Reduced expression of the co-regulator TLE1 in type 2 diabetes is associated with increased islet α-cell number

Sarah L. Armour1, Scott J. Anderson1, Sarah J. Richardson2, Yuchun Ding3, Chris Carey4, James Lyon5, Rashmi R. Maheshwari1, Najwa Al-Jahdami1, Natalio Krasnogor3, Noel G. Morgan2, Patrick MacDonald2, James A, M. Shaw1,6, Michael G. White*1

1 Institute of Cellular Medicine, Diabetes Research Group, Newcastle University Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH.

2 Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, Exeter, UK

3 Interdisciplinary Computing and Complex Biosystems (ICOS) Research Group, School of Computing, Newcastle University, Urban Sciences Building, 1 Science Square, Newcastle Helix, Newcastle upon Tyne NE4 5TG, UK.

4 Molecular Pathology Node Proximity Laboratory, Royal Victoria Infirmary, Queen Victoria road, Newcastle upon Tyne NE1 4LP

5 Department of Pharmacology, University of Alberta, Edmonton, AB, Canada; Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada

6 Institute of Transplantation, Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, NE6 BXH, UK

*Corresponding Author and Reprint requests: Michael, G White, Institute of Cellular Medicine, Diabetes Research Group, Newcastle University Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH. Email: michael.white3@ncl.ac.uk Tel: +44 191 222 7422. ORCID ID: https://orcid.org/0000-0001-9167-3320

Keywords: TLE1, bi-hormonal cell, glucagon, insulin, β-cell

Financial support: The study by Diabetes UK grant funding (16/0005434, 14/0005094 and 16/0005480) including a PhD Scholarship for SLA who also received a Visiting Scientist Award funded by the Islet Core (laboratory of PM) at the University of Alberta.

Disclosure Summary: JAMS has received travel funding form Novo Nordisk to attend the American Diabetes Association Annual Scientific Sessions and has participated in advisory boards for Medtronic and Sanofi. All other authors have nothing to disclose.

Abbreviations: Pdx-1, Pancreas/duodenum homeobox protein 1; Nkx6.1, NK6 homeobox 1; Nkx2.2, NK2 homeobox 2; TLE1, Transducin-Like Enhancer of Split 1; TLE3, Transducin-Like Enhancer of Split 3

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Abstract

β-cell dysfunction in type 2 diabetes (T2D) is associated with loss of cellular identity and mis-expression of alternative islet hormones, including glucagon. The molecular basis for these cellular changes has been attributed to dysregulation of core β-cell transcription factors, which regulate β-cell identity through activating and repressive mechanisms. The TLE1 gene lies near a T2D susceptibility locus and, recently, the glucagon repressive actions of this transcriptional co-regulator have been demonstrated in vitro. We investigated whether TLE1 expression is disrupted in human T2D, and whether this is associated with increased islet glucagon-expressing cells. Automated image analysis following immunofluorescence in donors with (n=7) and without (n=7) T2D revealed that T2D was associated with higher islet α/β cell ratio (Control: 0.7±0.1 vs T2D: 1.6±0.4; P<0.05) and an increased frequency of bi-hormonal (insulin+/glucagon+) cells (Control: 0.8±0.2% vs T2D: 2.0±0.4%, p<0.05). In non-diabetic donors, the majority of TLE1-positive cells were mono-hormonal β-cells (insulin+/glucagon−: 98.2±0.5%; insulin+/glucagon+: 0.7±0.2%; insulin−/glucagon+: 1.1±0.4%; p<0.001). TLE1 expression was reduced in T2D (Control: 36±2.9% vs T2D: 24±2.6%; p<0.05). Reduced islet TLE1 expression was inversely correlated with α/β cell ratio (r=−0.55; p<0.05). TLE1 knockdown in EndoC-βH1 cells was associated with a 2.5-fold increase in glucagon gene mRNA and mis-expression of glucagon in insulin-positive cells. These data support TLE1 as a putative regulator of human β-cell identity, with dysregulated expression in T2D associated with increased glucagon expression potentially reflecting β- to α-cell conversion.
Introduction

Type 2 diabetes (T2D) is characterised by the progressive loss of functional β-cell mass (1). Despite a relatively low frequency of apoptotic β-cells being reported, this decline in β-cell function has often been ascribed to irreversible β-cell death (2). However, evidence for the rapid restoration of long-term β-cell function in those with T2D following calorie restriction challenges this paradigm (3,4). As reported reductions in β-cell mass are believed insufficient to cause T2D (5), it is increasingly accepted that cellular dysfunction may play an important role in T2D pathogenesis.

Animal studies have demonstrated that β-cell identity is altered in response to metabolic stress, eventually leading to impaired insulin secretion (6,7). This is characterised by loss of core β-cell transcription factors, such as Pdx1 and Nkx2.2, and conversion towards other endocrine cell phenotypes, including glucagon-secreting ‘α-like’ cells (6-8). The molecular basis for these events has recently been attributed to the dual-functioning nature of these core β-cell regulators, which actively promote β-cell gene expression (e.g insulin) whilst concurrently repressing genes associated with alternative endocrine cell fates (e.g glucagon) (9-11). Thus, it has been proposed that metabolic stress-driven disturbances in the function/expression of these regulators leads to both β-cell dysfunction and mis-expression of non-β-cell hormones. Histological assessment of pancreatic tissue has provided circumstantial evidence for these events in human T2D, with increased α/β cell ratio and proportion of bi-hormonal (insulin⁺/glucagon⁺) cells reported, in addition to reduced expression of key β-cell transcription factors (e.g Nkx6.1 and PDX1) (8, 12-14).

Regulation of differentiation status through repressive mechanisms is well-recognised. The Transducin-Like Enhancer of Split (TLE) family (also known as the Groucho family) is a
widely studied group of co-regulators known to interact with a range of transcription factors, often converting them to repressors (15). Within islets, TLE3 has been shown to be critical for the maturation and maintenance of rodent beta cell identity, forming complexes with Nkx2.2 and Nkx6.1 to enable repression of alpha cell-related genes, including Glucagon and Arx (15-16). Recent indirect evidence suggests that TLE3 may also act as transcriptional activator in β-cells, consistent with a dual-functioning role (15). Another member of the TLE family, TLE1, is also strongly expressed in adult mouse β-cells and has been shown to function similarly (15). Thus, it has been proposed that TLE3 acts redundantly with TLE1 to actively maintain adult mouse β-cell identity.

In humans, TLE3 protein expression is undetectable in islets (17). TLE1, the gene encoding which lies near a T2D susceptibility locus (rs2796441), is highly expressed, however (17, 18). In light of this, the reported vulnerability of core β-cell regulators to metabolic stress, and the role of TLE1/3 in maintaining mono-hormonal rodent β-cell identity, we set out to test whether islet TLE1 expression is reduced in human T2D and whether this is associated with an increased proportion of glucagon-expressing cells.

**Methods**

*Pancreas donors*

Following ethical approval (University of Alberta; Pro00013094, Pro00001754) and informed consent from donor families, pancreata were procured from deceased organ donors and transported to the ADI IsletCore Laboratory (www.isletcore.ca), Edmonton in University of Wisconsin organ preservation solution at 4 °C. Standardised tissue blocks were obtained from pancreatic tail and paraffin-embedded. Seven donors with T2D and seven age-and BMI-matched control donors were studied. Donor characteristics are shown in Table 1. Detailed
methods for pancreas processing and biopsy collection are posted on the protocols.io repository (dx.doi.org/10.17504/protocols.io.x3mfq6).

**Immunofluorescence staining**

Tissue Sections (5 μm) were de-waxed in Histo-Clear II (National Diagnostics, Atlanta, USA) and rehydrated in decreasing ethanol concentrations. Heat-induced epitope retrieval (HIER) was performed by immersion in 10 mM citrate buffer (pH 6) in a pressure cooker and heating in a microwave oven for 20 minutes. Slides were blocked with 10% goat serum and subsequently incubated with mouse anti-TLE1 antibody (19) overnight at 4°C. This was detected with an anti-mouse Alexa Flour 555 Tyramide SuperBoost Kit (Thermo Fisher, UK), according to manufacturer’s instructions. Sections were then incubated with guinea pig anti-insulin (20) and rabbit anti-glucagon antibodies (21) overnight at 4°C followed by incubation with Alexa Fluor 488 conjugated anti-guinea pig (22) and Alexa Fluor 647 conjugated anti-rabbit secondary antibodies (23). Sections were counterstained with DAPI (Invitrogen, MA, USA) before being mounted with Vectorshield Mounting medium (Vector Laboratories, CA, USA). For EndoC-βH1 cells, the same protocol was applied with the omission of HIER and inclusion of a 20 minute 0.2% TritonX incubation at room temperature prior to blocking with 10% goat serum. All antibodies were validated in control pancreas sections and specificity confirmed by primary antibody omission.

**Image acquisition**

For pancreatic sections, slides were imaged using the Vectra 3 multispectral imaging platform (PerkinElmer, Waltham, MA) at 4x pixel resolution and regions of interest (pancreatic islets) identified using the Phenochart 1.0.7 software. Each region of interest
(ROI) were acquired at 20x resolution. For EndoC-βH1 cells, images were acquired using the Nikon A1+ point scanning confocal microscope.

**Image analysis**

Following capture on the Vectra 3, all images were spectrally unmixed and analysed using supervised machine learning (phenotyping function) within InForm2.3 (PerkinElmer, Waltham, MA), which segments cells based on nuclear signal (DAPI) and assigns phenotypes to all identified cells in the ROI based on IF characteristics as previously described (24). The phenotypes assessed were Ins+TLE1−Gcg−, Ins+TLE1+Gcg−, Ins+TLE1−Gcg+; Ins+TLE1+Gcg+, Ins−TLE1−Gcg−, Ins−TLE1+Gcg+, Ins−TLE1+Gcg−, Ins−TLE1−Gcg+ for each phenotype, development of cell-specific algorithms were generated based on an iterative training process (‘training phase’). Initially, a subgroup of cells (10-20 cells) of each phenotype were manually assigned, and the algorithms used to identify the phenotypes of all cells in each ROI (islets) in a ‘testing phase’. The accuracy was assessed by the user and subsequent training/testing phases were undertaken during which the algorithm accuracy was improved through manual correction. This cyclic process was undertaken a minimum of three times for each donor, resulting in 200-300 cells for the ‘training’ of each mono-hormonal (insulin or glucagon +/- TLE1) identity, and 10-30 cells for the rare bi-hormonal (insulin/glucagon co-expression) phenotype. The algorithm accuracy was confirmed by a second observer (MGW) before final phenotyping was conducted. Unique training and testing phases were performed for each individual donor. Co-expression of insulin and glucagon within single cells was confirmed by Nikon A1+ point scanning confocal microscope and NIS-Elements software for z-stack analysis (n=50 cells from R064 and R131).
Cell culture and transfections of EndoC-βH1 cells

Cells were obtained from Univercell-Biosolutions and cultured as previously described (25). For transfections, a protocol adapted from Marroqui et al (26) was employed. Cells were transfected with 30nmol/L of two different pre-designed siRNAs targeting TLE1 (S14167 and S14169; Ambion) or Silencer Select negative control #1 (4390843; Ambion) using Lipofectamine RNAiMAX in a two-step protocol. Briefly, 16 hours post plating, cells were exposed for 48 hours to 30nmol/L siTLE1 or siCTL, washed, and exposed again to the same siRNAs for a further 48 hours.

RNA extraction, cDNA synthesis and Quantitative Real Time PCR

RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit (RTN-350, Sigma-Aldrich) and On Column DNase-1 digestion set (DNASE-70, Sigma-Aldrich) according to the manufacturer’s instructions and quantified using NanoDrop (Thermo Scientific). cDNA was synthesised using ABI High Capacity cDNA kit (4368814, Applied Biosystems) as per manufacturer’s instructions. The cycle for cDNA synthesis was 25°C for 10 minutes followed by 37 °C for 120 minutes and finally 85 °C for 5 minutes.

For qRT-PCR, TaqMan® gene expression assays were used with Light Cycler® 480 Probes Master (4887301001, Roche) as per manufacturer’s instructions. qRT-PCR cycle was as follows: pre-incubation at 95 °C for 10 min, amplification for 15 sec at 95 °C followed by 1 min at 60 °C (x50), hold at 40 °C. The TaqMan® gene expression assays (Thermo Fisher) used were Rplp0 (Hs99999902_m1), TLE1 (Hs00270768_m1) and Glucagon (Hs01031536_m1). Data were normalised to Rplp0 reference gene and relative changes in gene expression were calculated using the ΔΔCt method.
Statistical analysis

Data are presented as mean±SEM or mean (range) and were compared using unpaired Student’s t-test, one-way ANOVA with Tukey post-hoc test or Pearson’s correlation coefficient, with p<0.05 considered significant.

Results

Details of the donors used in this study are summarised in Table 1. The study group comprised seven donors with T2D, with (mean±SEM) age 50.3±4.4 years, BMI 28.3±1.2 kg/m² and disease duration 5.9±2.4 years; in addition to seven age- and BMI-matched control donors without diabetes.

Insulin, glucagon and bi-hormonal (insulin+/glucagon+) cells were assessed in both groups by immunofluorescence staining (Fig 1A, B). Accurate identification of each phenotype, with confirmation that bi-hormonal cells expressed both insulin and glucagon, was validated by confocal microscopy with z-stack image analysis (Fig 1C). Quantitative image analysis was undertaken in 42 (32-58) islets in each of the donors with T2D and 48 (33-64) islets in each of the control donors (Table 1). Distribution of numbers of endocrine cells (sum of insulin/glucagon/bi-hormonal positive cells) in each islet section was comparable in all donors (Fig 1D). There was no significant difference in mean number of endocrine cells per islet between each group (T2D: 80±7 vs control: 83±11; p=0.87), supporting maintenance of endocrine cell number expressing the predominant hormones (insulin/glucagon) in T2D (Fig 1E). The percentage of insulin-positive cells per islet was significantly lower in those with T2D compared to the matched control group (T2D: 54±3.4% vs control: 65±2.6%; p<0.05), which was mirrored by a significant increase in the percentage of glucagon-positive cells (T2D: 44±3.5% vs control: 34±2.7%; p<0.05) (Figure 1F-G). This led to a significantly higher α/β
cell ratio in donors with T2D compared to the control group (T2D: 1.6±0.4 vs control: 0.7±0.1; p<0.05). The proportion of cells co-expressing insulin and glucagon was relatively low but bi-hormonal cells were more than twice as common in islets from donors with T2D (2.0±0.4%) compared with controls (0.8±0.2%; p<0.05) (Fig 1H).

Immunofluorescence staining for TLE1 in combination with insulin and glucagon demonstrated that TLE1 was expressed in the nuclei of cells within islets and the exocrine pancreas (Fig 2A). Within islets of control donors, the vast majority of TLE1-positive cells were mono-hormonal β-cells (insulin+/glucagon−: 98.2±0.5%; insulin+/glucagon+: 0.7±0.2%; insulin−/glucagon+: 1.1±0.4%; p<0.001) (Fig 2B).

The proportion of islet endocrine (sum of all phenotypes) cells expressing TLE1 was lower in donors with T2D (24±2.6%) in comparison to matched control donors (36±2.9%; p<0.05) (Fig 3A-C). 42.9±3.7% of β-cells (insulin+/glucagon−) expressed TLE1 in donors with T2D in comparison to 55.2±4.7% of control donors (p=0.06) (Fig 3D). Fewer bi-hormonal (insulin+/glucagon+) cells expressed TLE1 with no significant difference between donors with T2D (27±5.7% TLE+) and control donors (20±3.6% TLE+; p=0.3). Alpha-cells were very rarely TLE1-positive (T2D: 1.3±0.3%; Control 1.0±0.4%; p=0.5).

Reduced expression of islet TLE1 was associated with an increased α/β cell ratio, with a significant inverse correlation when considering donors both with and without diabetes (r=-0.56; p<0.05) (Fig 3E) and in a comparison of individual islets in all donors (r=-0.35; p<0.001; n=633). In support of a role of TLE1 in glucagon repression within β-cells, targeted siRNA mediated knockdown of TLE1 in the EndoC-βH1 cell line was associated with a greater than 2-fold increase in glucagon mRNA expression in comparison with cells
transfected with non-targeted siRNA (Fig 4A-B). Immunofluorescence staining confirmed expression of TLE1 in the majority of insulin-expressing EndoC-βH1 cells, with reduced protein expression evident following targeted knockdown (Fig 4C-D). Further, reduced TLE1 levels following targeted knockdown were associated with mis-expression of glucagon in a number insulin-expressing EndoC-βH1 cells, a phenotype that was not observed in cells transfected with non-targeted siRNA (Fig 4D-E).

**Discussion**

Compromised β-cell identity and mis-expression of non-β-cell hormones (e.g. glucagon) are emerging as important features of β-cell failure in T2D (6-8, 12-14). These changes appear to be underpinned by metabolic stress-driven dysfunction of a set of vulnerable β-cell regulators (e.g. PDX1 and Nkx2.2), leading to both silencing of β-cell genes and de-repression of alternative endocrine genes (9-11). In light of this newly recognised fragility of β-cell identity, and molecular control thereof, we assessed pancreata from donors with and without T2D for the expression of TLE1, a reported glucagon repressor encoded by a gene located near a newly identified T2D susceptibility locus (rs2796441) (15,18).

We report that nuclear TLE1 expression, which is predominantly within insulin-only positive cells of islets, is significantly reduced in islets of patients with T2D. Consistent with increased β- to α-cell conversion in T2D, we observed a higher α/β cell ratio and increased abundance of bi-hormonal cells in islets of donors with T2D, the former of which was negatively associated with TLE1 expression. Furthermore, the functionality of TLE1 as a glucagon repressor is supported by knockdown studies in the human EndoC-βH1 cell line, with reduced TLE1 expression leading to glucagon de-repression. These findings
demonstrate that TLE1 expression is disturbed in T2D in association with increased glucagon-positive cells, potentially reflecting β- to α-cell conversion.

These findings were derived from phenotypic analysis of donor pancreatic tissue samples using an automated, machine-learning approach, in which accuracy was enhanced through manual validation by two independent observers over several iterations. Whilst this provided an unbiased and efficient approach, our conclusions were derived from cross-sectional associations, an unavoidable limitation when studying human tissue pathologies. Animal studies have, however, identified a number of features that are testable in human samples, and provide support for our findings. For example, β-to α-cell conversion in response to hyperglycaemia involves a transitional intermediate characterised by cells co-expressing both hormones (7). We report an increased frequency of these bi-hormonal cells in the islets of patients with T2D, consistent with previous histology reports (12-13). Whilst we consider these cells to reflect ongoing β-to α-cell conversion, it cannot be excluded that these cells represent an intermediate of the reverse process. However, we believe this to be less likely, as the regenerative process to yield new β-cells has only been reported in response to near total β-cell ablation (27). In support of this, we report an increased islet α/β cell ratio in donors with T2D compared to controls, consistent with previous studies in other cohorts (13-14, 28).

Total islet endocrine cell number (insulin, glucagon and bi-hormonal cells) was comparable between the two groups, suggesting that β-cell loss through apoptosis is unlikely to be sufficient to account for these changes in islet α/β ratio.

TLE1 is expressed within both the endocrine and exocrine compartments of the human pancreas(17). In keeping with preliminary observations (15), we confirm that, within islets, TLE1 is expressed predominantly by insulin-positive cells, suggesting this co-regulator is
relatively β-cell specific. This supported by our findings that TLE1 is highly co-expressed with insulin in EndoC-βH1 cells, which have previously been reported as being immuno-negative for glucagon (29).

In rodents, another TLE member, TLE3, has been studied in the context of β-cell identity (15). Specifically, through forming complexes with β-cell regulators, such as Nkx2.2 and Nkx6.1, TLE3 functions to maintain mono-hormonal identity through repression of alpha-cell related genes, including glucagon. Subsequent studies, including in vivo expression analysis and overexpression studies in a mouse α-cell line, led the authors to propose that TLE3 functions redundantly with TLE1 (98% homology to humans) in maintaining β-cell identity (15). Here, we extend on these findings and demonstrate that knockdown of TLE1 in the human β-cell line, EndoC-βH1, causes de-repression of glucagon mRNA and mis-expression of glucagon in insulin-expressing cells. Functional similarities and conserved binding partners have been reported in other cell types (30), likely reflecting the highly conserved protein structures (31). Thus, whilst β-cell TLE1 binding partners have not been identified in this study, it is highly probable that interactions with several β-cell proteins will be conserved between the two TLE family members.

In line with established β-cell regulators (8,13,32), reduced islet TLE1 expression in patients with T2D suggests vulnerability to dysregulation following metabolic stress. Lineage tracing analysis has revealed that inactivation of several β-cell transcription factors, including established TLE3 binding partners, leads to both insulin secretory defects and de-repression of non-β-cell endocrine genes (9-11). Whilst our tissue analysis precludes confirmation of a direct functional role for TLE1 loss in de-repressing glucagon expression in T2D, the virtual absence of TLE1 expression in α-cells and inverse relationship between TLE1 expression
and α/β cell ratio is consistent with a role for TLE1 in glucagon repression in human β-cells, which is supported by TLE1 knockdown and overexpression studies reported here and by others (15).

Although considered primarily a co-repressor, TLE3 has been shown to be important in the activation of several genes during adipogenesis (33). Recent indirect evidence indicates a gene activation role for TLE3 in rodent beta-cells also (15). Whilst our analysis cannot confirm a dual-functioning role of TLE1, impaired insulin secretion following TLE1 knockdown has recently been reported in the EndoC-βH1 human beta cell line, indicating an activating role (34). Further studies in human islets, including knockdown and co-immunoprecipitation approaches, will be important in building on these novel observations and providing further molecular insights into TLE1 mediated control of human β-cell identity.

In conclusion, we have demonstrated reduced expression of the β-cell specific co-regulator, TLE1, in islets of patients with T2D. We propose TLE1 as a potential key regulator of β-cell identity, and that persistent metabolic stress leads to disturbances in its expression/function, leading to mis-expression of glucagon and potentially impaired insulin secretion.

**Acknowledgements:**

We would like to acknowledge the ADI IsletCore laboratory (www.isletcore.ca), Edmonton, for the provision of human pancreas samples. The authors thank the staff in the Newcastle MRC Pathology Node for granting access to the Vectra 3 imaging system. The authors gratefully acknowledge the Newcastle University BioImaging Unit for their support & assistance in this work. The research was supported by grants from Diabetes UK.
and an Early Career Grant from the Society for Endocrinology.

**Data Availability**

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.
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**Figure legends**

**Figure 1. Increased proportion of islet α-cells and bi-hormonal cells in T2D.** A-B: Representative images of immunofluorescence staining for insulin (green) and glucagon (glucagon) in pancreatic sections from a donor without (A) and an individual with (B) T2D. Scale bars represent 25 µm. C: Z-stack analysis of a magnified islet region representing the boxed area in panel B. Cells within the box are bi-hormonal co-expressing insulin and glucagon. Scale bars represent 50 µm. D: Total numbers of endocrine (insulin / glucagon/ bi-hormonal) cells per islet in each donor. Each point represents the cell number within a single islet in control (blue) and T2D (red) donors. E: Mean number of cells per islet in control (n=7) and T2D (n=7) donors. Each point represents a single donor. F-H: Percentage of each cell type expressed as a proportion all assessed phenotypes. Each point represents a single donor. Data are mean±SEM; *p<0.05 by unpaired Student’s t-test (n=7 donors in the control group, and n=7 donors in the T2D group).

**Figure 2. Most TLE1-positive cells within islets express insulin alone.** A: Representative image of immunofluorescence staining for insulin (green), glucagon (red) and TLE1 (white) in a donor without T2D. Scale bar represents 50 µm (top left panel) and 25 µm (top right panel). B: Distribution of endocrine phenotypes in TLE1-expressing cells (data are mean±SEM in the control donors (n=7); p<0.001 (one-way ANOVA with Tukey post-hoc test)).

**Figure 3. TLE1 expression is reduced in T2DM with lower expression associated with higher α/β cell ratio and glucagon gene derepression in vitro.** A-B: Representative islet from a donor without diabetes (A) and an individual with T2D (B) stained for insulin (green), glucagon (red) and TLE1 (white). Scale bar represents 50 µm. C: Proportion of islet endocrine cells expressing TLE1 (data are mean±SEM; n=7 donors for each group; p=0.06.
D: Proportion of β-cells (insulin+/glucagon−) expressing TLE1 (data are mean±SEM; n=7 donors for each group; *p<0.05 (unpaired Student’s t-test)). E: Inverse correlation between the percentage of TLE1-positive cells and α/β cell ratio (n=7 donors with T2D (black circles) and n=7 control donors (white circles); r=-0.56; p<0.05). F: Tle1 expression was silenced in INS-1E cells using targeted siRNA. Knockdown of Tle1 and de-repression of glucagon gene expression was confirmed by qRT-PCR, with data normalized to rplp0 and expressed as fold change relative to siRNA scrambled control transfections (data are mean±SEM; n=3 independent experiments; *p<0.001 vs scrambled control by unpaired Student’s t-test).

Figure 4. TLE1 inhibition induces glucagon mis-expression in insulin-expressing EndoC-βH1 cells. A-B: siRNA mediated knockdown of Tle1 and de-repression of glucagon gene expression by two independent targeted siRNAs by qRT-PCR, with data normalised to rplp0 (data are mean±SEM; n=3 independent experiments; *p<0.001 vs scrambled control by unpaired Student’s t-test). C-D: Representative immunofluorescent images of EndoC-βH1 stained for insulin, glucagon and TLE1 following transfection with siCTL (C) or siTLE1 (probe 2) (D). E: Magnified region representing the boxed area in panel D. Scale bars represent 100μm (C and D) and 50μm (E). Circles indicate insulin/glucagon co-expressing cells.
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F= Female, M= Male  
N/A= Not available; No. = Number  
* Pre-procurement blood glucose (mmol/L) - HbA1c not available.
Figure 1. Increased proportion of islet $\alpha$-cells and bi-hormonal cells in T2D
Figure 2. Most TLE1-positive cells within islets express insulin alone
Figure 3. TLE1 expression is reduced in T2DM with lower expression associated with higher α/β cell ratio and glucagon gene derepression in vitro.
Figure 4: TLE1 inhibition induces glucagon mis-expression in insulin-expressing EndoC-βH1 cells