Original article

MOLECULAR METABOLISM

Nor-1, a novel incretin-responsive regulator of insulin genes and insulin secretion*

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ABSTRACT

B-cell failure at the onset of type 2 diabetes is caused by a decline in β-cell function in the postprandial state and loss of pancreatic β-cell mass. Recently, we showed an association between increased insulin secretion and a single nucleotide polymorphism (SNP), SNP rs12686676, in the *NR4A3* gene locus encoding the nuclear receptor Nor-1. Nor-1 is expressed in β-cells, however, not much is known about its function with regard to insulin gene expression and insulin secretion. Nor-1 is induced in a glucose-/incretin-dependent manner via the PKA pathway and directly induces insulin gene expression. Additionally, it stimulates insulin secretion possibly via regulation of potentially important genes in insulin exocytosis. Moreover, we show that the minor allele of *NR4A3* SNP rs12686676 fully rescues incretin resistance provoked by a well-described polymorphism in *TCF7L2*. Thus, Nor-1 represents a promising new target for pharmacological intervention to fight diabetes.

Keywords Nor-1; Insulin gene expression; Insulin secretion; Incretin resistance; *TCF7L2*

1. INTRODUCTION

B-cell failure at the onset of type 2 diabetes (T2D) is caused by different mechanisms. On the one hand, β-cell function in the postprandial state declines and, on the other hand, pancreatic β-cell mass decreases over time [1,2]. Together with other factors, this promotes hyperglycemia. The main cause leading to T2D is overweight caused by western lifestyle (high-caloric diet and physical inactivity), however, a certain susceptibility to T2D is thought to be due to single nucleotide polymorphisms (SNPs). Interestingly, the majority of known T2D risk genes have an impact on the β-cell [3].

In 2009, we were able to show an association between insulin secretion and a common SNP in the *NR4A3* gene locus encoding the metabolically important nuclear receptor Nor-1 [4], i.e., SNP rs12686676. Our studies revealed increased insulin secretion in subjects carrying this SNP's minor allele.

The nuclear receptor 4A (NR4A) subgroup consists of Nur77 (encoded by *NR4A1*) [5], Nurr1 (encoded by *NR4A2*) [6], and Nor-1 (encoded by *NR4A3*) [7]. These nuclear receptors are thought to be ligand-independent and to function as early-response genes regulating important cellular processes, e.g., cell proliferation, differentiation, and survival. Due to their expression in tissues with high energy demand, such as liver [8], brain, skeletal muscle [7], and adipose tissue [9], the function of the NR4A members has been analyzed mainly in these tissues. Nur77 regulates hepatic gluconeogenesis [10] and induces genes associated with glucose metabolism in skeletal muscle [11]. Nor-1 is involved in the regulation of lipid and energy metabolism in skeletal muscle [12]. Nur77 and Nor-1 have been shown to be induced by insulin in skeletal muscle and are implicated in insulin resistance [13]. In the liver, *Nr4a3* is a CREB target and regulates hepatic glucose metabolism [10]. In most of these tissues, the members of the NR4A subgroup are induced via β-adrenoceptor activation which is tightly coupled to intracellular cAMP levels [14].

Upon expression, NR4A receptors can bind as monomers [15] and homodimers [16] to the octanucleotide 5′-AAAAGGTCA-3′ (NGFI-B response element, NBRE). Even though there are no endogenous ligands known up to now, several pharmacological compounds have been reported to activate the NR4A subgroup, like 6-mercaptopurine (6-MP) via the AF-1 domain [17] or 1,1-di(3′-indolyl)-1-(*p*-substituted phenyl)methanes via the aberrant C-terminal ligand-binding domain [18].

However, not much is known about the function of these nuclear receptors with regard to insulin gene expression and insulin secretion. We and others were able to show the expression of Nor-1 and Nur77 in β-cells [4,19,20]. Furthermore, our recent human study showed

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increased insulin secretion in individuals carrying the minor allele of SNP rs12686676 in *NR4A3* [4]. Therefore, the aim of our study was to determine the molecular role of Nor-1 in glucose-stimulated insulin secretion (GSIS) and insulin gene expression.

2. MATERIAL AND METHODS

2.1. Cells

INS-1E cells (kindly provided by Dr. C. B. Wollheim, University of Geneva, Switzerland) were cultured in a humidified atmosphere containing 5% CO_2 in RPMI 1640 (11 mM glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 60 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 U/ml streptomycin. The following treatments were performed prior to lysis: forskolin (10 μM, 90 min, 3 h, and 24 h), dibutyryl-cAMP (db-cAMP, 1 mM, 90 min), exendin-4 (10 nM, 90 min), glucose (22.5 mM, 90 min), H89 (10 μM, 30 min pre-incubation), 6-MP (50 μM, 48 h).

2.2. Rat islets

Rat islets were isolated from 6-week-old male Wistar rats (Charles River Laboratories, Wilmington, MA, USA). Islets were isolated by collagenase (3 mg/ml, Serva, Heidelberg, Germany) infusion of the pancreas followed by digestion for 10 min at 37 °C and separated from exocrine tissue first by centrifugation through a Histopaque 1.077 g/ml (Sigma-Aldrich, St. Louis, MO, USA) cushion and then by manually collecting islets under the dissection microscope [21]. Islets were cultured overnight in RPMI 1640 containing 5.6 mM glucose. The following treatments were performed prior to lysis: forskolin (1 μM, 90 min), exendin-4 (10 nM, 90 min), glucose (22.5 mM, 90 min), H89 (10 μM, 30 min pre-incubation), 6-MP (50 μM, 24 h). Following the 6-MP treatment, batches of 10 islets each were pre-incubated for 1 h at 37 °C in buffer containing 2.8 mM glucose. For measurement of basal insulin secretion, the islets were incubated for 1 h at 37 °C in buffer containing 2.8 mM glucose. All animal procedures were approved by local government authorities for animal research according to the guidelines of laboratory animal care.

2.3. Human islets

Frozen human islets from 8 donors were purchased from ProCell Biotech (Newport Beach, Ca, USA). Islets were obtained as frozen pellets, resuspended in RLT (Qiagen, Hilden, Germany) buffer and subjected to RNA isolation.

2.4. RNA interference

Small interfering RNA (siRNA) oligonucleotides targeting *Nr4a3*, *Creb1*, and *Glp1r* were purchased as siGENOME-SMART-pool (Thermo Scientific, Rockford, IL, USA). As control, we used an unrelated siRNA targeting firefly luciferase in all experiments as reported earlier [22]. Transfection was performed with Dharmafect 4 (Dharmacon, Lafayette, CO, USA) according to the instructions of the manufacturer. 24 h after transfection, the cells either were immediately harvested or the medium was changed. Then, cells were either harvested at the indicated time point or were further incubated with 6-MP for 48 h or with forskolin for 24 h.

2.5. Quantitative real-time RT-PCR (reverse transcription PCR)

Cells and islets were washed once with PBS, lysed with RLT buffer and homogenized using QIAshredder (Qiagen). Total RNA (RNeasy Mini Kit, Qiagen) isolation, transcription to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Indianapolis, IN, USA) and RT-PCR were performed as described before [23]. The following primer sequences (TIB Molbiol, Berlin, Germany) were used to amplify the indicated genes. Human genes: *RPS13* 5′-CCCCACTTGGTTGAAGTTGA-3′ and 5′-ACACCATGTGAATCTCTCAGGA-3′; *NR4A1* 5′-GCCCATGTCGACTCCAAC-3′ and 5′-ACTCATTTGATAGTCAGGGTTCG-3′; *NR4A2* 5′-GCCCATGTCGACTCCAAC-3′ and 5′-ACTCATTTGATAGTCAGGGTTCG-3′; *NR4A3* 5′-ACACCCAGAGATCTTGATTATTCC-3′ and 5′-GTAGAATTGTTGCACATGCTCAG-3′; and 5′-CACAATGCCACGCTTCTG-3′. Rat genes: *Rps13* 5′-CTGACGACGTGAAGGAACAA-3′ and 5′-TCACAAAACGGACCTGTGC-3′; *Nr4a1* 5′-TGCTCTGGTCCTCATCACTG-3′ and 5′-ACAGCTAGCAATGCGGTTC-3′; *Nr4a2* 5′-CCACGTGCACTCCAATCC-3′ and 5′-TAGTCAGGGTTTGCCTGGAA-3′; *Nr4a3* 5′-TGCCTGTCAGCACTGAGTATG-3′ and 5′-GCTGCTTGTGATCTTGTTGC-3′; *Glp1r* 5′-CTGCTTTGTCAACAATGAGGTC-3′ and 5′-GTCCCTCTGGATGTTCAAGC-3′; *Creb1* 5′-GACGGAGGAGCTTGTACCAC-3′ and 5′-GCATCTCCACTCTGCTGGTT-3′; *Ins1* 5′-AGACCATCAGCAAGCAGGTC-3′ and 5′-CTTGGGCTCCCAGAGGAC-3′; *Ins2* 5′-CGAAGTGGAGGACCCACA-3′ and 5′-TGCTGGTGCAGCACTGAT-3′. PCR conditions can be provided upon request. All RNA data are presented relative to the housekeeping gene *Rps13* using the ΔΔCt method.

2.6. Microarray analysis

INS-1E cells were transfected with control or *Nr4a3* siRNA as described earlier and harvested 72 h after transfection. Total RNA was isolated from four independent experiments, replicate samples were pooled according to the test condition and subjected to microarray analysis using GeneChip Rat Gene 1.0 ST Arrays from Affymetrix (Santa Clara, CA, USA). The microarray analysis was performed by MFT Services (Tübingen, Germany), an official Affymetrix service provider. Genes with a more than 2-fold and less than 0.5-fold change in expression were taken into account and the expression level of candidate genes was verified by RT-PCR in individual samples.

2.7. Intracellular insulin content

INS-1E cells were transfected with siRNA and kept in culture medium. The medium was changed to low-glucose medium (RPMI 1640, 2.8 mM glucose) 24 h prior to extraction. Cells were counted, washed once with PBS and subjected to acid ethanol (0.18 mol/l HCl in 70% ethanol) extraction. Extraction was performed overnight at 4 °C. After centrifugation, supernatants were frozen at −20 °C. For activation of Nor-1, INS-1E cells were incubated for 48 h with 6-MP (50 μM) in culture medium, counted, and subjected to acid ethanol extraction. Intracellular insulin content was quantified by ELISA (Mercodia AB, Uppsala, Sweden).

2.8. Insulin secretion

Cells were washed three times with Krebs–Ringer Buffer (KRB) containing 2.8 mM glucose and pre-incubated for 3 h with the same buffer. For basal insulin secretion, cells were incubated for 1 h in the same buffer. GSIS was measured in cells incubated with KRB containing 11 mM glucose. Cells were subjected to acid ethanol extraction, and intracellular as well as secreted insulin were quantified by ELISA (Mercodia). Basal rat islet insulin secretion was measured accordingly, except with 1 h pre-incubation in 2.8 mM Glc. Secreted insulin was normalized for intracellular insulin content.

2.9. Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using the MAGnify Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, Nor-1 antibody (H7833) was purchased from R&D Systems (Minneapolis, MN, USA) and coupled to Dynabeads. INS-1E cells were incubated with 6-MP (50 μM) for 48 h to achieve

binding of Nor-1 to DNA binding sites. Cells were washed with PBS, trypsinized and counted. Nor-1 binding to chromatin was fixed with 37% formaldehyde. After cell lysis, chromatin shearing and isolation, chromatin from 200,000 cells was used for chromatin immunoprecipitation. Crosslinking was reversed and DNA extracted according to the manufacturer's instructions. The following primers (Invitrogen) were used to amplify Nor-1 binding sites in *Ins1* and *Ins2* using quantitative real-time RT-PCR: *Ins1* NBRE I 5′-CTTCGTTGTGACCTATTTTGGATGA-3′ and 5′-GGTTCAGTAACAAATGCCTGGAG-3′; *Ins1* NBRE II 5′-GGAAGGCAACTGATTTC-TTTGAGTTA-3′ and 5′-GATACAGATCGGAAAAGAAGAGGTCA-3′; *Ins1* NBRE III 5′-ATCCCACACCATCCTGCAAT-3′ and 5′-CTTAGTTGGCCCACAAAAATCTT-3′; *Ins2* NBRE I 5′-GGGAAGAAATTGGGCTTGGT-3′ and 5′-TTCAGAGCACTAAA-GGTCACTTGGAT-3′; *Ins2* NBRE II 5′-GATGTGCACCTTTGGGGTTTA-3′ and 5′-AACTCCAAGCAAAGAGAGGGATTACT-3′. PCR products were also analyzed by agarose gel electrophoresis.

2.10. Western blot

Cells were washed with PBS, and nuclear proteins were isolated using nuclear and cytoplasmic extraction reagents (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific). Nuclear lysates were centrifuged at 13,000*g* for 10 min, and the protein concentration was measured in the supernatant using the Bradford protein assay (Bio-Rad, Richmond, CA, USA). The same amount of protein was loaded in each lane of a SDS-PAGE gel. After separation, proteins were transferred to nitrocellulose membranes (Amersham Life Sciences Inc., Arlington Hights, IL, USA) and incubated with Nor-1 (1:500, H7833, R&D Systems) antibody or Histone H3 (1:1000, 3H1, Cell Signaling Technologies Inc., Beverly, MA, USA) antibody. Histone H3 was used as housekeeping protein for normalization of Nor-1 protein content. After three washes, membranes were incubated with goat anti-rabbit IgG–Peroxidase antibody (Sigma-Aldrich). The proteins were detected using the ECL system (Amersham Life Sciences Inc.). For quantification, we used EasyWin32 Herolab (Wiesloch, Germany) Software.

2.11. Luciferase reporter gene assays

A 190-bp sequence encompassing the Nor-1 binding site of the human insulin gene was amplified from genomic DNA obtained from human myotubes. A 207-bp sequence encompassing the rat binding site II of *Ins1* was amplified from genomic DNA from INS-1E cells. The following primers (Invitrogen) were used: 5′-GGAGACCCTCTCCCTGACC-3′ and 5′-GGGACCCCGACTCTGACTTA-3′ for the human sequence, 5′-GCCAC-TTTGCTGAAGTTGTTT-3′ and 5′-CTGCCCACTCTCTCCCTACTT-3′ for the rat sequence. Both fragments were cloned into the luciferase reporter vector pGL3-Promoter (Promega, Madison, WI, USA). The vector harboring the human *Nr4a3* gene was purchased from Origene (Rockville, MD, USA). HEK293 cells were seeded at 5000 cells per well in a 96-well plate, transfected with Lipofectamine (Invitrogen) 24 h after seeding and assayed 48 h after transfection using Dual-Glo Luciferase Assay reagent (Promega) according to the manufacturer's instructions.

2.12. Lentiviral transduction

Control vector, vector coding for rat *Nr4a3*, 3rd Generation Packaging Mix, Lentifectin, Polybrene and ViralPlus Transduction enhancer were purchased from Applied Biological Materials Inc (Vancouver, Canada). Virus production in HEK293FT cells (Invitrogen) and infection of INS-1E cells was performed according to the manufacturer's instructions.

2.13. Human data

Data from 1454 non-diabetic participants of the **Tü**bingen **F**amily (TÜF) Study for type 2 diabetes were analyzed in this study [24]. Data from repeated sampling oral glucose tolerance tests (OGTT; 0, 30, 60, 90, and 120 min) and *TCF7L2* rs7903146 and *NR4A3* rs12686676 genotypes were available from earlier studies [4,25]. Details on genotyping as well as on associations of these variants with insulin secretion and other traits were reported recently [4,25]. Insulin secretion during OGTT was estimated by the insulinogenic index as described previously [26].

2.14. Statistical analysis

For all statistical analysis, the Software package JMP 8.0.2 (SAS Institute, Cary, NC, USA) was used. If not indicated otherwise, data are provided as relative values with control conditions chosen as reference point. Data are given as mean $\pm$ SEM. Two-group comparisons were performed using matched pairs Student's *t*-test. With regard to human data, we stratified the cohort for the *TCF7L2* SNP rs7903146 and *NR4A3* SNP rs12686676 genotypes using dominant inheritance models. Differences in insulin secretion between the *NR4A3* genotypes were tested using multiple linear regression analysis with gender, age, and BMI as covariates. For all analyses, p-values$\leq$0.05 were considered statistically significant.

3. RESULTS

3.1. Nor-1 is a novel regulator of insulin genes

All mechanistic investigations in this study were performed in INS-1E, a commonly used model of insulin-secreting cells, and important findings were validated in primary pancreatic islets. First, we determined the expression of *Nr4a1*, *Nr4a2*, and *Nr4a3* using RT-PCR. Expression of *Nr4a1* in INS-1E cells was 12-fold higher than that of *Nr4a3* which in turn was expressed 2-fold higher than *Nr4a2* (Figure 1A). The isoform with the strongest expression in rat and human islets was *Nr4a1* as well (Figure 1B and C). In rat islets, *Nr4a2* expression was 5.5-fold higher than that of *Nr4a3* (Figure 1B). Human islets expressed *Nr4a2* and *Nr4a3* at similar levels (Figure 1C).

Since the *NR4A3* gene product Nor-1, but none of the other two family members, revealed an association with insulin secretion in humans [27], we intended to analyze whether Nor-1, as a transcription factor, affects insulin gene expression. Up to now, there are no physiological activators known for Nor-1. Therefore, we decided to use the pharmacological activator 6-MP. Activation of Nor-1 by 6-MP (48 h) induced a more than 2-fold increase in rat *Ins1* and *Ins2* gene expression (Figure 2A). To verify whether this gene induction is reflected at the protein level, we measured the intracellular insulin content in 6-MP-treated cells. Upon 6-MP treatment, the intracellular insulin content doubled compared to control cells (Figure 2B). Treatment of isolated rat islets with 6-MP for 24 h resulted in increased expression of *Ins1* and *Ins2* as well (Figure 2C).

In the next step, *Nr4a3* expression was down-regulated via RNA interference. Using small interfering RNA (siRNA), its expression was reduced by 75% after 24 h and by 80% after 72 h compared to control (Figure 2D). *Nr4a3* down-regulation reduced the expression of *Ins1* by 35% after 24 h and by 55% after 72 h, while the expression of *Ins2* was reduced by 30% and 50%, respectively (Figure 2D). *Nr4a2* down-regulation did not affect *Ins1* or *Ins2* expression ($p\geq$0.06; n=4) excluding an involvement of this transcription factor in the regulation of insulin genes. However, *Nr4a1* down-regulation reduced the expression of *Ins1* and *Ins2* by 15% (p=0.0151 and p=0.0076, respectively; n=4) after 24 h. Since the effect of *Nr4a1* down-regulation was much lower compared to that of *Nr4a3* down-regulation and no associations between common genetic variation in the *NR4A1* locus and insulin

secretion was detected in humans [27], we focused on *NR4A3* in all subsequent experiments. At 72 h after *Nr4a3* siRNA transfection, the effect on insulin gene expression was strongest. Therefore, we analyzed whether the intracellular insulin content was changed at this time point. However, we were not able to show a reduction in intracellular insulin content ($p=0.8$; $n=4$). Moderate lentiviral overexpression of *Nr4a3* resulted in a trend for increased *Ins1* expression and in significantly increased expression of *Ins2* (Figure 2E).

Since 6-MP is an activator of all three NR4A members, we down-regulated *Nr4a3* prior to 6-MP treatment. In these cells, 6-MP-induced *Ins1* and *Ins2* expression levels were markedly decreased (Figure 2F) excluding compensation of lost *Nr4a3* expression by *Nr4a1* or *Nr4a2* and further strengthening the prominent role of *Nr4a3* in the regulation of *Ins1* and *Ins2* expression.

Upon their expression, *NR4A* members bind to NBRE sites. *In silico* analysis of the rat insulin loci for putative NR4A binding sites displayed three NBREs in the *Ins1* locus and two in *Ins2* (Figure 3A). The human gene locus harbors one NBRE that represents a perfect consensus sequence (Figure 3A). The binding of Nor-1 to the rat insulin genes was analyzed by chromatin immunoprecipitation. Nor-1 preferentially binds to NBREII (Cp-value: 21.08 ± 1.99) and weaker to NBREI (Cp-value: 34.74 ± 2.09) and III (Cp-value: 33.76 ± 2.10) in *Ins1*, while it shows comparable binding to both sites in *Ins2* (Cp-values: 32.39 ± 1.65 and 34.08 ± 1.83, respectively). Qualitative data are shown in Figure 3B. Overexpression of a luciferase reporter vector containing either the human NBRE or the NBRE II of rat *Ins1* resulted in an increased luciferase activity compared to control (Figure 3C). *Nr4a3* co-transfection in both cases further increased luciferase activity (Figure 3C). These data indicate a direct role of Nor-1 in the transcriptional regulation of insulin genes.

3.2. Nor-1 regulates insulin secretion

Since we demonstrated an association between *NR4A3* SNP rs12686676 and oral glucose-stimulated insulin secretion in humans [4], we analyzed GSIS in cells treated with *Nr4a3* siRNA. Down-regulation of *Nr4a3* did not affect basal insulin secretion ($p=0.4$), however, it reduced GSIS by 13% compared to cells treated with control siRNA (Figure 4A). Furthermore, exendin-4-enhanced GSIS was reduced by 18% compared to control cells (Figure 4A). Activation of *Nr4a3* by 6-MP for 48 h resulted in a 2-fold increased insulin level in the supernatant (Figure 4B). 6-MP treatment of INS-1E cells and isolated rat islets resulted in increased basal insulin secretion as well (Figure 4C).

72 h after transfection with *Nr4a3* siRNA the intracellular insulin content was unchanged. Therefore, we hypothesized that Nor-1, as a transcription factor, may regulate the expression of additional genes involved in insulin secretion. To this end, we performed exploratory microarray analysis using mRNA from control cells and from cells treated with *Nr4a3* siRNA. We found 313 genes to be differently regulated by *Nr4a3* down-regulation (Supplementary Table S1, fold-change >2 or <0.5, respectively). At least six of these genes are potentially related to insulin secretion according to current literature. Therefore, we verified their altered expression by RT-PCR. Four of the six genes were down-regulated: *Vamp3* by 70%, *Syt11* by 70%, *Hpca* by 40%, and *Nlgn3* by 85% (Figure 4D). Two of the six genes were up-regulated by *Nr4a3* down-regulation: the expression of *Casr* was induced 5.5-fold, that of *Gpr39* approximately 2.5-fold (Figure 4D). By *in silico* analysis of the 5′- and 3′-flanking regions of all these genes, we identified putative NBREs in each of these genes.

3.3. Nor-1 expression is regulated by the PKA pathway in β-cells

One of the major second messengers in β-cells is cAMP. It is produced upon activation of adenylyl cyclase after stimulation of the cells with diverse stimuli, such as glucose or insulinotropic enterohormones, i.e., incretins [28,29]. In the β-cell, cAMP has been shown to induce insulin expression via CREB [29]. Pharmacological activation of the PKA pathway via db-cAMP induced an 8-fold increase in *Nr4a3* expression, while forskolin, an adenylyl cyclase activator, induced a 70-fold increase (Figure 5A). The forskolin effect was reflected at the protein level: treatment for 90 min or 3 h induced 4-fold and 3-fold increments in nuclear Nor-1 protein content, respectively (Figure 5B). In primary rat islets, forskolin treatment induced a 50-fold increase in *Nr4a3* expression (Figure 5C). Glucose induced a modest, but not significant, increase in *Nr4a3* expression ($p=0.12$), while exendin-4 alone induced a 4-fold increase in INS-1E cells (Figure 5D). The strongest effect was seen with a combination of exendin-4 and glucose (5.5-fold, Figure 5D). Similar effects were seen in isolated rat islets. Exendin-4 alone induced a 2.5-fold induction in *Nr4a3* expression, while co-incubation of cells with exendin-4 and glucose induced a 12-fold increase (Figure 5E). Down-regulation of glucagon-like peptide 1 receptor (*Glp1r*) in INS-1E cells resulted in a blunted induction of *Nr4a3* expression induced by glucose and exendin-4 (Figure 5F). Inhibition of the PKA pathway via the specific PKA inhibitor H89 reduced exendin-4/glucose-induced *Nr4a3* expression by 45% in INS-1E cells (Figure 5G) and by 30% in rat islets (Figure 5H). Next, the impact of Creb1 on PKA-induced stimulation of *Nr4a3* expression was examined. The expression of *Creb1* mRNA was reduced by 90% via RNA interference (Figure 5I). Upon *Creb1* knock-down, *Nr4a3* expression was reduced by 40% (Figure 5I). Hence, via activation and inhibition of the PKA pathway, we were able to demonstrate the involvement of this pathway in *Nr4a3* gene induction in β-cells and islets.

3.4. *Nr4a3* plays a role in PKA-dependent insulin gene induction

To understand the role of *Nr4a3* in the PKA pathway, we down-regulated its expression for 72 h and, in parallel, activated the pathway for 24 h

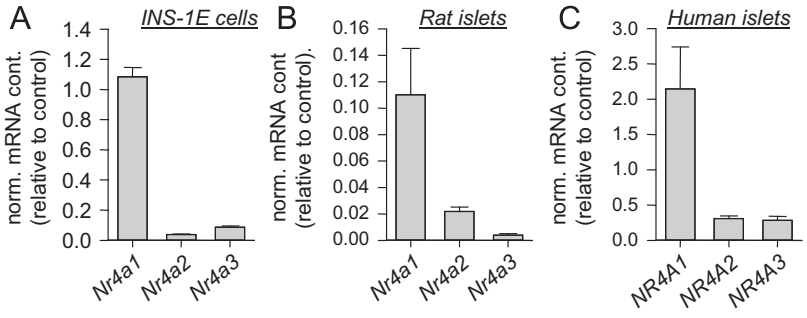

Figure 1: Expression of the Nr4a family members in INS-1E cells and primary islets. Expression of the indicated genes was measured in INS-1E cells ($n=3$), rat islets ($n=8$) and human islets ($n=8$). Data are given as mean ± SEM.

with forskolin. Forskolin treatment significantly induced *Ins1* expression, and this was blunted in cells treated with *Nr4a3* siRNA (Figure 6). Even though we did not observe forskolin-induced *Ins2* expression, down-regulation of *Nr4a3* resulted in a significant reduction of *Ins2* expression in the presence or absence of forskolin (Figure 6). Similar results were seen after 48 h of forskolin treatment. These data further underline the role of Nor-1 as a novel important link between the PKA pathway and insulin gene expression.

3.5. *NR4A3* expression in healthy versus diabetic donors

Islets from diabetic donors showed somewhat lower *NR4A3* and *INS* expression. This, however, did not reach significance in this small cohort (Supplementary Figure S1).

3.6. Genetic interaction between *NR4A3* and *TCF7L2* in humans

Recent genome-wide association studies revealed more than 50 type 2 diabetes risk genes [30–33] with *TCF7L2* variant rs7903146 being the strongest predictor for the onset of the disease [34]. We and others have shown resistance to the incretin glucagon-like peptide-1 in subjects carrying the *TCF7L2* risk allele [25,35]. Since we provide evidence of a new mechanism for incretin-dependent induction of insulin expression and secretion, we analyzed whether the aforementioned variant in the *NR4A3* gene (SNP rs12686676) is able to aggravate or compensate for the secretion-compromising effect of the *TCF7L2* risk allele. We therefore tested for gene–gene interactions between the *TCF7L2* SNP rs7903146 and *NR4A3* SNP rs12686676 with regards to insulin secretion during an OGTT. *NR4A3* minor allele carriers show increased insulin secretion (as reported earlier [4] and shown in Figure 7A in a somewhat smaller cohort additionally genotyped for rs7903146). The

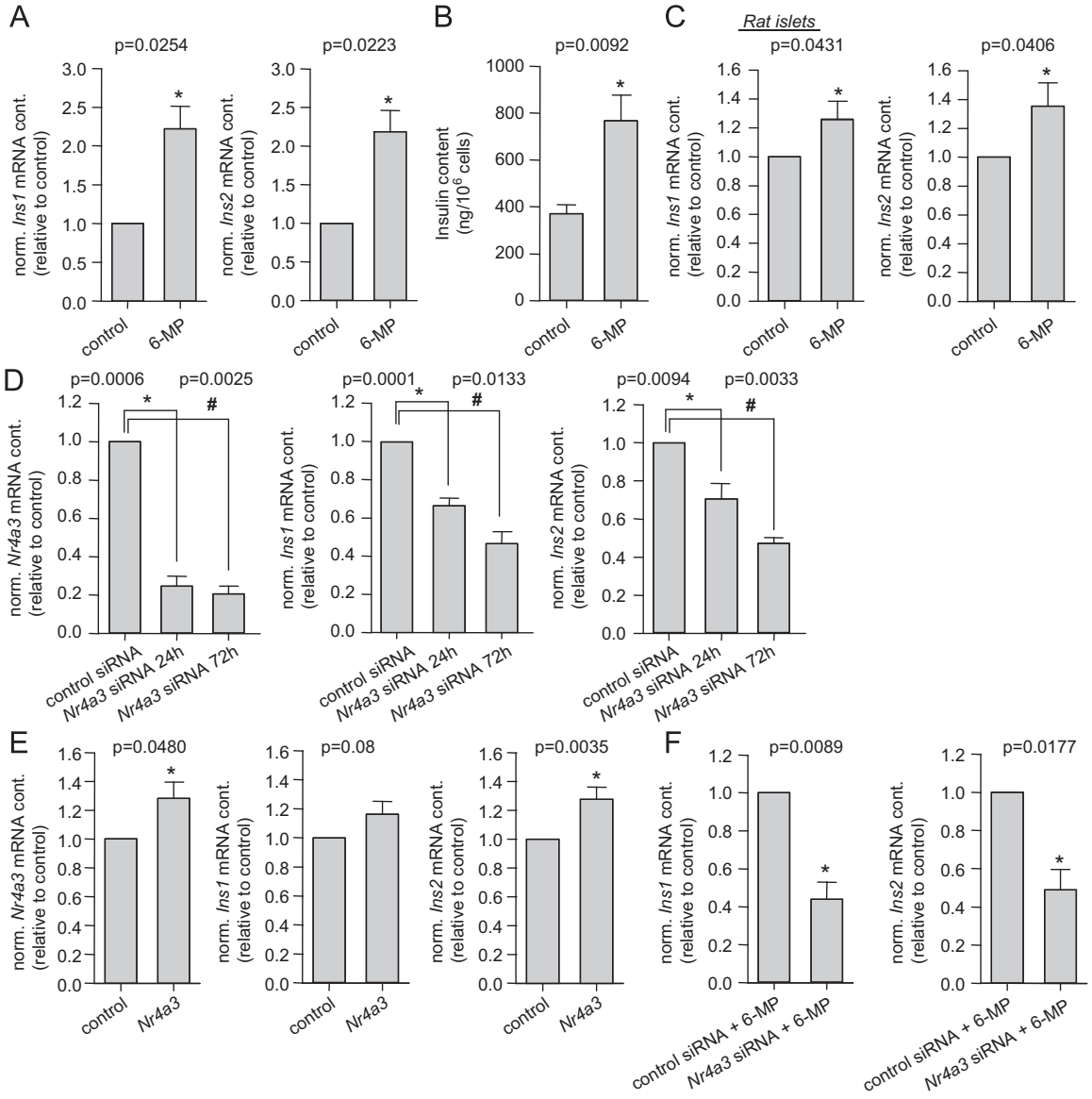

Figure 2: Nor-1-regulated expression of insulin genes. Expression of the rat insulin genes *Ins1* and *Ins2* (A) and intracellular insulin content (B) after incubation of INS-1E cells with 50 μM 6-MP for 48 h. Expression of the rat insulin genes *Ins1* and *Ins2* (C) after incubation of purified rat islets with 50 μM 6-MP for 24 h. After 24 h and 72 h treatment with *Nr4a3* siRNA, the expression of *Nr4a3*, *Ins1* and *Ins2* (D) were measured in INS-1E cells. Expression of *Nr4a3*, *Ins1* and *Ins2* in *Nr4a3*-overexpressing INS-1E cells (E). *Ins1* and *Ins2* gene expression after *Nr4A3* down-regulation with siRNA for 72 h and 6-MP (50 μM, 48 h) treatment of INS-1E cells (F). Data are given as mean ± SEM. Two-group comparisons were performed using matched pairs Student's *t*-test ($n \geq 3$).

well-described effect of the *TCF7L2* risk allele on insulin secretion is also evident in our cohort (Figure 7B, [25]). The minor *NR4A3* allele has no effect in *TCF7L2* wildtype allele carriers that do not display compromised insulin secretion, but compensates for incretin resistance induced by the *TCF7L2* risk allele (Figure 7C).

4. DISCUSSION

The impact of dysregulated insulin secretion gets more and more important for understanding the mechanisms leading to T2D. The majority of known T2D risk genes is associated with β-cell function suggesting that β-cell dysfunction is predominantly determined by genetics, while insulin resistance seems more prone to environmental influences [3]. We previously linked the polymorphism rs12686676 in the human *NR4A3* gene, encoding for Nor-1, to increased insulin secretion in humans [4] revealing it as a beneficial minor allele. In the present study, we investigated the molecular function of Nor-1 in β-cells and introduce it as a novel transcriptional regulator of insulin genes and insulin secretion. Additionally, we provide a link to the best described T2D risk allele in *TCF7L2* which is associated with reduced insulin secretion and reduced insulin gene expression [36].

Nor-1 and its isoforms Nur77 and Nurr1 are expressed in INS-1E cells, primary rat and human islets. Pharmacological activation of Nor-1 by 6-MP resulted in a strong induction of both insulin genes, *Ins1* and *Ins2*, in rat insulin-secreting cells, while down-regulation of *Nr4a3* resulted in reduced insulin expression showing that Nor-1 regulates the expression of these genes. The 6-MP effect on *Ins1* and *Ins2* expression was verified in isolated rat islets. Moderate overexpression of Nor-1 via lentiviral infection induced *Ins2* expression significantly and showed a tendency towards increased *Ins1* expression. Since down-regulation of *Nr4a3* prior to 6-MP treatment prevented the expression of the insulin genes, we can assume that loss of Nor-1 is not compensated by either Nur77 or Nurr1, which are also activated by 6-MP [17]. Nevertheless, down-regulation of *Nr4a1* also resulted in a weak reduction of *Ins1* and *Ins2* gene expression. This shows that Nur77, which is encoded by *Nr4a1*, might have a minor impact on insulin gene expression. The physiological relevance of this finding however appears questionable since we were not able to show an association between common genetic variation in *NR4A1* and insulin secretion in humans [27]. The 6-MP-induced increase in insulin expression is reflected at the protein level. Acute down-regulation of *Nr4a3* (72 h) did not result in reduced intracellular insulin content, probably due to the long half-life of 3–5 days of insulin granules [37]. The intracellular insulin content might however be reduced under chronic impairment of *Nr4a3* expression. This could be the case in T2D, as it was shown that obese or diabetic rodent models express lower levels of *Nr4a3* and *Nr4a1* in muscle and adipose tissue [13]. Under the same conditions, the levels of all NR4A members are elevated in liver [10]. A human study with morbidly obese patients showed a high expression of all NR4A members that was normalized after weight loss [38]. These contradictory findings may be due to species- or tissue-specific differences in *NR4A3* expression and therefore need further clarification. We tried to investigate *NR4A3* expression in human islets from healthy versus diabetic donors, but did not obtain a significant difference.

Interestingly, altered *Nr4a3* gene expression also affected insulin secretion. This cannot be explained by changes in intracellular insulin content, since the insulin content was not changed by *Nr4a3* down-regulation. Down-regulation of *Nr4a3* reduced glucose- and incretin-induced insulin secretion, while basal insulin secretion was unchanged. On the other hand, 6-MP-dependent activation of Nor-1 induced insulin secretion, and 6-MP treatment of isolated rat islets resulted in increased basal insulin secretion as well. Since Nor-1 is a transcription factor, we analyzed whether the expression of proteins involved in insulin exocytosis is affected by *Nr4a3* down-regulation. We found four proteins to be down-regulated (*Vamp3*, *Nlgn*3, *Syt11* and *Hpca*) while two others were induced (*Gpr39* and *Casr*). Cellubrevin (encoded by *Vamp3*) is attached to the insulin granules by a C-terminal transmembrane domain and is, as a part of the SNARE complex, involved in Ca^{2+}-dependent insulin secretion [39,40]. Neuroligin-3 (encoded by *Nlgn3*) has been shown to be expressed in human and rat islets, and overexpression of the protein increased basal insulin secretion. However, an influence on GSIS was not analyzed [41]. Also involved in Ca^{2+}-dependent insulin secretion are members of the synaptotagmin (Syt) family. The expression of several Syt isoforms has been shown in different insulin-secreting cells, and two isoforms, Syt III and Syt VII, have been shown to co-localize with insulin granules and to play a role in Ca^{2+}-dependent insulin release [42]. Consequently, synaptotagmin VII null mutant mice revealed impaired insulin secretion [43]. Even though up to now, there are no data suggesting an involvement of Syt XI (encoded by *Syt11*) in insulin secretion, our data indirectly suggest that Syt XI is a novel member of this protein family being involved in the exocytosis of insulin. Hippocalcin (encoded by *Hpca*) is a member of the Ca^{2+} sensor protein subfamily of visinin-like proteins. Visinin-like protein-1, another member of the same family, has been shown to regulate insulin secretion in primary mouse β-cells [44]. Surprisingly, the down-regulation of *Nr4a3* also induced the expression of two genes related to insulin secretion suggesting that Nor-1 may also function as a gene repressor. GPR39 (encoded by *Gpr39*) seems to play a role in insulin secretion in knock-out mice under increased insulin demand, i.e., in the state of insulin resistance [45]. In human islets, activation of the calcium-sensing receptor (CasR, encoded by *Casr*) induced a rapid but relatively transient stimulation of insulin secretion in the absence of glucose suggesting that CasR is linked to the secretory process [46]. The results of our analysis indicate that several proteins related to insulin secretion are regulated by Nor-1, and changes in their expression could possibly explain our results showing reduced insulin secretion after *Nr4a3* down-regulation as well as increased insulin secretion in subjects carrying a SNP in this gene. Further studies are however needed to corroborate this.

In the β-cell, cAMP is one of the major second messengers and regulates insulin secretion in a PKA-dependent as well as a PKA-independent manner (for review, see [47]), and cAMP has also been shown to induce insulin expression via CREB [48]. Here, we demonstrate a novel cAMP/PKA/CREB-dependent player inducing insulin expression. B-cell specific activation of the PKA pathway by the incretin mimetic exendin-4 induced *Nr4a3* expression in INS-1E cells as well as in isolated rat islets. The strongest effect was seen with a combination of exendin-4 and glucose reflecting the amplifying effect of glucose on the incretin effect in β-cells [49]. The expression of *Nr4a3* was reduced by pharmacological inhibition of this pathway in INS-1E cells as well as in primary rat islets. Additionally, we found *Nr4a3* to be a CREB target gene in insulin-secreting cells. Here, we show that Nor-1 directly binds to regulatory sequences in the insulin genes. Insulin gene expression is induced upon Nor-1 activation via 6-MP and reduced upon down-regulation of *Nr4a3* expression. Therefore, elevated cAMP levels not only regulate insulin gene expression directly via CREB binding to insulin genes but also via CREB-dependent *Nr4a3* induction. Therefore, Nor-1 represents a novel link in the PKA pathway inducing insulin genes. Since down-regulation of *Nr4a3* had such a strong effect on insulin gene

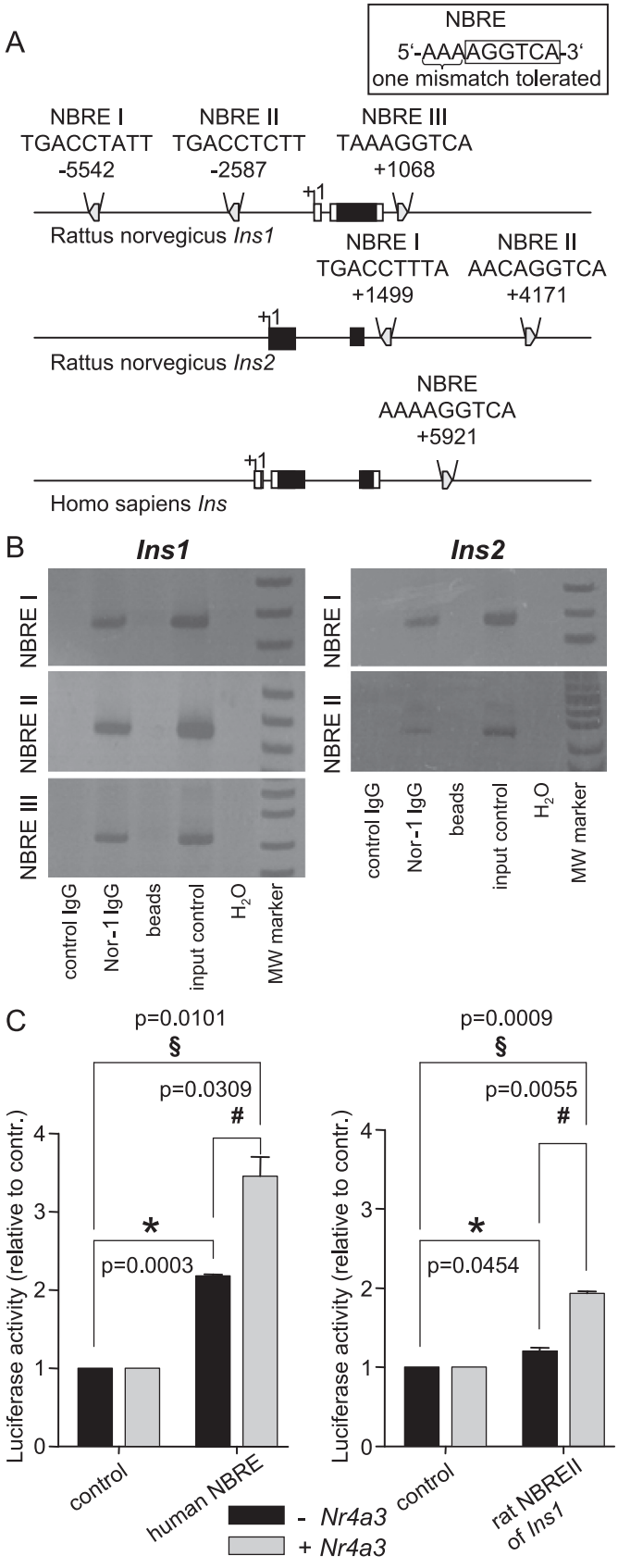

Figure 3: Nor-1 binding to insulin genes. *In silico* analysis of putative Nor-1 binding sites in the rat insulin genes *Ins1* and *Ins2* and in the human insulin gene *Ins* (A). Qualitative chromatin immunoprecipitation: Nor-1 binding to NBREs in *Ins1* and *Ins2*. PCR reaction was stopped after 36 cycles for *Ins1* NBRE I, 25 cycles for *Ins1* NBRE II, 36 cycles for *Ins1* NBRE III, 35 cycles for *Ins2* NBRE I, and 34 cycles for *Ins2* NBRE II, respectively (B). HEK293 cells were transiently transfected with luciferase reporter vectors containing the human NBRE or rat NBRE II of *Ins1* with or without *Nr4a3* co-transfection. Luciferase activity was measured 48 h after transfection. Data are given as mean ± SEM. Two-group comparisons were performed using matched pairs Student's *t*-test ($n=3$).

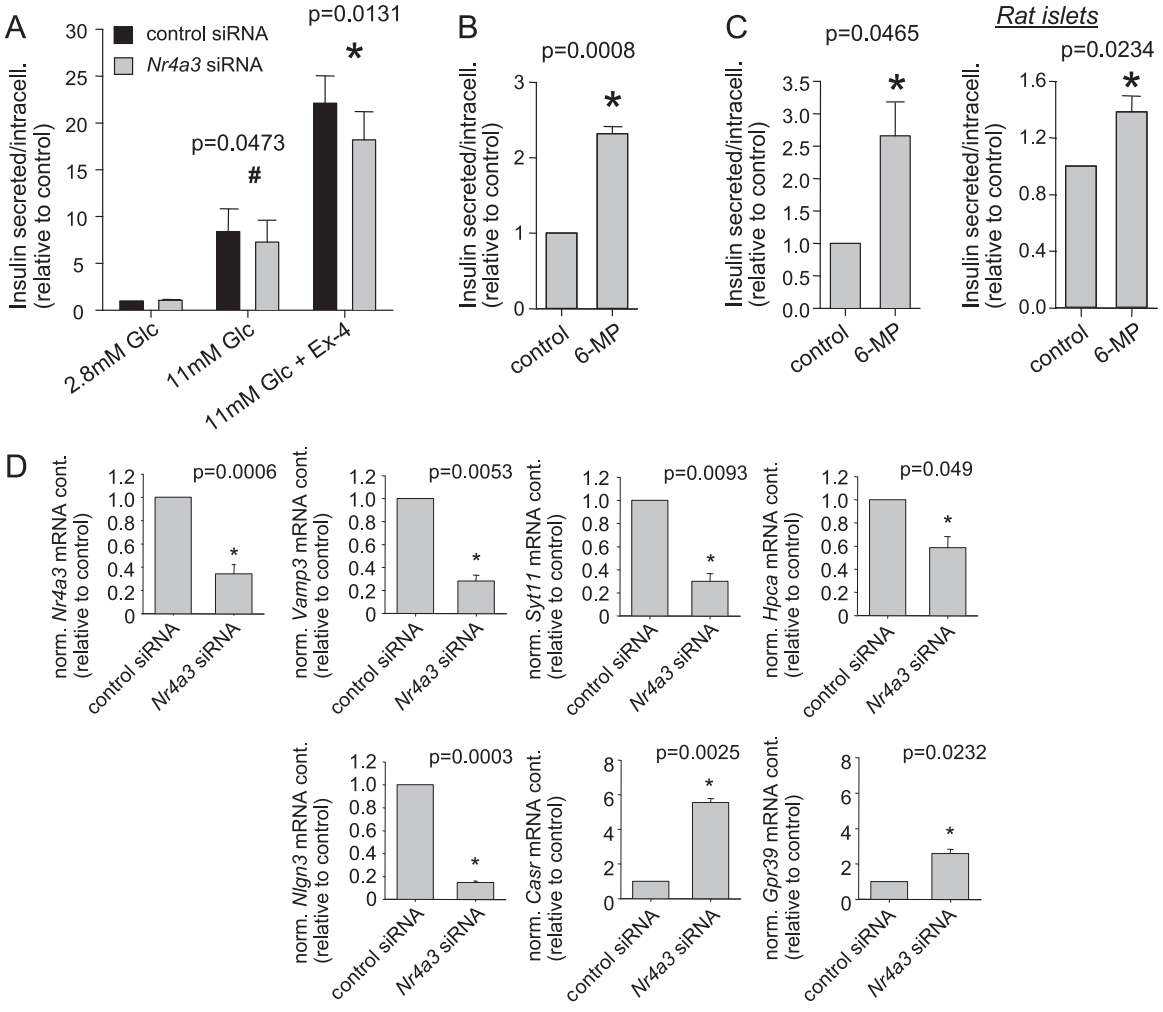

Figure 4: Nor-1 and insulin secretion. INS-1E cells were transfected with *Nr4a3* siRNA 72 h prior to treatment. Basal, glucose- and incretin-stimulated insulin secretions as well as intracellular insulin content were measured. Secreted insulin was normalized for intracellular insulin content. Cells were pre-incubated with 2.8 mM glucose (Glc) for 3 h prior to incubation with indicated glucose concentrations in the presence or absence of exendin-4 (Ex-4) (A). Insulin concentration was measured in cell culture supernatant after incubation of INS-1E cells with 50 μM 6-MP for 48 h (B). Basal insulin secretion at 2.8 mM glucose after incubation of purified rat islets or INS1E cells with 50 μM 6-MP for 24 h (C). Regulation of several genes related to insulin exocytosis by *Nr4A3* down-regulation for 72 h (D). Data are given as mean ± SEM. Two-group comparisons were performed using matched pairs Student's *t*-test ($n \geq 3$).

expression, it is conceivable that Nor-1 plays an important role in the PKA/CREB pathway leading to insulin gene expression. Since it is possible to activate Nor-1 by external stimuli (6-MP), it represents a potential therapeutic target for the treatment of incretin resistance. Therefore, it would be worthwhile to develop new Nor-1 activators with fewer side effects than 6-MP.

Genetic variation in *TCF7L2* is the most important genetic risk factor for type 2 diabetes known up to now and is involved in incretin- and glucose-mediated insulin secretion [25,50,51]. In the present study, we introduce a novel incretin-dependent mechanism of insulin secretion. Therefore, we intended to analyze whether the effects of the *NR4A3* and *TCF7L2* SNPs, with regard to insulin secretion in humans are influencing each other or if they are even counteracting each other. By stratification of our cohort according to *TCF7L2* SNP rs7903146, we indeed were able to show that the SNP in *NR4A3* is counteracting the effect of the *TCF7L2* SNP. Based on our findings of Nor-1's function, we hypothesize that the SNP in *NR4A3* that associates with improved insulin secretion can circumvent and compensate for the impaired incretin response of *TCF7L2* risk allele carriers, e.g., by directly increasing the expression of insulin genes and insulin secretion-related genes (Figure 8). The effect of *NR4A3* SNP rs12686676 is only visible in subjects carrying the *TCF7L2* risk allele since incretin signaling is intact in *TCF7L2* non-risk allele carriers. These genetic findings underline the importance of Nor-1's connection to incretin signaling in humans and render Nor-1 a promising new target for pharmacological intervention. Moreover, this finding may help to create personalized therapies for type 2 diabetes and support the efforts which have been started with regard to *TCF7L2* [52]. Nevertheless, our understanding of the role of Nor-1 in human insulin gene expression and insulin secretion is still limited and needs further clarification.

5. CONCLUSIONS

In this study, we introduce the novel transcriptional regulator of insulin genes, Nor-1. Its expression is induced via the PKA

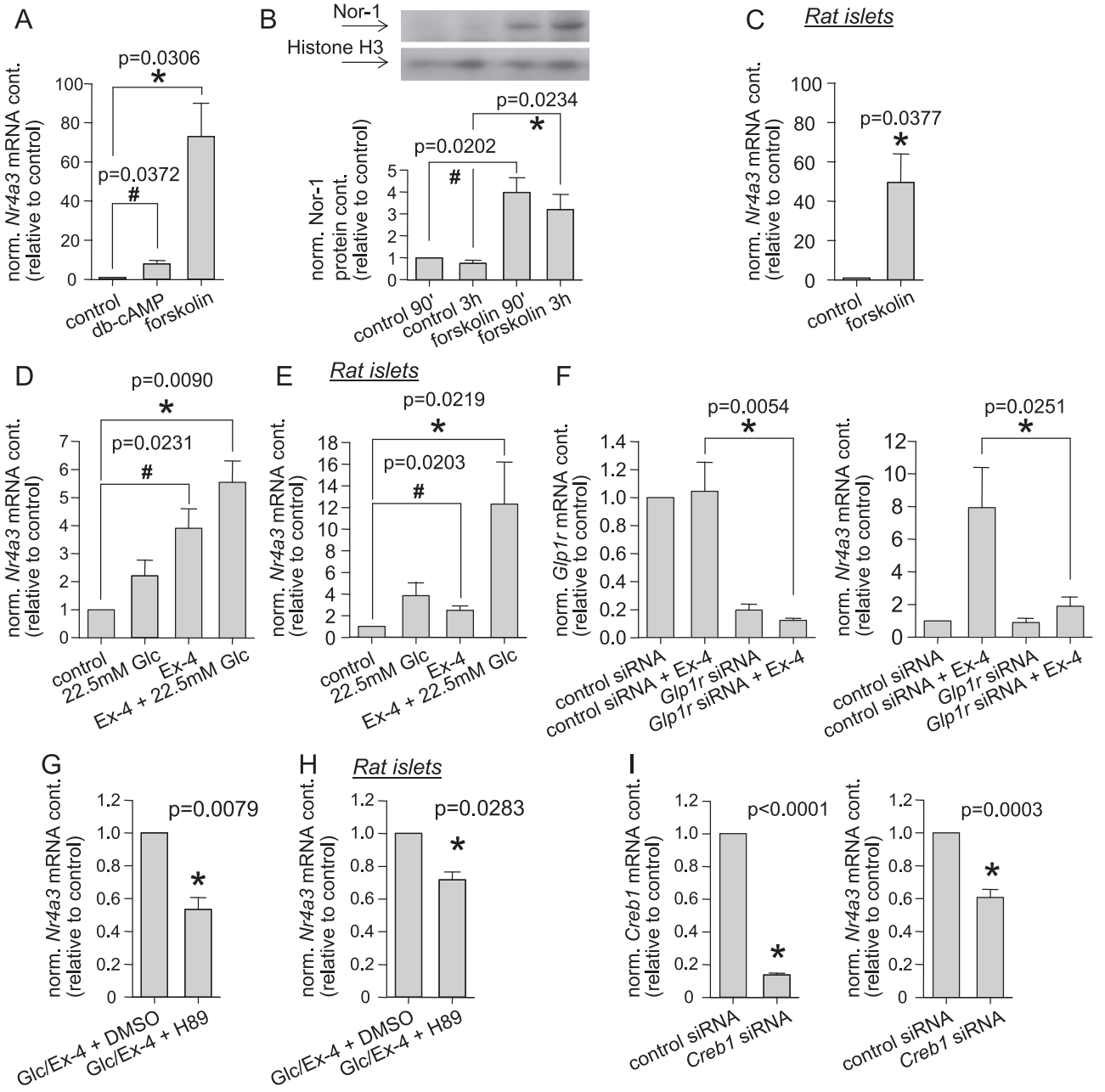

Figure 5: PKA pathway-regulated *Nr4a3* expression. *Nr4a3* expression after incubation of INS-1E cells for 90 min with 1 mM db-cAMP or 10 μM forskolin (A). Nor-1 and Histon H3 protein content was visualized by Western blotting after 90 min and 3 h of forskolin treatment. Nor-1 protein content was normalized by the housekeeping protein Histone H3. (B). *Nr4a3* expression after incubation of rat islets with 1 μM forskolin for 90 min (C). *Nr4a3* expression was measured after treatment of INS-1E cells (D) or purified rat islets (E) for 90 min with 10 nM exendin-4 (Ex-4) in the absence or presence of 22.5 mM glucose (Glc). INS-1E treated for 24 h with *Glp1r* siRNA and for 90 min with 10 nM exendin-4 in the presence of 22.5 mM glucose were lysed, and *Glp1r* and *Nr4a3* expression were measured (F). *Nr4a3* expression after pre-incubation with 10 μM H89 for 30 min prior to incubation with 10 nM exendin-4 in the presence of 22.5 mM glucose for 90 min: INS-1E cells (G) and purified rat islets (H). *Nr4a3* and *Creb1* expression were determined after down-regulation of *Creb1* by siRNA for 24 h in INS-1E cells (I). Data are given as mean ± SEM. Two-group comparisons were performed using matched pairs Student's *t*-test ($n \geq 3$).

pathway in insulin-secreting cells and primary pancreatic islets in an incretin- and glucose-dependent manner. It directly regulates the expression of insulin genes and insulin secretion. Since Nor-1 has also been shown to play a major role in metabolism in other tissues, it might be an interesting new pharmacological target to fight type 2 diabetes. Especially patients with impaired insulin gene expression or impaired insulin secretion, for example *TCF7L2* SNP carriers, might profit from pharmacological activation of Nor-1.

AUTHOR CONTRIBUTIONS

A.-M.O. performed the *in vitro* experiments, analyzed the data, and wrote the manuscript. O.R., C.H., I.T. provided technical support and analyzed the data. R.G., M.H., S.H.-S., A.B., F.M. collected and analyzed the human data. S.U. contributed to the design of the *in vitro* experiments. N.S., A.F. and H.-U.H. designed and carried out the human study. H.S. designed the *in vitro* experiments, analyzed the data, and edited the manuscript. All authors discussed the results and commented on the manuscript.

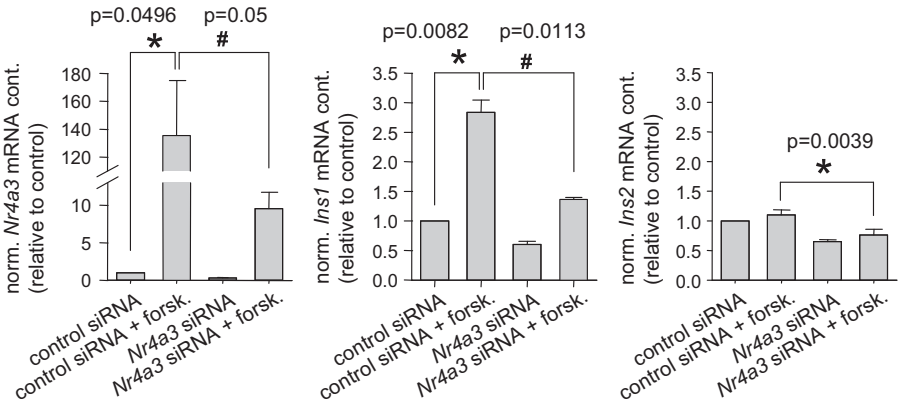

Figure 6: Role of *NR4A3* in PKA-dependent insulin gene expression. INS-1E cells were treated for 72 h with *Nr4a3* siRNA. Cells were incubated with forskolin (forsk., 10 μM) 24 h prior to lysis. *Nr4a3*, *Ins1* and *Ins2* expression were measured. Data are given as mean ± SEM. Two-group comparisons were performed using matched pairs Student's *t*-test ($n=3$).

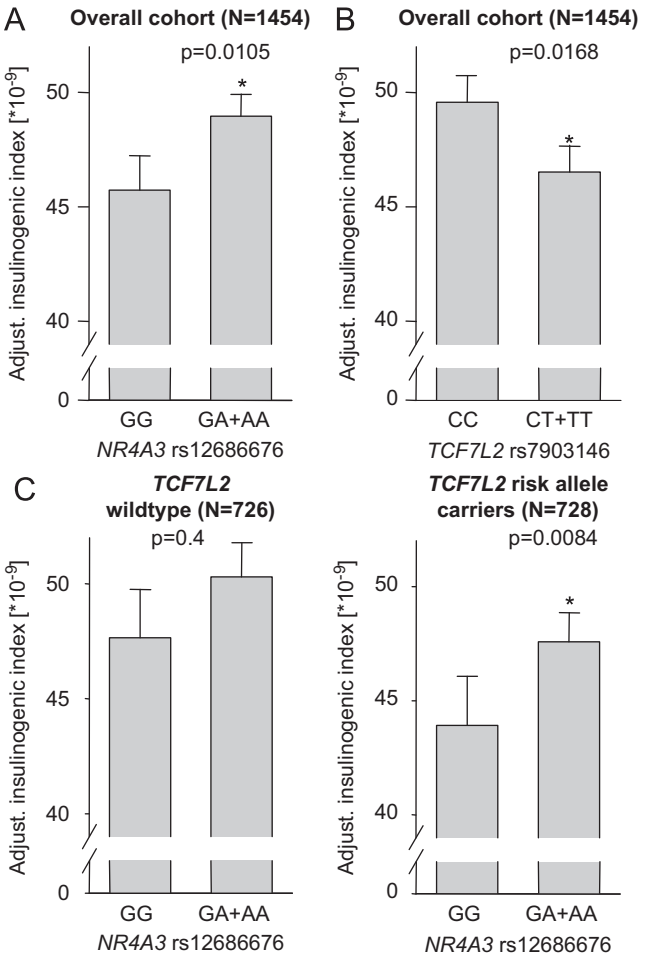

Figure 7: Gene–gene interaction between *TCF7L2* and *NR4A3*. The effects of *NR4A3* SNP rs12686676 and *TCF7L2* SNP rs7903146 in our overall cohort are shown in (A) and (B), respectively. Thereafter, we stratified our cohort according to *TCF7L2* SNP rs7903146 in order to assess the effect of *NR4A3* SNP rs12686676 in both subgroups separately (C). Both SNPs, *TCF7L2* SNP rs7903146 and *NR4A3* SNP rs12686676, are presented in the dominant inheritance model. We adjusted for possible confounders (gender, age, and BMI). Data are given as mean ± SEM.

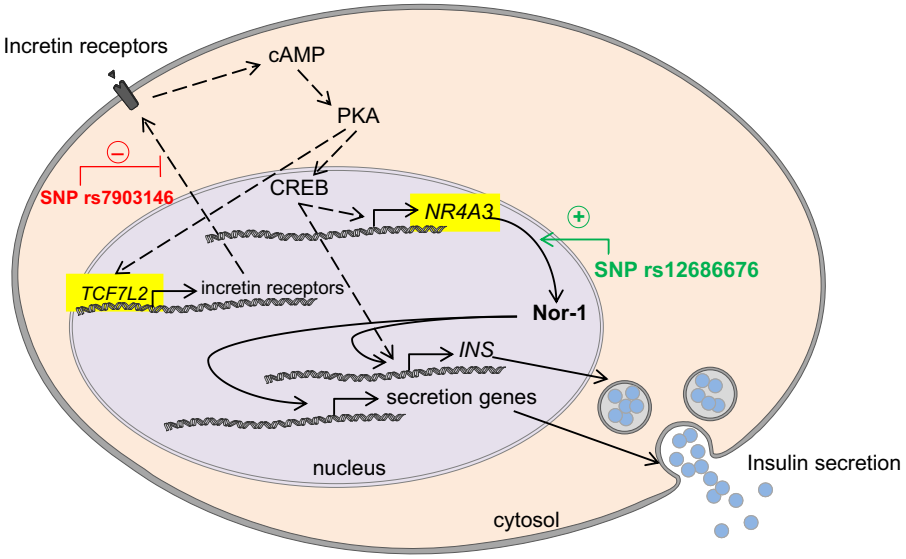

Figure 8: Role of Nor-1 in incretin-induced insulin expression and secretion and hypothetical localization of the *TCF7L2* and *NR4A3* SNP effects. The well-known *TCF7L2* SNP rs7903146 leads to impaired incretin response, inhibited PKA pathway, and reduced insulin secretion (dashed lines). Based on our molecular data, we propose a gain-of-function mechanism in *NR4A3* SNP rs12686676 carriers independent of incretin action. Increased Nor-1 expression enhances insulin secretion in these subjects probably by induction of genes encoding for exocytosis proteins and maybe by increasing intracellular insulin content thereby bypassing the defect caused by the *TCF7L2* SNP (solid lines).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

APPENDIX A. SUPPORTING INFORMATION

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.molmet.2013.06.003.

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