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

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

[a] Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates
[b] Lund University Diabetes Center, Malmoe, Lund University, Sweden
[c] Endocrinology and Diabetes, University of Michigan Medical School, Ann Arbor, USA
[d] Finnish Institute for Molecular Medicine (FIMM), Helsinki University, Finland
[e] 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_{1c}. 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 β-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 (β and α) 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 β cells and its overexpression enhanced insulin secretion, suggesting that *FTO* could play an important role in β-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 (J. Taneera).

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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 β 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_{1c} levels (Fadista et al., 2014a). Still, the functional role of *FTO* and *ARL15* in pancreatic β cells remains unclear.

Pancreatic β-cells are specialized cells that regulate glucose homeostasis. Defects of insulin secretion in β-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 β-cells. Here we used the stable β-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 *Apa*I 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_2 in RPMI 1640 culture medium containing 11 mM D-glucose (Gibco, Life Technologies) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/mL streptomycin, 10 mM glutamine, 1 mM sodium pyruvate, 10 mm HEPES, 50 µM β-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_2PO_4, 1.16 mM MgSO4, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$ 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 µM Forskolin). Secreted insulin was measured from the supernatant using Coat-a-Count Insulin radioimmunoassay kit (RIA) (Siemens), High Range Rat Insulin ELISA (Mercodia, 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® (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® 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% CO2 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-µL reaction volume containing TaqMan master mix (Applied Biosystems, USA). The rat HPRT (Applied Biosystem) was used to normalize gene expression by the ΔΔCt method, where the final normalized quantity was expressed as 2^-(Ct target - Ct control).

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 ± 10, BMI 26.2 ± 3.8, HbA_{1c} 5.7 ± 0.5) and 29 T2D donors (10 females, 19 males, age 61.1 ± 10, BMI 27.6 ± 4.5, HbA_{1c} 6.8 ± 0.8). Purity of islets was assessed by dithizone staining and was 70% ± 17% in the nondiabetic and 67% ± 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, 1×10^5 cells (72-h post-transfection) were re-suspended in 500 µl of Annexin-V (1X) Binding Buffer (BD, USA). A 5 µl of Annexin V-FITC and 5 µl Propidium Iodide was added and incubated at room temperature for 10 min in the dark. Analysis with