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The orphan nuclear receptor Nor1/Nr4a3 is a negative regulator of β-cell mass

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The Nr4a subfamily of nuclear receptor comprises three members in mammalian cells: Nur77/Nr4a1, Nurr1/Nr4a2, and Nor1/Nr4a3. Nr4a proteins play key roles in the regulation of glucose homeostasis in peripheral metabolic tissues. However, their biological functions in β-cells remain relatively uncharacterized. Here we sought to investigate the potential role of Nor1 in the regulation of β-cell mass and, in particular, β-cell survival/apoptosis. We used histological analysis to examine the consequences of genetic deletion of either *Nur77* and *Nor1* on β-cell mass, investigated the expression patterns of Nr4as in human islets and INS cells and performed gain- and loss-of-function experiments to further characterize the role of Nor1 in β-cell apoptosis. Surprisingly, *Nor1* knockout mice displayed increased β-cell mass, whereas mice with genetic deletion of *Nur77* did not exhibit any significant differences compared with their WT littermates. The increase in β-cell mass in *Nor1* knockout mice was accompanied by improved glucose tolerance. A gene expression study performed in both human islets and INS cells revealed that *Nor1* expression is significantly increased by pro-inflammatory cytokines and, to a lesser extent, by elevated concentrations of glucose. *Nor1* overexpression in both INS and human islet cells caused apoptosis, whereas siRNA-mediated *Nor1* knockdown prevented cytokine-induced β-cell death. Finally, *Nor1* expression was up-regulated in islets of individuals with type 2 diabetes. Altogether, our results uncover that Nor1 negatively regulates β-cell mass. Nor1 represents a promising molecular target in diabetes treatment to prevent β-cell destruction.

Type 2 diabetes is characterized by progressive deterioration of both β-cell mass and function, resulting in a relative deficit in insulin secretion (1, 2). Autopsy studies have indeed reported significant reductions in β-cell mass in patients with type 2 diabetes (3, 4), an effect that has been largely attributed to an increase in β-cell apoptosis (3, 5). Several environmental factors might contribute to β-cell death in diabetes (5), including elevated circulating levels of long-chain fatty acids, chronic hyperglycemia, and production of pro-inflammatory cytokines (6). Despite recent progress in this field, the instructive signals and the precise intracellular pathways that orchestrate β-cell death remain elusive.

The Nr4a family of orphan nuclear receptors comprises three members in mammalian cells: Nur77/Nr4a1, Nurr1/Nr4a2, and Nor1/Nr4a3 (7). To date, no ligand has been identified for Nr4as (8). Thus, their activity has been suggested to be regulated at the level of expression (consistent with their designation as immediate-early genes) as well as through post-translational modifications and proteasome-dependent degradation (9–15). Nr4as have been implicated in numerous pathologies such as Parkinson disease, cancer, and inflammatory diseases (16, 17). Importantly, the potential involvement of Nr4as in metabolic diseases has received increasing attention (reviewed in Ref. 16). This stems from several key observations: Nr4as expression varies in animal models of obesity and/or diabetes (18–20), their expression is increased in adipose tissue of obese patients compared with lean subjects (21), Nr4a expression is modulated by physical activity and nutritional interventions (16, 22–24), and Nr4as have been shown to exert tissue-specific functions, resulting in the regulation of glucose homeostasis (18, 20, 25–32).

The biological roles of Nr4as in pancreatic β-cells remain relatively unexplored but have recently attracted interest. A previous publication has suggested that Nr4as expression in the pancreas is restricted to endocrine cells (26). Further, Nur77 was induced by cytotoxic concentrations of free fatty acids to mediate some of the deleterious effects of lipotoxicity in MIN6 cells (26). Importantly, Nur77 and Nor1 were found to regulate different transcriptional targets in MIN6 cells, suggesting nonredundant functions for the two Nr4a members (26). More recently, the homeodomain transcription factor Nkx6.1 has been characterized as a transcriptional regulator of *Nur77* and *Nor1* (33). The authors went on to show that the proliferative action of Nkx6.1 is blunted in *Nur77* knockout animals.

Here we sought to investigate the potential role of Nor1 in pancreatic β-cell mass. We thus examined the cross-sectional β-cell area in animals with genetic deletion of either *Nur77* or

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This article contains Fig. S1.
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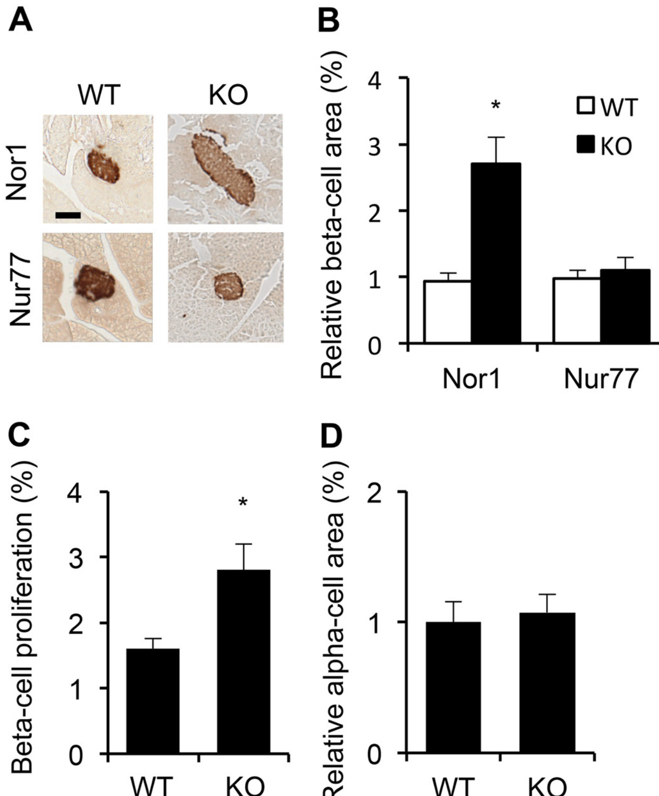

Figure 1. Nor1 knockout mice display increased β-cell mass. *A*, images show representative insulin immunostaining in WT, *Nor1*$^{-/-}$ and *Nur77*$^{-/-}$ pancreata. *B*, quantification of cross-sectional β-cell areas. Results are represented as -fold-change over WT animals. $n = 6$ for each genotype. *C*, β-cell proliferation was evaluated by counting the number of Ki67-positive nuclei. $n = 4$ for each genotype. *D*, quantification of cross-sectional α-cell area in WT and *Nor1* knockout mice. $n = 4$ for each genotype. All results are represented as means ± S.E.; *, $p < 0.05$ *versus* WT animals. *Scale bar* = 100 μm.

Nor1. Surprisingly, β-cell mass was significantly increased in *Nor1* knockout mice. This prompted us to further characterize the function of Nor1 in β-cells by studying its regulation and potential implication in β-cell death.

Results

Nor1 knockout mice display increased β-cell mass and improved glucose homeostasis

To investigate the potential roles of Nr4a members in the regulation of β-cell mass, we measured the cross-sectional β-cell areas in pancreata harvested from whole-body *Nor1* or *Nur77* knockout mice compared with WT littermates. Because homozygous deletion of *Nurr1* is embryonic lethal, we could not directly compare the consequences of loss of *Nurr1*. Fig. 1*A* shows typical immunohistochemistry images for each phenotype. Our morphological study indicated that *Nor1* knockout mice have increased β-cell mass compared with their WT littermates (Fig. 1*B*). Indeed, the β-cell area was ~2.5-fold greater in *Nor1* knockout mice *versus* control animals. This phenotype was unique to Nor1 because *Nur77* knockout islets did not present any significant difference compared with their respective controls. In addition, *Nor1* knockout animals consistently displayed a 50% increase in the number of replicating (Ki67-positive) β-cells compared with control animals (Fig. 1*C*). α-Cell mass remained unchanged in *Nor1* knockout mice (Fig. 1*D*).

We next investigated the tissue distribution of Nor1 in the pancreas by immunofluorescence in mice (Fig. 2). Our results indicate that, in WT pancreata, Nor1 is most strongly expressed in islets and co-localizes with both insulin (Fig. 2*A*) and glucagon (Fig. 2*B*). The signal for Nor1 was absent in tissue sections from *Nor1* knockout mice (Fig. 2*D*), confirming the specificity of the detected signal.

We next sought to test whether the increase in β-cell mass in *Nor1* knockout animals translated into improved glucose tolerance. We thus measured nonfasting blood glucose and also performed an i.p. glucose tolerance test in *Nor1* knockout mice *versus* controls. Nonfasting blood glucose was significantly lower in *Nor1* knockout mice with an average of 4.7 mM compared with 5.5 mM in WT mice (Fig. 3*A*). Also, mice with genetic deletion of *Nor1* displayed improved glucose tolerance, an effect that was not observed in *Nur77* knockout mice (Fig. 3, *B* and *C*, respectively). Indeed, blood glucose was significantly lower 15, 30, and 45 min following glucose injection in *Nor1* knockout *versus* WT mice. After 120 min, the area under the curve was significantly reduced in *Nor1*-knockout mice *versus* controls (Fig. 3*D*), whereas it was unchanged in Nur77 knockout animals compared with their respective controls. The effect of Nor1 on islet mass and glucose homeostasis could not be accounted for by differences in body weight (Fig. 3*E*) because this parameter was similar in *Nor1* knockout *versus* control mice. These results are consistent with a hitherto uncharacterized role for Nor1 in the regulation of β-cell mass.

Nor1 expression is up-regulated by pro-inflammatory cytokines

Although Nor1 has been extensively studied in muscle (34), its regulation and biological functions remain relatively unexplored in β-cells. We therefore studied the expression profile of Nor1 and explored the mechanisms by which the nuclear receptor could exert its effect on β-cell mass.

To investigate the regulation of *Nr4a* gene expression, we exposed INS cells to either a cytokine mixture comprising IL-1β and IFNγ or elevated concentrations of glucose (glucotoxicity) for various periods of time ranging from 0 to 24 h and measured the expression of all three members of the *Nr4a* family by qPCR.[3] Cytotoxic concentrations of cytokines induced a rapid and transient increase in the expression of *Nor1*, with a maximum induction of ~14-fold at 4 h (Fig. 4*A*). Interestingly, this effect was relatively specific to *Nor1*. Indeed, *Nur77* was increased by a much lesser extent compared with *Nor1* (~2-fold at 1 h), whereas *Nurr1* expression remained unaltered. Fig. S1 expresses the results differently to illustrate the relative expression of each Nr4a member before (Fig. S1*A*) and after cytokine treatment (Fig. S1*B*). High glucose induced an ~5-fold increase in *Nor1* expression after 8 h (Fig. 4*B*). Again, this effect of glucose was specific to *Nor1* because *Nur77* and *Nurr1* mRNA levels were not significantly altered. We confirmed the up-regulation of Nor1 by cytokines at the protein level. Expo-

[3] The abbreviations used is: qPCR, quantitative PCR.

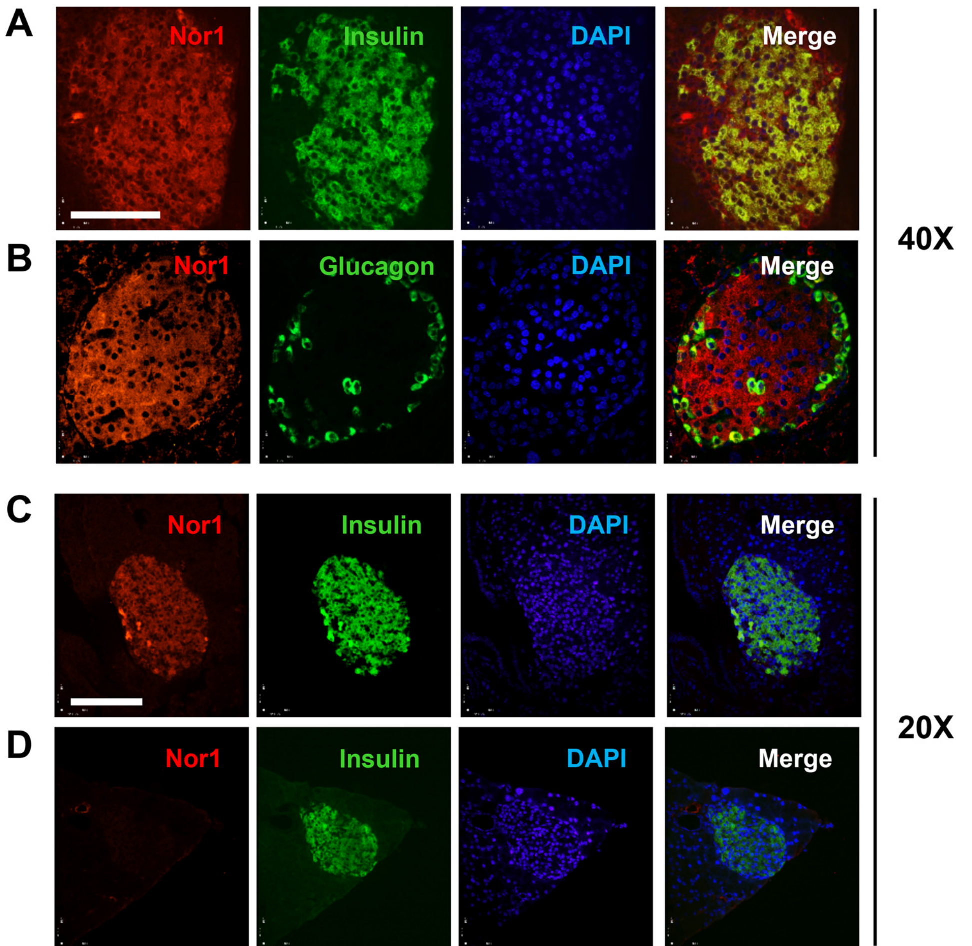

Figure 2. Nor1 expression in pancreatic islets. *A*, double immunofluorescence staining for Nor1 (*red*) and insulin (*green*) in WT mice. *B*, double immunofluorescence staining for Nor1 (*red*) and glucagon (*green*) in WT mice. *C* and *D*, double immunofluorescence staining for Nor1 and insulin in WT (*C*) and *Nor1*$^{-/-}$ animals (*D*). *Scale bars* = 100 μm. *DAPI*, 4′,6-diamidino-2-phenylindole.

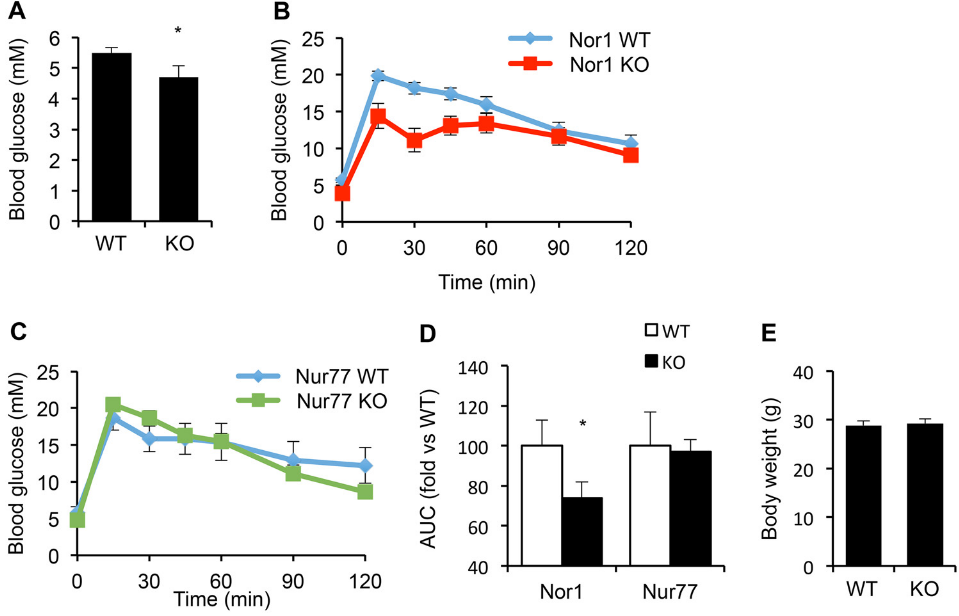

Figure 3. Invalidation of *Nor1* improves glucose tolerance. *A*, random-fed blood glucose in WT *versus Nor1* knockout mice. *B* and *C*, glucose tolerance test in *Nor1* (*B*) and Nur77 knockout mice. *C*, area under the curve for each genotype. *D*, body weight of 6-month-old WT and *Nor1* knockout mice. *E*, body weight of WT and *Nor1* knockout mice. All results are represented as means ± S.E.; $n = 10$ mice in each group. *, $p < 0.05$ *versus* WT animals; *AUC*, area under the curve.

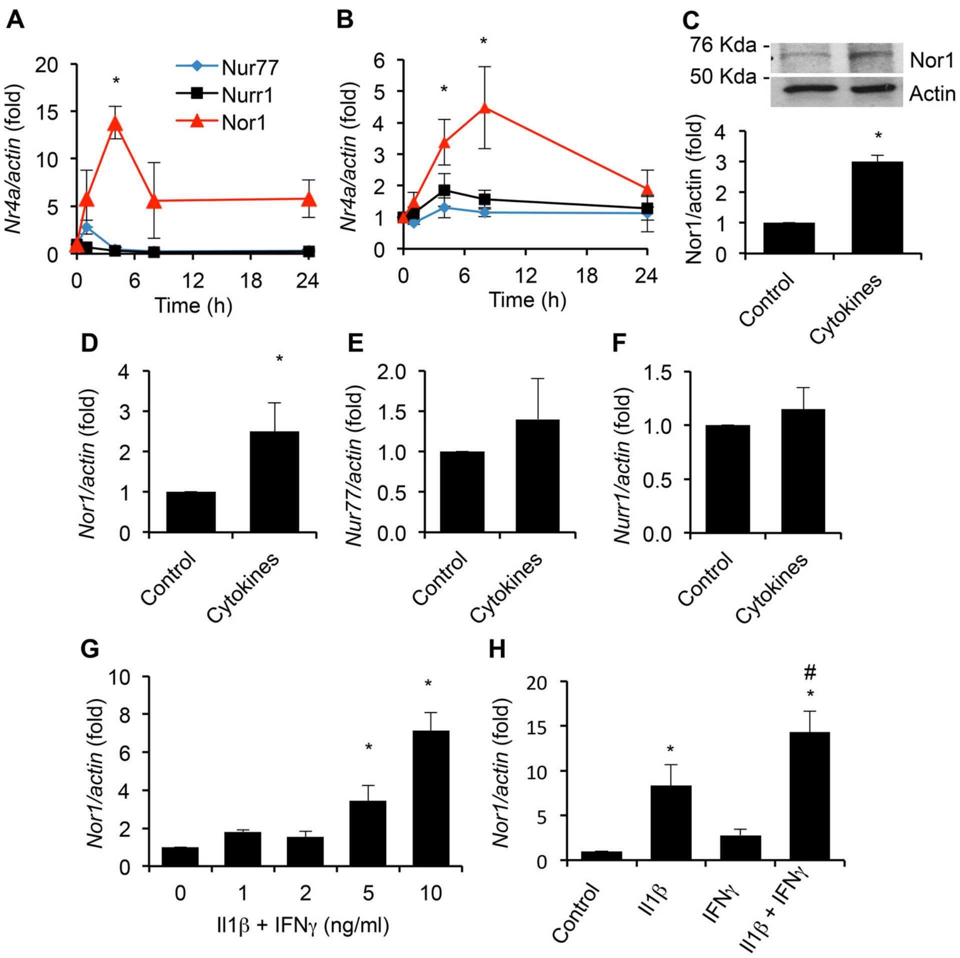

Figure 4. Nor1 is up-regulated by pro-inflammatory cytokines in INS and human islet cells. *A* and *B*, mRNA levels of *Nor1*, *Nur77*, and *Nurr1* were determined by qPCR in INS832/13 cells exposed to cytokines (10 ng/ml IL-1β and 10 ng/ml IFNγ, *A*) or an elevated concentration of glucose (25 mM, G25, *B*) for the indicated periods of time ($n = 5$). Results were normalized to actin and are expressed as -fold change over nonstimulated cells. *C*, Nor1 protein levels were determined in INS832/13 cells incubated for 6 h in the presence or absence of cytokines ($n = 4$). A representative image is shown. *D–F*, the effect of cytokines on *Nor1*, *Nur77*, and *Nurr1* expression was determined by qPCR in human islets after 8 h of treatment ($n = 3$). *G*, dose–response relationship between *Nor1* gene expression and cytokine concentrations. INS832/13 cells were exposed to increasing concentrations of IL-1β and IFNγ for 4 h ($n = 5$). *H*, the effects of 10 ng/ml IL-1β and IFNγ were tested separately or in combination for 4 h ($n = 5$). All results are represented as means ± S.E.; *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* IL-1β.

sure of INS cells to cytokines for 6 h caused a 3-fold increase in Nor1 protein levels (Fig. 4*C*).

We next sought to confirm the effects of cytokines on *Nor1* mRNA expression in human islets. Consistent with our previous results obtained in INS cells, cytokines up-regulated *Nor1* expression in human islets (Fig. 4*D*), albeit to a lesser extent. On the contrary, both *Nur77* and *Nurr1* mRNA levels remained unchanged (Fig. 4, *E* and *F*).

To further characterize the effects of cytokines on *Nor1* expression, we tested increasing concentrations of IL-1β and IFNγ and evaluated the respective contribution of each cytokine singly. *Nor1* expression was increased in a dose-dependent manner in response to the cytokine mixture in INS cells (Fig. 4*G*). Also, when both cytokines were used separately, the effect of IL-1β on Nor1 expression was greater than that of IFNγ alone (~6-fold *versus* ~1.5-fold, respectively) (Fig. 4*H*).

Altogether, these results identify *Nor1* as a cytokine-responsive gene in β-cells and thus raise the possibility that Nor1 could exert pro-apoptotic effects in β-cells. Indeed, such a pro-apoptotic action of Nor1 would dovetail with the increased β-cell mass observed in Nor1$^{-/-}$ animals.

Nor1 is a novel mediator of β-cell death

To investigate the potential pro-apoptotic role of Nor1 in β-cells, we sought to perform gain-of-function and loss-of-function studies using Nor1 overexpression and siRNA-mediated knockdown, respectively. Fig. 5*A* shows that Nor1 protein levels were increased by 3- to 4-fold in INS cells overexpressing Nor1-GFP or Nor1-FLAG compared with control cells. We then transfected INS cells with either Nor1-FLAG or a control vector before incubating them in the presence or absence of cytokines. Nor1 overexpression induced a 2-fold increase in apoptosis (Fig. 5, *B* and *C*), and this effect was not additive to that of cytokines. The pro-apoptotic action of Nor1 was confirmed in human islets. Human islet cells were transfected with either Nor1-GFP or a control GFP plasmid. Then we measured apoptosis by counting the number of TUNEL-positive (red) nuclei in the transfected cell population only (green). Ectopic expression of Nor1 increased apoptosis by 5-fold (Fig. 5, *D* and *E*).

To further test the hypothesis that Nor1 represents a novel mediator of cytokine-induced β-cell apoptosis, we performed

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Figure 5. Nor1 plays a critical role in INS and islet cell apoptosis. *A*, Nor1 protein levels were evaluated in nontransfected (*NT*) INS832/13 cells or 24 h after transfection with a control GFP plasmid, Nor1-GFP, or Nor1-FLAG ($n = 5$). A representative immunoblots is shown. Quantification results are represented as means ± S.E.; *, $p < 0.05$ *versus* control GFP. The *arrows* indicate the predicted Nor1 bands, with Nor1-GFP migrating at a higher molecular weight. *B* and *C*, apoptosis was assessed by TUNEL assay in INS832/13 cells transfected with Nor1 (*black columns*) or a control vector (*white columns*) and subsequently incubated in the presence or absence of cytokines for 24 h ($n = 3$). *D* and *E*, apoptosis was measured by TUNEL assay in dispersed human islets 24 h after transfection with Nor1-GFP or GFP alone. The results are represented as the percentages of TUNEL-labeled nuclei in the population of GFP-positive cells ($n = 3$). *DAPI*, 4′,6-diamidino-2-phenylindole. *F*, *Nor1*, *Nur77*, and *Nurr1* expression was evaluated by qPCR in INS832/13 cells 48 h after transfection with a control siRNA or two different Nor1-specific siRNA sequences ($n = 3$). *G* and *H*, siRNA-mediated knockdown of Nor1 prevented cytokine-induced apoptosis. Apoptosis was determined by TUNEL in INS832/13 cells transfected with control or Nor1-specific siRNAs and incubated in the presence (*black columns*) or absence (*white columns*) of cytokines as described above ($n = 3$). *I*, the effects of cytokines on islet cell apoptosis was also investigated in intact WT and Nor1-KO mouse islets *ex vivo* by measuring the fragmentation of DNA using an ELISA assay ($n = 5$). *J* and *K*, siRNA-mediated knockdown of Nor1 prevented cytokine-induced apoptosis in human islet cells. *L*, Nor1 caused cytochrome *c* release. Cells were treated as described in *B*, fractionated into cytoplasmic and mitochondrial fractions, and analyzed by ELISA ($n = 4$). All results are represented as means ± S.E.; *, $p < 0.05$ *versus* control; # $p < 0.05$ *versus* PCMV-Nor1-FLAG without cytokines. *Scale bars* = 50 μm.

siRNA-mediated *Nor1* knockdown (Fig. 5*F*). Cytokine-induced β-cell apoptosis was completely abolished by two different siRNA sequences (Fig. 5, *G* and *H*), indicating that Nor1 is necessary for the pro-apoptotic action of cytokines in β-cells. We next sought to confirm this result in isolated mouse islets from WT and Nor1 KO mice. Thus, intact islets were cultured *ex vivo* in the presence or absence of cytokines, and DNA fragmentation was measured by ELISA (Fig. 5*I*). The results show that Nor1 KO islets were resistant to cytokine-mediated apoptosis. siRNA-mediated knockdown of Nor1 in human islets also prevented cytokine-induced apoptosis like it did in INS cells (Fig. 5, *J* and *K*).

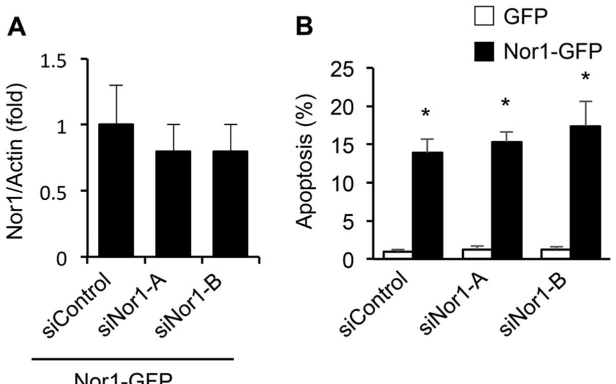

Figure 6. Nor1 re-expression following siRNA-mediated knockdown causes apoptosis in INS cells. *A* and *B*, cells were transfected with Nor1 siRNAs or siControl, followed by overexpression using Nor1-GFP. GFP was used as a control. Results shown are means ± S.E.; *, $p < 0.05$ *versus* GFP controls.

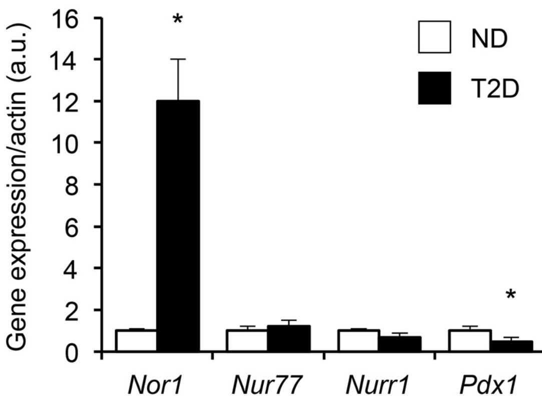

Figure 7. Nor1 is up-regulated in type 2 diabetic islets. We compared *Nor1*, *Nur77*, *Nurr1*, and *Pdx1* mRNA expression in isolated human islets of healthy non-diabetic (*ND*) or type 2 diabetic (*T2D*) donors ($n = 7$). Islets were procured the morning after their isolation and handpicked, and mRNAs were isolated immediately. We only used islet preparations of regular morphology (circularity and size). Results shown are means ± S.E.; *, $p < 0.05$.

To gain insight into the molecular mechanisms by which Nor1 causes β-cell apoptosis, we tested the effects of Nor1 on cytochrome *c* release in β-cells. Indeed, it was suggested previously that Nor1 could translocate to the mitochondria and activate the intrinsic (mitochondrial) pathway of apoptosis in thymocytes (35). Fig. 5*L* shows that ectopic expression of Nor1 induced cytochrome *c* release from the mitochondria to the same extent as cytokines. This result suggests that Nor1-mediated β-cell apoptosis involves the intrinsic pathway as well. Future studies should delve deeper into the mechanisms by which Nor1 promotes β-cell death.

We next sought to perform a "gene rescue" experiment in which we performed siRNA-mediated gene knockdown as described above, followed by Nor1 overexpression to re-establish Nor1 expression levels (Fig. 6*A*). It could be observed that the two siRNA sequences failed to prevent apoptosis when Nor1 was re-expressed to apoptotic levels (Fig. 6*B*). This experiment limits the possibility that our results were due to off-target effects.

Nor1 expression is increased in type 2 diabetes

Because IL-1β is up-regulated in diabetic islets (36), we reasoned that Nor1 could be increased in islets of patients with type 2 diabetes. Fig. 7 shows that *Nor1* mRNA levels are increased by ~12-fold in human islets obtained from patients with type 2 diabetes compared with healthy individuals. This change was specific to *Nor1* because the two other Nr4a members, *Nur77* and *Nurr1*, were not significantly altered in type 2 diabetic islets. As a control, we measured the expression of *Pdx1*, which was significantly decreased by 50% in diabetic islets. We thus postulate that the up-regulation of *Nor1* in type 2 diabetic islets could contribute to β-cell demise and may represent a target for therapeutic interventions.

Discussion

The biological roles of Nr4as in metabolic diseases are gathering increasing interest (16, 37). Although their roles in liver and muscle have been studied extensively, their biological actions in pancreatic β-cells remain relatively unexplored.

Here we demonstrate that adult *Nor1* knockout mice show increased β-cell mass and that this phenotype is not reiterated by genetic deletion of *Nur77*. The increase in β-cell mass in *Nor1* knockout animals was accompanied by lower blood glucose in the random fed state and improved glucose tolerance during ipGTT. We believe that the effect of Nor1 on β-cell mass involves, at least in part, an increase in pancreatic β-cell replication because we detected an increase in Ki67 staining in *Nor1* knockout mice. This distinct phenotype of *Nor1* knockout mice is consistent with the unique DNA-binding properties of Nor1, which support distinct biological functions for Nor1 compared with its two Nr4a orthologs. Indeed, Nur77 and Nurr1 can bind the Nur-responsive element as homodimers (38, 39), whereas Nor1 displays a much lower affinity for the Nur-responsive element, resulting in a negligible transcriptional activation (38, 39). In addition, both Nur77 and Nurr1, but not Nor1, can form heterodimers or interact with retinoid X receptors to target DR5 elements (8, 40). Thus, Nor1 displays distinct binding characteristics to DNA regulatory elements and cofactors. This dovetails with a previous report demonstrating that Nur77 and Nor1 regulate distinct sets of genes in β-cells (26).

Because our study employed whole-body *Nor1* knockout animals, we cannot rule out the possibility that some of the effects of Nor1 on pancreatic β-cell mass are secondary to changes in other tissues. We believe this is unlikely because our results were reiterated *in vitro* using gain- and loss-of-function experiments in both INS cells and human islet tissues. Importantly, *Floxed-Nor1* mice have not yet been generated. This precludes tissue-specific deletion of Nor1 in β-cells, and whole-body *Nor1*-KO mice represent the best available model.

Our expression study shows that Nor1 expression is rapidly increased by cytokines. Indeed, cytokine treatments elicited an ~14-fold and ~3-fold increase in Nor1 expression in INS cells and human islets, respectively. High glucose also induced Nor1 expression, but its effect was less pronounced and occurred later. Interestingly, high glucose was found to stimulate production of IL-1β in β-cells (36). Because here we demonstrate that IL-1β is a key inducer of *Nor1* expression in β-cells, this raises the possibility that glucose induces *Nor1* expression via production of pro-inflammatory cytokines. Be that as it may, the up-regulation of *Nor1* upon cytokine treatments hinted at a

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pro-apoptotic role for Nor1 in β-cells, an effect that, over time, could in part explain the increase in β-cell mass observed in *Nor1* knockout mice.

Our functional assays using gain and loss of function for Nor1 in INS cells and human islets demonstrated a critical role for Nor1 in β-cell apoptosis. This holds great clinical significance because Butler *et al.* (3) showed that type 2 diabetic patients present a lower β-cell mass compared with healthy subjects and that this reduction was associated with increased rates of apoptosis. Because we detected an increase in *Nor1* expression in type 2 diabetic islets, our study strongly suggests that Nor1 could participate in the β-cell demise that precedes diabetes onset. To unravel a potential mechanism by which Nor1 could promote β-cell apoptosis, we tested its potential effect on the intrinsic pathway of apoptosis. Nor1 was found to provoke cytochrome *c* release in INS cells, suggesting that Nor1-mediated β-cells apoptosis could involve mitochondrial stress. This is reminiscent of its role in thymocytes, where Nor1 has been demonstrated to relocate to the mitochondria and to associate with Bcl family members to cause apoptosis (35). Our findings, describing Nor1 as a negative regulator of β-cell mass expansion, conflict with those presented in a recent study (33). Indeed, Tessem *et al.* (33) characterized Nur77 and Nor1 as transcriptional targets of Nkx6.1 in β-cells. They also showed that both Nur77 and Nor1 were necessary for Nkx6.1-mediated β-cell proliferation in rat islets via a mechanism that could implicate transcriptional repression of *p21*. The causes of this discrepancy are unknown. However, although the authors confirmed their results in *Nur77* knockout mice, they did not study *Nor1* knockout animals.

SNPs located within the *Nor1* locus have been associated with increased insulin secretion and a higher insulinogenic index in diabetic as well as nondiabetic patients (29, 31). At this point, it is unclear whether the improvement in insulin secretion in individuals bearing this mutation results from improved islet function, increased β-cell mass, or both. Further, some controversies reside over the role of Nor1 in insulin secretion. In one study, Nor1 has been suggested to mediate Exendin4-induced potentiation of glucose-induced insulin secretion (29). Conversely, another study characterized Nor1 as a negative regulator of insulin secretion (41).

In conclusion, our work shows that expression of the nuclear receptor Nor1 is up-regulated in type 2 diabetes islets and that Nor1 plays a key role in β-cell apoptosis. We propose that Nor1 represents an interesting molecular target for therapeutic intervention.

Experimental procedures

Animals

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies, with approval from the Animal Care and Use Committee of Laval University and the University of Alberta. Efforts were made to minimize animal suffering and to reduce the number of animals used. *Nur77*$^{-/-}$ mice were graciously provided by Dr. Jeff Milbrandt (University of Washington) (42). *Nor1*$^{-/-}$ mice were from Dr. Yves Labele (St-François d'Assise Hospital, Quebec City, QC, Canada). *Nur77*$^{-/-}$ mice were maintained on a C57/B6N background. *Nor1*$^{-/-}$ mice were maintained on a 129S4/SV background. 6-month-old male mice were used in this study. The mice were housed in a temperature-controlled environment on a 12-h light/dark cycle with *ad libitum* access to food and water. Access to food was removed 12 h prior to glucose challenge. Blood glucose was measured with FreeStyle Lite test strips (Abbott, Alameda, CA). Plasma insulin was measured using a commercial ELISA kit (Millipore, Etobicoke, ON, Canada).

Immunostaining

Pancreata were fixed and embedded in paraffin. 5- to 10-μm sections were deparaffinized and rehydrated before microwave antigen retrieval in citrate buffer. Slides were then blocked in 0.5% BSA and 4% donkey serum in 0.1% PBST (0.1% (v/v) Triton in PBS) for 1 h at room temperature, followed by overnight incubation at 4 °C with mouse anti-Nor1 (Abnova, 1:100, RRID AB_922071), guinea pig anti-insulin (Dako, Burlington, ON, Canada; 1:800, RRID AB_10013624), rabbit anti-Ki67 (Millipore, 1:50, RRID AB_2142366), or rabbit anti-glucagon (Dako, 1:800, RRID AB_1841774) antibodies. The next day, slides were incubated for 1 h at room temperature with anti-mouse Alexa Fluor 568, anti-guinea pig Alexa Fluor 488, anti-rabbit Alexa Fluor 568, or anti-rabbit Alexa Fluor 488 secondary antibodies (1:250, Life Technologies). Slides were mounted with Fluoromount-G® containing 4′,6-diamidino-2-phenylindole (Southern Biotech, Birmingham, AL). Images were analyzed using a spinning disk ERS confocal microscope (PerkinElmer Life Sciences, Guelph, ON, Canada) and Volocity imaging software.

Cell culture

INS832/13 cells (43) (passages 52–78) were grown in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37 °C in a humidified 5% CO_2 atmosphere. Cells at 80% confluence were treated in serum-free RPMI with a mixture of cytokines (IL-1β and IFNγ, both at 10 ng/ml) or with 25 mM glucose. All cell culture reagents were purchased from Life Technologies.

Human islets

Human islets were purchased from the Alberta Diabetes Institute Islet Core at the University of Alberta with the assistance of the Human Organ Procurement and Exchange Program and the Trillium Gift of Life Network, who provide donor pancreata for research. Islets are obtained the next morning after their isolation. For mRNA expression in response to cytokines and apoptosis assays, islets from seven different healthy lean donors between 48 and 74 years of age were used. We used islet preparations of similar morphology (including size and circularity) and purity of more than 95%. Islets were cultured in 11 mM glucose DMEM supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin, and 50 μM β-mercaptoethanol at 37 °C in a humidified 5% CO_2 atmosphere. Our study was approved by the Human Research Ethics Board at the University of Alberta. The study abided by the Declaration of Helsinki principles.

qPCR

RNAs were extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse-transcribed using the SuperScript First-Strand Synthesis System (Life Technologies). For real-time PCR, we used RT^2 SYBR Green ROX qPCR Mastermix (Qiagen) and an ABI 7900HT instrument (Life Technologies). Results were analyzed using SDS software 2.3. Actin was chosen as gene of reference. Results are expressed as -fold change ($2^{(-\Delta\Delta Ct)}$) compared with the indicated control condition.

Western blotting

Cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, and protease inhibitors (cOmpleteMini, Roche Applied Sciences). Protein concentrations were determined by BCA protein assay. Equal amounts of heat-denaturated proteins from each treatment group were run on Novex gels (Life Technologies) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight with a mouse anti-Nor1 antibody (1:1000). The next day, membranes were incubated with horseradish peroxidase–linked secondary antibodies and exposed to ECL reagents. Band density was determined using the ChemiDoc MP Imaging System and Image Lab software 4.1 (Bio-Rad, Mississauga, ON, Canada). Membranes were stripped and incubated with an anti-Actin antibody (Santa Cruz Biotechnology, Dallas, TX; RRID AB_626630) to confirm equal loading. Blots were developed on film, and band density was determined using ImageJ software.

Plasmid transfection

pCMV-Nor1-GFP and pCMV-Nor1-FLAG were obtained from Origene (Rockville, MD). pCMV-mCherry was a kind gift from Dr. Peter Light (University of Alberta, Edmonton, AB, Canada). INS832/13 cells were transfected via nucleofection (Lonza, Allendale, NJ). Human islet cells were dispersed using 0.05% trypsin–EDTA and transfected the following day using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Cells were assayed 24 h post-transfection.

siRNA-mediated knockdown

Nor1-specific siRNAs were from Life Technologies. The sequences used were RSS329251 (hereafter called siNor1-A) and RSS367608 (siNor1-B). siNor1 and control siRNAs were transfected using Lipofectamine RNAiMax following the manufacturer's protocol.

Apoptosis

Apoptosis was assessed by TUNEL assay using either TMR Red (human islets) or fluorescein green (INS cells) *in situ* cell death detection kits (Roche Applied Science), following manufacturer's protocol. Images were captured at ×40 magnification using a Zeiss COLIBRI inverted fluorescence microscope. At least 2000 cells were analyzed for each experimental condition in at least three independent experiments. In human islet studies, the results are presented as the percentage of apoptotic cells (red nuclei) in the transfected population only (green, GFP-positive cells). For the determination of apoptosis in WT *versus* Nor1-KO islets, 40 intact islets of similar size were incubated in the presence or absence of cytokines for 24 h in 12-well plates. Histone-associated DNA fragments were quantified using the Cell Death Detection ELISA Plus Kit (Roche, Laval, QC, Canada) according to the manufacturer's protocol. This kit allows specific determination of mono- and oligonucleosomes generated by apoptotic cleavage of DNA.

Cytochrome c release

Cells were fractionated into nuclear, cytosolic, and mitochondrial fractions using a cell fractionation kit (Abcam, Toronto, ON, Canada). Cytochrome *c* release from the mitochondria to the cytoplasm was subsequently evaluated using a commercial ELISA kit (Abcam) and an EnVision 2104 Multilabel reader (PerkinElmer Life Sciences).

Statistical analysis

Data are presented as means ± S.E. Statistical analyses were performed with GraphPad Prism® using analysis of variance methods followed by Bonferroni's post test. $p < 0.05$ was considered statistically significant.

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