



## Silencing of the *FTO* gene inhibits insulin secretion: An *in vitro* study using GRINCH cells



Jalal Taneera<sup>a,b,\*</sup>, Rashmi B. Prasad<sup>b</sup>, Sarah Dhaiban<sup>a</sup>, Abdul Khader Mohammed<sup>a</sup>, Leena Haataja<sup>c</sup>, Peter Arvan<sup>c</sup>, Mawieh Hamad<sup>a</sup>, Leif Groop<sup>b,d</sup>, Claes B. Wollheim<sup>b,e</sup>

<sup>a</sup> Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates

<sup>b</sup> Lund University Diabetes Center, Malmö, Lund University, Sweden

<sup>c</sup> Endocrinology and Diabetes, University of Michigan Medical School, Ann Arbor, USA

<sup>d</sup> Finnish Institute for Molecular Medicine (FIMM), Helsinki University, Finland

<sup>e</sup> Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland

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### ABSTRACT

Expression of fat mass and obesity-associated gene (*FTO*) and ADP-ribosylation factor-like 15 (*ARL15*) in human islets is inversely correlated with HbA<sub>1c</sub>. However, their impact on insulin secretion is still ambiguous. Here in, we investigated the role of *FTO* and *ARL15* using GRINCH (Glucose-Responsive Insulin-secreting C-peptide-modified Human proinsulin) clonal rat  $\beta$ -cells. GRINCH cells have inserted GFP into the human C-peptide insulin gene. Hence, secreted CpepGFP served to monitor insulin secretion. mRNA silencing of *FTO* in GRINCH cells showed a significant reduction in glucose but not depolarization-stimulated insulin secretion, whereas *ARL15* silencing had no effect. A significant down-regulation of insulin mRNA was observed in *FTO* knockdown cells. Type-2 Diabetic islets revealed a reduced expression of *FTO* mRNA. In conclusion, our data suggest that fluorescent CpepGFP released from GRINCH cells may serve as a convenient marker for insulin secretion. Silencing of *FTO* expression, but not *ARL15*, inhibits insulin secretion by affecting metabolic signaling.

### 1. Introduction

The *FTO* gene has been identified as an obesity-susceptibility gene in multiple populations (Frayling et al., 2007; Dina et al., 2007; Scuteri et al., 2007; Scott et al., 2007; Peeters et al., 2008). The gene was first discovered in mice where a deletion of 1.6 Mbp in chromosome 8 was reported. Mice heterozygous for the deletion displayed a malformation of forelimbs (fused toes) (Peters et al., 1999), while homozygous mice died during mid-gestation of malformations of the head and face, with defect in the central nervous system (CNS) development and growth retardation. *FTO* acts as a 2-oxoglutarate (2OG)-dependent nucleic acid demethylase (Gerken et al., 2007), therefore, it is suggested that *FTO* might down-regulate genes involved in metabolism and lead to obesity.

*FTO* has been shown to play a vital role in obesity, appetite and energy homeostasis (Church et al., 2009). The gene is ubiquitously expressed in the brain, pancreatic endocrine cells ( $\beta$  and  $\alpha$ ) and adipose tissue (Frayling et al., 2007; Gao et al., 2010; Stratigopoulos et al., 2008; Taneera et al., 2012). Its expression is highest in the hypothalamic region that regulates energy intake and expenditure (Poritsanos et al., 2011). Fasted mice exhibit a significant reduction of *FTO*

expression in the hypothalamic regions (Poritsanos et al., 2011). These data suggest that variation in *FTO* resulting in decreased expression or activity might provide a signal that promotes feeding and obesity. Moreover, inactivation of the *FTO* gene in mice was shown to protect from obesity, substantiating a central role of *FTO* in the control of energy expenditure (Fischer et al., 2009; Merkestein et al., 2014).

In humans, several studies have demonstrated single nucleotide polymorphisms (SNPs) in the *FTO* gene that are associated with increased body mass index (BMI) (Frayling et al., 2007; Scuteri et al., 2007). Many studies have shown a vital role for *FTO* in the development of obesity, regulation of appetite and energy homeostasis (Church et al., 2009). Moreover, Genome-wide association studies (GWAS) have identified genetic variants in the *FTO* gene that are associated with increased risk of type 2 diabetes (T2D) (Frayling et al., 2007; Yajnik et al., 2009). Furthermore, the *FTO* protein was reported to have a rapid turnover in clonal pancreatic  $\beta$  cells and its overexpression enhanced insulin secretion, suggesting that *FTO* could play an important role in  $\beta$ -cell function (Russell and Morgan, 2011).

*ARL15* belongs to the ADP-ribosylation factor (ARF) family. It has been shown that *ARL15* regulates membrane trafficking, lipid

\* Corresponding author. Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates.  
E-mail address: [Jtaneera@sharjah.ac.ae](mailto:Jtaneera@sharjah.ac.ae) (J. Taneera).

### Abbreviations

FTO	Fat mass and obesity-associated
ARL15	ADP-ribosylation factor-like 15
CHL1	Cell adhesion molecule L1 like
GFP	Green fluorescence Protein
RIA	Radioimmunoassay
2OG	2-oxoglutarate
SNPs	Single nucleotide polymorphisms
GRINCH	Glucose-Responsive Insulin-secreting C-peptide-modified Human proinsulin

composition and the insulin signaling pathway (Donaldson and Jackson, 2011). Several studies reported a strong association of *ARL15* with increased risk of T2D, fasting insulin level and even  $\beta$  cell proliferation (Scott et al., 2012; Thomsen et al., 2016). Also, it has been reported that *ARL15* acts as insulin-sensitizing protein to phosphorylate the insulin receptor, (IR), the insulin receptor substrate-1 (IRS1) and AKT kinase in the insulin pathway (Zhao et al., 2017). Recently, using RNA-sequencing gene expression from human islets we reported that both *FTO* and *ARL15* are inversely correlated with HbA<sub>1c</sub> levels (Fadista et al., 2014a). Still, the functional role of *FTO* and *ARL15* in pancreatic  $\beta$  cells remains unclear.

Pancreatic  $\beta$ -cells are specialized cells that regulate glucose homeostasis. Defects of insulin secretion in  $\beta$ -cells represent the culprit of T2D, a disease that is expected to affect more than 640 million people by 2040 (<http://www.diabetesatlas.org>). Therefore, there is an urgent need to understand mechanisms of dysregulated insulin secretion in humans. One way to achieve this is to develop experimental models that mimic real human pancreatic  $\beta$ -cells. Here we used the stable  $\beta$ -cell line called GRINCH, derived from INS-1 cells but additionally expressing a human proinsulin, in which the superfolder GFP is contained within the C-peptide (Haataja et al., 2013).

In this study, we harness GRINCH cells to investigate the influence of expression silencing of *FTO* and *ARL15* on glucose-stimulated insulin secretion. Our data indicate that *FTO* expression is reduced in islets from T2D organ donors and that its silencing attenuates glucose-stimulated insulin secretion. In contrast, knockdown of *ARL15* did not alter insulin secretion.

## 2. Materials and methods

### 2.1. GRINCH cells

The establishment of GRINCH cells has been described elsewhere (Haataja et al., 2013). Briefly, cDNA encoding superfolder GFP (sfGFP) was amplified and ligated into the *ApaI* site located at the C-peptide encoding sequence to make hPro-CpepSfGFP. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in RPMI 1640 culture medium containing 11 mM D-glucose (Gibco, Life Technologies) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50  $\mu$ M  $\beta$ -mercaptoethanol (all from Sigma) and 0.1 mg/ml G418 (CalBiochem, Germany). INS-1 (832/13) cells were cultured under the same conditions but without G418.

### 2.2. Cell culture and insulin secretion assay

Confluent plates containing (GRINCH or INS-1 (832/13) cells were washed with 1 mL pre-warmed Secretion Assay Buffer (SAB), pH 7.2 (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.16 mM MgSO<sub>4</sub>, 20 mM HEPES, 2.5 mM CaCl<sub>2</sub>, 25.5 mM NaHCO<sub>3</sub> and 0.2% Bovine Serum Albumin) containing 2.8 mM glucose. The cells were then pre-incubated for 2 h in fresh 2 mL SAB with 2.8 mM glucose. Thereafter,

separate wells were incubated for 1 h in 1 mL SAB containing either 2.8 mM glucose plus secretagogue (16.7 mM glucose, 1 mM 3-isobutylmethylxanthine (IBMX) and 10  $\mu$ M Forskolin). Secreted insulin was measured from the supernatant using Coat-a-Count Insulin radioimmunoassay kit (RIA) (Siemens), High Range Rat Insulin ELISA (Merckodia, Uppsala, Sweden) and the GFP signals using TECAN plate-reader (Infinite M200, Switzerland). The fluorescence intensity in the plate-reader was adjusted for excitation wavelength 489 nm and emission wavelength 527 nm.

### 2.3. RNA interference

GRINCH (passage nr 14) or INS-832/13 (passage nr 59) cells were cultured as previously described (Taneera et al., 2012) and transfected using Lipofectamine RNAiMAX transfection reagent<sup>®</sup> (Invitrogen). siRNA sequences against *CHL1* (S 44135 and S 44134), *FTO* (S 146052 and S146053) and *ARL15* (s187063) were purchased from ThermoFisher. For control purposes, a previously described control sequence Silencer<sup>®</sup> Negative Control #2 from Ambion was employed. Cells were cultured in RPMI 1640 medium for 72 h at 37 °C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> in the presence of 40 nM siRNA in 24-well cell culture microplates (250 000 cells/well). Three days after transfection, insulin secretion measurements were performed by RIA or ELISA and by TECAN plate-reader.

### 2.4. RT-qPCR

Total RNA was isolated with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA quality and concentration were measured using an Agilent 2100 bioanalyzer (Bio-Rad, Hercules, CA, USA) and Nanodrop ND-1000 equipment (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using RevertAid H Minus First Strand cDNA synthesis Kit (Fermentas, Life Sciences). Analysis of gene expression after knockdown was performed using the TaqMan qPCR with an ABI Prism 7900 HT System (Applied Biosystems, USA) using gene-specific primer probes for *CHL1* (Rn01420997\_m1), *FTO* (Rn01538186\_m1), *ARL15* (Rn01494252\_m1), *Ins1* (Rn02121433\_g1), *Ins2* (Rn01774648\_g1) and human *Ins* (Hs02741908\_m1). (Applied Biosystems, USA) in triplicates on 384-well plate. 1.2 ng cDNA was used per well in 10- $\mu$ L reaction volume containing TaqMan master mix (Applied Biosystems, USA). The rat HPRT (Applied Biosystem) was used to normalize gene expression by the  $\Delta\Delta$ Ct method, where the final normalized quantity was expressed as 2<sup>-(Ct target - Ct control)</sup>.

### 2.5. RNA sequencing and human pancreatic islets

Human Islets from 204 cadaver donors (162 nondiabetic and 29 diabetic) of European ancestry were provided by the Nordic Islet Transplantation Program and processed as previously described (Taneera et al., 2015). Islets were obtained from 162 nondiabetic donors (65 females, 97 males, age 57.9  $\pm$  10, BMI 26.2  $\pm$  3.8, HbA<sub>1c</sub> 5.7  $\pm$  0.5) and 29 T2D donors (10 females, 19 males, age 61.1  $\pm$  10, BMI 27.6  $\pm$  4.5, HbA<sub>1c</sub> 6.8  $\pm$  0.8). Purity of islets was assessed by dithizone staining and was 70%  $\pm$  17% in the nondiabetic and 67%  $\pm$  20% in the diabetic islets (p = 0.45). RNA sequencing was done using Illumina's TruSeq RNA Sample Preparation Kit. Detailed materials and methods for RNA-seq were previously described (Fadista et al., 2014b). All procedures were approved by the ethics committees at Uppsala and Lund University.

### 2.6. Apoptosis analysis

For apoptosis analysis, 1  $\times$  10<sup>5</sup> cells (72-h post-transfection) were re-suspended in 500  $\mu$ l of Annexin-V (1X) Binding Buffer (BD, USA). A 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l Propidium Iodide was added and incubated at room temperature for 10 min in the dark. Analysis with

Flowcytometer (FACSaria III, BD) was performed using FITC channel (515 nm) and PI at PE detector (585 nm), 30000 events are collected for the analysis. Compensation was performed by using the single color controls with Annexin FITC and PI.

### 2.7. Western blot analysis

Cells were lysed in ice-cold NP-40 (1.0% NP-40, 150 mM of NaCl, 50 mM of Tris-Cl, pH 8.0) lysis buffer containing protease cocktail inhibitor tablets (Sigma) 72 h post-transfection. Protein concentrations were quantified using the standard Bradford method (Bio-Rad, CA, USA). A 30 µg sample of protein lysate was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad) and then blocked by 5% skimmed milk powder for 1 h at room temperature, washed with (TBST), and the blot was probed with FTO (1:1000; #Ab92821 Abcam, UK) antibody overnight at 4 °C. The secondary (anti-mouse and anti-rabbit) antibodies were then reacted with the membrane at 1:1000 dilutions for 1 h at room temperature. Chemiluminescence was detected using the ECL kit (Thermo Fisher). Protein band quantification was carried out using the Bio-Rad Image Lab software (ChemiDoc™ Touch Gel and Western Blot Imaging System; Bio-Rad). β-actin was used as a normalization control.

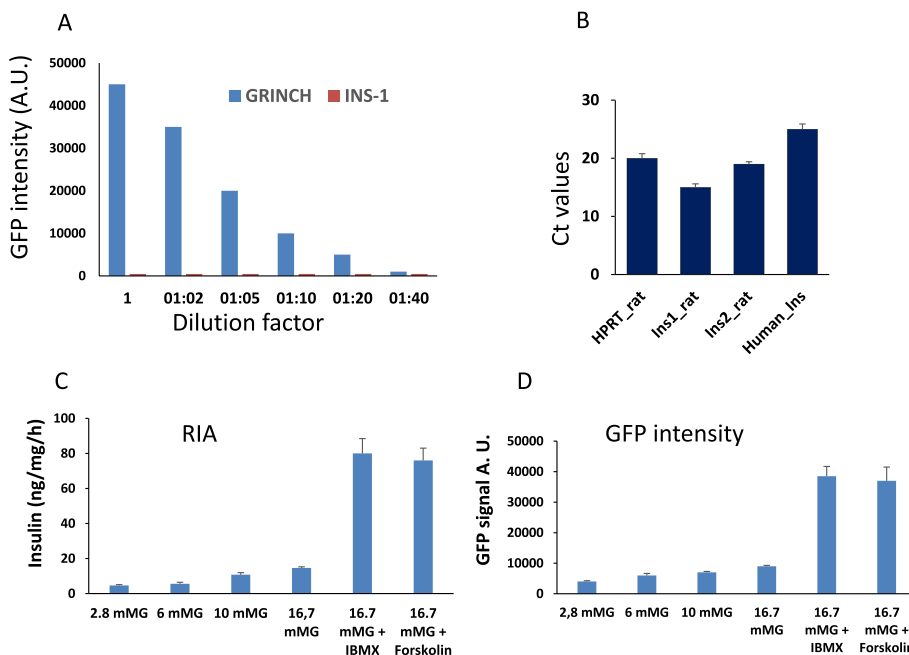
### 2.8. Statistical analysis

Data are presented as means ± S.D. Differences in expression levels were analyzed by student's t-test or nonparametric Mann-Whitney tests.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. GFP signal intensity in GRINCH cells

To measure CpepGFP signal intensity of GRINCH cells, we prepared lysate dilutions from GRINCH cells, with regular INS-1 (832/13) cells used as a negative control. Lysates from GRINCH cells exhibited high GFP intensity, which is gradually reduced with higher dilution (Fig. 1-A). We were able to detect CpepGFP signals from the lysate preparation even at dilution factor 1:40 compared to INS-1 (832/13) cell lysate, which showed no GFP signal intensity.



**Fig. 1.** GFP signal intensity, *Ins* genes expression and insulin secretion in GRINCH cells. (A) Sonicated lysates from GRINCH and regular INS-832/13 cells were diluted in H<sub>2</sub>O and CpepGFP signals were measured using the TECAN plate-reader. (B) Ct values of qRT-PCR demonstrate that GRINCH cells express both rat (*Ins1* and *Ins2*) and human insulin genes. HPRT1 was used as a house keeping gene. (C, D) GRINCH cells were stimulated with 2.8, 6, 10, 16.7 mM glucose, 16.7 mM glucose + 1 mM IBMX or 10 µM Forskolin for 1 h. Supernatants containing secreted insulin were measured by RIA (C) and CpepGFP signals using TECAN plate-reader method (D).

### 3.2. GRINCH cells expressed a human and rat insulin gene cDNA along with endogenous rat insulin

As previously described, INS-1 (832/13) cells were derived from INS-1 cells and engineered to stably express human insulin cDNA (Asfari et al., 1992). Moreover, the human insulin content of GRINCH and INS-1 (832/13) cells was similar and both exhibited stimulus-dependent secretion (Haataja et al., 2013). To confirm expression of human and rat insulin mRNA in GRINCH cells, RT-qPCR was performed using distinct sets of amplifying primers. As expected, both human and rat insulin mRNA was expressed in GRINCH cells. The Ct values for the rat *Ins1* was 13, rat *Ins2* 19 and for the human *Ins* gene was 25 (Fig. 1-B).

### 3.3. Insulin secretion in GRINCH cells

To test secretagogue-stimulated insulin secretion in GRINCH cells, cells were seeded (700 000 cell/well) and grown overnight. The next day, cells were stimulated with 2.8, 6, 10, 16.7 mM glucose, 1 mM IBMX or 10 µM Forskolin for 1 h. The supernatant containing secreted insulin was analyzed using RIA and by measuring the GFP signals in a TECAN plate-reader. RIA measurements showed a 3-fold increase between the basal level (2.8 mM Glucose) and secretion level (16.7 mM Glucose) and almost 6-fold between the 16.7 mM glucose and 16.7 mM glucose with IBMX or Forskolin (Fig. 1-C). Using the GFP signal as a surrogate marker to measure insulin secretion, we observed a 2-fold increase between the basal and stimulating section level (16.7 mM) and an almost 5-fold increase between 16.7 mM glucose and IBMX or Forskolin (Fig. 1-D). Insulin secretion measured by RIA and plate-reader were highly correlated ( $R^2 = 0.99$ ) (data not shown). The percentage of insulin release measured as a fraction of insulin content was 1.8% using CpepGFP signal assay, while it was 2% using the ELISA assay after 16.7 mM glucose stimulation.

### 3.4. GRINCH cells as a tool to study pancreatic β cell function

The *CHL1* gene encodes for a neural adhesion molecule, it has been suggested that *CHL1* might be involved in the development of islet structure and determine the distribution of β, α, and δ cells in pancreatic islets. Previously, we reported that expression of *CHL1* in human islets is strongly related to HbA<sub>1c</sub> and insulin secretion and decreased in

islets and  $\beta$  cells from patients with T2D (Taneera et al., 2012). Importantly, we showed that siRNA gene silencing of *CHL1* in INS-1 (832/13) cells significantly reduced insulin secretion.

Thus, to validate GRINCH cells as a tool for functional studies of pancreatic  $\beta$  cells, we silenced the expression of *CHL1* in GRINCH cells. Knockdown efficacy measured by RT-qPCR after 48 h revealed almost 50% decreased expression of *CHL1* (Fig. 2-A). The supernatants containing secreted insulin from transfected cells were analyzed after glucose stimulation using ELISA assay and TECAN plate-reader for CpepGFP signal intensity. We noticed a significant reduction in insulin secretion (45%,  $p < 0.05$ ) in silenced cells compared to negative control siRNA using ELISA assay (Fig. 2-B), whereas a 37% reduction in insulin secretion was observed using the CpepGFP signal measurement ( $p < 0.05$ ) (Fig. 3-C). These results further validate the use of GRINCH cells for screening of altered insulin secretion by monitoring fluorescence.

### 3.5. Silencing of T2D candidate genes

Fadista et al. have identified a list of 17 established T2D and glycemic associated loci (*PCSK1*, *SLC30A8*, *G6PC2*, *IKBKAP*, *FTO*, *VPS13C*, *PDX1*, *ARL15*, *ADAMTS9*, *JAZF1*, *RASGRP1*, *PROX1*, *SLC2A2*, *GRB14*, *AMT*, *PTPRD* and *IRS1*) whose gene expression proxies in human islets using RNA-sequencing are associated with HbA<sub>1c</sub> levels (Fadista et al., 2014b). Of the 17 genes, 2 genes (*ARL15* (ADP-ribosylation factor-like 15) and *FTO*) were selected for further investigation in GRINCH cells.

Specifically, siRNA against *FTO* and *ARL15* were used to silence the expression of each gene in GRINCH cells. Transfection efficacy measured by RT-qPCR after 48 h resulted in almost a 90% decreased expression of *FTO* and 70% for *ARL15* (Fig. 3-A). Similar reduction of *FTO* protein expression was observed by western blot analysis. (Fig. 3-B). The supernatants containing secreted insulin from transfected cells were analyzed after glucose stimulation using ELISA and TECAN plate-reader for GFP signal intensity. Insulin secretion measurement after 72 h in *FTO* knockdown cells (measured by ELISA) in response to 16.7 mM glucose showed almost 50% reduction ( $p < 0.5$ ) and 40% reduction ( $p < 0.05$ ) as measured by CpepGFP compared to negative control cells (Fig. 3-B and C). As the first siRNA silencing of *FTO* (si*FTO*-1) revealed a decrease in insulin secretion, we further confirmed the finding using another siRNA (si*FTO*-2) (Fig. 3-B and C). Moreover, *FTO* knockdown reduced the first phase of insulin secretion as measured at 10 min after glucose stimulation compared to negative control cells (Supplementary Fig. 1).

Silencing of *ARL15* showed no impact on insulin secretion compared to siRNA negative control measured by ELISA or GFP intensity (Fig. 3-B and C).

To elucidate how siRNA gene silencing of *FTO* contributes to pancreatic  $\beta$ -cell failure, we performed several functional studies to identify the possible mechanism.

First, to explore whether the exocytosis machinery is defective upon silencing of *FTO*, we stimulated insulin exocytosis with depolarizing (35 mM) KCl, and this showed no difference between transfected cells

and controls (Fig. 3-D and E).

Next, we stained transfected cells with Annexin-V/PI to investigate the cell death of INS-1 (832/13) cells following siRNA silencing. Since Annexin-V is monitored using the FITC channel we could not use the GRINCH cells because of fluorescence overlap. As shown in Fig. 4-A, the ratio of the apoptotic cells between *FTO* knockdown cells and siRNA negative control were similar. The percentage of early apoptotic cells (0.2% vs. 0.3%) or late apoptotic cells (0.1% vs. 0.2%) between *FTO* knockdown cells and control siRNA (Fig. 4-A).

Finally, we analyzed the mRNA expression level of *Ins1* and *Ins2* genes in transfected GRINCH cells. qRT-PCR analysis showed a significant decrease of both *Ins1* ( $p = 0.01$ ) and *Ins2* ( $p = 0.02$ ) mRNA expression in transfected cells compared to negative siRNA control (Fig. 4-B). This was further confirmed by measuring the GFP signal from cell lysates which showed almost 30% decrease in *FTO* transfected cells (Fig. 4-C).

### 3.6. Expression of *FTO* in human islets

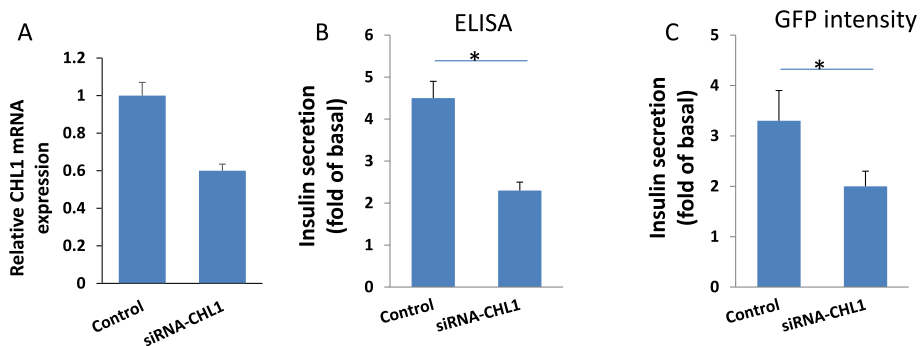
Using RNA-sequencing data from human pancreatic islets, we investigated the expression of *FTO* in diabetic ( $n = 30$ ) and nondiabetic donors ( $n = 172$ ). *FTO* expression was found to be lower in T2D islets ( $p = 0.058$ , adjusted for age, sex and BMI) than in non-diabetic islets (Fig. 5-A). Moreover, stratifying the donors into normal glucose tolerant (NGT; HbA<sub>1c</sub> < 6.0) and T2D (HbA<sub>1c</sub> < 6.5) showed that mean expression of *FTO* was significantly lower in T2D donors ( $p = 0.04$ ; adjusted to age, sex and BMI) (Fig. 5-B).

## 4. Discussion

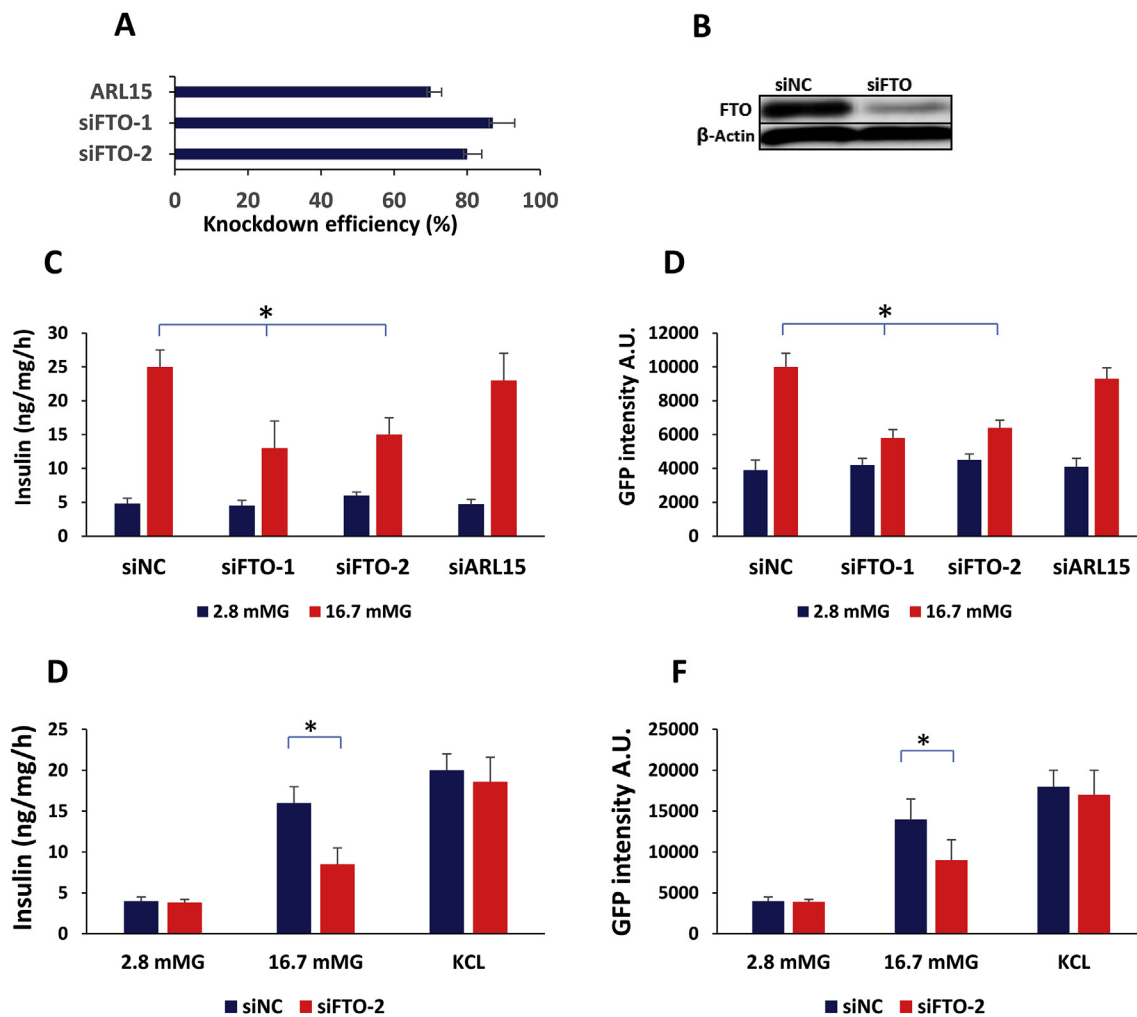
Several studies have suggested a potential role for *FTO* in insulin secretion (Russell and Morgan, 2011; Kirkpatrick et al., 2010). Kirkpatrick et al., reported that *FTO* mRNA level was reduced in purified human  $\beta$ -cells from a small number of T2D patients compared with non-diabetic controls (Kirkpatrick et al., 2010). Fadista et al. found *FTO* to be suppressed in islets of T2D patients (21), which we confirm in a larger number of organ donor islets. It is likely that the reported increase in DNA methylation of the *FTO* gene contributes to the lower mRNA expression (Dayeh et al., 2014).

However, the role of *FTO* in insulin secretion is controversial. Here, we demonstrate that siRNA silencing of *FTO* results in reduced glucose-stimulated insulin secretion without affecting the stimulant action of depolarizing potassium concentrations. This indicates that the exocytotic machinery is not altered by *FTO* silencing. In agreement with our finding, Russell et al. showed that conditional expression of the *FTO* gene product in INS-1 cells enhanced first phase insulin secretion stimulated with 20 mM glucose (Russell and Morgan, 2011). However, the increase in insulin secretion in this case was attributed to the exocytosis machinery rather than upregulated insulin biosynthesis.

Inconsistent with our findings and those of Russell and Morgan (Russell and Morgan, 2011) was the observation that *FTO* overexpression in MIN6 clonal mouse  $\beta$ -cells by lentivirus resulted in a



**Fig. 2. CHL1 knockdown in GRINCH cells.** (A) Knockdown efficiency of siRNA of *CHL1* in GRINCH cells as determined by RT-qPCR. (B) Insulin secretion as assessed by RIA in response to 2.8 mM and 16.7 mM glucose at 72 h post siRNA transfection during 1-h static incubation. Insulin secretion expressed as a fold increase (insulin secreted at 16.7 mM at 2.8 mM glucose). (C) Insulin secretion measured as GFP intensity by plate-reader. Bars represent mean  $\pm$  SD. \* $p < 0.05$ .

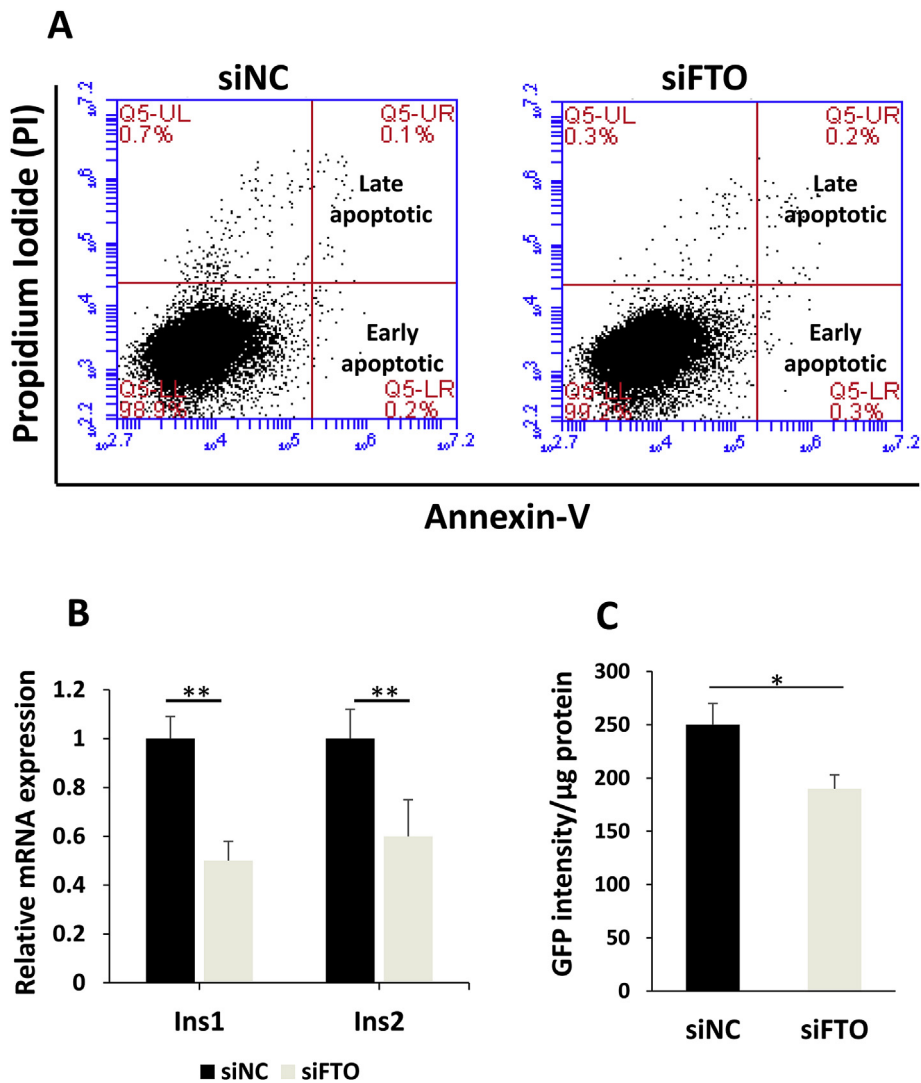


**Fig. 3.** *FTO* and *ARL15* knockdown in GRINCH cells. (A) knockdown efficiency of siRNA for *ARL15* (s187063), *FTO*-1 (s146052) and *FTO*-2 (s146053) in INS-1 (832/13) cells. (B) Western blot bands of *FTO* protein expression relative to the endogenous control protein  $\beta$ -actin in *FTO* knockdown cells and cells transfected with negative siRNA control. (C) Insulin secretion in response to 2.8 mM and 16.7 mM glucose 72 h post siRNA transfection measured during 1-h static incubation. Cells transfected with siRNA against *FTO* showed almost a 50% reduction in insulin secretion in both siRNAs, whereas those with *ARL15* showed no effect compared to siRNA negative control (NC). (D) Insulin secretion measured by Cpep-GFP intensity by plate-reader showed a 40% reduction with *FTO* transfected cells but not *ARL15*. (E & F) Transfected cells with siRNA against *FTO*-2 were stimulated by a depolarizing agent (35 mM KCl) and measured by ELISA and GFP intensity. No significant differences between transfected and control cells were observed. Insulin secretion was normalized for cell count. Data are shown from three independent experiments for each siRNA. Bars represent mean  $\pm$  SD. \* < 0.05, \*\* < 0.01.

significant reduction in insulin secretion in the presence of 20 mM glucose or KCl (Fan et al., 2015). Interestingly, silencing of *FTO* in MIN6 cells revealed no effect on insulin secretion. A possible explanation for the discrepancy could relate to the different approach for *FTO* silencing by siRNA, vs. overexpression by lentivirus. INS-1 cells and GRINCH cells were derived from a rat insulinoma, while MIN6 cells are of murine origin. Therefore, species-related variations could underlie the discrepant results. Species-specific results regarding the role of *FTO* have indeed been reported. For example, while fasted mice exhibit a significant decrease in hypothalamic *FTO* expression (independently of leptin levels) as compared to fed controls (Gerken et al., 2007), fasted rats show increased *FTO* expression in the hypothalamus (Fredriksson et al., 2008). Moreover, loss of *FTO* function in mice reduces fat mass through increased energy expenditure but not decreased energy intake (Church et al., 2009). It has been suggested that *FTO* impacts feeding behavior by affecting neuropeptide Y expression in the hypothalamus in rodents (Alkan et al., 2017). In man, *FTO*-SNPs are associated with appetite and food intake but not energy expenditure (Fischer et al., 2009; Do et al., 2008). Thus, it is likely that the discrepancy between species reflects differences of *FTO* actions.

GWAS have implicated *ARL15* in several metabolic traits (Scott et al., 2012; Thomsen et al., 2016). Despite the fact that *ARL15* expression was correlated with HbA<sub>1c</sub> levels, our results showed that gene silencing of *ARL15* in INS-1 cells had no effect on insulin secretion. During the preparation of this study, Rocha et al. reported studies on *ARL15* in insulin signal transduction and adipocytes differentiation in 3T3-L1 preadipocytes (Rocha et al., 2017). In *ARL15* knockdown cells, there was no effect on insulin-stimulated phosphorylation of AKT or ERK nor on insulin-stimulated glucose uptake. However, significant impairment of adiponectin secretion was observed.

Here we report the use of CpepGFP signal intensity from GRINCH cells as a new tool to assay pancreatic  $\beta$ -cell function. GRINCH cells were developed to study proinsulin trafficking, processing and secretion from pancreatic  $\beta$ -cells. The superfolder GFP inserted in human proinsulin C-peptide showed excellent glucose-stimulated insulin secretion upon exposure to high glucose doses and unique feature based on the ability to exhibit a recoverable CpepGFP signal (Haataja et al., 2013). This feature of GRINCH cells allowed us to exploit the CpepGFP signal in GRINCH cells as a marker of  $\beta$ -cell function. We noticed that secretion data from stimulated GRINCH cells measured by GFP-plate

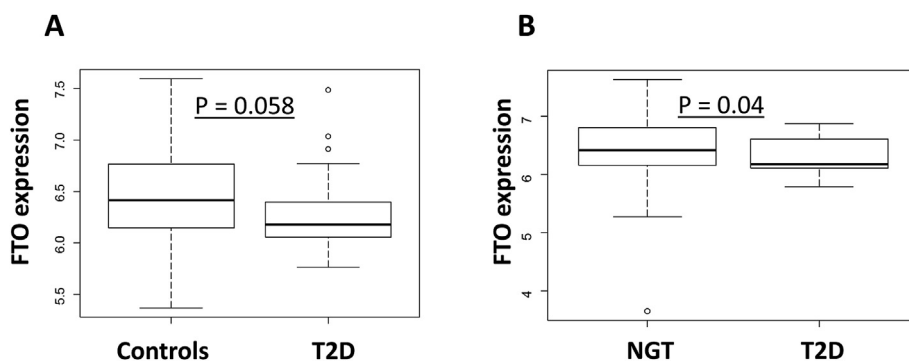


**Fig. 4. Assessment of apoptosis in INS-1 cells.** (A) Cell transfected with siRNA negative control or siRNA against *FTO* were stained with Annexin V-FITC and PI and analyzed by flow cytometry. (B) qPCR expression analysis of *Ins1* and *Ins2* genes in GRINCH cells transfected with negative control or siRNA against *FTO*. Cell lysate from transfected cells (with negative control or *FTO*) were used to measure GFP intensity as indicator for insulin content. Bars represent mean  $\pm$  SD. \* < 0.05.

reader assay were overall similar to results obtained with RIA or ELISA, except for a marginally smaller fold-stimulation. However, such a difference between the assays was not statistically significant ( $p > 0.05$ ). Herein, we have used relatively early passage number of GRINCH cells, stability of the CpepGFP over multiple passages need to be tested.

Several reports have described surrogate markers for insulin

secretion in pancreatic  $\beta$ -cell lines by fused GFP or luciferase (Burns et al., 2015; Pouli et al., 1998). Recently, Bruns et al. reported a luminescent insulin secretion assay that enables large-scale investigation of  $\beta$ -cell function (Burns et al., 2015). Specifically, Bruns et al. inserted a Gaussia luciferase into the C-peptide portion of proinsulin, and they found that upon stimulation with secretagogues,  $\beta$ -cells expressing this



**Fig. 5. *FTO* gene expression in human pancreatic islets.** *FTO* mRNA expression revealed lower level in pancreatic islets from T2D donors compared to those from non-diabetic controls (B), the reduced expression was significant when donors were stratified for NGT vs T2D.

reporter secreted luciferase in close correlation with insulin. In the case of GRINCH cells, insulin secretion measurement is performed directly by plate-reader assay of the supernatants after secretagogues simulations while the luciferase activity requires adding coelenterazine substrate to the supernatant before read-out.

Out of the 17 established T2D genes whose gene expression proxies are associated with HbA1c, eight genes (*SLC30A8* (Wijesekara et al., 2010), *G6PC2* (Pound et al., 2013), *PCSK1* (Ravassard et al., 2011), *PDX1* (Stoffers et al., 1997), *VPS13C* (Grarup et al., 2011), *RASGRP1* (Taneera et al., 2012), *SLC2A2* (Guillam et al., 1997), and *IRS1* (Meur et al., 2010)) were excluded as they have already been implicated for a role in insulin secretion. Additionally, previously we showed that gene silencing of *ADAMTS9* and *JAZF1* has no impact on insulin secretion (Taneera et al., 2015). For the other 7 genes, the selection criteria included expression in  $\beta$ -cells based upon previously published RNA-sequencing expression data from sorted human pancreatic endocrine cells (Blodgett et al., 2015). The *FTO*, *IKBKAP* and *ARL15* genes were found to have the highest expression in sorted  $\beta$ -cells. *ARL15* is a GTP-binding protein that has been shown to influence adiponectin levels (Richards et al., 2009). *IKBKAP* encodes a scaffold protein and a regulator for 3 different kinases involved in pro-inflammatory signaling. Mutations in this gene have been associated with familial dysautonomia (Slaugenhaupt et al., 2001). Based on gene function, only *FTO* and *ARL15* were selected for functional studies in GRINCH cells.

**Conclusions** We demonstrate the validity of GRINCH cells as a new tool to study pancreatic  $\beta$ -cell secretion. The cells retain insulin secretion responses to glucose and exhibit similar physiologic characteristics comparable to those of INS-1 (832/13) cells. This tool may serve as a rapid, and inexpensive method for screening of compounds, in order to investigate molecular mechanisms influencing insulin secretion. Knockdown of *FTO* expression in GRINCH cells inhibits insulin secretion, whereas no effect for *ARL15* was observed. The reduced insulin secretion in *FTO* knockdown cells was associated with decreased insulin mRNA levels while cell viability was preserved. Additional work should pin-point the exact role of the *FTO* gene as a regulator of pancreatic  $\beta$ -cell function.

## Conflicts of interest

The authors declare that there is no duality of interest associated with this manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2018.06.003>.

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