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Abstract

The loss or dysfunction of the pancreatic endocrine β-cell results in diabetes. Recent innovative therapeutic approaches for diabetes aim to induce β-cell proliferation in vivo by pharmacological intervention. Based on the finding that overexpression of the transcription factor Nkx6.1 in islets in vitro increases β-cell proliferation while maintaining β-cell function, Nkx6.1 has been proposed as a potential target for diabetes therapy. However, it is unknown whether elevated Nkx6.1 levels in β-cells in vivo have similar effects as observed in isolated islets. To this end, we sought to investigate whether overexpression of Nkx6.1 in β-cells in vivo could increase β-cell mass and/or improve β-cell function in normal or β-cell-depleted mice. Using a bigenic inducible Cre-recombinase-based transgenic model, we analyzed the effects of Nkx6.1 overexpression on β-cell proliferation, β-cell mass, and glucose metabolism. We found that mice overexpressing Nkx6.1 in β-cells displayed similar β-cell proliferation rates and β-cell mass as control mice. Furthermore, after partial β-cell ablation, Nkx6.1 overexpression [...]

Reference

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Transgenic Overexpression of the Transcription Factor Nkx6.1 in β-Cells of Mice Does Not Increase β-Cell Proliferation, β-Cell Mass, or Improve Glucose Clearance

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The loss or dysfunction of the pancreatic endocrine β-cell results in diabetes. Recent innovative therapeutic approaches for diabetes aim to induce β-cell proliferation in vivo by pharmacological intervention. Based on the finding that overexpression of the transcription factor Nkx6.1 in islets in vitro increases β-cell proliferation while maintaining β-cell function, Nkx6.1 has been proposed as a potential target for diabetes therapy. However, it is unknown whether elevated Nkx6.1 levels in β-cells in vivo have similar effects as observed in isolated islets. To this end, we sought to investigate whether overexpression of Nkx6.1 in β-cells in vivo could increase β-cell mass and/or improve β-cell function in normal or β-cell-depleted mice. Using a bigenic inducible Cre-recombinase-based transgenic model, we analyzed the effects of Nkx6.1 overexpression on β-cell proliferation, β-cell mass, and glucose metabolism. We found that mice overexpressing Nkx6.1 in β-cells displayed similar β-cell proliferation rates and β-cell mass as control mice. Furthermore, after partial β-cell ablation, Nkx6.1 overexpression was not sufficient to induce β-cell regeneration under either nondiabetic or diabetic conditions. Together these results demonstrate that sustained Nkx6.1 overexpression in vivo does not stimulate β-cell proliferation, expand β-cell mass, or improve glucose metabolism in either normal or β-cell-depleted pancreata. Thus, raising cellular Nkx6.1 levels in β-cells in vivo is unlikely to have a positive impact on type 2 diabetes. (Molecular Endocrinology 25: 1904–1914, 2011)

One promising approach to treat diabetic patients with residual β-cell mass comprises the targeted expansion of remaining β-cells to reconstitute a functional β-cell mass. Evidence from several recent β-cell ablation studies has highlighted that increased proliferation of residual β-cells is the predominant mechanism through which β-cell mass is restored in response to partial β-cell ablation (1–7). Likewise, the adaptive expansion of β-cells has been well documented under conditions of metabolic stress, such as pregnancy or insulin resistance (8–15).

Analysis of human and rodent pancreatic tissue has revealed that β-cell mass is established and maintained by balancing β-cell proliferation and apoptosis (16–21). Specifically, β-cell proliferation is regulated by the cell cycle activators cyclin D2, D1, and CDK4. Overexpression of constitutively active Akt or activated CDK4 has been shown to increase proliferation, whereas loss of CDK4 decreases proliferation in vivo (22, 23). β-Cell replication is negatively regulated by the cell cycle inhibitors p21, p27, p16INK4a, and p19Arf (24–27). Moreover, p16INK4a has been shown to be an age-dependent inhibi-
tor of β-cell proliferation (28). The combined interactions of these and other factors provide tight regulation of the β-cell cycle.

Recent studies have implicated the transcription factor Nkx6.1 in the maintenance of β-cell mass by regulating β-cell proliferation (29). Using adenovirus-mediated overexpression of Nkx6.1 in isolated human and rat islets, Schisler et al. (29) demonstrated that Nkx6.1 increases β-cell proliferation in vitro, whereas knockdown of Nkx6.1 with a small interfering RNA has the opposite effect. Stimulation of β-cell proliferation upon Nkx6.1 overexpression was shown to be associated with increased expression of positive regulators of cell cycle progression, including several regulatory kinases as well as cyclins A2, E1, B1, and B2, of which cyclins A2 and B1 were shown to be directly regulated by Nkx6.1 (29). In addition to stimulating β-cell proliferation, gain- and loss-of-function studies in isolated islets and insulinoma cell lines have further revealed that Nkx6.1 improves glucose-stimulated insulin secretion (GSIS) (29, 30). Its rare property of simultaneously stimulating β-cell proliferation and β-cell function has made Nkx6.1 an attractive pharmacological target for restoring euglycemia in diabetic patients. However, it remains to be tested whether Nkx6.1 the overexpression in vivo evokes similar effects as those observed in vitro.

Using genetic approaches in mice, our laboratory has uncovered an essential developmental role for Nkx6.1 in the allocation of multipotent pancreatic progenitors to an endocrine fate (31–34). As one constituent of these studies, we have recently generated mice which conditionally express Nkx6.1 and green fluorescent protein (GFP) upon Cre-recombinase-mediated excision of an upstream LacZ cassette (34). In the present study, we used this model to examine the effects of Nkx6.1 overexpression on β-cell proliferation and glucose metabolism in vivo.

Results

Robust in vivo induction of Nkx6.1 overexpression in β-cells of adult mice

Based upon in vitro manipulation of Nkx6.1 expression in insulinoma cell lines and isolated rat and human islets, it has been suggested that Nkx6.1 is a key modulator of β-cell proliferation and function (29, 30). To investigate whether Nkx6.1 functions in a similar manner in β-cells in vivo, we assessed whether transgenic Nkx6.1 overexpression in mature β-cells increases β-cell mass or improves cell function. To overexpress Nkx6.1 in β-cells, conditional Nkx6.1 gain-of-function (Nkx6.1OE) and Pdx1-CreERTM mice were crossed to generate double-transgenic mice. In these mice, tamoxifen administration results in Cre-mediated recombination of the Nkx6.1OE transgene in β-cells and simultaneous induction of Nkx6.1 and GFP expression (Fig. 1A). nBecause endogenous Nkx6.1 in β-cells precludes immunohistochemical detection of Nkx6.1 expression from the transgene, GFP serves as a marker to assess recombination efficiency. Three-week-old Pdx1-CreERTM; Nkx6.1OE mice received six ip injections of tamoxifen over a 2-wk period and pancreatic Nkx6.1 expression was analyzed 1 wk after the final injection (Fig. 1A).

To assess whether tamoxifen administration induces efficient recombination of the Nkx6.1OE transgene, we performed immunohistochemistry for Nkx6.1 in conjunction with insulin and GFP. Although no GFP+ cells were detected in corn oil-injected Pdx1-CreERTM; Nkx6.1OE control mice after tamoxifen administration, on average 42.9 ± 5.5% of the insulin+ cells recombined the Nkx6.1OE transgene as revealed by GFP labeling within the insulin+ cell population (Fig. 1, B–D, and Supplemental Fig. 1). To demonstrate that Nkx6.1 is expressed from the Nkx6.1OE transgene, we tested whether delta and acinar cells, in which the Pdx1-CreERTM transgene is active (35–37), ectopically express Nkx6.1. Consistent with the reported expression domain of the Pdx1-CreERTM transgene, exocrine acinar cells and somatostatin+ delta cells occasionally expressed GFP and Nkx6.1 (Supplemental Fig. 2, A and B). To verify Nkx6.1 overexpression in β-cells, we also quantified Nkx6.1 expression in isolated islets. Quantitative RT-PCR analysis and Western Blotting revealed a 4.8-fold increase in Nkx6.1 transcript and 10.9-fold increase in Nkx6.1 protein levels, respectively, in double-transgenic, tamoxifen-treated mice compared with controls (Fig. 1, E and F). Given the recombination efficiency of 42.9% in β-cells, these results imply that the recombination of the Nkx6.1OE transgene results on average in a 20-fold increase of Nkx6.1 levels in targeted β-cells. However, the level of induction varied between animals and some mice displayed only a 3-fold increase in Nkx6.1 expression. To demonstrate that the level of Nkx6.1 overexpression has biological effects, we compared mRNA levels of the Nkx6.1 target gene MafA (38) in isolated islets from tamoxifen-treated Pdx1-CreERTM; Nkx6.1OE and control mice. We found that the MafA levels were 12.5-fold higher in islets from the Nkx6.1-overexpressing mice (Supplemental Fig. 2C), showing that the induction of the Nkx6.1OE transgene affects gene transcription in β-cells.
Overexpression of Nkx6.1 does not increase β-cell replication, β-cell mass, or improve glucose clearance in vivo

Recent in vitro studies demonstrated an 8-fold increase in 5-bromo-2-deoxyuridine (BrdU) incorporation in isolated islets upon adenovirus-mediated Nkx6.1 overexpression in rat islets (29). To address whether increased Nkx6.1 expression can stimulate β-cell proliferation in vivo, we compared β-cell proliferation rates based on Ki67 staining between tamoxifen-treated Pdx1-CreERTM; Nkx6.1OE and control mice (Fig. 2, A–D). This analysis revealed a similar β-cell proliferation index for control and tamoxifen-treated, double-transgenic mice (Fig. 2C). Likewise, GFP+ targeted β-cells and GFP− non-targeted β-cells in tamoxifen-treated Pdx1-CreERTM; Nkx6.1OE mice showed no difference in the frequency of Ki67 staining (Fig. 2D), suggesting that up-regulated Nkx6.1 expression does not increase β-cell proliferation.
Furthermore, Nkx6.1 overexpression in vivo did not result in a significant increase in mRNA levels of cell cycle regulators in islets (Fig. 2E).

The results of our in vivo study are thus in contrast to the recent finding that 80 h of Nkx6.1 overexpression in isolated islets in vitro is sufficient to substantially increase β-cell proliferation and to expand the β-cell population by 31% (29). To determine whether a transient short-term effect on β-cell proliferation by Nkx6.1 up-regulation might have remained unidentified 3 wk after initiating recombination of the Nkx6.1OE transgene, we quantified β-cell mass 1 wk after the administration of the last tamoxifen dose. Concordant with β-cell proliferation being unchanged in Pdx1-CreERTM; Nkx6.1OE mice, we observed no difference in β-cell mass between Nkx6.1-overexpressing and control siblings (Fig. 2F). These results were confirmed using two different Nkx6.1OE founders. Thus, up-regulation of Nkx6.1 expression in β-cells in mice does not stimulate β-cell proliferation or increase β-cell mass.

Although evidence from a number of in vitro studies suggests that Nkx6.1 overexpression in β-cells enhances GSIS (29, 30), the function of Nkx6.1 in β-cells in vivo remains unexamined. Therefore, to determine whether Nkx6.1 overexpression in vivo affects glucose homeostasis, we performed ip glucose tolerance tests (IPGTT) and insulin secretion assays on cohorts of control and tamoxifen-treated mice. We performed ip glucose tolerance tests (IPGTT) and insulin secretion assays on cohorts of control and tamoxifen-treated Nkx6.1OE mice, we observed no difference in β-cell mass between Nkx6.1-overexpressing and control siblings (Fig. 2F). These results were confirmed using two different Nkx6.1OE founders. Thus, up-regulation of Nkx6.1 expression in β-cells in mice does not stimulate β-cell proliferation or increase β-cell mass.

To determine whether in vivo counterregulatory mechanisms mask an effect of Nkx6.1 overexpression on GSIS, we isolated islets 7 d after tamoxifen-mediated Nkx6.1 induction in vivo and performed in vitro GSIS assays (Fig. 3D). We observed no significant difference in insulin secretion between islets from Nkx6.1-overexpressing and control mice at 2.75, 11, or 16.75 mM glucose concentrations, suggesting that Nkx6.1 overexpression does not increase GSIS. However, insulin mRNA and protein levels were elevated in islets from Nkx6.1-overexpressing mice, both after overnight and after 7 d of culture (Fig. 3, E and F). Together these findings suggest that Nkx6.1 overexpression stimulates insulin expression without affecting GSIS.

**Nkx6.1 overexpression does not enhance β-cell regeneration or rescue glucose intolerance after β-cell ablation**

Having found that Nkx6.1 overexpression in β-cells of the healthy pancreas does not affect β-cell mass, we next sought to examine whether Nkx6.1 up-regulation could stimulate β-cell regeneration in an injured state, when β-cell mass is reduced beyond a threshold to fully meet the metabolic demand. We first exploited a recently generated genetic diphtheria toxin (DT)-inducible β-cell ablation model, in which injection of DT results in cell-autonomous, efficient apoptosis of β-cells in a highly reproducible manner shortly after DT administration (39). In this model, the rat insulin promoter (RIP) drives expression of the human DT receptor (DTR). Because the RIP-DTR transgene integrated on the X-chromosome, DT injection into hemizygous females allows for ablation of approximately 50% of β-cells, whereas male mice injected with DT exhibit almost complete loss of β-cells (39). We generated an experimental cohort of female mice carrying Pdx1-CreERTM, Nkx6.1OE, and RIP-DTR transgenes as well as control RIP-DTR cohorts (Fig. 4A). Mice of the control RIP-DTR cohort subjected to DT (RIP-DTRDT) were

![FIG. 3. β-Cell-specific Nkx6.1 overexpression in adult mice does not alter GSIS in vivo or in vitro. A and B, Glucose clearance, as measured by IPGTT, is similar in control and TM-injected double-transgenic mice at both 2 (A) and 8 (B) months of age (n = 8). In vivo (n = 5; 8 wk old mice) (C) or in vitro (n = 16; 6 wk old mice) (D) GSIS is similar between TM-injected double-transgenic and control mice. E and F, Insulin mRNA (E) and protein (F) measurements in isolated islets from 6-wk-old mice after overnight (0 d) or after 7 d of culture reveal increased insulin transcript and protein levels in Nkx6.1OE-overexpressing islets compared with control islets (n = 4). Values are shown as mean ± SEM.](image-url)
directly compared with the experimental cohort to investigate the effect of Nkx6.1 overexpression on β-cell proliferation, β-cell mass, and glucose clearance after β-cell ablation. The control RIP-DTR cohort not treated with DT (RIP-DTR\textsuperscript{No DT}) was used to assess the effects resulting from DT-induced β-cell ablation.

Before DT administration, Nkx6.1 overexpression was induced via six ip tamoxifen injections administered over a 2-wk period (Fig. 4A). To induce β-cell ablation at 6 wk of age, the experimental Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} cohort were injected with a single dose of DT; pancreata were harvested after 2 wk, when β-cell ablation was complete (Fig. 4A). To verify the efficiency of β-cell ablation in this model, we compared β-cell mass in mice that received DT and those that did not. As expected, the two cohorts treated with DT exhibited a 65–72% reduction in β-cell mass (Fig. 4, B–E). Moreover, we observed no significant difference in β-cell mass between the experimental Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} cohort and the control RIP-DTR\textsuperscript{DT} cohort (Fig. 4E). Thus, Nkx6.1 overexpression in β-cells does not confer protection from DT-induced apoptosis.

To confirm that the β-cell ablation regimen did not attenuate all of the transgene-expressing β-cells, we performed coimmunofluorescence analysis for Nkx6.1, insulin, and GFP. As expected, we observed similar recombination efficiencies of the Nkx6.1\textsuperscript{OE} allele in the β-cell-ablated Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} mice as observed in Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE} animals (compare Figs. 1C and 4D), supporting the finding that DT-induced injury did not preferentially spare the transgene-expressing cells compared with unrecombined β-cells.

It has been reported that a loss of 50–70% β-cell mass induces glucose intolerance in mice (39). As anticipated, control RIP-DTR\textsuperscript{No DT} mice were euglycemic, whereas control RIP-DTR\textsuperscript{DT} mice were glucose intolerant, even after a 3-month recovery period (Fig. 4F). To investigate whether Nkx6.1 overexpression in β-cells can compensate for β-cell loss and restore normal glucose tolerance after an injury, we also performed IPGTT on the Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} cohort. Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} and RIP-DTR\textsuperscript{DT} control mice displayed a similar degree of glucose intolerance (Fig. 4F). Thus, overexpression of Nkx6.1 cannot compensate for a β-cell deficit to restore glucose tolerance after a partial β-cell ablation.

Next, we sought to examine whether sustained Nkx6.1 expression would aid in the regeneration of β-cell mass after partial β-cell ablation. A previous report using a bICIC tetraacycline-inducible model of DT-mediated β-cell ablation has shown that β-cell proliferation peaks between 7 d and 2 wk after initial exposure to DT (3). Therefore, β-cell proliferation rates were similarly assessed 14 d after DT treatment. Although a 2.5-fold increase in β-cell proliferation was detected in experimental Pdx1-Cre\textsuperscript{ERTM}; Nkx6.1\textsuperscript{OE}; RIP-DTR\textsuperscript{DT} mice compared with control RIP-DTR\textsuperscript{No DT} animals, the proliferation rate was not significantly different from that seen in the control RIP-DTR\textsuperscript{DT} cohort (Fig. 5, A–D). This finding suggests that DT treatment alone promotes β-cell proliferation shortly after β-cell ablation, and increased Nkx6.1 expression does not significantly affect β-cell proliferation after β-cell depletion.

It would be expected that if Nkx6.1 overexpression manifests in an elevated β-cell proliferation rate over an extended period, this might result in an expansion of...
β-cell mass and therefore recovery of β-cell mass after their depletion. To test this hypothesis, β-cell proliferation and mass was quantified in both Pdx1-CreERTM; Nkx6.1OE; RIP-DTRDT and control RIP-DTRDT cohorts 12 wk after ablation. β-Cell proliferation rates were similar in all cohorts, independent of whether they received DT treatment or overexpressed Nkx6.1 (Fig. 6D).

Neither cohort subjected to DT exhibited any significant recovery of β-cell mass 3 months after injury (Fig. 6E), showing that Nkx6.1 overexpression does not stimulate β-cell regeneration after substantial β-cell injury.

To exclude the possibility that Nkx6.1 is no longer expressed from the Nkx6.1OE allele during the period over which β-cell regeneration was examined, we performed immunohistochemical analysis for Nkx6.1, insulin, and GFP (Fig. 6, A–C). Consistent with our previous observations, we found that approximately 40% of the insulin+ cells in mice carrying Pdx1-CreERTM and Nkx6.1OE transgenes expressed GFP. This result both confirms that the Nkx6.1OE transgene remains active and also demonstrates that recombined, Nkx6.1-overexpressing β-cells are not afforded a selective advantage over unrecombined β-cells. To confirm this finding, we isolated islets from mice in each cohort 3 months after β-cell ablation (Fig. 6F). Control RIP-DTRDT mice displayed reduced Nkx6.1 expression compared with control RIP-DTRNo DT animals, consistent with the specific loss of β-cells in islets of the DT-treated animals, compared with the other endocrine cell subtypes. On the other hand, experimental Pdx1-CreERTM; Nkx6.1OE; RIP-DTRDT mice exhibited a 5-fold increase in islet Nkx6.1 expression compared with control RIP-DTRDT mice, verifying maintenance of Nkx6.1 overexpression in the β-cells of Pdx1-CreERTM; Nkx6.1OE; RIP-DTRDT mice throughout the postablation period.

Because hyperglycemia has been shown to stimulate β-cell proliferation (1, 40), we next sought to determine whether elevated Nkx6.1 expression levels in β-cells are sufficient to promote β-cell replication in a diabetic mouse model. To test this hypothesis, we used Pdx1-Nkx6.1 mice, which express both Nkx6.1 and LacZ in an islet-specific manner during adulthood (Fig. 7A). As an
Nkx6.1 overexpression does not enhance β-cell proliferation, β-cell mass, or rescue injury-induced hyperglycemia after STZ-induced β-cell ablation. A, Schematic of the experimental design to test the effects of Nkx6.1 overexpression in the STZ-induced β-cell ablation model. B, Western blot analysis demonstrates higher Nkx6.1 protein levels in islets from Pdx1-Nkx6.1 mice compared with islets from wild-type mice (n = 6). HDAC1 serves as loading control. C, Before STZ treatment, Pdx1-Nkx6.1 mice and wild-type littermates display identical blood glucose levels in IPGTT (n = 15). D, Pdx1-Nkx6.1 and wild-type mice were both diabetic after 1 wk of STZ treatment (n = 10). E and F, X-gal, immunoperoxidase staining for BrdU, and eosin staining of pancreatic sections in 7-wk-old mice reveal reduced islet size (black dotted line) after STZ-induced β-cell ablation in wild-type (E) or Pdx1-Nkx6.1 mice (F). Persistence of X-gal+ cells 1 wk after β-cell ablation indicates expression of the Pdx1-Nkx6.1 transgene (F). Islet proliferation rates (G) and β-cell mass (H) are equivalent in wild-type and Pdx1-Nkx6.1 mice 1 wk after STZ treatment (n = 3). I, STZ-treated wild-type and Pdx1-Nkx6.1 mice both exhibit hyperglycemia after a 2-month recovery period (n = 5). Scale bars, 100 μm. Filled oval (A), Internal ribosome entry site. Values are shown as mean ± SEM.

FIG. 7. Nkx6.1 overexpression does not enhance β-cell proliferation, β-cell mass, or rescue injury-induced hyperglycemia after STZ-induced β-cell ablation. A, Schematic of the experimental design to test the effects of Nkx6.1 overexpression in the STZ-induced β-cell ablation model. B, Western blot analysis demonstrates higher Nkx6.1 protein levels in islets from Pdx1-Nkx6.1 mice compared with islets from wild-type mice (n = 6). HDAC1 serves as loading control. C, Before STZ treatment, Pdx1-Nkx6.1 mice and wild-type littermates display identical blood glucose levels in IPGTT (n = 15). D, Pdx1-Nkx6.1 and wild-type mice were both diabetic after 1 wk of STZ treatment (n = 10). E and F, X-gal, immunoperoxidase staining for BrdU, and eosin staining of pancreatic sections in 7-wk-old mice reveal reduced islet size (black dotted line) after STZ-induced β-cell ablation in wild-type (E) or Pdx1-Nkx6.1 mice (F). Persistence of X-gal+ cells 1 wk after β-cell ablation indicates expression of the Pdx1-Nkx6.1 transgene (F). Islet proliferation rates (G) and β-cell mass (H) are equivalent in wild-type and Pdx1-Nkx6.1 mice 1 wk after STZ treatment (n = 3). I, STZ-treated wild-type and Pdx1-Nkx6.1 mice both exhibit hyperglycemia after a 2-month recovery period (n = 5). Scale bars, 100 μm. Filled oval (A), Internal ribosome entry site. Values are shown as mean ± SEM.

In the current study, we investigated whether β-cell-specific Nkx6.1 overexpression in vivo can stimulate β-cell proliferation and enhance β-cell function, as demonstrated in vitro for rat islets (29, 30). Although we were able to achieve up-regulation of Nkx6.1 target genes in pancreatic β-cells in vitro, we failed to detect an increase in β-cell proliferation in Nkx6.1-overexpressing mice either under basal conditions or after β-cell depletion. Moreover, Nkx6.1 overexpression did not affect glucose tolerance in vivo, alter GSIS in vitro, or improve impaired glucose clearance in a glucose-intolerant or diabetic mouse model. Our data support the notion that Nkx6.1 overexpression alone is insufficient to promote β-cell mass expansion or improve glucose metabolism in vivo.

Based on the previous observations by Schisler et al. (29, 30) that Nkx6.1 overexpression in isolated rat islets promotes β-cell proliferation, increases β-cell mass, and improves GSIS, we anticipated that Nkx6.1 overexpression in mice in vivo would elicit similar effects. Such a finding would have provided an important proof of concept that stimulation of Nkx6.1 activity or Nkx6.1-controlled pathways could constitute a potentially valid therapeutic strategy for novel treatments of diabetes. The failure of Nkx6.1 expression to either enhance β-cell proliferation or β-cell function in vivo may be due to a variety of causes. These differences may be partly due to metabolic feedback mechanisms that exist in vivo but are lacking in vitro. Alternatively, species differences between mice and rats could explain the divergent findings. Consistent with this notion, the molecular machinery of cell cycle regulation has been shown to differ significantly between murine and human β-cells (41). Finally, it is possible that the levels of Nkx6.1 overexpression achieved...
with the Nkx6.1<sup>OE</sup> transgene in vivo differed from the levels achieved with adenoviral misexpression in isolated islets. Because the study by Schisler et al. (29) did not quantify the extent of Nkx6.1 overexpression, it is difficult to determine whether significant differences in Nkx6.1 levels may account for the observed discrepancy in response between in vitro and in vivo Nkx6.1 overexpression in β-cells.

To ensure that the phenotypes observed in our study were consistent within a broad range of Nkx6.1 protein levels, experiments were independently verified using two Nkx6.1<sup>OE</sup> founders displaying islet Nkx6.1 protein levels ranging between 2.9- and 19.7-fold above control levels. We note that our previous studies have shown that the levels of Nkx6.1 expression achieved with the Nkx6.1<sup>OE</sup> and Pdx1-Nkx6.1 transgenes are sufficient to evoke significant biological responses in embryonic pancreatic cells (33, 34). Moreover, in this study we demonstrate that Nkx6.1 overexpression with the Pdx1-CreERT<sup>TM</sup> transgene results in a robust increase in expression levels of the Nkx6.1 target gene MafA in islets. Because supraphysiological concentrations of a transcription factor result in nonspecific effects on gene transcription (42–44), we purposely included mice in which Nkx6.1 levels were only 2- to 3-fold increased.

Although we were unable to detect a positive effect of Nkx6.1 overexpression on glucose clearance in a glucose-intolerant or diabetic model, Nkx6.1 overexpression simultaneously did not exhibit an obviously deleterious effect on β-cell function. By contrast, we found that elevated Nkx6.1 expression positively affected insulin gene transcription and protein expression. This suggests that β-cells are able to maintain normal function in the presence of elevated levels of Nkx6.1. In this respect, Nkx6.1 differs from other β-cell transcription factors such as Pax4, Pax6, and Hnfα, which all induce diabetes when transgenically overexpressed in β-cells (45–47). Interestingly, Nkx6.1 has recently been shown to be one of the first transcription factors to become activated when α-cells spontaneously convert into β-cells in response to near-complete β-cell ablation (39). Although it remains to be explored whether Nkx6.1 could induce or aid in α-to-β-cell transdifferentiation, our findings suggest that its misexpression in fate-converted β-cells will not negatively impact β-cell function.

The observation that mice display unique regenerative responses based on the extent of β-cell ablation and the specific nature of the insult (3–5, 39) suggests that the pancreas possesses a spectrum of divergent cellular responses, which can be activated by β-cell injury. In our study we used a genetic DT-inducible β-cell ablation model (39), which manifested in a 71% reduction in β-cell mass. In this model, we failed to observe any recovery in β-cell mass or amelioration of the glucose-intolerant phenotype over a 3-month recovery period. In contrast, data generated using a similar tetracycline-dependent bigenic DT-inducible β-cell ablation model revealed a reconstitution of β-cell mass after a 70–80% reduction in β-cell mass (3). Although both studies were similar in regard to the type of β-cell injury and age of the mice, one significant difference between the two models is that mice in our model were glucose intolerant but not diabetic, whereas Nir et al. (3) reported overt hyperglycemia in their model. Likewise, severe hyperglycemia was observed in two other genetic models of cell-autonomous β-cell ablation, which also displayed cell mass recovery after injury (4, 5). Cumulatively, the findings in these different models suggest that elevated blood glucose levels could be a key stimulus for β-cell regeneration. This notion is consistent with the observation that prolonged glucose infusion over a 1-wk period increases β-cell mass (40) and that glucose stimulation shortens the quiescent period of β-cells between cell divisions (1).

In summary, our study shows that stimulation of Nkx6.1 expression in β-cells in mice does not promote β-cell regeneration under either normal conditions or after β-cell injury. In addition, our data suggest that unless accompanied by hyperglycemia, 50–70% of β-cell ablation alone does not provide a sufficient stimulus to induce β-cell regeneration. Given the hope that Nkx6.1 could be a suitable target for stimulating both β-cell expansion and function in vivo, our findings have important implications for diabetes therapy.

Materials and Methods

Mice

Generation of Pdx1-Nkx6.1<sup>OE</sup>, Pdx1-CreERT<sup>TM</sup>, and RIP-DTR mice has been previously reported (33, 34, 39, 48). Except for DT-induced β-cell ablation studies, in which female heterozygous mice were used, male mice were used in all experiments. Mice were maintained on a CD1 (Charles River Laboratories, Wilmington, MA) background, except for the RIP-DTR mice, which were maintained on a C57BL/6J (Jackson Laboratories, Bar Harbor, ME) background.

Tamoxifen (Sigma, St. Louis, MO) was dissolved in corn oil (Sigma) and six doses of 2 mg per 40 g body weight were administered over a 2-wk period by sc injection. One hundred twenty-five nanograms of diphtheria toxin (Sigma) dissolved in sterile saline were administered by ip injection. For BrdU labeling, mice received one ip injection of 100 μg/g body weight BrdU (Fisher Scientific, Fair Lawn, NJ) 6 h before the animals were killed. Mice were fasted for 4 h before receiving an ip injection
of 40 mg/kg body weight STZ dissolved in citrate buffer (pH 4.5) for 5 consecutive days.

**Immunofluorescence analysis, X-gal staining, morphometry, and cell quantification**

Tissue preparation, immunohistochemistry, X-gal staining, and \(\beta\)-cell mass measurements were performed as previously described (2, 7, 33). Briefly, the entire pancreas was fixed overnight in 4% paraformaldehyde in PBS at 4 C and embedded in Tissue Tek OCT embedding medium (Sakura Finetek, Tokyo, Japan) or paraffin for BrdU detection. Each pancreas was subsequently sectioned, and six representative sections were stained with the following antibodies: guinea pig anti-insulin (Dako, Carpinteria, CA) at 1:5000, rabbit antisomatostatin (Dako) at 1:200, rabbit anti-Ki67 (Lab Vision, Fremont, CA) at 1:200, mouse anti-Nkx6.1 (Beta Cell Biology Consortium; clone 2023, National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases) at 1:500, rat anti-GFP (gift from Professor Maike Sander, Department of Cellular and Molecular Medicine, The University of California, San Diego, CMM-East 2051, La Jolla, California 92093-0695. E-mail: masander@ucsd.edu) for anti-GFP antibodies, and P. Serup (Hagedorn Research Institute, Gentofte, Denmark) for anti-Nkx6.1 and C. Kioussi (Oregon State University, Corvallis, OR) for anti-GFP antibodies, and P. Chessler and V. Cirulli for providing help with islet isolation and GSIS studies.

For \(\beta\)-cell mass measurements, images covering an entire pancreas section were tiled using a Zeiss AxioObserver.Z1 microscope (Carl Zeiss, New York, NY) with the Zeiss ApoTome module. The insulin\(^-\) area and total pancreas area were measured using ImagePro Plus 5.0.1 software (Media Cybernetics, Silver Spring, MD) from at least six sections per mouse, and \(\beta\)-cell mass was calculated as follows: (insulin\(^-\) area/total pancreatic area) multiplied by pancreatic weight. Recombination rates were calculated by counting GFP and insulin copositive cells. \(\beta\)-Cell proliferation rates were assessed by counting Ki67 and insulin copositive cells or BrdU\(^+\) islet cells relative to total insulin\(^-\) or islet area, respectively. For all data points, a minimum of 50 islets were analyzed per mouse.

**Islet isolation, GSIS assay, immunoblotting, ELISA, and mRNA quantification**

Islet isolations, culture, and GSIS assays were performed as previously described (7, 49). Islets from four mice per genotype were isolated and divided into four pools per mouse for GSIS assays and insulin measurements. For insulin measurements, islet protein was extracted in acidified ethanol and insulin protein levels determined by ELISA (ALPCO, Windham, NH). Insulin content was separately determined in the supernatant and the islet cell fraction and the percentage of secreted insulin calculated as: 100(secreted insulin/secreted insulin + islet cell insulin), as previously described (50). For Western blot analysis, primary antibodies against Nkx6.1 (gift from P. Serup, Hagedorn Research Institute) at 1:2000 or histone deacetylase (HDAC)-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 were used. For each measurement, islets were pooled from three mice and three independent measurements performed for each genotype or experimental condition. The bands were quantified using the LI-COR infrared imaging system (LI-COR Biosciences, Lincoln, NE). For quantitative RT-PCR analysis, the isolated islets were pooled from three mice. Two independent samples were analyzed in triplicate by quantitative RT-PCR using SYBR green (Applied Biosystems Inc., Foster City, CA) as described (34). Oligonucleotide sequences are available upon request.

**IPGTT and in vivo insulin secretion assay**

Mice were fasted for 16 h and blood glucose levels recorded (Bayer Contour glucometer; Bayer, Tarrytown, NJ) before an ip injection of 1.5 mg/g body weight dextrose in sterile water. Blood glucose levels were analyzed at 20, 40, 60, 90, and 120 min after the injection. For in vivo insulin secretion assays, blood serum was collected for insulin and glucose measurements after 16 h of fasting and 30 min after ip dextrose administration. To account for the variability of blood glucose concentrations within the cohort, the serum insulin concentration was normalized to the simultaneously recorded blood glucose level of each mouse.

**Statistical analysis**

All values are shown as mean ± SEM; \(P\) values were calculated using an unpaired Student’s \(t\) test; \(P < 0.05\) was considered significant.

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