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Modeling human pancreatic beta cell dedifferentiation

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ABSTRACT

Objective: Dedifferentiation could explain reduced functional pancreatic β-cell mass in type 2 diabetes (T2D).
Methods: Here we model human β-cell dedifferentiation using growth factor stimulation in the human β-cell line, EndoC-βH1, and human pancreatic islets.
Results: Fibroblast growth factor 2 (FGF2) treatment reduced expression of β-cell markers, (*INS, MAFB, SLC2A2, SLC30A8*, and *GCK*) and activated ectopic expression of *MYC, HES1, SOX9*, and *NEUROG3*. FGF2-induced dedifferentiation was time- and dose-dependent and reversible upon wash-out. Furthermore, FGF2 treatment induced expression of *TNFRSF11B,* a decoy receptor for RANKL and protected β-cells against RANKL signaling. Finally, analyses of transcriptomic data revealed increased FGF2 expression in ductal, endothelial, and stellate cells in pancreas from T2D patients, whereas FGFR1, SOX,9 and HES1 expression increased in islets from T2D patients.
Conclusions: We thus developed an FGF2-induced model of human β-cell dedifferentiation, identified new markers of dedifferentiation, and found evidence for increased pancreatic FGF2, FGFR1, and β-cell dedifferentiation in T2D.

Keywords Beta-cell; Dedifferentiation; Type 2 diabetes; Human

1. INTRODUCTION

Type 2 diabetes develops as a consequence of a combination of insulin resistance and insufficient β cell mass. The decline in β cell mass has been attributed to a decrease in both β cell number and function [1]. It has been suggested that decreased β cell mass is the result of premature programmed cell death and research efforts focused on increasing β cell survival [2]. However, recent data challenge the β cell death hypothesis. These data suggest that β cell dedifferentiation represents an alternative mechanism to explain loss of functioning β cells [3]. During dedifferentiation, β cells do not undergo apoptosis. Rather, they lose identity and function with a decrease in expression of key β cell markers such as genes encoding major transcription factors, e.g. MafA, and proteins implicated in glucose-stimulated insulin secretion, e.g. Slc2a2 (Glut2) [4].
This concept of β cell dedifferentiation is now strongly supported by experiments performed in several murine models and based on lineage-tracing of β cells [3,5]. On the other hand, data on human β cell dedifferentiation remain scarce and mainly based on experiments performed on static models such as human pancreatic tissue sections. For example, studies at the electron microscopy level showed that pancreata from type 2 diabetic donors contain degranulated β cells that are depleted of insulin [6]. Pancreatic sections of type 2 diabetic patients demonstrated a 3-fold increase in the number of endocrine cells (Chromogranin A-positive) that stain negative for insulin, glucagon, somatostatin, or pancreatic polypeptide [7]. Such dedifferentiated human β cells, however, expressed the endocrine progenitor cell marker aldehyde dehydrogenase 1A3 (ALDH1A3) [7].
Our study aimed at developing a dynamic model of human β cell dedifferentiation. Access to preparations of primary human β cells is limited and human-translatable models to study their dedifferentiation are lacking. Here, we used EndoC-βH1, a recently developed functional human β cell line that was generated by lentiviral transduction of human fetal pancreas with oncogenes expression driven by the insulin promoter [8]. This line represents an efficient tool to study human β cells in pathophysiological conditions [8–10].
Our work specifically aimed at: i). Discovering molecules and pathways that induce human β cell dedifferentiation ii). Discovering new positive markers of human β cell dedifferentiation iii). Defining whether human β cell dedifferentiation is a reversible process and iv). Identifying new evidences of dedifferentiation in human islets from type 2 diabetic individuals. Here, we discovered that two members of the fibroblast

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growth factor family, fibroblast growth factor 1 (FGF1) and fibroblast growth factor 2 (FGF2), induce human β cell dedifferentiation, in a reversible manner. We also identified several transcription factors, SOX9, HES1, MYC, and TNFRSF11B, as new positive markers of human β cell dedifferentiation and showed that pancreata from type 2 diabetic patients display sustained expression of both FGF2/FGFR1 and our novel markers of dedifferentiation.

2. METHODS

2.1. Culture of human β cell lines and treatments

EndoC-βH1 cells were cultured in low-glucose (5.6 mM) Dulbecco's modified Eagle's medium (DMEM; Sigma—Aldrich) containing 2% BSA fraction V (Roche), 50 μM 2-mercaptoethanol (Sigma—Aldrich), 10 mM nicotinamide (Calbiochem), 5.5 μg/ml human transferrin (Sigma—Aldrich), 6.7 ng/ml sodium selenite (Sigma—Aldrich), and 100 units/mL Penicillin and 100 μg/mL Streptomycin (ThermoFischer Scientific) as previously described [8]. Cells were seeded at a density of 9.10^4 cells/cm^2 on Matrigel (1.2%; Sigma—Aldrich) and Fibronectin (3 μg/ml; Sigma—Aldrich) -coated plates and cultured at 37 °C in 5% CO_2. EndoC-βH1 cells were treated with either recombinant human FGF1 (100 ng/mL) plus heparin sodium salt (2 μg/mL) (both from Sigma—Aldrich), recombinant human FGF2 (100 ng/mL, Peprotech), recombinant human Soluble RANK Ligand Protein (Merck Millipore), recombinant Human Osteoprotegerin/TNFRSF11B Protein (R&D Systems), or U0126 (Calbiochem).

2.2. Human islets

Human islets were obtained from 8 donors (from M.A., Paris; LM and PM, Pisa; and commercially from Prodo Laboratories Inc.). They were cultured during three days in medium supplemented with either FGF2 or with the FGF2 carrier (PBS-BSA 0.2%).

2.3. RNA isolation, reverse transcription, and real-time PCR

RNeasy Micro kit (Qiagen) was used to extract total RNA from EndoC-βH1 cells and from human islets. Maxima First Strand cDNA synthesis (ThermoFischer) was used to synthesize cDNA. Quantitative RT-qPCR was performed using Power SYBR Green mix (Applied Biosystems) with a QuantStudio analyzer (Applied Biosystems). Custom primers were designed with NCBI Primer-BLAST [11] and their efficiency was determined following serial dilutions of cDNA samples from EndoC-βH1-cells or human islets. Cyclophilin-A transcript levels were used for normalization. The list of primers is presented in Table S1.

2.4. Transcriptome analysis

Total RNA was isolated using RNeasy Micro kit (Qiagen). The quality of the RNA was assessed by a Fragment Analyzer (Advanced Analytical Technologies). One microgram of total RNA was used for each library. The RNA samples were processed with Illumina TruSeq Stranded mRNA Library prep kit following the manufacturer's recommendations. Libraries were quantified with Qubit HS (ThermoFisher) and Fragment Analyzer (Advanced Analytical Technologies). Indexed libraries were pooled and sequenced on an Illumina NextSeq 500 high-output run using paired-end chemistry with 75 bp read length.

2.5. Processing of RNA-Seq data and differential expression analysis

Paired-end reads were generated using an Illumina NextSeq 500 and processed using the RNA-seq pipeline implemented in the bcbio-nextgen project [https://bcbio-nextgen.readthedocs.org/en/latest/]. Reads were aligned to the human genome version hg38 using the Hisat2 aligner [12].

Counts of reads aligning to known genes are generated by featureCounts [13]. Differential expression analysis was performed using DESeq2 [14]. All data are MIAME compliant, and the raw data have been deposited in a MIAME compliant database (GEO, accession number GSE103383).

2.6. Analysis of published transcriptomes

Raw data from GSE25724 microarray study was downloaded from the GEO database. Differential gene expression was assessed as previously described [15,16]. The moderated t-statistics within the limma package were used to obtain p-values for differential expression of genes under investigation [17]. If multiple probes mapped to the same gene, only the probe with smallest p-value was retained. Given the small number of individual hypothesis tests, we did not further adjust p-values for multiple testing.

For the single-cell RNA sequencing studies (E-MTAB-5061), we downloaded fastq files from the GEO database and aligned them to human genome version hg38 using the hisat2 aligner [12], followed by quantitation using the Salmon algorithm [18] and cpm (counts per million) normalization. Differential expression analysis was carried out using the MAST (model-based analysis of single-cell transcriptomics) R-package [19]. In addition to the number of detected genes per cell and disease condition, we included ethnicity and gender as covariates in the model to prevent erroneous differential expression calls due to different ethnicity or gender distributions in the control and case group. P-values were then derived from a likelihood ratio test carried out with the generalized linear model parameters of the case and control group.

2.7. Immunostaining of EndoC-βH1 cells and primary pancreatic islets

EndoC-βH1 cells were cultured on 12-mm Matrigel/fibronectin-coated glass coverslips. They were treated with FGF2 for 3 days, fixed for 1 h with 4% paraformaldehyde, permeabilized, and stained with a rabbit anti-SOX9 antibody (1/500; Millipore) that was revealed with a Goat anti-Rabbit IgG Alexa Fluor 488 conjugate (Life Technologies). Human pancreatic islets were treated for 72 h with FGF2 and fixed in 3.7% formaldehyde prior to embedding in paraffin. Sections (4-μm thick) were prepared and processed, as described [20]. The sections were stained with the following antibodies: mouse anti—chromogranin A (1/50; Dako Cytomation); rabbit anti-SOX9 antibody (1/500; Millipore). The secondary antibodies were Goat anti-Rabbit IgG Alexa Fluor 488 conjugate (Life Technologies) and Goat anti-mouse IgG Alexa Fluor 594 conjugate (Jackson ImmunoResearch). Digital images of EndoC-βH1 cells were captured using a cooled 3-chip charge-coupled device camera (Hamamatsu C5810; Hamamatsu) that was attached to a fluorescent microscope (Leica; Leitz).

2.8. Western blot analyses

Protein lysates prepared in RIPA buffer were subjected to immunoblotting. The following antibodies were used: MAFA (1/500; a gift from Dr. Rezania, BetaLogics Venture, New Jersey, USA), SOX9 (1/500; Millipore), HES1 (1/1,000, Cell-Signaling), ZNT8 (1/1,200, clone 17H2.4 generously provided by Dr. Davidson, Barbara Davis Center, Denver, Colorado), PDX1 (1/2,000; [20]), phospho-P38 MAPK (1/1,000; Cell Signaling), P38 MAPK (1/1,000, Cell Signaling), and TUBULIN (1/2,000; Sigma—Aldrich). Species-specific HRP-linked secondary antibodies (Cell Signaling) were added on the membrane and visualization was performed on an ImageQuant LAS 4000

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following ECL exposure (GE Heatlhcare). Quantifications were normalized by TUBULIN expression.

2.9. Luciferase reporter assays

EndoC-βH1 cells were transfected with 950 ng insulin promoter driven reporter and 50 ng pGL4.72-TK[hRlucCP] (Renilla luciferase, Promega) per well of 12 well plate using 3 μl of Lipofectamin2000 (Life Technologies) [21]. Three hours later, the transfection medium was changed for culture medium containing either the carrier (PBS-BSA 2%), or FGF1 (100 ng/mL) or FGF2 (100 ng/mL). Luciferase activities were measured 72 h later using the Dual-Luciferase reporter assay system (Promega) following manufacturer's instructions. Firefly luciferase activities were normalized to Renilla luciferase activities to correct for variation in transfection efficiency.

2.10. Insulin content

EndoC-βH1 cells were lysed by overnight incubation at −20 °C in ethanol (75%)/HCl (1.5%), centrifuged at 2,000rpm for 15 min at 4 °C and stored at −20 °C. Insulin contents were measured in triplicate by ELISA according to the manufacturer's instructions using the human insulin kit (Mercodia).

2.11. Zinc staining

EndoC-βH1 cells were seeded in 96 wells plates and allowed to attach for 24 h before treatment with 100 ng/mL FGF2. After 72 h, cells were treated with 30 mM KCl for 15 min to induce acute degranulation. They were rinsed, and fresh media containing 100 ng/mL FGF2 was added for another 72 h. Following treatment, cells were rinsed in PBS and stained with the zinc-specific fluorescent probe Zinpyr-1 (5 μM, Cayman Chemical), which stains primarily zinc-enriched secretory granules in beta cells [22]. Cells were then observed using confocal microscopy using a 488 nm laser line for excitation and 525/50 nm emission wavelength. Image analysis was performed using the Colombus software (PerkinElmer).

2.12. Statistical analysis

Data are expressed as mean ± SD. Analyses were carried out using SigmaStat® 3.5 (Systat Software, San Jose, CA, 8 USA). Differences between groups were evaluated using non-parametric tests, namely Mann—Whitney U test for non-paired analyses and Wilcoxon test for analysis of paired data. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. FGF2 treatment decreases *INS* and *MAFA* expression in EndoC-βH1

We first tested whether EndoC-βH1 could be used to discover compounds that modulate β cell differentiation status. For this purpose, we treated EndoC-βH1 with molecules acting through different pathways: ligands of receptor tyrosine kinases (FGF1, FGF10, IGF1, EGF), a G-protein coupled receptor ligand (Exendin-4), a ROCK-1 inhibitor (Y-27632), an activator of the WNT/β catenin pathway (R-Spondin) and a modulator of the TGF-beta signaling (Noggin). We measured the expression of *INS* and *MAFA*, a β cell specific transcriptional activator. We observed that following 72 h treatment, FGF1 induced a 2-fold decrease in *INS* mRNA levels while *MAFA* mRNA levels dropped down by more than 10 fold (Figure 1A,B).

FGF1 is a member of the Fibroblast Growth Factor family that signals through each of the 7 FGF receptors (FGFR) [23]. Interestingly, the effect of FGF1 on *INS* and *MAFA* mRNA levels was mimicked by FGF2 (Figure 1C), which belongs to the same subfamily of FGFs, but not FGF10 (Figure 1A,B), which belongs to the FGF3, 7 and 22 subfamily [24]. Both FGF1- and FGF2-treated cells showed a reduction in the activity of the human insulin promoter as compared to control cells, supporting a role for both factors as negative regulators of *INS* gene transcription (Figure 1D). RT-qPCR analyses indicated that EndoC-βH1 mainly express *FGFR1c*, *FGFR3b*, and *FGFR4* (Figure 1E). As FGF2 signals preferentially through the c-forms of FGFRs [23], it can be postulated that in EndoC-βH1, FGF1 and FGF2 act through FGFR1c to modulate *INS* and *MAFA* gene expression. Finally, FGF treatment did not significantly modify cellular growth and survival during the 3-days culture period (Figure 1F—H).

3.2. Decreased expression of several master β cell genes following FGF1 and FGF2 treatments

We treated EndoC-βH1 with FGF2 and performed global transcriptomic analyses by RNA-Seq at different time points (24 h-144 h treatments). We first searched for genes implicated in β cell function, with decreased expression following treatment with FGF2. As expected, *INS* and *MAFA* mRNA levels decreased. This was also the case for transcription factors expressed in β cells such as *MAFB*, *PAX6*, *NEUROD1*, *RFX6*, *INSM1* but also for factors implicated in insulin processing and secretion such as *PCSK2*, *SLC2A2 GCK*, and *SLC30A8* (ZNT8) (Figure 2A and Table S2). These data were confirmed by RT-qPCR using either FGF2 or FGF1 (Figure 2B). Both FGF1 and FGF2 repress the expression of β cell specific genes in a time- and concentration-dependent manner (Figs. S1 and S2). Following treatment with either FGF1 or FGF2, we also observed a sharp decrease in total cellular insulin content as measured by ELISA (Figure 2C), while western blot analyses indicated decreased levels of both the transcription factor MAFA and the β cell enriched zinc transporter ZNT8 (Figure 2D). Interestingly, we could also measure the functional consequences of decreased ZNT8 expression, as shown by a significant reduction in granular zinc content in EndoC-βH1 (Figure 2E). Of note, while the expression of several β specific markers collapsed, other β or endocrine markers remained expressed following FGF treatment. Similarly, the transcription factor PDX1 shows limited decrease at the RNA and protein level (Figure 2F,G). This is also the case for β cell-specific marker such as IAPP and endocrine markers such as *CHROMOGRANIN A* and *ABCC8* (Figure 2F and Table S2). Taken together, while keeping their global endocrine feature, EndoC-βH1 loose a number of β cell-specific markers following FGF treatment.

3.3. FGFs treatment induces ectopic gene expression in EndoC-βH1

Our global transcriptomic analysis indicated that in parallel to the extinction of a number of β cell specific genes, treatment of EndoC-βH1 with FGF2 sharply induced ectopic gene expression. This was the case for three transcription factors: *SOX9*, *HES1*, and *MYC* (Figure 3A). We confirmed these transcriptomic data using FGF1 and FGF2 by RT-qPCR (Figure 3B) and by western Blot and immunostaining (Figure 3C,D). Both FGF1 and FGF2 induced ectopic gene expression in a time- and concentration-dependent manner (Figs. S1 and S2). This effect was blocked when the cells were pre-treated with the MEK inhibitor U0126 (Fig. S3). FGF2 treatment also increased ectopic expression of *GAST (gastrin)*, which encodes a peptide hormone found in islets during fetal life, but absent during adult life [25] and *PYY*, an anorexigenic hormone usually not found in human β cells but rather produced by intestinal L cells [26] (Figure 3E,F). Interestingly, FGF treatment also induced the expression of the pancreatic endocrine progenitor marker *NEUROG3* also usually absent from mature human β cells [27] (Figure 3E,F). Induction of endocrine progenitor markers was also observed by comparison with a published list of pancreatic

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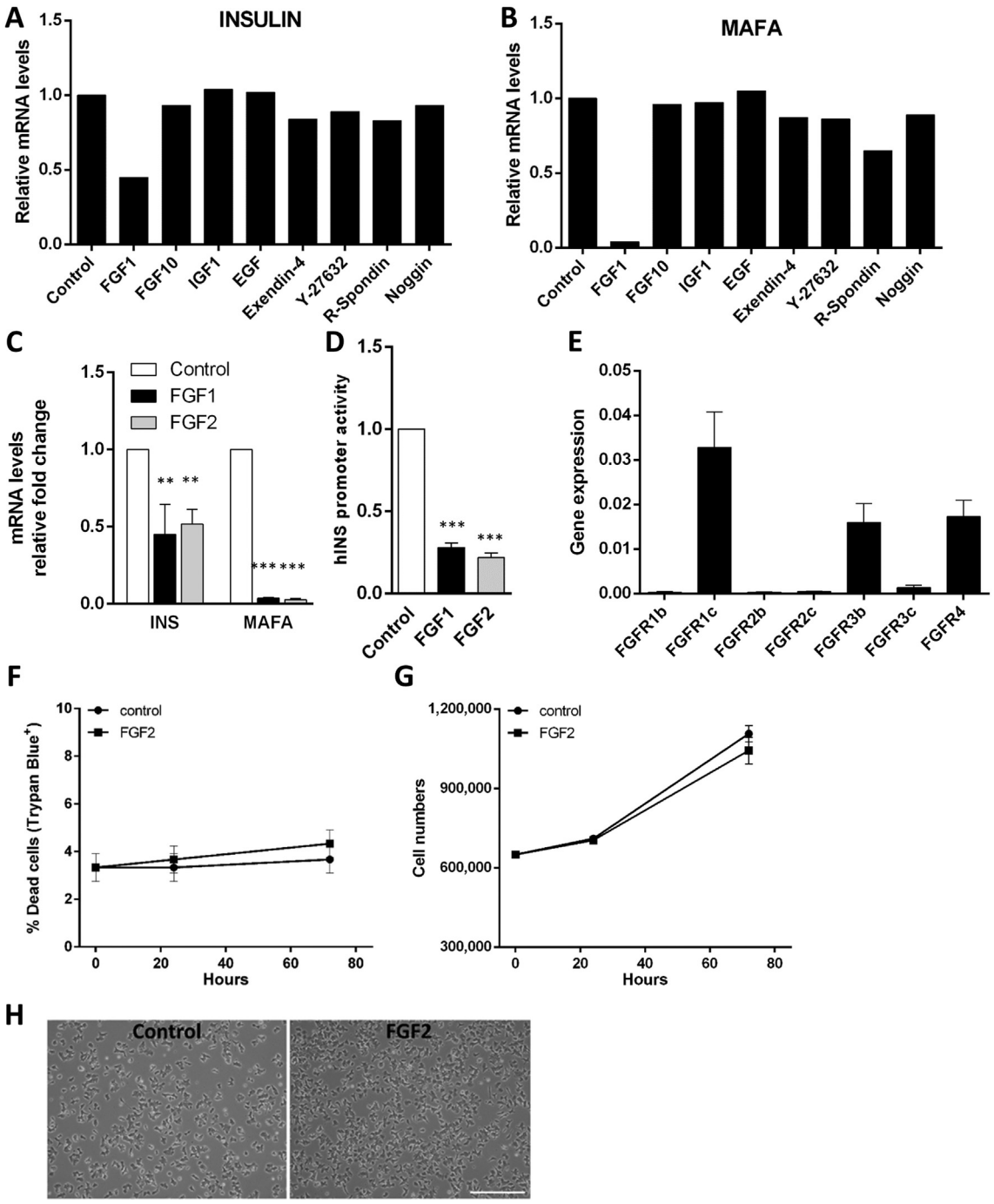

Figure 1: FGF1 and FGF2 treatments decrease *INS* and *MAFA* expression in EndoC-βH1. (A, B) EndoC-βH1 cells were exposed to the indicated treatments for 3 days. *INSULIN* and *MAFA* mRNA were measured by RT-qPCR. **(C)** Both FGF1 and FGF2 decrease *INS* and *MAFA* mRNA levels. **(D)** Human insulin promoter (HIP) activity was determined after transient transfection of EndoC-βH1 cells with the reporter vector HIP-Luc2CP followed by 3 days treatment with FGF1 or FGF2. **(E)** Expression by qPCR of human *FGFR* isoforms in EndoC-βH1 cells. **(F, G, H)** A 72 h treatment of EndoC-βH1 cells with FGF2 does not modify cell survival, growth or morphology (scale bar: 100 μm). Data are represented as mean ± SD. n = 5 biological replicates. **p < 0.01, ***p < 0.001.

endoderm enriched genes (Table S3) [28] (Figure 3G, horizontal axis). Moreover, temporal analysis revealed that loss of β cell specific genes (Table S4) preceded the induction of progenitor cell genes: β cell genes expression decreased as early as 24 h treatment, while progenitor genes were induced from 72 h and more intensely at 144 h in parallel with a collapse of β cell genes (Figure 3G).

On the other hand, the expression of acinar (*AMY2B*, *CPA1*) and duct (*CFTR*, *SPP1*) markers was very low in control condition and not induced by FGF treatment (Fig. S4). A lack of induction was also observed for endocrine markers such as *GCG*, *NPY*, and *GHRL* with the exception of *SST*, whose expression was induced by FGF1 and FGF2 (Figure 3H).

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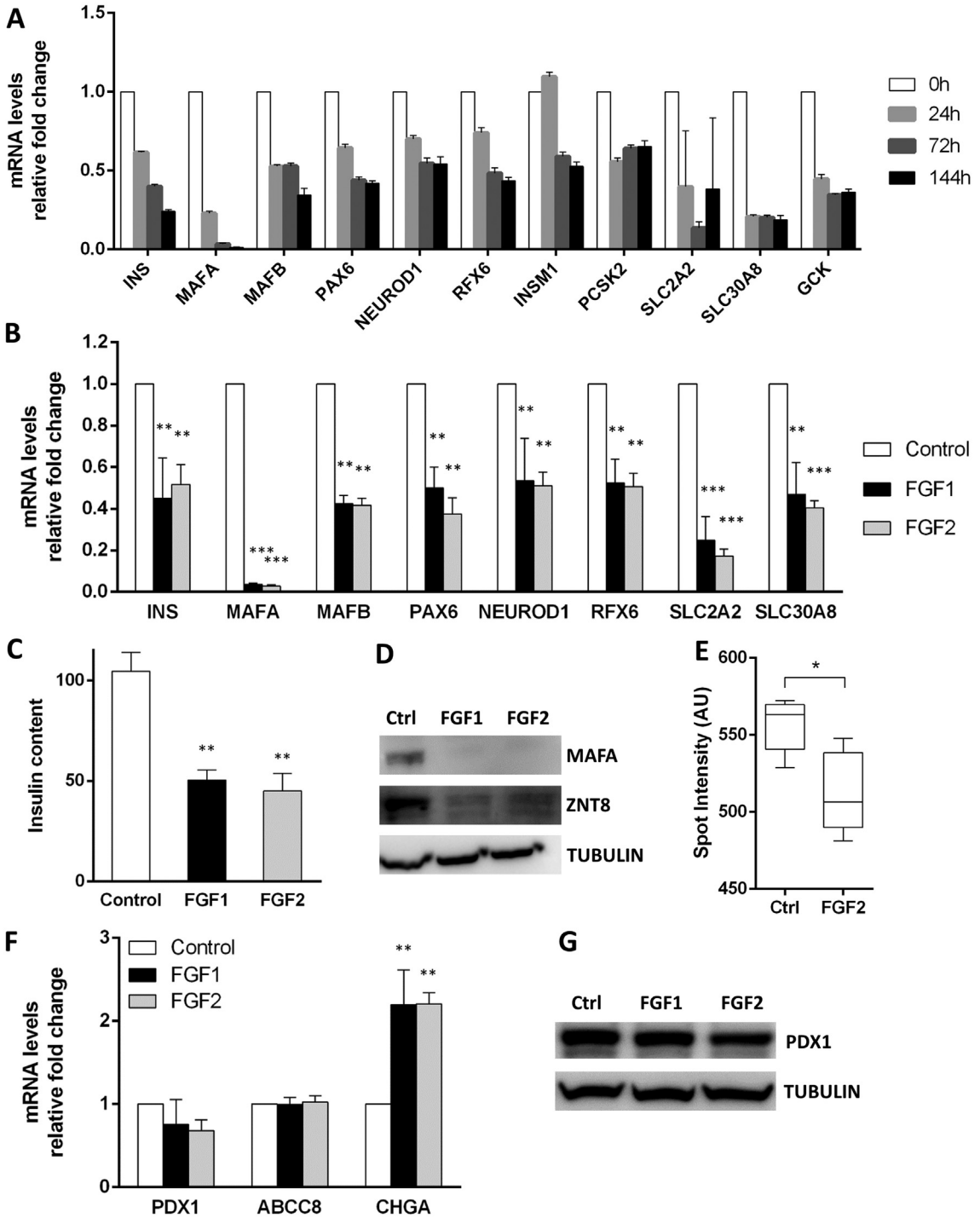

Figure 2: FGF1 and FGF2 treatments decreased the expression of several master β cell genes. (A) mRNA levels of β cell markers in EndoC-βH1 are decreased by FGF2 in a time-dependent manner as assessed by RNA-Seq. **(B)** Similar results were obtained using either FGF1 or FGF2 as measured by RT-qPCR. **(C)** Insulin content (ng per 10^6 cells) after 6 days of treatment with FGF1 or FGF2 determined by ELISA. **(D)** Western-Blot analyses of MAFA and ZNT8 levels after 3 days of treatment with FGF1 or FGF2. **(E)** Quantification of granular zinc staining using the zinc-specific fluorescent probe Zinpyr-1. **(F)** FGF1 and FGF2 treatments do not decrease *PDX1*, *ABCC8*, and *CHGA* mRNA levels as assessed by RT-qPCR. **(G)** Western-Blot analyses of PDX1 levels after three days of treatment with FGF1 or FGF2. Data are represented as mean ± SD. n = 5 biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. AU: Arbitrary Units.

3.4. Dedifferentiation is a reversible state

We treated EndoC-βH1 for 3 days with FGF2 to induce dedifferentiation. We next performed a chase culture period of 11 days in the absence of FGF2 in order to address whether dedifferentiation represents an irreversible new state or whether it can be reversed. As expected, *INS* and *MAFA* mRNA levels dramatically decreased while

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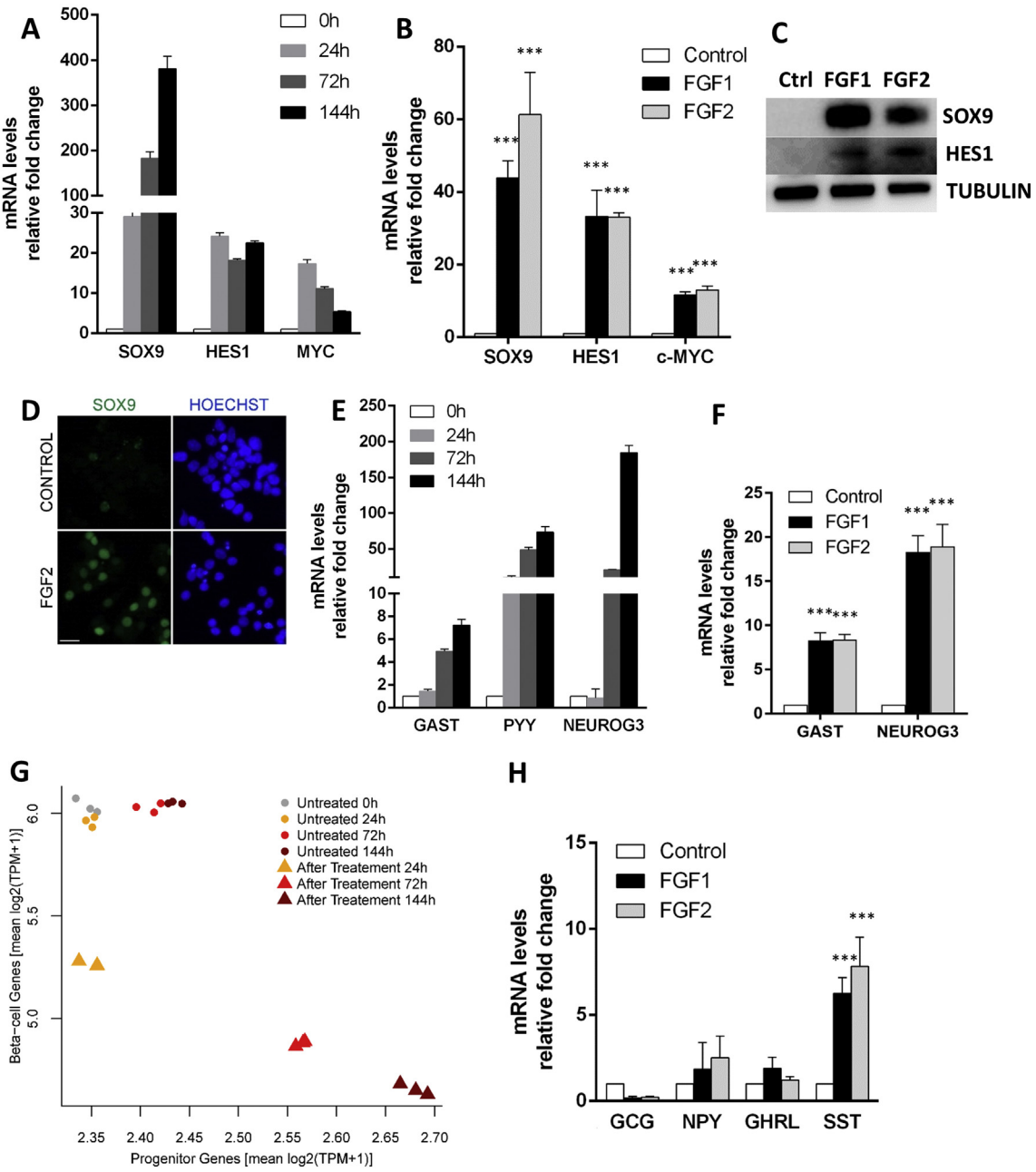

Figure 3: FGFs treatments induce ectopic gene expression in EndoC-βH1. **(A)** mRNA levels of *SOX9*, *HES1*, and *MYC* in EndoC-βH1 are induced by FGF2 in a time-dependent manner as assessed by RNA-Seq. **(B)** Similar results were obtained by RT-qPCR using either FGF1 or FGF2 (72 h treatment). **(C)** Western-Blot analyses of SOX9 and HES1 levels after three days of treatment with FGF1 or FGF2. **(D)** Immunofluorescence analysis of SOX9 expression EndoC-βH1 cells. Nuclear expression of SOX9 (in green) is observed following 72 h treatment with FGF2. Nuclei are stained with Hoechst 33342 stain (blue). Scale bar, 15 μm. **(E)** mRNA levels of *GAST*, *PYY* and *NEUROG3* in EndoC-βH1 are induced by FGF2 in a time-dependent manner as assessed by RNA-Seq. **(F)** *GAST* and *NEUROG3* data were confirmed by RT-qPCR using either FGF1 or FGF2 (72 h treatment). **(G)** Average expression (TPM) of β cell specific genes *vs.* progenitor genes (genes lists in Tables S2 and S3) assessed by RNA-Seq after treatment of EndoC-βH1 with FGF2. **(H)** The expression pancreatic hormones (*GCG*, *NPY*, *GHRL*) are not induced by a 72-hour treatment with either FGF1 or FGF2, with the exception of SST. Data are represented as mean ± SD. n = 5 biological replicates $^{**}p < 0.01$, $^{***}p < 0.001$.

SOX9 and *HES1* mRNA levels were strongly induced following 72 h or 14 days of treatment with FGF2 (Figure 4). Importantly, when FGF2 was removed at day 3 and EndoC-βH1 further cultured for 11 days in the absence of FGF2, *INS*, *MAFA*, *SOX9,* and *HES1* mRNA levels returned to control values, demonstrating that dedifferentiation represents a reversible state.

3.5. Dedifferentiated β cells express TNRFSF11B (OPG)

By mining our transcriptomic data, we discovered that FGF2 treatment induced the expression of *TNRFSF11B* mRNA levels (Figure 5A). Such an induction of *TNRFSF11B* by FGF2 was confirmed by RT-qPCR in EndoC-βH1 (Figure 5B). TNRFSF11B/Osteoprotegerin (OPG) is a secreted decoy receptor that competes for the binding of RANKL

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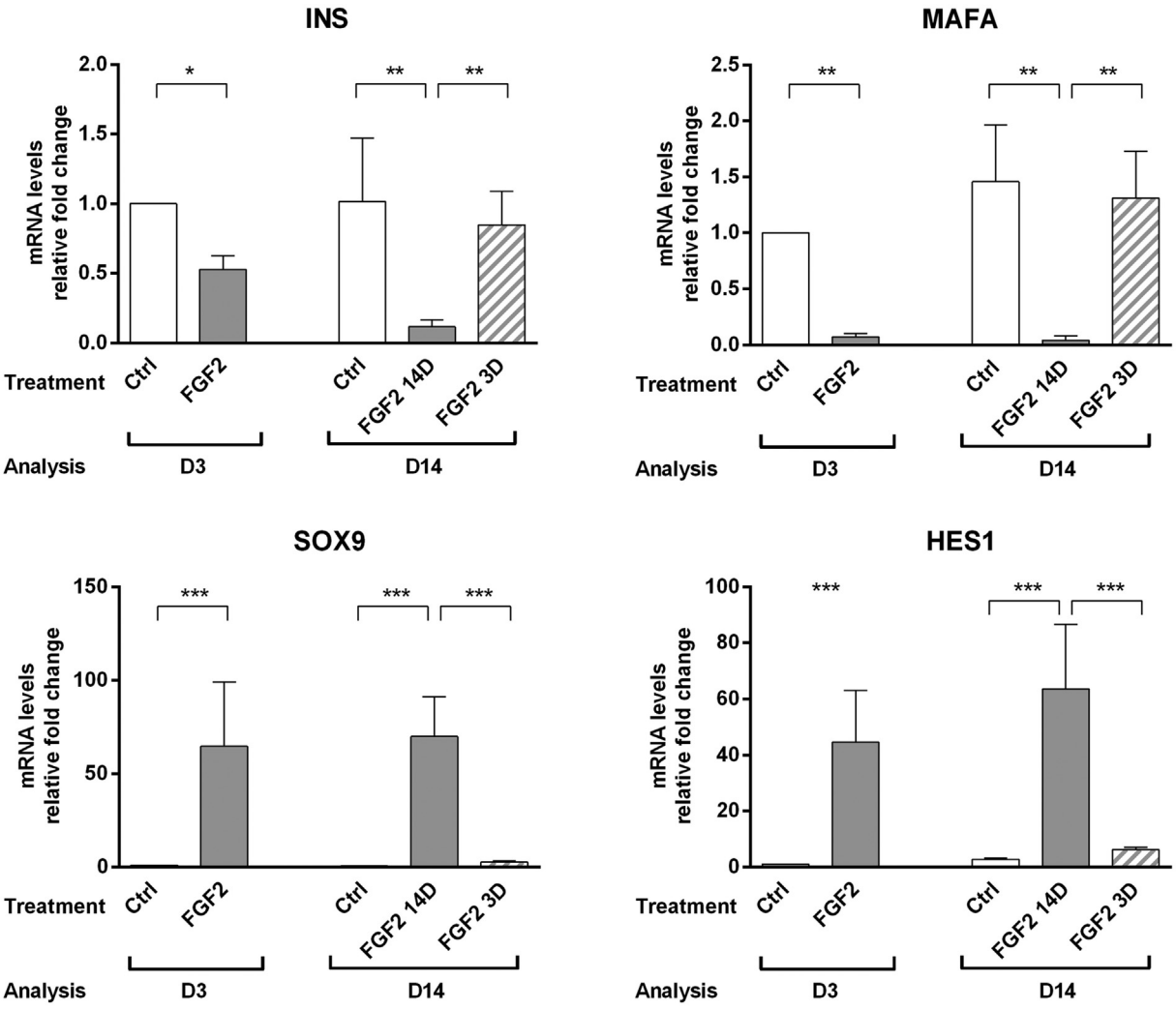

Figure 4: Dedifferentiation is a reversible state. EndoC-βH1 cells were cultured for 3 or 14 days in control conditions (white columns), with FGF2 (gray columns) or for 3 days with FGF2 followed by 11 days without FGF2 (dashed columns). INS, MAFA, SOX9 and MAFA levels were analyzed by RT-qPCR at day 3 and day 14. Data are represented as mean ± SD. n = 5 biological replicates. **p < 0.01, ***p < 0.001.

(TNFSF11) to its receptor RANK (TNFRSF11A). Transcriptomic and qPCR data indicated that EndoC-βH1 expressed RANK, but not RANKL (Figure 5C,D). RANK expressed in EndoC-βH1 was functional as exogenous RANKL phosphorylated P38 in a dose-dependent manner (Figure 5E). Moreover, exogenous OPG blocked the phosphorylation of P38 by RANKL (Figure 5F). Remarkably, FGF2 that induced OPG expression also prevented the P38 phosphorylation by RANKL (Figure 5G).

3.6. Human pancreatic islets respond to FGF2 in a similar manner to EndoC-βH1

We next tested the effect of FGF2 on primary human islet cell differentiation status. We treated human islets with FGF2 for 72 h. As observed with EndoC-βH1, following FGF2 treatment, *MAFA* and *PAX6* mRNA levels significantly decreased, while the expression of β cell markers such as *PDX1* and *ABCC8* was notably stable (Figure 6A). Decreased MAFA and stable *PDX1* protein levels were observed in parallel (Figure 6B). We next tested whether FGF2 treatment induced SOX9 expression in human islet cells. First, RT-qPCR confirmed increase of *SOX9* and *HES1* mRNA levels in pancreatic islets upon FGF2 treatment, although to lower extents than in EndoC-βH1 (Figure 6A). Double immunostaining for Chromogranin A and SOX9 indicated mutually exclusive expression in control pancreatic islets: Chromogranin A was observed in endocrine pancreatic islets cells, whereas almost no SOX9 staining was detected (Figure 6C). By contrast, FGF2-treated islets displayed an increased SOX9 staining and revealed cells that were double positive for both Chromogranin A and SOX9 (Figure 6C), thus confirming SOX9 induction in primary human islet cells, similarly to observations in EndoC-βH1 cells. Furthermore, human islets expressed mRNA for both *RANK* and *RANKL* (Fig. S5) and showed FGF2 induction of *TNRFSF11B* (Figure 6D).

3.7. Upregulation of the FGF2 pathway and dedifferentiation markers in human type 2 diabetes

Finally, we analyzed previously published transcriptomic data to ask whether the pancreatic FGF pathway and dedifferentiation markers were activated in patients with type 2 diabetes. Remarkably, single cells RNA-Seq data [27] revealed an increase of *FGF2* expression in

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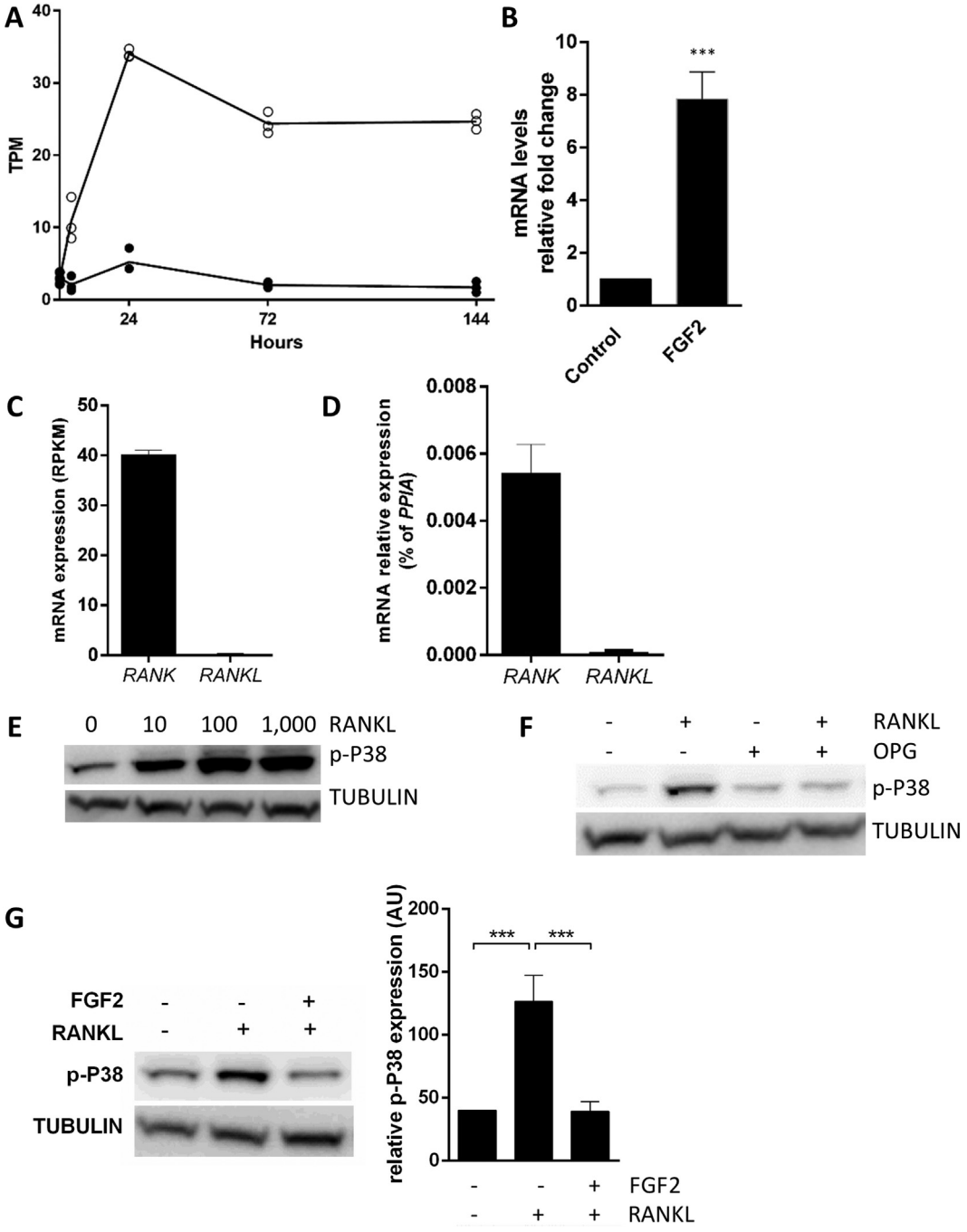

Figure 5: FGF2-induced OPG (TNFRSF11B) blunts RANKL (TNFSF11) signaling. (A) Time dependent induction of *OPG* mRNA by FGF2 assessed by RNA-Seq in EndoC-βH1. Closed circles: control; open circles: FGF2. **(B)** RT-qPCR analysis of OPG expression following 72 h treatment with FGF2 of EndoC-βH1. **(C, D)** *RANK* and *RANKL* expression in EndoC-βH1 assessed by RNA-Seq (C) and RT-qPCR (D). **(E)** RANKL phosphorylates P38 in EndoC-βH1 in a concentration-dependent manner (15min treatment). **(F)** Exogenous OPG blocks the phosphorylation of P38 by RANKL in EndoC-βH1. **(G)** Western-Blot and quantification of phospho-P38 after 15min RANKL (100 ng/mL) incubation, in control EndoC-βH1 or EndoC-βH1 pre-treated by FGF2 during three days. Data are represented as mean ± SD. n = 5 biological replicates. $^{**}p < 0.01$, $^{***}p < 0.001$. For Western Blot, representative blot of n = 5 experiments.

pancreatic ductal, endothelial and pancreatic stellate cells from type 2 diabetic patients (Figure 7A). Moreover, analysis of microarray islets data [29] revealed increased of *FGFR1* mRNA levels in human islets from type 2 diabetic compared with non-diabetic individuals, and also *SOX9* and *HES1* expression (Figure 7B—D).

4. DISCUSSION

Here, we validated the recently developed human β cell line EndoC-βH1 as a model system to analyze human pancreatic β cell dedifferentiation. As EndoC-βH1 were derived from a human fetal pancreas, they might

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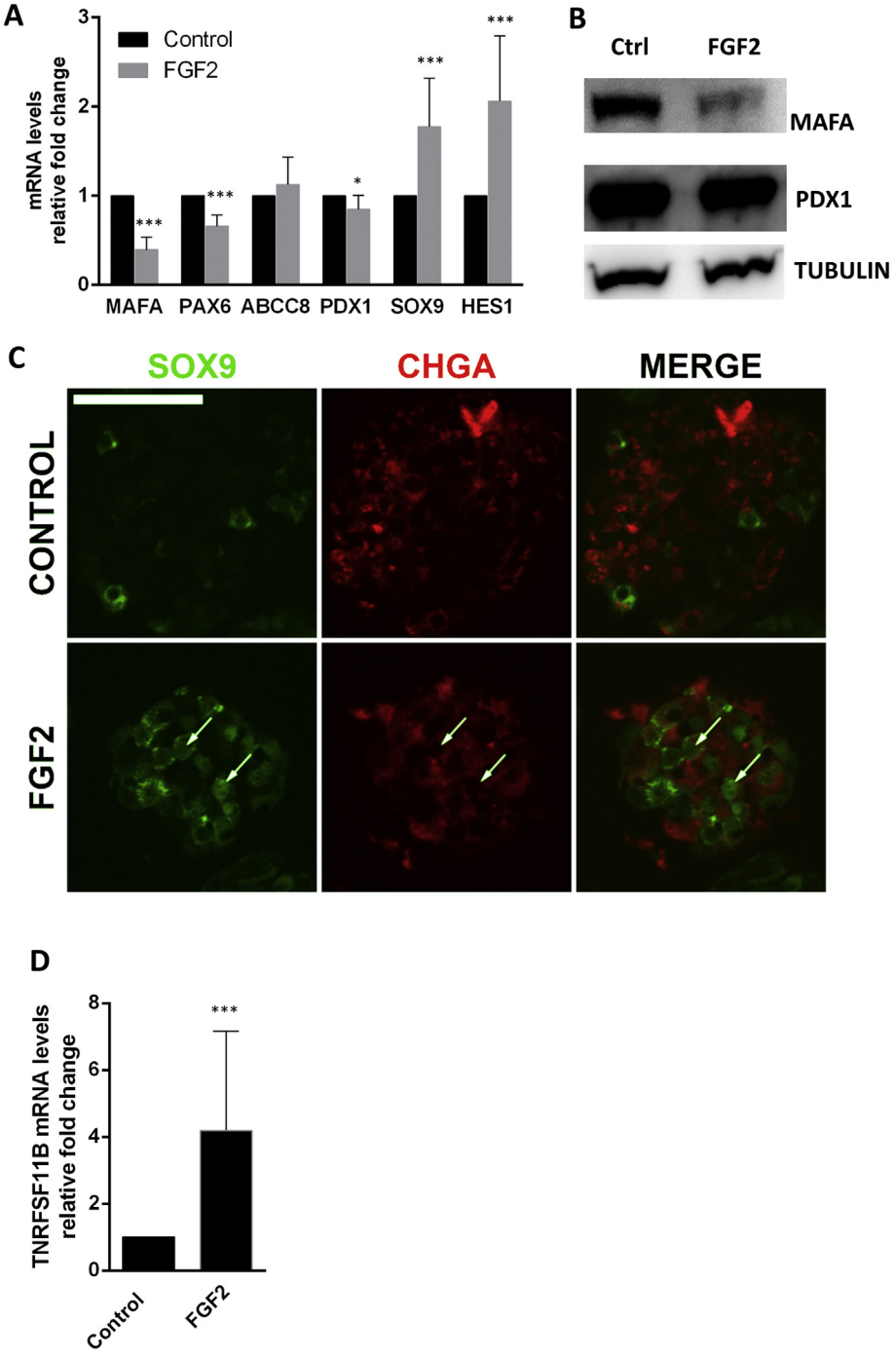

Figure 6: Human pancreatic islets respond to FGF2 in a similar manner than EndoC-βH1. Human islets were exposed to FGF2 for 3 days. **(A)** *MAFA*, *PAX6*, *PDX1*, and *ABCC8* mRNA levels were measured by RT-qPCR. **(B)** MAFA and PDX1 levels were analyzed by Western Blot. **(C)** SOX9 (green) in and CHGA (red) were analyzed by immunohistochemistry. Scale bar: 60 μm. **(D)** RT-qPCR analysis of *OPG* expression following 72 h treatment of human islets with FGF2. Data are represented as mean ± SD. n = 5 biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001.

be more prone to dedifferentiation to a developmental phenotype. Confirmation of our data using human islets, thus, was of importance. We discovered that following FGF1 and FGF2 treatment, the expression of many β cell specific markers is decreased. We also discovered new positive markers of human β cell dedifferentiation, which translated to primary human islets. Importantly, the dedifferentiation process observed following FGF treatment was reversible. Our data also indicated that human β cell dedifferentiation was paralleled by an increased expression of *TNFRSF11B,* which encodes a decoy receptor that blocks the RANK-RANKL interaction. Finally, we highlighted in transcriptomic from type 2 diabetic islets an increase of the FGF2 pathway as well as positive dedifferentiation markers.

Original Article

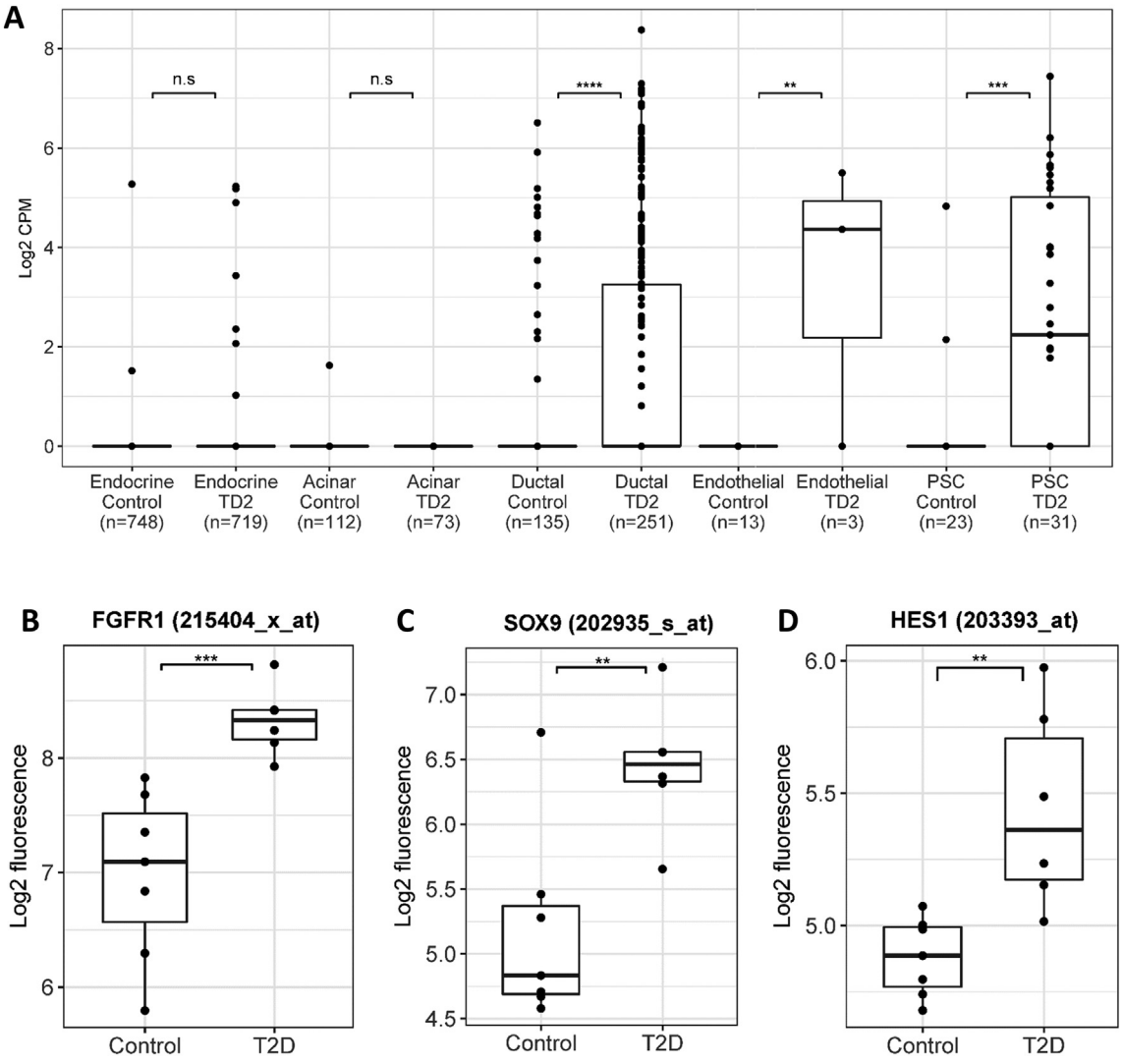

Figure 7: FGF pathway and dedifferentiation markers in type 2 diabetic islets. (A) *FGF2* expression levels across pancreatic cell types from healthy and type 2 diabetic individuals [27]. **(B)** *FGFR1*, **(C)** *SOX9,* and **(D)** *HES1* mRNA expression in type 2 diabetic and non-diabetic isolated human islets assessed by microarray analysis [29]. **$p < 0.01$, ***$p < 0.001$.

By comparing various culture conditions, we discovered that treatment with either FGF1 or FGF2 decreased the expression of a large set of β cell specific markers in a time- and concentration-dependent manner. These markers included the transcription factor MAFA whose loss of function in murine models leads to a decreased β cell identity [30,31], and PAX6, recently identified as a key factor that maintains β cell identity by repressing genes of other islet cell types in rodents [32].

4.1. Dedifferentiation through FGFR1c

We propose that FGF1 and FGF2 act through FGFR1c to induce dedifferentiation. Indeed, while FGF1 can signal through each of the 7 FGFR, FGF2 specifically binds the c forms of FGFR, namely FGFR1c, FGFR2c, and FGFR3c [23]. As our data indicate that EndoC-βH1 cells mainly express FGFR1c among the FGF2 receptors, we can propose that FGF2 binds FGFR1c to induce dedifferentiation. Interestingly, we have evidence that the FGF2 actions required the activation of the MAPK signaling because dedifferentiation is inhibited by a pretreatment with a MAPK inhibitor.

4.2. New positive markers of β cell dedifferentiation

Dedifferentiated rodent β cells express a number of non-β cell genes [3,30,33]. On the other hand, the number of markers with increased expression in dedifferentiated human β cells that could be used as positive markers of dedifferentiation is extremely limited. A recent study reported ALDH1A3 as the first positive marker of dedifferentiated human beta cells from type 2 diabetic patients [7]. Our global transcriptomic analyses of EndoC-βH1 cells treated with FGF2 allowed us to discover additional markers of dedifferentiation. We found SOX9, HES1, MYC, GAST, PYY, and NEUROG3 as potential new positive markers of dedifferentiation. SOX9 and HES1 are transcription factors that are absent from human pancreatic endocrine cells but expressed by exocrine cells in the adult pancreas [27]. Here, we demonstrate that treatment with FGFs induces SOX9 expression in both human β cell lines and in primary endocrine cells (Chromogranin A-positive) from human islets. This induction was not paralleled by an activation of pancreatic exocrine markers such as AMY2B, CPA1, CFTR or SPP1, suggesting that FGF treatment does not trans-differentiate β cells towards an exocrine phenotype.

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4.3. Dedifferentiated β cells revert to progenitor-like cells

SOX9 is expressed by pancreatic progenitor cells during prenatal life [34]. Interestingly, we observed that FGF treatment induces the expression in β cells of a set of markers that are normally expressed either in pancreatic endocrine progenitors, or in β cells during early life. This is for example the case for NEUROG3, a marker of endocrine progenitor cells. While induction of Neurog3 expression has been described in dedifferentiated β cells in rodents [3,35], determining whether NEUROG3 levels increase when human β cells dedifferentiate had been technically challenging [4]. Here, we observed that NEUROG3 expression is induced in a model of FGF-mediated human β cell dedifferentiation. In parallel to NEUROG3 induction, we also observed an activation of gastrin expression following FGF treatment. Gastrin is a stomach hormone known for a long time to be transiently expressed during fetal and perinatal life in the rat pancreas with expression decreasing thereafter [25]. Interestingly, it was recently demonstrated that gastrin is re-expressed in β cells in rodent and human with type 2 diabetes [36]. Therefore, gastrin may represent a novel biomarker of dedifferentiated human β cells. Taken together, we propose that upon dedifferentiation, human β cells express markers of pancreatic progenitors, such as SOX9 and HES1, of endocrine progenitors such as NEUROG3 and of perinatal β cells such as gastrin.

4.4. β cell dedifferentiation is reversible

The vast majority of data related to β cell dedifferentiation has been generated from *in vivo* mice models; thus, the process of dedifferentiation has been analyzed at a limited number of time points [30,33]. While it has been proposed that loss of differentiation markers precedes the expression of markers of progenitor cells, this hypothesis has not been fully validated [3]. Here, by constructing and validating an *in vitro* model of human β cell dedifferentiation, we were able to efficiently study dedifferentiation at different time points. Using our model, we demonstrated that loss of expression of β cell specific markers such as MAFA, ZNT8, GCK or SLC2A2, clearly preceded the induction of non-β cell genes such as neurogenin3 or gastrin. Whether loss of expression of β cell specific genes is necessary for the induction of non-β cell genes remains to be determined. Of interest, we also observed that when EndoC-βH1 cells were first treated with FGFs that was followed by a washout treatment, the dedifferentiation state was reverted. It should now be tested on human islet preparations. Accordingly, rodent data indicate that dedifferentiated β cells can redifferentiate to mature insulin-positive β cells following insulin therapy [5] or diet [37,38]. Human data also support the concept of reversibility of dedifferentiated β cells [39,40]. The mechanisms that allow rodent cells to redifferentiate into β cells remain partly unexplored, and human data are even scarcer. Our FGF-dependent model of human β cells dedifferentiation-redifferentiation should permit to progress on this topic and to specify additional ways to efficiently reverse dedifferentiation in human β cells.

4.5. Dedifferentiation state alters extracellular signaling

Dedifferentiation could also represent a mechanism to protect β cells against the deleterious effects of extracellular signals. OPG is a soluble decoy receptor that binds RANKL and inhibits its interaction with its receptor, RANK [41]. OPG is expressed by pancreatic islet cells, and up-regulated in models of β cell proliferation [42,43]. OPG has also recently been described as a target of lactogen in β cells and is required for lactogen-induced β cell proliferation [44]. Here, we demonstrate that human β cells express a functional RANK signaling pathway. Interestingly, FGF2 induces the expression of OPG in EndoC-βH1 and in human islets and blunts the RANK-RANKL signaling pathway. Taken together, induction of OPG during dedifferentiation could have a protective effect against deleterious signaling through the RANK-signaling pathway.

4.6. FGF2 and dedifferentiation during type 2 diabetes

In this work, to model dedifferentiation and to discover positive markers of human β cell dedifferentiation, we have used FGF treatment. The FGF pathway previously has been demonstrated to be involved in maintaining β cell mass in mice [45]. Interestingly, FGF1 is selectively induced in visceral fat in response to a high-fat diet in mice [46], and peri- and intra-pancreatic visceral fat correlates with age and body mass index, two major risk factors for type 2 diabetes [47]. Moreover, our analysis of available transcriptomic data [27] indicates that FGF2 expression is increased in several non-islets cell types in T2D pancreata, including ductal, endothelial, and pancreatic stellate cells, while FGFR1 is increased in islets from T2D patients. We thus hypothesize that an excess pancreatic FGF can contribute to β cell dedifferentiation, worsening type 2 diabetes. Intriguingly, FGF1 levels are elevated in newly diagnosed cases of type 2 diabetes as compared to normal glucose tolerance controls [48].

4.7. Conclusion

In conclusion, this work models β cell dedifferentiation in a human context. It implicates FGF signaling in this process. Our model, characterization of dedifferentiation markers, and identification on FGF2 as a modulator, could lead to new interventions to protect human β cells against dedifferentiation.

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DECLARATIONS OF INTEREST

None.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.02.002.

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