV: CCTTACG5CATGCCTCCTAAT|
| Human ZnT8 | FOR: CTGTCATGAAGGCCTCCTCC|
|            | REV: AGGGCTAGACAAAACACAG  |
| Glu-rtTA   | FOR: CATAAACGGGGCTCTGGAATCTCAATGGAATCGG |
|            | REV: GCCGAGATGCGACCTTAAAGCCCTCCTGCGATGAGA |
| Luciferase | FOR: CAAACTGCTAAAGGCTATAGAGAAGAGA |
|            | REV: ATTGATTTACGCCCATATCGTITT |

**Table 2 Antibodies**

| Antibody       | Species | Company   | Dilution |
|----------------|---------|-----------|----------|
| ZnT8           | Rabbit  | Mellitech | 1:200    |
| Glucagon       | Mouse   | Sigma     | 1:1000   |
| Glucagon       | Rabbit  | Santa Cruz| 1:200    |
| Insulin        | Guinea pig | Santa Cruz| 1:200    |
| Antibody       | Species | Company   | Dilution |
| Anti-mouse Alexa 568 | Goat   | Invitrogen| 1:1000   |
| Anti-rabbit Alexa 488 | Goat   | Invitrogen| 1:1000   |
| Anti-guinea pig Alexa 488 | Donkey | Invitrogen| 1:1000   |
| Anti-mouse Alexa 488 | Donkey | Invitrogen| 1:1000   |
| Anti-rabbit 568 | Goat    | Invitrogen| 1:1000   |

**Methods**

Glucagon secretion was measured from islets essentially as described in detail in [17]. In brief, 18 size-matched islets per condition were incubated with constant agitation for 1 h in 0.5 mL Krebs-HEPES bicarbonate buffer at 37 °C with either 1 mM or 17 mM glucose, and total and secreted glucagon measured at the end of the incubations by radioimmunoassay (Millipore). Details of intraperitoneal glucose (1 g/kg; IPGTT) and insulin (0.5 U/kg; IPITT) tolerance tests, and hypoglycemic clamps are provided in [17].

**Statistics**

Student’s t test was used to identify differences between two independent variables and assessments between multiple variables assessed by two-way ANOVA (with suitable correction from multiple tests), followed by Bonferroni’s post hoc test. All analyses were performed using GraphPad Prism 6.0, and $p < 0.05$ was considered significant. Values are presented as means ± S.E.

**Results**

**Expression of human ZnT8 in transgenic islets**

Initially to check that the administration of doxycyclin was effective in inducing the overexpression of ZnT8, total RNA was extracted from isolated islets incubated overnight with doxycyclin (5 μg/mL). qPCR analysis revealed expression of the human form of ZnT8 in the mice containing both the overexpressing transgene and...
the Glu-rTA but not in the control mice carrying only the ZnT8 transgene (Fig. 1b). We tested both founders that carried the transgene and observed that only founder 2 (#31 from [23]) expressed detectable levels of human ZnT8. Consequently, offspring from this founder was used in all in experiments.

In order to obtain a more accurate assessment of the degree of overexpression in a pure α-cell population and as opposed to islets, which are largely composed of β-cells [28], isolated islets were dissociated and stained with glucagon for fluorescence-activated cell sorting (FACS). RNA was then extracted from the sorted cells to check for expression of the human ZnT8 in the α-cells obtained from transgenic and control animals (Fig. 1c). Whilst essentially absent from non-transgenic islets, hZnT8 mRNA was readily detected in islets from transgenic animals.

**Intraperitoneal glucose and insulin tolerance are unaltered in αZnT8Tg mice**

To assess whether glucose homeostasis in transgenic mice was altered, intraperitoneal glucose tolerance tests (IPGTTs) were performed on male (Fig. 2a) and female (Fig. 2b) mice aged 8 weeks. In neither case were any differences in the excursions in blood glucose observed between control and transgenic animals at any time point during the tests.

Insulin sensitivity was similarly determine by intraperitoneal insulin tolerance tests (ITTs) were performed on transgenic and control male (Fig. 2c) and female (Fig. 2d) mice at the age of 14 weeks. Again, no differences were observed between the responses of αZnT8Tg and control mice.

**Trangenic mice require an elevated glucose infusion rate (GIR) and display impaired glucagon secretion during hypoglycemic clamps**

ITTs as described above could only be used safely (i.e. without life-threatening hypoglycaemia) to provide a limited decrease in blood glucose levels (to ~ 4 mM), above the usual threshold for substantial glucagon release in the mouse [29]. In order to elicit a more marked, but stable, decrease in blood glucose levels, where glucagon release is likely to be more strongly stimulated, hypoglycaemic clamps [17] were performed. Male mice were thus rendered hypoglycaemic by continuous intravenous administration of insulin (Fig. 3a). Under these conditions ZnT8-overexpressing mice

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**Fig. 2** Glucose and insulin tolerance in αZnT8Tg mice. Intraperitoneal glucose tolerance tests were performed on control and transgenic male (a) and female (b) mice at 8 weeks of age. n = 8–10 mice/genotype. Animals were fasted for 16 h before injection of 1 g/kg glucose. Blood glucose was sampled after venesection of the tail vein at the indicated times, and quantified using an automated glucometer (AccuCheck, Roche). c, d Insulin sensitivity. Intraperitoneal insulin tolerance tests performed on 12 week old male (c) and female (d) mice fasted for 5 h and injected with 0.5U/kg insulin, n = 9–10 mice per genotype. Blood sampling was performed as in (a, b)
required a significantly higher glucose infusion rate (GIR) to maintain hypoglycaemia compared to control mice (Fig. 3b), suggesting an impairment in glucagon release. Plasma glucagon levels were consequently measured prior to the onset of hypoglycaemia and at 120 min. after the beginning of insulin infusion (Fig. 3c). Whilst no differences were observed between glucagon levels in control and transgenic mice at the earlier time point, concentrations of the hormone at 120 min were ~45 % lower (p < 0.01) in transgenic versus control mice (Fig. 3c).

**Glucagon secretion is impaired in islets isolated from αZnT8Tg mice**

We sought next to determine whether the impaired glucagon secretion observed in vivo in αZnT8Tg mice chiefly reflected a cell autonomous effect in the α-cell, or a more complex set of changes in the autocrine response to hypoglycaemia, potentially involving multiple tissues (brain, adrenals etc.) [30, 31]. Correspondingly, we observed that when isolated islets were incubated in 1 mM glucose (a concentration in the range which strongly stimulates glucagon release from α-cells) [29], those overexpressing ZnT8 secreted significantly less (~20 %, p < 0.05) glucagon that control islets (Fig. 4). At 17 mM glucose the responses did not differ between islets expressing ZnT8 selectively in the α-cell and control islets.

**Impact of ZnT8 over-expression on granular and cytosolic free Zn²⁺ concentrations in clonal α-cells**

In order to determine whether ZnT8 overexpression may act via changes in subcellular free Zn²⁺ concentrations, we monitored the latter in secretory granules (Fig. 5).
using the granule-accumulated Zn\(^{2+}\) probe, Zinpyr4 [17] or in the cytosol with the recombinant-targeted Förster resonance energy-based probe, eCALWY4 [27]. Since individual \(\alpha\) cells could not readily be identified in primary mouse islets from the transgenic animals, these experiments were performed using the clonal \(\alpha\)-cell line, \(\alpha\)TC1-9 [29]. Whereas granular Zn\(^{2+}\) was significantly increased in the presence of exogenously-expressed ZnT8, as indicated by a substantial increase in the fluorescence of Zinpyr4 (Fig. 5), free Zn\(^{2+}\) concentrations tended to be lowered in the cytosol after ZnT8 overexpression (Fig. 6).

**Discussion**

In this report our aim was to overexpress ZnT8 in the pancreatic \(\alpha\)-cell in order to investigate the effect on glucagon secretion. The results, which demonstrate interesting parallels with respect to our findings in \(\alpha\)ZnT8 null animals [17], reveal that overexpression of ZnT8 in the \(\alpha\)-cell has no effect on glucose homeostasis or insulin sensitivity, as assessed by intraperitoneal tolerance tests. By contrast, increased \(\alpha\)-cell ZnT8 levels strongly affect responses to hypoglycaemia, as studied in hyperinsulinaemic clamps, as well as glucagon secretion in response to low glucose both in vivo and in vitro. The findings from the transgenic mouse with respect to glucagon release are thus reciprocal to those obtained in \(\alpha\)ZnT8 null mice and further reinforce the view that ZnT8 has an important role in the \(\alpha\)-cell in regulating glucagon secretion.

Measured by qPCR in FACS-purified \(\alpha\)-cells from \(\alpha\)ZnT8Tg mice, human ZnT8 mRNA levels were approximately 0.6 times those of \(\beta\)-actin (\(\text{Actb}\)) mRNA (Fig. 1c). This value is similar to that measured for the murine ZnT8 homologue [17]. Assuming similar amplification efficiencies of the primers used for human and rodent ZnT8, and homogeneous expression across the entire \(\alpha\)-cell population, the above measurement implies roughly equivalent levels of mouse and human ZnT8 mRNA in transgenic \(\alpha\)-cells, i.e. a doubling of the endogenous level in wild type mice. We note, however, that though the more active (W325) human ZnT8 variant
was used in the present studies [7], the relative transporter activities of this versus the rodent (Q325) variant are not known, precluding a more accurate estimate of the extent of the increase in ZnT8 activity in αZnT8Tg versus control α-cells.

Likewise, efforts to quantify the degree of human ZnT8 expression in the α-cell at the protein level were unsuccessful. These included immunostaining followed by islet dissociation, FACS analysis, and confocal imaging. Both approaches revealed limited affinity or selectivity of the available antibodies towards human ZnT8 (Table 2). Thus, staining of islets did not reveal human ZnT8-positive populations in the face of high background staining (results not shown). Likewise, under FACS analysis, the entire population of islet cells from either control or αZnT8Tg mice was found to be positive for human ZnT8 (results not shown). Similar findings were made over a range of primary and secondary antibody concentrations, and suggest at least partial reactivity of the anti-human antibody towards murine ZnT8. An anti-c-Myc antibody, aimed to detect the epitope tag present at the C-terminus of the ZnT8 transgene was also tested. Again, the staining was too weak across a range of primary antibody concentrations for meaningful quantitation (results not shown).

The results here complement those of a similar recent study from our laboratory [23]. In this earlier study, human ZnT8 was over-expressed in the adult β-cell using an insulin promoter-dependent Tet-On system. In the latter model, glucose-induced insulin secretion from βZnT8Tg mice was impaired whereas Zn$^{2+}$ release during stimulated exocytosis was elevated. A similar increase in granular Zn$^{2+}$ content and secretion of these ions might reasonably be expected from αZnT8Tg α-cells especially since targeted deletion of ZnT8 led to a reduction of granular Zn$^{2+}$ levels in both the α [17] and β [23] cell. Likewise, changes in cytosolic free Zn$^{2+}$ cells levels may occur after ZnT8 overexpression (as observed in β cells after the deletion of the transporter) [32]. Measurements using targeted Zn$^{2+}$ probes [33] revealed the expected changes in compartmentalised Zn$^{2+}$ upon ZnT8 over-expression in the α cell line αTC1-9 (Figs. 5 and 6). Thus, it seems likely that ZnT8 over-expression also increased granular Zn$^{2+}$ in the primary α cell in the transgenic mice. Future studies, in which the latter mouse line is crossed to a reporter strain allowing the expression and identification of a fluorescent marker in the α cell [17], will be required to confirm these observations.
In addition to changes in cell function, we do not exclude the possibility that subtle changes (lowering) in α cell mass may also contribute to decreased glucagon release in αZnT8Tg mice, potentiating the impact on the acute release of the hormone observed after islet isolation (Fig. 4). Importantly, such changes may lead to alterations in the activity of signalling pathways the control cell survival [33]. Similarly, changes in the expression of genes involved in glucose sensing by α-cells (e.g. glucokinase, Gck, or subunits of ATP-sensitive K⁺ channels, i.e. Kcnj11 or Abcc8, etc.) [34, 35] may also contribute to altered glucagon release from αZnT8Tg mouse islets. We note, however, that alterations in the latter were not observed after ZnT8 ablation [8] or overexpression [23] from β cells. Changes in the expression of genes controlling glucagon synthesis or processing of the prohormone (e.g. Pcsk1 or Pcsk2) would appear unlikely given unaltered total glucagon levels in αZnT8Tg islets (not shown).

Interestingly, over-expression of ZnT8 in the β cell also substantially inhibited glucose-induced insulin release both in vivo and from isolated islets [23]. In both β and α cells this might reflect slower dissolution of crystalline or other higher-order forms of the cargo hormones within secretory granules, impairing the formation and subsequent release of dimers or monomers through a diffusion-limiting pore [36]. βZnT8Tg mice nonetheless displayed enhanced glucose clearance compared to wild-type animals [23]. This change may be the consequence of increased Zn²⁺ release and an insulinomimetic action of these ions on target tissues [33], as well as impaired clearance of insulin by the liver [10]. By contrast, the impaired hypoglycemic response observed in αZnT8Tg mice was largely in line with the observed decrease in plasma glucagon levels, arguing that any increase in Zn²⁺ co-release from the α cell is insufficient to compensate for the lowered levels of the latter hormone. In any case, we note that glucagon and Zn²⁺ are reported to exert opposing effects on glycolysis in the isolated liver cells, whilst exerting a similar, activating effect on glycogen breakdown [37].

Conclusions

In direct contrast to the activating effect of ZnT8 deletion in α cells [17], overexpression of the transporter in these cells inhibits glucagon release under hypoglycaemic conditions. The present results thus reinforce the view that ZnT8 inhibition may be useful as a means to enhance glucagon release and hypoglycemic responses in the context of T1D. Conversely, activation of ZnT8 in the α-cell, and inhibition of glucagon secretion, may be beneficial in T2D, complementing the effect of ZnT8 overexpression in β cells to enhance insulin action [23]. Our findings also suggest that differences in the impact on T2D risk between rare [12] and more common [2] variants in the human SLC30A8/ZnT8 gene [13] might be explained in part by varying actions on glucagon release.

Abbreviations

FACS, fluorescence-activated cell sorting; GIR, glucose infusion rate; rTtTA, reverse tetracyclin transactivator; IPITT, intraperitoneal glucose and insulin tolerance tests, respectively; T1D, T2D, type 1 and type 2 diabetes mellitus, respectively; TMEM, N,N,N'-tetrakis (2-pyridylmethyl) ethylenediamine

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Authors’ contributions

GAR designed and coordinated the study, wrote the manuscript. AS performed mouse crosses, glucose tolerance and glucagon secretion studies. EP performed hypoglycemic clamps. PC performed zinc imaging, analysed data. SML performed hypoglycemic clamps. JG undertook glucagon measurements. JL supervised glucagon measurements and edited the manuscript. CM supervised clamps, analysed data, edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All in vivo procedures were approved by a local ethical committee (Animal Welfare and Ethics Review Board, AWERB) and conducted according to UK Home Office regulations (Animal Scientific Procedures Act of 1986, Home Office Project License number PPL 70/06608, holder Dr Isabelle Leclerc), in the Central Biological Services (CBS) unit at the Hammersmith Campus of Imperial College London.

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