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How to cite

ASRIH, Mohamed et al. Growth differentiation factor-15 prevents glucotoxicity and connexin-36 downregulation in pancreatic beta-cells. In: Molecular and cellular endocrinology, 2022, vol. 541, p. 111503. doi: 10.1016/j.mce.2021.111503

This publication URL: https://archive-ouverte.unige.ch/unige:169219
Publication DOI: 10.1016/j.mce.2021.111503

Molecular and Cellular Endocrinology 541 (2022) 111503

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Growth differentiation factor-15 prevents glucotoxicity and connexin-36 downregulation in pancreatic beta-cells

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ARTICLE INFO

Keywords:
Connexin-36
GDF15
Glucotoxicity
INS-1E
Pancreatic beta-cell dysfunction

ABSTRACT

Pancreatic beta cell dysfunction is a hallmark of type 2 diabetes. Growth differentiation factor 15 (GDF15), which is an energy homeostasis regulator, has been shown to improve several metabolic parameters in the context of diabetes. However, its effects on pancreatic beta-cell remain to be identified. We, therefore, performed experiments using cell models and histological sectioning of wild-type and knock-out GDF15 mice to determine the effect of GDF15 on insulin secretion and cell viability. A bioinformatics analysis was performed to identify GDF15-correlated genes. GDF15 prevents glucotoxicity-mediated altered glucose-stimulated insulin secretion (GSIS) and connexin-36 downregulation. Inhibition of endogenous GDF15 reduced GSIS in cultured mouse beta-cells under standard conditions while it had no impact on GSIS in cells exposed to glucolipotoxicity, which is a diabetogenic condition. Furthermore, this inhibition exacerbated glucolipotoxicity-reduced cell survival. This suggests that endogenous GDF15 in beta-cell is required for cell survival but not GSIS in the context of glucolipotoxicity.

1. Introduction

Obesity is a major public health concern that is predicted to affect more than 30% of the global population by 2025 (Collaboration, 2016). Being overweight is a critical risk factor for developing cardiovascular diseases and type 2 diabetes (Collaborators et al., 2017). Lifestyle changes are the first-line therapeutic approach to prevent life-threatening events of metabolic disorders. Indeed, to date efficient pharmacological treatments are lacking. There is, therefore, a need for the development of pharmacologically efficient and safe therapies. Recently, growth differentiation factor-15 (GDF15), which is a member of the transforming growth factor-beta (TGF-beta) superfamily has emerged as a potential candidate for the treatment of obesity and diabetes (Xiong et al., 2017).

GDF15 is a secreted protein that is widely expressed in mammalian tissues (Mullican and Rangwala, 2018; Chung et al., 2017). Its expression and secretion are dramatically enhanced upon injury, physical exercise, and several disease states including obesity and diabetes (Mullican and Rangwala, 2018; Galliera et al., 2014; Dostalova et al., 2009). Administration of GDF15 to obese mice, rats and monkeys significantly improves multiple metabolic parameters and reduces body weight (Xiong et al., 2017). Furthermore, transgenic mice over-expressing *Gdf15* exhibit obesity resistance and improved insulin sensitivity (Macia et al., 2012; Chrysovergis et al., 2014). In contrast, *Gdf15* knock-out mice exhibit increased food intake, body weight and adipose tissues development (Tsai et al., 2013). Together, these studies

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https://doi.org/10.1016/j.mce.2021.111503
Received 22 February 2021; Received in revised form 26 October 2021; Accepted 29 October 2021
Available online 8 November 2021

provide evidence for a major role of GDF15 in the regulation of body weight and energy balance. Therefore, GDF15 could be a potential therapeutic target for type 2 diabetes although the underlying mechanisms remain unclear. Indeed, even though GDF15 was discovered twenty years ago, its receptor has only been recently identified (Mullican et al., 2017; Yang et al., 2017).

Type 2 diabetes is a chronic multifactorial disease characterized by high blood glucose level, insulin resistance and impaired insulin secretion due to pancreatic beta-cell dysfunction (van Greevenbroek et al., 2013). Furthermore, chronic exposure of pancreatic beta-cells to increased levels of glucose and lipids, a condition defined as glucolipotoxicity, appears to be a deleterious event in diabetes that contributes to and accelerates the loss of beta-cell mass and function (Poitout et al., 2010). In this context palmitate induces connexin-36 (CX-36) down-regulation and beta-cell uncoupling while CX-36 overexpression protects against cell uncoupling (Allagnat et al., 2008; Carvalho et al., 2012; Klee et al., 2011).

Although GDF15 has been shown to protect against diet-induced obesity and insulin resistance, the effect of this cytokine on pancreatic beta-cell function and cell viability in the context of glucolipotoxicity has not been investigated to date. Therefore, we aimed to 1) evaluate the impact of GDF15 on INS-1E and isolated mouse pancreatic beta-cell function; 2) assess the effect of endogenous *Gdf15* depletion on islets using pancreatic histological sections from wild-type and *Gdf15* knock-out mice; 3) test whether the effect of GDF15 could be mediated by CX-36 modulation and identify a correlation between *Gdf15* and various genes involved in metabolism, inflammation and pancreatic cell viability through a bioinformatics analysis.

2. Materials and methods

2.1. Animals

Ten to Twelve weeks-olds transgenic C57Bl/6J-Tgv (GLU-Venus and INS-Cherry) mice that express the Venus and mCherry fluorochromes driven by glucagon (Gcg) and insulin (Ins2) gene promoters respectively were bred in conventional housing with a 12h/12h light/dark cycle and subjected to experimental procedures. The ethics committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). G*df15* mice were housed in a controlled environment (12h/12h light/dark cycle; humidity: 50%–60%; ambient temperature: 22± 2°C) and fed a normal chow diet in a specific pathogen-free animal facility at the Chungnam National University Hospital (CNUH) Preclinical Research Center, South Korea. All of the *Gdf15* mice were handled according to institutional guidelines, and all of the experiments were approved by the Institutional Review Board of CNUH (CNUH-017-A0048).

2.2. Primary mouse beta-cells sorting and culture

Islets were isolated by collagenase digestion of pancreas from male adult mice using standard procedures as previously described (Gosmain et al., 2007). Beta-cells were separated from non-beta-cells by fluorescence-activated cell sorting (FACS) (Dusaulcy et al., 2019), after which the cells were cultured in 804G ECM-coated plates (Parnaud et al., 2008) in DMEM supplemented with 11 mM glucose, 0.05 mg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium-pyruvate, and 10% fetal bovine serum (FBS), at 37°C in 5% CO_2. The cells were allowed to adhere for 24h, after which they were incubated in FBS-deprived medium (11 mM glucose, 0.05 mg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium-pyruvate, and 1% FBS) with 0.5% BSA or 0.5 mM palmitate/BSA complex and 20 mM glucose for 72h.

2.3. INS-1E cell culture

INS-1E cells, were cultured in RPMI-1640 containing 5% FBS, 11 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM mercaptoethanol, at 37°C in 5% CO_2. The cells were left allowed to adhere for 24h before initiating experiments. For cells treatments, INS-1E cells were incubated in normal growth medium with 0.5% BSA or 0.5 mM palmitate/BSA complex and 20 mM glucose for 24, 48, or 72h.

2.4. Cell viability

Cell viability was determined using PrestoBlue reagent (Thermo Fisher Scientific). INS-1E cells or pancreatic mouse beta-cells cultured in standard media containing 20 mM glucose were exposed either to 0.5% BSA or 0.5 mM palmitate/BSA in the presence of increased human recombinant GDF15 (R&D) doses (1, 10, and 100 nM) for 72h after which 50 μL of PrestoBlue reagent was added. The plates were incubated for a further 2h at 37°C and absorbance was measured at 570 nm with a reference wavelength of 600 nm.

2.5. RNA extraction and quantitative real-time PCR

Total RNA from cultured INS-1E cells or mouse pancreatic beta-cells was isolated according to the manufacturer's instructions using a Qiagen RNeasy mini kit (#74104; Qiagen, Hilden, Germany). Reverse transcription of RNA was performed with a PrimeScript™ RT reagent Kit (Takara Bio Inc., France), according to the manufacturer's instructions. Quantitative real-time PCR was performed with a total reaction volume of 20 μL using SYBR Green Master Mix (Roche, Rotkreuz, Switzerland) and run on a Roche Light Cycler LC480 Sequence Detection System. Rps 29 was used as the reference gene for both the INE-1E and the primary beta-cells. The data were analyzed using the ΔΔCt method. The primer sequences are listed in Table 1 (Supplementary data).

2.6. ELISA

GDF15 protein levels in the supernatant as well as in the cell lysate were measured using a mouse/rat ELISA kit (R&D MGD150) according to the manufacturer's instructions.

2.7. Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) was assayed as previously described (Ward et al., 2017). Briefly, INS-1E or mouse pancreatic beta-cells were exposed to palmitate/BSA (0.5 mM) and glucose (20 mM) in the presence or absence of human recombinant GDF15 (100 nM) for 72h prior to the GSIS experiments. The cells were then preincubated in Krebs–Ringer bicarbonate HEPES buffer, 0.5% BSA (KRBH) containing 2.8 mM glucose for 2h, followed by successive 1-h incubations in KRBH containing 2.8 mM glucose and KRBH containing 16.7 mM glucose. Total insulin was extracted with an acid/ethanol mixture to determine the insulin content. Insulin was measured with an ELISA kit (Mercodia AB).

2.8. Immunofluorescence and immunohistochemical staining

For immunofluorescent staining, INS-1E cells were cultured for 72h on glass coverslips without treatment or in the presence of either GDF15 (100 nM) or palmitate (0.5 mM) and glucose (20 mM) or combination of all three treatments, after which cells were fixed for 20min in 70% ethanol at -20°C, rinsed in PBS, and permeabilized for 15 min in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100. The coverslips were incubated overnight at 4°C in the presence of a polyclonal rabbit antibody against rat CX-36 (#36–4600, 1:100, Thermo Fischer Scientific). After repeated washes, the cells were counterstained

Table 1
Primer sequences used for the Light Cycler ® PCR assays.

Target	Species	Forward	Reverse
GDF15	Rat	CCAGCTGTCCGGATACTCAG	GGTAGGCTTCGGGGAGACC
GDF15	Mouse	ATACTCAGTCCAGAGGTGAGATTGGG	TCGGTGCACGCGGTAGGCTT
RPS 29	Mouse/Rat	GCCAGGGTTCTCGCTCTTG	GGCACATGTTCAGCCCGTAT

with goat anti-rabbit Alexa Fluor 488 antibody (#A-11008 1:500, Thermo Fischer Scientific). DNA was labeled with TO-PRO-3 iodide (Invitrogen). Confocal images of the fluorescence staining were obtained with a Carl Zeiss LSM800 microscope. The image luminosity and contrast were digitally enhanced with ImageJ, taking care to apply the same linear adjustments to images from different experimental groups (Asrih et al., 2011). Quantification of the fluorescence intensity and the total CX-36 granule area per cell was analyzed using algorithms devised for MetaMorph® Software (Molecular Devices) at the Geneva University School of Medicine Bioimaging Facility.

Pancreatic tissue samples from three wild-type (WT) and three *Gdf15* knockout (*Gdf15*−/−) mice were excised and fixed in 10% PBS-buffered formalin for 24h. After paraffin embedding and sectioning (5 μm), the tissues were either stained with hematoxylin and eosin (H&E) as previously described (Wakae-Takada et al., 2013), or subjected to double-label immunohistochemistry for insulin and glucagon staining similarly to previous methods (Kushner et al., 2002). Section images were obtained with AxioScanZ.1; Zen3 and ImageJ software were used to analyze islet parameters including the mean cell number per islet, the cell number per islet corrected for the surface area expressed as μm^2, the mean islet size expressed as μm^2, and the mean islet number per mm^2 of pancreas. Each analysis was performed on three different islets per sections per animal of three animals per group, thus resulting in nine islets per group.

2.9. GDF15 silencing via si-RNA transfection

A specific si-RNA sequence was designed for *Gdf15* to target mouse mRNA sequences (Silencer Select Predesigned siRNA numbers s202691; Ambion) as well as universal scrambled si-RNA (Silencer Control Negative Control 1; Ambion). Pancreatic mouse beta-cells were transfected with either 100 nM of *Gdf15* or scrambled si-RNAs using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. After 72h of treatment, samples were subjected to qPCR, determination of cell viability and measurement of glucose-stimulated insulin secretion.

3. Identification of GDF15-correlated genes involved in metabolism, inflammation and cell viability

The transcriptomic datasets of human pancreatic islets were obtained from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/). For the correlation analysis, expression data from GSE53949 were log10 transformed (Cheadle et al., 2003) and data from GSE118230 were log2 transformed and normalized as described previously (Zwiener et al., 2014). The pre-processed expression data was then converted into a Z-score (Cheadle et al., 2003; Zwiener et al., 2014). To evaluate the correlations between GDF15 and genes involved in metabolism, inflammation, and cell survival in human pancreatic islet, we computed Pearson's correlation and visualized in scatter plot using RStudio (4.0.2) with R package ggplot2, ggpubr, ggscatter, and ggExtra.

4. Statistical analysis

The data are presented as means ± the SEM. The statistical calculations were carried out with GraphPad Prism 6 software (Prism 6; GraphPad Software, Inc, La Jolla, USA). The statistical significance of differences was determined by unpaired Student's t-tests, and one-way or two-way ANOVA. A p-value < 0.05 was considered statistically significant.

5. Results

5.1. Glucolipotoxicity upregulates GDF15 in INS-1E and primary pancreatic beta-cells

INS-1E and primary mouse beta-cells were exposed to high levels of glucose and palmitate to evaluate the impact on *Gdf15* gene expression and protein levels. This treatment significantly increased GDF15 levels in cell culture supernatants of INS-1E cells (Fig. 1a). The intracellular GDF15 content was also measured (Fig. 1b) and the amount of GDF15 released was estimated as the percentage of the GDF15 content (Fig. 1c). We observed that high levels of glucose and palmitate enhanced both the GDF15 content and release (Fig. 1b and c). In parallel, we isolated mouse beta-cells and subjected them to similar conditions. Palmitate and high glucose treatment increased GDF15 protein release (Fig. 1d). However, the intracellular GDF15 content was not altered in mouse beta-cells that has been subjected to glucolipotoxicity (Fig. 1e) although it did increase GDF15 release (Fig. 1f). To determine whether this discrepancy with INS-1E cells could be due to differential regulation at the RNA level, we measured the *Gdf15* gene expression level in INS-1E and pancreatic mouse beta-cells exposed to palmitate and high glucose. This treatment enhanced the *Gdf15* mRNA level in both cellular models compared to their control counterparts (Fig. 1g and h). Importantly, glucolipotoxicity has been shown to contribute to pancreatic beta-cell dysfunction (Poitout et al., 2010). This effect was assessed on INS-1E cells by evaluation of the cell viability. Reduced cell viability was observed in treated compared to untreated cells at 48 and 72h (Fig. 1i).

5.2. GDF 15 does not prevent glucolipotoxicity-reduced cell viability

The potential effect of GDF15 on cell survival was assessed using PrestoBlue reagent. After exposure to palmitate (0.5 mM) and high glucose (20 mM), the cell viability was significantly reduced compared to that of unexposed cells. This reduction in cell viability was observed in INS-1E and isolated mouse beta-cells (Fig. 2a and b). Additionally, when cells were concomitantly exposed to high glucose and palmitate and treated with increasing doses of GDF15 (1, 10, and 100 nM) no protective or additive deleterious effect of GDF15 was observed on INS-1E (Fig. 2a) as well as on isolated primary mouse beta-cells (Fig. 2b).

5.3. GDF15 prevents the adverse effects of glucolipotoxicity on glucose-stimulated insulin secretion

In addition to inducing a pro-apoptotic effect, prolonged exposure to glucolipotoxicity alters the secretory function of pancreatic beta-cells (Kim and Yoon, 2011). To investigate whether GDF15 can prevent the glucolipotoxicity effect on GSIS, INS-1E cells were concomitantly exposed to palmitate (0.5 mM) and high glucose (20 mM) in the absence and presence of recombinant GDF15 (100 nM), followed by assessment of glucose-stimulated insulin secretion. We found that GDF15 treatment attenuated the glucolipotoxicity-induced decrease in insulin secretion (Fig. 3a), while the insulin content remained unaffected (Fig. 3b). Although INS-1E cells are a valuable model to study insulin secretion, they exhibit a number of differences in their responsiveness compared to isolated primary beta-cells (Skelin et al., 2010). Therefore, primary

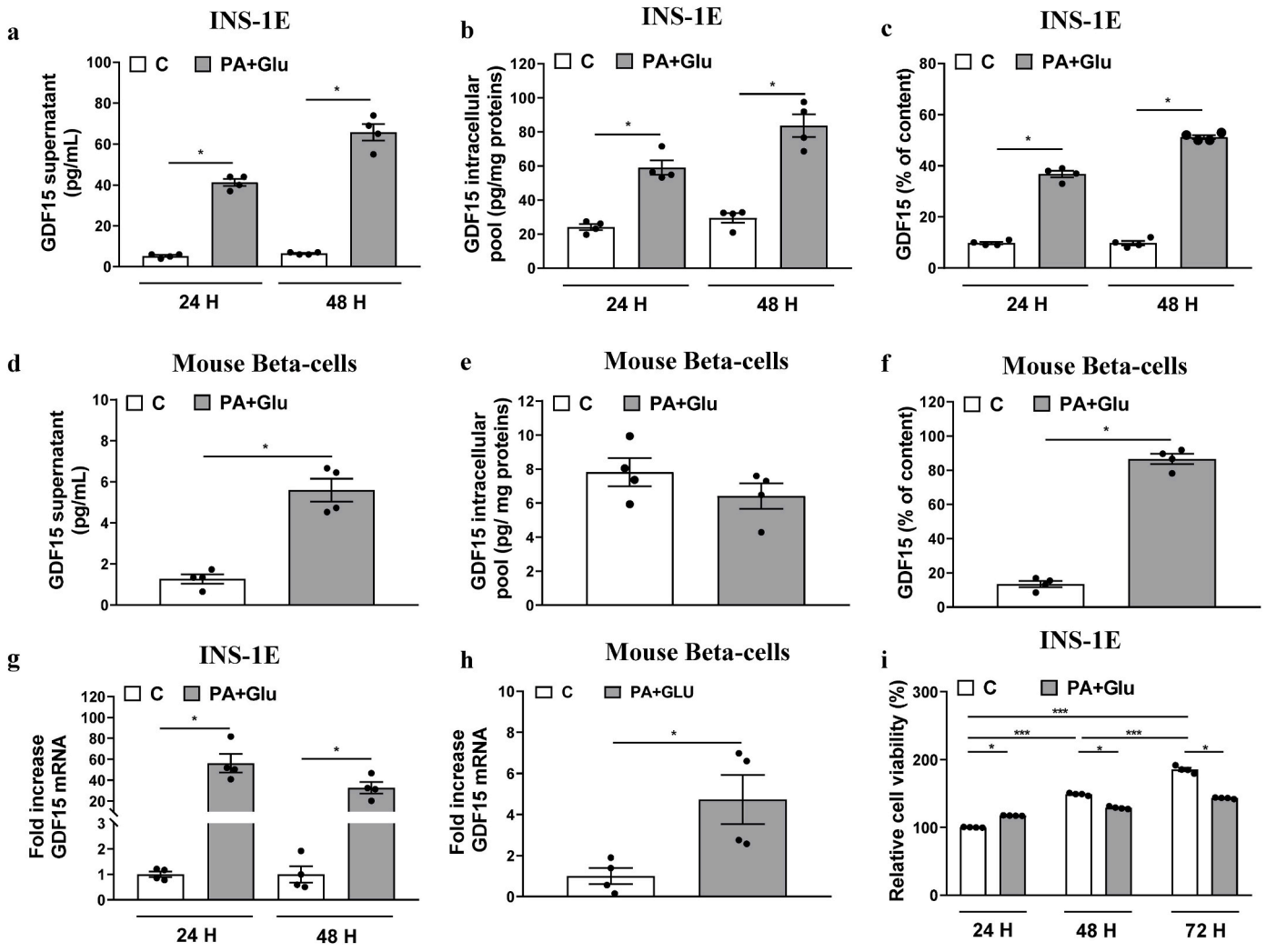

Fig. 1. GDF15 expression and release is upregulated in INS-1E and mouse pancreatic beta-cells exposed to high levels of glucose and palmitate. INS-1E and primary isolated mouse beta-cells were cultured in the absence (C) or the presence of palmitate (0.5 mM) and high glucose (20 mM) for 24, 48 and 72h. A) GDF15 protein level in the supernatant. B) GDF15 intracellular protein level. C) GDF15 release, expressed as the percentage of the total GDF15 content, was measured in INS-1E cells cultured for 24 and 48h. D) GDF15 protein level in the supernatant. E) GDF15 intracellular protein level in primary mouse beta-cells. F) GDF15 release from primary cells, expressed as the percentage of the total content, was measured in primary mouse beta-cells cultured for 72h. G) GDF15 mRNA level in INS-1E cells cultured for 24 and 48h. H) GDF15 mRNA level in mouse primary beta-cells exposed for 72h. A) Relative cell viability at 24, 48 and 72h in INS-1E cells. The statistical analyze were performed using an unpaired Student's t-test or two-way ANOVA followed by Tukey's post hoc test for multiple comparisons. The data are expressed as the means ± the SEM of n = 4 experiments. *$p < 0.05$, ***$p < 0.001$.

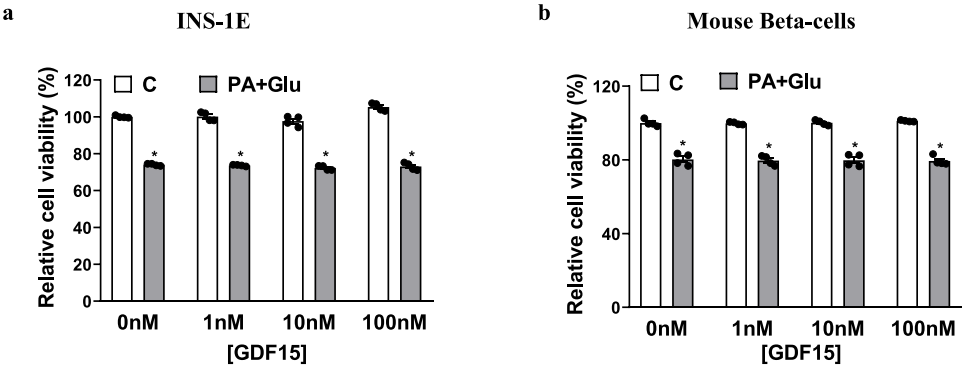

Fig. 2. GDF15 does not prevent glucotoxicity-mediated altered viability of INS-1E and mouse pancreatic beta-cells. INS-1E and primary mouse beta-cells were concomitantly cultured with palmitate (0.5 mM) and high glucose (20 mM) in the absence or presence of increasing doses of GDF15. After 72h of treatment, the cells were exposed 2h to the PrestoBlue cell viability reagent and the absorbance was measured at 570 and 600 nm. A) Relative cell viability of INS-1E cells. B) Relative cell viability of primary mouse beta-cells. The statistical analyses were performed using an unpaired Student's t-test for each GDF15 concentration. The data are expressed as means ± the SEM of n = 4 experiments. *$p < 0.05$.

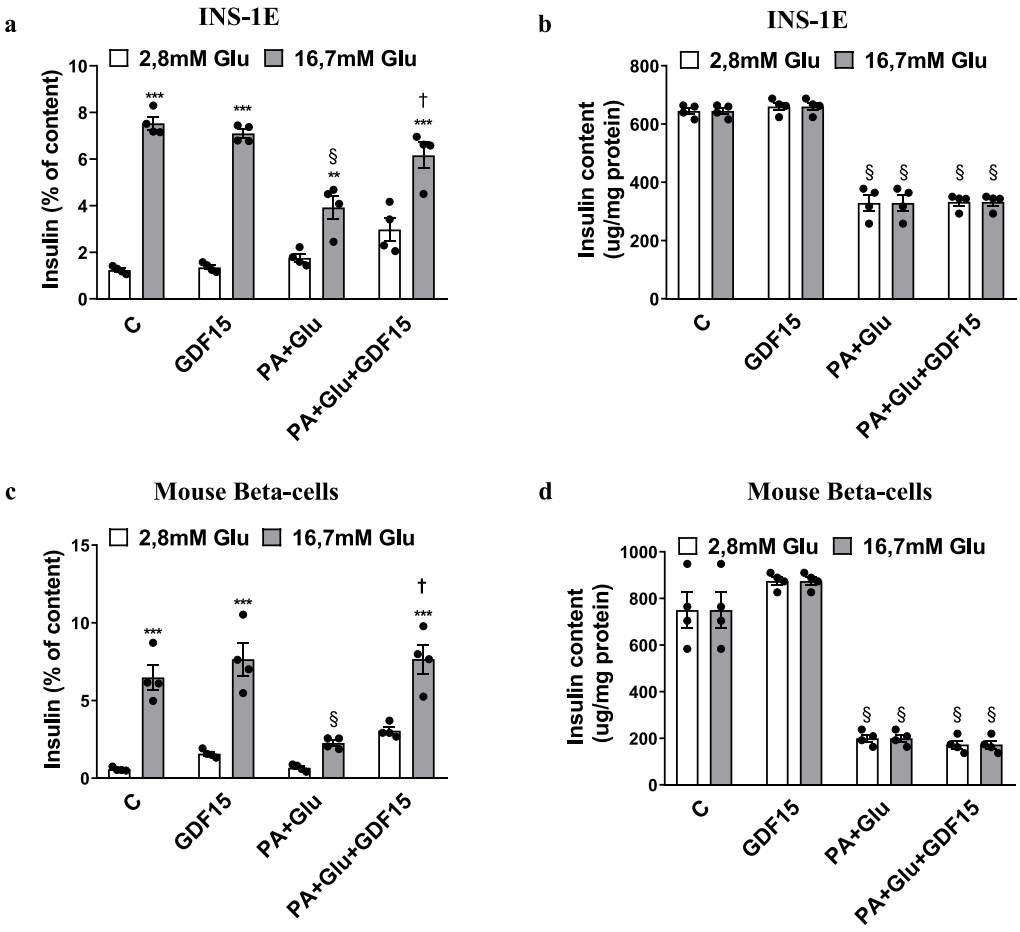

Fig. 3. GDF15 abrogates altered glucose-stimulated insulin secretion in INS-1E and mouse pancreatic beta-cells exposed to high levels of palmitate and glucose. INS-1E and primary mouse beta-cells were left untreated or treated concomitantly with palmitate (0.5 mM) and high glucose (20 mM) in the absence or presence of recombinant GDF15 (100 nM). After 72h of treatment, the cells were exposed for 1 h to 2.8 mM glucose (open bars) followed by 1 h in 16.7 mM glucose (grey bars). The level of insulin was then measured by ELISA. A) Glucose-stimulated insulin secretion (GSIS) in INS-1E. B) Total insulin content in INS-1E cells. C) GSIS and D) Total insulin content in primary mouse beta-cells. The statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc multiple comparisons. The data are expressed as means ± the SEM of n = 4 experiments. ***p < 0.001; **p < 0.01 for basal glucose stimulation (2.8 mM) versus high glucose (16.7 mM); §p < 0.05 for unexposed (C) *versus* exposure to palmitate/glucose; †p < 0.05 for palmitate/glucose (open bars) *versus* palmitate/glucose and GDF15 followed by 1 h of exposure to 16.7 mM glucose (grey bars).

mouse pancreatic beta-cells were exposed to similar conditions (*i.e.*, untreated or exposed to palmitate and high glucose). Similar, to its effect on INS1-E cells, GDF15 could counteract the adverse effect of glucolipotoxicity on GSIS (Fig. 3c). As observed for INS-1E cells, the insulin content of primary beta-cells was not affected by GDF15 treatment (Fig. 3d).

5.4. Endogenous GDF15 is involved in glucose-stimulated insulin secretion in beta-cells

GDF15 is likely to have a major role in glucolipid metabolism, which is known to influence pancreatic beta-cell function and survival (Poitout et al., 2010). Therefore, we examined the role of endogenous GDF15 on GSIS and cell viability. Endogenous *Gdf15* expression was down-regulated using an si-RNA approach in mouse pancreatic beta-cells. The effectiveness of the *Gdf15* si-RNA was validated by qPCR. The designed *Gdf15* si-RNA significantly reduced *Gdf15* gene expression (Fig. 4a). *Gdf15* inhibition exacerbated the reduction in cell viability caused by glucolipotoxicity without affecting basal cell survival (Fig. 4b). In addition to altering cell survival, chronic exposure of beta-cells to glucolipotoxicity also increased *Gdf15* gene expression and release, in addition to reducing GSIS. Furthermore, pancreatic beta-cells exposed to recombinant GDF15 exhibited an improved GSIS response under high palmitate and glucose conditions. In light of these results, we investigated the role of endogenous *Gdf15* on GSIS. We found that inhibition of *Gdf15* reduced GSIS in primary beta-cells cultured under standard conditions (Fig. 4c). Surprisingly, inhibition of endogenous *Gdf15* in mouse beta-cells concomitantly exposed to high glucose and palmitate did not further reduce GSIS due to glucotoxicity (Fig. 4c). Moreover, inhibition of the endogenous *Gdf15* had no impact on the total insulin content (Fig. 4d).

5.5. GDF15 prevents glucolipotoxicity induced connexin-36 downregulation

Cellular communication and coupling represent key processes in insulin secretion (Benninger and Piston, 2014). Connexins are important mediators of cell coupling. Therefore, we evaluated the effect of GDF15 on CX-36 levels. We compared immunolabelling of CX-36 in INS-1E cells exposed to GDF15 in the presence or absence of palmitate and glucose versus untreated cells. Immunofluorescence staining revealed a punctate/granular pattern of CX-36 in INS-1E cell (Fig. 5a). The CX-36 fluorescence signal intensity and the total granule area were similar in cells whether or not they had been exposed to GDF15 (Fig. 5b and c). However, palmitate and glucose treatment significantly decreased the CX-36 signal compared to untreated cells. This effect was abrogated when the cells were concomitantly exposed to palmitate, glucose and GDF15.

5.6. GDF15 deficient mice display neither islet hypertrophy nor hyperplasia

To test whether a lack of *Gdf15* (*Gdf15*-KO) could influence the cell number per islet, the islet size or the islet number per pancreatic section, the pancreas from wild-type (WT) and *Gdf*15-knock-out (*Gdf15*-KO) male mice were stained with hematoxylin-eosin (HE) and analyzed. *Gdf15*-KO mice displayed a slight and statistically insignificant increase in the total cell number per islet (Fig. 6a lower panel and Fig. 6b). Of note, when corrected for surface area, this difference became negligible (Fig. 6a lower panel and Fig. 6c). Moreover, *Gdf15*-KO islets were

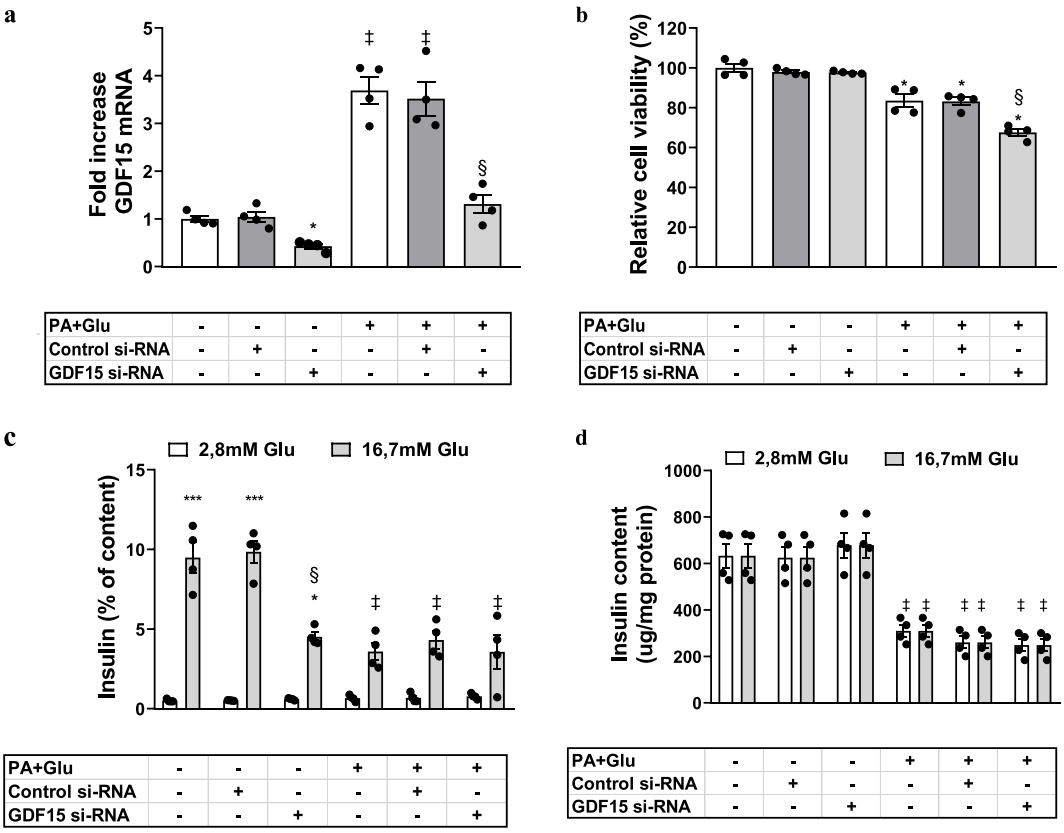

Fig. 4. ***Gdf15*** **downregulation by si-RNA affects cell viability and GSIS in mouse pancreatic beta-cells.** Isolated primary mouse beta-cells were left untreated or treated with control siRNA or *Gdf15* siRNA or concomitantly cultured with high glucose (20 mM) and palmitate (0.5 mM) in the absence or presence of *Gdf15* siRNA. A) After 72h of treatment, inhibition of *Gdf15* was confirmed by qPCR; The mean ± the SEM of n = 4 experiments using one-way ANOVA followed by Brown-Forsythe's post hoc test for multiple comparisons. *p < 0.05 for *Gdf15* siRNA *versus* control, ‡p < 0.05 for palmitate/glucose *versus* untreated; §p < 0.05 for palmitate/glucose in the absence or the presence of *Gdf15* siRNA. B) Another sample of cells was also treated for 72h and the cell viability was determined. The mean ± the SEM of n = 4 experiments using one-way ANOVA followed by Brown-Forsythe's post hoc test for multiple comparisons. *p < 0.05 for palmitate/glucose in the presence or absence of GDF15 siRNA versus untreated in the presence or absence of GDF15 siRNA; §p < 0.05 for palmitate/glucose versus palmitate/glucose and GDF15 siRNA. C-D) In parallel, an additional sample of cells, subjected to similar treatments for 72h, was exposed 1h to 2.8 mM glucose followed by 1h at 16.7 mM glucose. Insulin secretion and the total insulin content were measured by ELISA. The mean ± the SEM of n = 4 experiments using two-way ANOVA followed by Tukey's post hoc test for multiple comparisons. *p < 0.05, ***p < 0.001 for basal glucose stimulation (2.8 mM) *versus* high glucose (16.7 mM); §p < 0.05 for untreated versus treated with GDF15 siRNA followed by 1h exposure to 16.7 mM glucose (black bars). ‡p < 0.05 for the presence versus the absence of palmitate/glucose treatment followed by 1h exposure to 16.7 mM glucose.

moderately larger than those of WT mice (Fig. 6a lower panel and Fig. 6d). Together these data suggest that depletion of *Gdf15* does not promote islets hyperplasia or hypertrophy under basal conditions. In addition, *Gdf15* deficiency did not affect the mean islets number per unit of surface area (Fig. 6e), as indicated by representative HE images (Fig. 6a upper panel). Although no differences were observed in the total cell number per islet, we assessed whether depletion of *Gdf15* could selectively affect a specific cell type number or size, e.g., only alpha- or beta-cells. As shown in Fig. 7a, the number of either alpha- or beta-cells was relatively similar in WT and *Gdf15*-KO mice. This observation was confirmed by a quantitative analysis (Fig. 7b, d). Because the size of the considered islets for our analysis could introduce a degree of bias, for instance, a large islet contains more cells, the cell number was reported per unit of islet surface area. This approach revealed that *Gdf15*-KO mice exhibited a slightly lower but not significantly different number of alpha- and beta-cells per islet (Fig. 7c, e).

5.7. Analysis of GDF15 correlation using transcriptome datasets from pancreatic islets

We next sought to investigate the relationship between GDF15, and other candidate genes involved in either metabolic, inflammatory response, or cell viability. This analysis was performed at the transcriptome level using GSE53949 and GSE118230 datasets from pancreatic islets. The data were collected from a control group and from palmitate-treated pancreatic islets. The results indicate that the expression levels of ABCC8 and GLP1-R, which play an important role in insulin secretion, negatively correlated with GDF15 in pancreatic islets cultured under control and palmitate conditions (Fig. 8 I a, b and II a, b). On the other hand, P2RX1, which is also involved in insulin secretion exhibited a stronger positive correlation with GDF15 in the palmitate group compared to the control group (Fig. 8 II c). SIRT1, which is a major metabolic sensor and effector, positively correlated with GDF15 in the control group, while in the palmitate group the correlation was negative (Fig. 8 II d).

FOXO3, which has an anti-oxidative function and BCL-2, which is an anti-apoptotic factor, were downregulated in the palmitate group compared to the control group. Additionally, a positive correlation between FOXO3, BCL-2 and GDF15 was observed, albeit only in the palmitate group (Fig. 8 Id, e). Interestingly, BAX, which is a key component in the induction of apoptosis, was not correlated with GDF15 in the control or in the palmitate group (Fig. 8 Ic). SOD1, which exerts an anti-oxidative action, was downregulated in the palmitate group and exhibited a strong negative correlation with GDF15 in both groups, although it was not the case in the control group (Fig. 8 II e). S100A8 and TNF, which are two modulators of inflammation, were positively

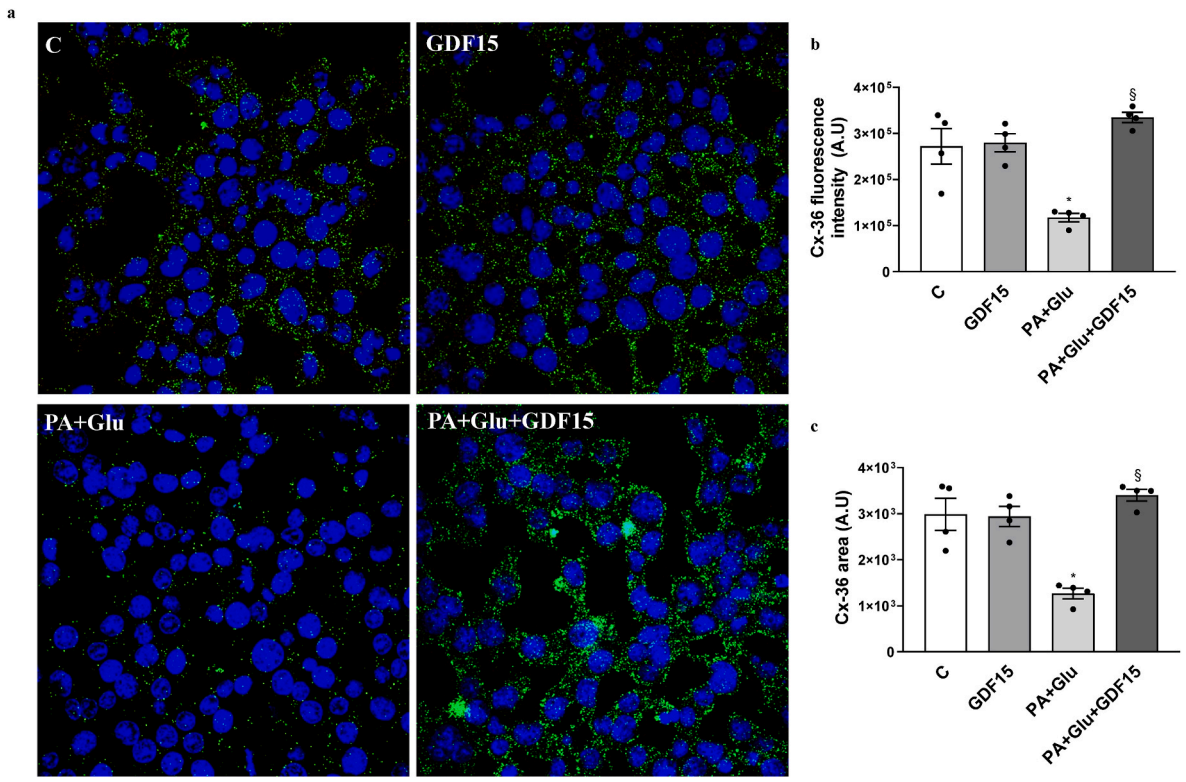

Fig. 5. GDF15 treatment prevents glucose- and palmitate-induced connexin-36 downregulation in INS-1E cells. A) Representative images of connexin-36 (CX-36) immunostaining in INS-1E cells left untreated or exposed to either GDF15 (100 nM) or palmitate (0.5 mM) and glucose (20 mM) or a combination of palmitate, glucose and GDF15 for 72h. B–C) Morphometric analysis of confocal images of CX-36 immunolabeling of INS-1E cells. B) Fluorescence intensity of the CX-36 signal expressed as arbitrary units (A.U). C) Relative area of CX-36 labeling per image. The statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons. The data are expressed as means ± the SEM of n = 4 experiments. *p < 0.05 palmitate/glucose versus untreated; §p < 0.05 exposure to palmitate/glucose versus exposure to palmitate/glucose/GDF15.

correlated with GDF15 (Fig. 8 I f, II f).

6. Discussion

GDF15, which has recently been reclassified as a member of the glial cell-derived neurotrophic factor (GDNF) family, exerts pleiotropic effects. These comprise regulation of body weight and energy homeostasis, as shown by several studies in animals and humans (Xiong et al., 2017; Macia et al., 2012; Chrysovergis et al., 2014; Baek and Eling, 2019). It has been reported that mice overexpressing *Gdf15* exhibit increased expression of genes that regulate lipolysis and oxidative metabolism (Chrysovergis et al., 2014). Furthermore, elevated GDF15 has been associated with increased insulin and glucose levels (Adela and Banerjee, 2015). It has therefore, been proposed that GDF15 can act as a marker of insulin resistance and glucose intolerance. This has led to several studies focused on the effect that GDF15 exerts on insulin target organs such as skeletal muscle and adipose tissue (Tsai et al., 2018). The role of GDF15 on the pancreas, which is the organ that secretes insulin, was recently investigated (Schernthaner-Reiter et al., 2019). These authors have shown that beta-cell function is an independent predictor of GDF15 concentration in obese patients (Schernthaner-Reiter et al., 2019). In another recent study, GDF15 was shown to protect from cytokines-induced islets dysfunction in-vitro, in addition to preserving pancreatic function *in-vivo* (Nakayasu et al., 2020). However, the impact of GDF15 on beta-cell function and survival in the context of glucotoxicity has not been extensively investigated to date. Here, we show that a pharmacological dose of GDF15 improves GSIS in the context of chronic exposure to palmitate and high glucose without preventing the deleterious effect of glucolipotoxicity on cell viability. Furthermore, endogenous *Gdf15* downregulation by si-RNA exacerbated glucolipotoxicity-reduced cell viability. However, no effects on GSIS were observed in primary mouse beta-cells pre-exposed to glucolipotoxicity conditions. On the other hand, *Gdf15* inhibition resulted in reduced GSIS in mouse beta-cells under standard conditions.

In patients with type 2 diabetes, insulin resistance and circulating GDF15 are correlated (Vila et al., 2011). Moreover, a high glucose and free fatty acid environment represent a risk factor for the development of insulin resistance and altered beta-cell function. Together, these results suggest that glucolipotoxicity may induce GDF15. We, therefore, examined the impact of palmitate and high glucose on GDF15 release and content in INS-1E and mouse pancreatic beta-cells. Although INS-1E cells have the advantage of unlimited growth in cell culture, they exhibit differences in their insulin-secretory responsiveness to glucose compared to normal beta-cells (Skelin et al., 2010). Therefore, these two cellular models were selected to perform our study. We observed that exposure to glucolipotoxicity elevated the *Gdf1*5 mRNA level and its release in both cellular models. Our *in-vitro* results are in line with data showing an increased level of GDF15 in diabetic and obese patients with high levels of circulating lipids and glucose (Baek and Eling, 2019). In addition to increasing the level of GDF15, concomitant chronic exposure to palmitate and glucose had a cytotoxic effect. It decreased the viability of both cell types (INS-1E and pancreatic mouse beta-cells). This effect has already been described as a glucolipotoxicity phenomenon (Li et al., 2018).

Resistance to growth factors could arise in the context of altered metabolism, although pharmacological doses of this factor may induce an effect, as it has been observed in the case of fibroblast growth factor 21 (FGF21) (Fisher et al., 2010). Therefore, we postulated that despite potential resistance to the endogenous GDF15 under our conditions, pharmacological doses of GDF15 may prevent altered cell viability due

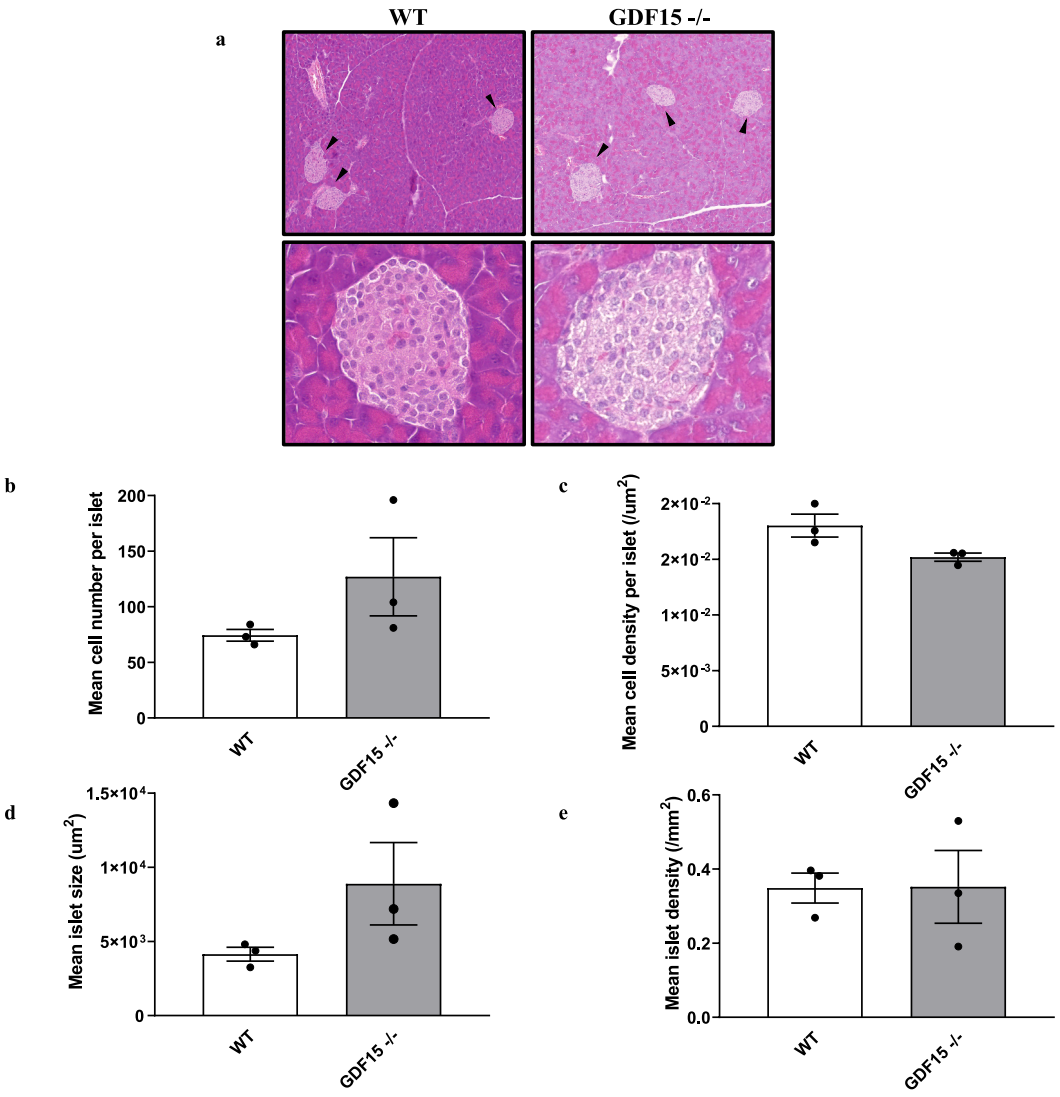

Fig. 6. Quantitative islet histomorphometry of eosin-hematoxylin-stained pancreatic sections of wild-type (WT) and *Gdf15* knockout (GDF15−/−) mice. A) Representative images of islet cells and pancreatic sections of wild-type (WT) and *Gdf15* knockout (GDF15−/−) mice stained with eosin-hematoxylin. Arrows indicate islets in sections. B-E) Morphometric analysis of eosin-hematoxylin-stained pancreas sections, three sections of three wild-type and three *Gdf15* knockout mice were examined. B) Mean cell number per islet. C) Mean cell number per square micrometer of islet. D) Mean islet size per square micrometer of pancreatic section. E) Mean islet number per square millimeter of pancreatic section. The statistical analyses were performed using an unpaired Student's t-test. The data are reported as means ± the SEM of n = 3 sections per group.

to glucolipotoxicity. However, GDF15 was unable to prevent altered cell viability induced by glucolipotoxicity in both INS-1E cells and in mouse pancreatic beta-cells. In contrast, inhibition of endogenous *Gdf1*5 by si-RNA in mouse pancreatic beta-cells exacerbated the glucolipotoxicity effects on cell survival. On the other hand, this downregulation had no effect on cell survival under standard culture conditions. These results were confirmed by analysis of the cell number on histological sections of wild-type and *Gdf15*-KO mice. Altogether, our results suggest that endogenous *Gdf15* is necessary to prevent reduction of cell survival under conditions of high glucose and palmitate-induced stress, while exposure of these cell types to a pharmacological dose of GDF15 failed to abrogate the adverse effects of glucotoxicity on cell viability. This discrepancy could be due to differential intracellular pathways involved in the mechanism of action for endogenous versus exogeneous GDF15. For instance, it has been suggested that pro-GDF15, which is an immature form of native GDF15, may exhibit biological activities that differ from those of the GDF15 receptor-mediated effects (Min et al., 2016). Nevertheless, additional experiments are required to further elucidate our observations and the hypothesis proposed by Min et al. (2016).

In addition to altered cell survival, glucotoxicity perturbs the secretory function of beta-cells (Kim and Yoon, 2011). We, therefore, investigated whether GDF15 could prevent such dysfunction. We showed that a pharmacological dose of GDF15 prevented reduction of high glucose-stimulated insulin secretion in primary beta-cells under conditions of glucolipotoxicity. This effect was also seen in INS-1E cells. Moreover, GDF15 did not impact the total insulin content in both cell models. Thus, GDF15 prevented a reduction of GSIS in cells pre-exposed to high glucose and palmitate. Interestingly, the gap junction protein CX-36 contributes to cell coupling and control of insulin secretion (Allagnat et al., 2008). In addition, its downregulation has been involved in the pathogenesis of beta-cell dysfunction, thereby leading to type 2 diabetes (Carvalho et al., 2012). Furthermore, the development of type 2 diabetes is associated with deleterious effects of cytokines and hyperlipidemia on islets. In this context, palmitate and cytokines downregulate CX-36 levels, potentially leading to beta-cell uncoupling (Allagnat et al., 2008; Klee et al., 2011) while CX-36 overexpression protects against cytokine-driven adverse effects (Klee et al., 2011). Therefore, we tested whether GDF15 could prevent reduction of GSIS

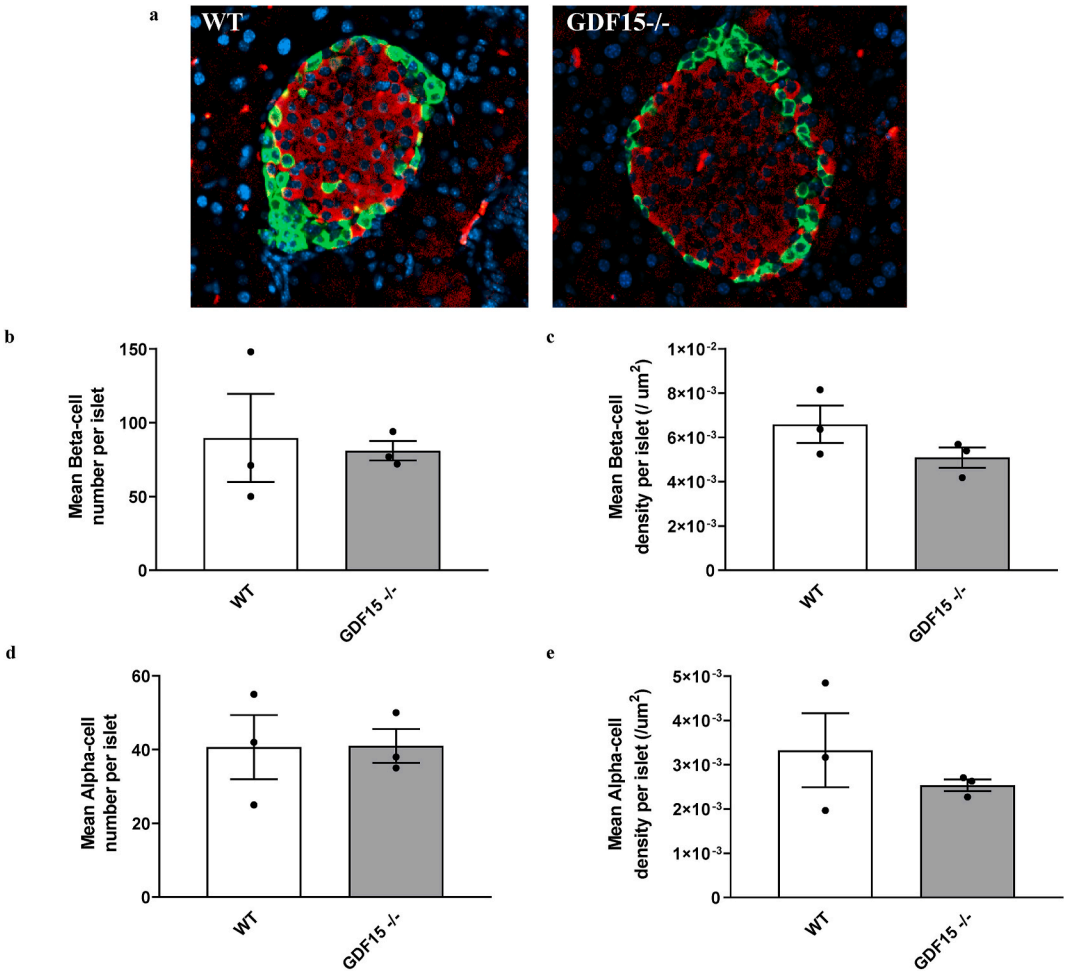

Fig. 7. Immunofluorescence staining of alpha-cells (green) and beta-cells (red) on pancreatic sections of wild-type (WT) and *Gdf15* knockout (GDF15−/−) mice. A) Representative images of islets from wild-type (WT) and *Gdf15* knockout (GDF15−/−) mice; alpha-cells and beta-cells were stained green and red, respectively. B-E) Morphometric analysis of pancreatic section, three sections of three wild-types and three *Gdf15* knockout mice were examined. B) Mean beta-cell number per islet. C) Mean beta-cell number *per* square micrometer of islet. C) Mean alpha-cell number *per* islet. D) Mean alpha-cell number per square micrometer of islet. Statistical analyses were performed using an unpaired Student's t-test. The data are reported as means ± the SEM of n = 3 sections per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

under palmitate/glucose conditions through an increase in CX-36 expression. Our data indicate that palmitate/glucose reduced CX-36 level in INS-1E cells compared to untreated cells. However, when concomitantly exposed to palmitate and glucose as well as GDF15, the level of CX-36 was not reduced. Altogether, these results suggest that GDF15 may prevent the deleterious effects of palmitate and glucose on GSIS through modulation of CX-36 expression.

Using two distinct transcriptomic datasets we sought to investigate the relationship between GDF15, and other candidates involved in either metabolism, inflammatory responses, or viability. We found that there was a positive correlation between GDF15 and inflammatory genes such as TNF and S100A8. BCL-2 and FOXO3, which are two anti-apoptotic modulators also positively correlated with GDF15. Therefore, one could speculate that GDF15 expression increases in the context of palmitate-induced inflammation to protect cells from apoptosis by elevation of BCL-2 and FOXO3 levels. This hypothesis is supported by recent data (Luan et al., 2019).

Additionally, GDF15 expression in islets exposed to palmitate negatively correlated with metabolic effectors such as SIRT1, GLP1R, ABCC8, and P2RX. This suggests a link between these genes and GDF15. Although GDF15 has been implicated in metabolic functions, the signaling pathway and mechanism of action remain poorly understood (Mullican et al., 2017; Yang et al., 2017; Tsai et al., 2018). In this context, our analysis provides new candidates that could be involved in the action of GDF15 on metabolism.

Interestingly, the profile of GSIS in cells depleted of *Gdf15* was different relative to GDF15 treated cells. Indeed, inhibition of endogenous *Gdf15* in mouse beta-cells pre-exposed to high glucose and palmitate had no effect on GSIS. However, when the cells were cultured under standard conditions, we observed a reduction in high glucose-stimulated insulin secretion when *Gdf15* was downregulated. These results suggest that endogenous *Gdf15* is involved in the mechanism of GSIS under control conditions, while it might not be required for GSIS in cells that under conditions of glucolipotoxicity. Another explanation could be the fact that palmitate and high glucose treatment may have blunted the effect of GDF15-downregulation on GSIS.

In summary, concomitant exposure of beta-cells to palmitate and glucose appears to result in an increase in GDF15 expression and release. Pharmacological treatment of beta-cells with recombinant GDF15 prevents reduction of GSIS glucose and palmitate, while it does not affect cell survival. This protective effect may be mediated by an increase in CX-36. Interestingly, downregulation of GDF15 by si-RNA decreased high glucose-stimulated insulin secretion, while exacerbation of reduced GSIS in the presence of glucose and palmitate was not observed. In addition, endogenous GDF15 inhibition exacerbated decreased -beta-cell viability. These findings, suggest differential roles of endogenous

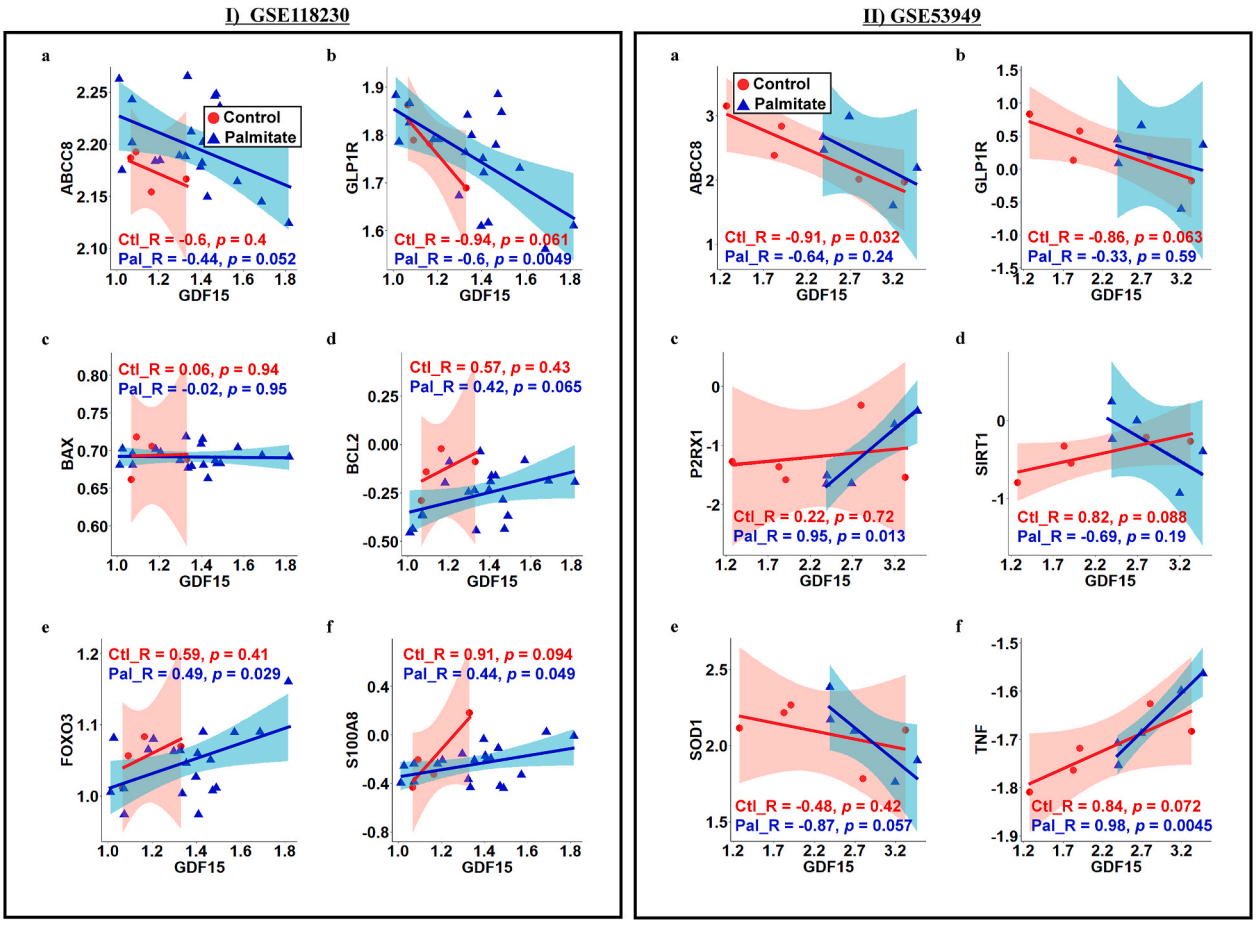

Fig. 8. Correlation between GDF15 and transcripts related to metabolism, inflammation and cell viability. Scatter plots describing correlations of GDF15 with genes involved in metabolism, inflammation, and cell survival in human pancreatic islets treated either with vehicle (Ctl in red) or palmitate (Pal in blue) from GSE118230 (left panel, n = 24) and GSE53949 (right panel, n = 10). All the expression levels of GDF15 and other genes were represented as Z-scores (arbitrary unit). All correlations (95% CI in red and blue shade) were evaluated by Pearson's correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and exogenous GDF15 on GSIS and cell survival.

CRediT authorship contribution statement

Mohamed Asrih: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Approval of the version. **Rodolphe Dusaulcy:** Formal analysis. **Yvan Gosmain:** Approval of the version. **Jacques Philippe:** Writing – review & editing, Approval of the version. **Emmanuel Somm:** Writing – review & editing, Approval of the version. **François R. Jornayvaz:** Writing – review & editing, Approval of the version. **Baeki E. Kang:** Data curation, Formal analysis. **Yunju Jo:** Data curation, Approval of the version. **Min Jeong Choi:** Data curation, Formal analysis, Approval of the version. **Hyon-Seung Yi:** Data curation, Formal analysis, Approval of the version. **Dongryeol Ryu:** Data curation, Formal analysis, Writing – review & editing, Approval of the version. **Karim Gariani:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Approval of the version.

Acknowledgment

This project was supported by the Helmut Horten Foundation (Lugano, Switzerland), the Raymond Berger Foundation (Lausanne, Switzerland), the Hejlt Foundation (Geneva, Switzerland) and the de Reuter Foundation to Karim Gariani (Geneva, Switzerland). François Jornayvaz is supported by a grant from SNSF Swiss National Science Foundation (#189003).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2021.111503.

Declaration of interests and contributions

The authors declare that they have no conflicts of interest with the subject matter that could prejudice the impartiality of the findings presented here. Mohamed Asrih and Karim Gariani performed the experiments, analyzed the data, prepared the figures and wrote the manuscript. Baeki E. Kang, Min Jeong Choi, Hyon-Seung Yi, **Yunju Jo,** and Dongryeol Ryu were involved in the bioinformatics analysis, and they generated the data for Fig. 8. Rodolphe Dusaulcy and Yvan Gosmain provided technical support and they proofread the manuscript. Emmanuel Somm, François Jornayvaz, and Jacques Philippe contributed to discussions, and they reviewed the manuscript.

Data availability

The data are available from the corresponding author upon reasonable request.

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