

In Vivo Conditional Pax4 Overexpression in Mature Islet β -Cells Prevents Stress-Induced Hyperglycemia in Mice

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OBJECTIVE—To establish the role of the transcription factor Pax4 in pancreatic islet expansion and survival in response to physiological stress and its impact on glucose metabolism, we generated transgenic mice conditionally and selectively overexpressing Pax4 or a diabetes-linked mutant variant (Pax4R129W) in β -cells.

RESEARCH DESIGN AND METHODS—Glucose homeostasis and β -cell death and proliferation were assessed in Pax4- or Pax4R129W-overexpressing transgenic animals challenged with or without streptozotocin. Isolated transgenic islets were also exposed to cytokines, and apoptosis was evaluated by DNA fragmentation or cytochrome C release. The expression profiles of proliferation and apoptotic genes and β -cell markers were studied by immunohistochemistry and quantitative RT-PCR.

RESULTS—Pax4 but not Pax4R129W protected animals against streptozotocin-induced hyperglycemia and isolated islets from cytokine-mediated β -cell apoptosis. Cytochrome C release was abrogated in Pax4 islets treated with cytokines. Interleukin-1 β transcript levels were suppressed in Pax4 islets, whereas they were increased along with NOS2 in Pax4R129W islets. Bcl-2, Cdk4, and c-myc expression levels were increased in Pax4 islets while MafA, insulin, and GLUT2 transcript levels were suppressed in both animal models. Long-term Pax4 expression promoted proliferation of a Pdx1-positive cell subpopulation while impeding insulin secretion. Suppression of Pax4 rescued this defect with a concomitant increase in pancreatic insulin content.

CONCLUSIONS—Pax4 protects adult islets from stress-induced apoptosis by suppressing selective nuclear factor- κ B target genes while increasing Bcl-2 levels. Furthermore, it promotes dedifferentiation and proliferation of β -cells through MafA repression, with a concomitant increase in Cdk4 and c-myc expression. *Diabetes* 60:1705–1715, 2011

Diabetes is a disease characterized by high levels of circulating blood glucose. The etiology involves insufficient release of insulin from pancreatic islet β -cells and resistance of target tissues to the action of the hormone. The two most

common forms of diabetes are type 1 diabetes characterized by a destruction of β -cells (1) and type 2 diabetes typified by β -cell failure combined with insulin resistance (2). Factors such as the environment and genetic predisposition are key determinants that influence development and progression of the disease. Genetic studies including linkage analysis, candidate gene approaches, and more recently, genome-wide association studies (GWAS) have identified at least 40 loci affecting risk of type 1 diabetes and 27 type 2 diabetes susceptibility genes (3–5). Although GWAS have been a powerful approach to yield new diabetogenes, susceptible gene loci for which functions may be altered by environmental factors such as pregnancy and obesity remain to be identified.

One such susceptibility gene locus not highlighted by GWAS encodes the islet β -cell transcription factor Pax4. Expression of the *pax4* gene is mandatory for the development and maturation of β -cells (6). Although detectable, *Pax4* expression was found to be low in adult β -cells (7). Forced expression of *Pax4* in embryonic α -cells induced a complete phenotypic change toward β -cells indicating that Pax4 is a master regulator of the β -cell genetic program (8).

Mutations and polymorphisms in the *pax4* gene have been associated with both type 1 and type 2 diabetes in several populations, contrasting with other diabetogenes for which association has only been linked to one or the other form of diabetes (7,9). Interestingly, we found that Pax4 expression is increased in type 2 diabetic islets, an effect that is most likely mediated by high blood glucose levels (10). Together, these studies suggest that Pax4 may function as a survival and/or proliferation gene allowing mature islets to adapt in response to physiological cues. Consistent with this premise, Pax4 mRNA levels were increased in islets cultured in the presence of glucose, betacellulin, activin A, and glucagon-like peptide-1 (10). Ectopic expression of mouse Pax4 in human or rat islets and in the mouse MIN6 cell line conferred protection against cytokine-mediated cell death and promoted replication (11,12). A diabetes-linked mutant variant R121W, identified in the Japanese population (13,14), was less efficient in protecting human islets against cytokines (11).

Although these in vitro studies suggest a fundamental role of Pax4 in β -cell survival and replication, the impact of Pax4 in vivo and its relation to diabetes remains to be established. Herein, we have generated two transgenic mouse lines that conditionally express Pax4 or its mutant variant R121W (PAX4R129W in mice) in β -cells. Our results demonstrate that conditional overexpression of Pax4 in adult β -cells protects transgenic animals against streptozotocin (STZ)-induced hyperglycemia and isolated islets against cytokines, while animals expressing the mutant variant were susceptible to developing hyperglycemia

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and β -cell death by both treatments. Long-term expression of Pax4 in animals repressed MafA and insulin, resulting in blunted glucose-induced insulin secretion suggesting de-differentiation of β -cells.

RESEARCH DESIGN AND METHODS

Transgenic animals. The pIRES2-DsRedexpress (Clontech) vector was used for the generation of the inducible Pax4 or the mutant variant Pax4R129W cDNA cassette. The final construct contained the tetracycline responsive promoter, the rabbit β -globin intron followed by the Pax4 or Pax4R129W coding sequence. A myc-epitope and polyhistidine tag were added for detection purposes. DsRedexpress was included in the constructs in order to follow Pax4 induction using noninvasive *in vivo* imaging. Pax transgenic animals were crossed to RIPrtTA mice to generate double transgenic descendants with conditional expression of Pax4 or Pax4R129W specifically in β -cells. Induction of transgene expression was achieved by providing 1 g/L of doxycycline (Sigma-Aldrich) in the drinking water. The GLUT2 knockout mouse has been described elsewhere (15). The Geneva Veterinary Cantonal Office and the CABIMER animal committee approved all experiments.

Glucose and insulin measurements. Animals fasted overnight or for 4 h and were injected intraperitoneally with 2 g glucose/kg body wt or with 1 unit insulin/kg body wt Actrapid (Novo Nordisk Pharma). Blood was collected from tail vein, and glucose levels were measured with a Precision Xceed glucometer. Plasma insulin levels were measured at 0 and 15 min using a mouse insulin ELISA kit (Merckodia, Uppsala, Sweden). Pancreatic insulin content was determined using an insulin enzyme immunoassay kit (SPI-BIO; Bertin Pharma Biotech Division, Brunswick, Basel, Switzerland).

STZ treatment. STZ was prepared in 0.1 mmol/L sodium citrate at pH 4.5 and administered by intraperitoneal injection (200 mg/kg body wt). Blood glucose was determined before STZ injection and then three times per week.

Mouse islet isolation. Pancreatic islets were isolated by collagenase (Roche, Switzerland) digestion, handpicked and maintained in 11.1 mmol/L glucose/RPMI-1640 (Invitrogen, Switzerland) supplemented with 10% FCS (Brunswick), 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma, St-Gallen, Switzerland).

Quantitative real time PCR. Total RNA from islets was extracted using the RNeasy Micro Kit (Qiagen), and quantitative real time PCRs (Q-RT-PCRs) were performed as previously described (16). Primer sequences were designed using the Primer Express Software (Applied Biosystems) and can be obtained upon request.

Transfection studies. Transient transfections were performed as previously described (11). The luciferase reporter constructs, pFOX-Luc and pFOX-MafAR3-Luc (region 3 of the MafA promoter specifying β -cell expression), were provided by Dr. R. Stein (Vanderbilt University, Nashville, TN).

Cytokine treatment, apoptosis, and nitrite measurements. Islets were isolated from transgenic animals that had received 1 g/L doxycycline in drinking water for 1 month. Islets were cultured for 48 h in the presence of 1 μ g/L doxycycline prior to the addition of 0.25 ng/mL interleukin (IL)-1 β , 9.1 ng/mL tumor necrosis factor- α , and 100 ng/mL γ -interferon (R&D Systems). Apoptosis was evaluated 24 h posttreatment using the Cell Death ELISA Plus kit (Roche). Alternatively, cell death was estimated by cytochrome C release. Dispersed islets were concentrated by cytospin onto glass slides, and immunofluorescence was performed using anticytochrome C serum (BD Biosciences) and antiserum for the mitochondrial marker TOM 20 (Santa Cruz). Nitrite production in culture media was determined using the Griess reaction (Sigma).

Proliferation assay. Mice were injected with 1 mL BrdU solution/100 g body wt (Roche); 24 h later, they were killed. Proliferation was detected by immunohistochemistry on pancreatic sections.

Immunohistochemistry. For paraffin sections, pancreata were dissected and fixed in 4% paraformaldehyde. Dehydration, embedding, and sectioning were performed at either the Geneva or CABIMER Histology platform. Sections were rehydrated in ethanol and blocked in PBS containing 1% BSA and 0.1% Tween. The following primary polyclonal antibodies were used: guinea pig anti-insulin, mouse anti-glucagon, rabbit anti-pdx1 (1:200; provided by C. Wright, Vanderbilt University), rabbit anti-GLUT2 (1:200), and rabbit anti-MafA (1:200; Bethyl). A mouse monoclonal BrdU was also used. The following secondary antibodies were added: Alexa Fluor 568 goat anti-guinea pig, Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit. For cryostat sections, pancreata were frozen in liquid nitrogen prior to sectioning and mounting. Sections were fixed in 4% paraformaldehyde, and immunodetections were performed with the following primary polyclonal antibodies: rabbit anti-Pax4 (provided by Dr. Sosa-Pineda) and guinea pig anti-insulin. Secondary antibodies used were as follows: Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-guinea pig.

Immunoblot analysis. Islets were harvested in lysis buffer (0.05 mol/L TRIS-HCl, pH 7.5, and 62.5 mmol/L EDTA), cells disrupted by sonication, and proteins resolved by 10% PAGE. Gels were processed for immunoblotting using standard

procedures. The following primary polyclonal antibodies were used: rabbit anti-Pax4, goat DsRed (Santa Cruz), and rabbit TFIIIE- α (Santa Cruz). A goat anti-rabbit IgG antiserum conjugated to horseradish peroxidase (GE Healthcare) was then added, and immunoreactive products were visualized by chemiluminescence (Pierce, Rockford, IL).

Statistical analysis. Results are expressed as means \pm SEM. Statistical differences were estimated using the unpaired *t* test, ANOVA with Bonferroni post hoc test, or nonparametric Mann-Whitney test.

RESULTS

Pax4 overexpression in adult pancreatic β -cells does not impact glucose homeostasis. To study *in vivo* the role of Pax4 and the impact of a mutant variant associated with diabetes in β -cell plasticity, we generated inducible double transgenic mouse lines expressing RIP-rtTA along with either Pax4/DsRedexpress or Pax4R129W/DsRedexpress. For simplicity, double transgenic rtTA/Pax4 and rtTA/Pax4R129W animals are denoted as Pax4 and Pax4R129W, respectively, while control animals comprising single transgenic littermates are referred to as ST. Q-RT-PCR revealed that both Pax4 and Pax4R129W transcripts were expressed to similar levels exclusively in islets of doxycycline-treated transgenic Pax4 animals, whereas expression of the transgenes was not detected in either control ST mice or in double transgenics that had not been provided with doxycycline (Fig. 1A). Western blotting confirmed expression of Pax4 and DsRedexpress in islets of the doxycycline-treated animals (Fig. 1B). Immunohistochemistry using either Pax4 or c-myc antisera further substantiated specific expression of Pax4 in islets and revealed colocalization with insulin in most but not all β -cells, suggesting β -cell-restricted expression of the transgene (Fig. 1C and D).

In parallel, induction of DsRedexpress was monitored by noninvasive *in vivo* imaging in Pax4 transgenic animals. Fluorescence was detected in the abdominal region of anesthetized living animals treated with doxycycline for 48 h (Supplementary Fig. 1A). Imaging of extracted organs confirmed expression of DsRedexpress specifically in the pancreas of treated animals, while other organs exhibited background fluorescence (Supplementary Fig. 1B).

Pax4 transgenic animals that received doxycycline for 1 month exhibited normal glucose tolerance and insulin sensitivity (Fig. 1E and F). Consistent with this finding, glucose-induced insulin secretion was normal in islets isolated from Pax4-expressing mice compared with control ST islets (245 ± 56 vs. 246 ± 55 ng/mL). Similar results were obtained with Pax4R129W animals (data not shown). These results demonstrate that conditional expression of Pax4 can be specifically achieved in islet β -cells and that its forced expression does not alter glucose homeostasis in double transgenic animals.

Overexpression of Pax4 but not Pax4R129W protects mice against STZ-induced hyperglycemia. To assess the protective role of Pax4 *in vivo*, doxycycline-treated Pax4 or ST animals were challenged with a single high dose of STZ to destroy β -cells. ST animals rapidly developed hyperglycemia while Pax4-overexpressing animals remained normoglycemic throughout the experiment (Fig. 2A). For confirmation that Pax4 was mediating the protection against STZ-induced hyperglycemia, doxycycline was removed on day 27 and animals in group A received, 14 days later, a second STZ injection. Group A animals developed hyperglycemia similar to that observed in ST animals, whereas Pax4 animals that did not receive a second STZ injection (group B) remained normoglycemic (Fig. 2A). In an independent set of experiments, animals continuously exposed to doxycycline and rechallenged

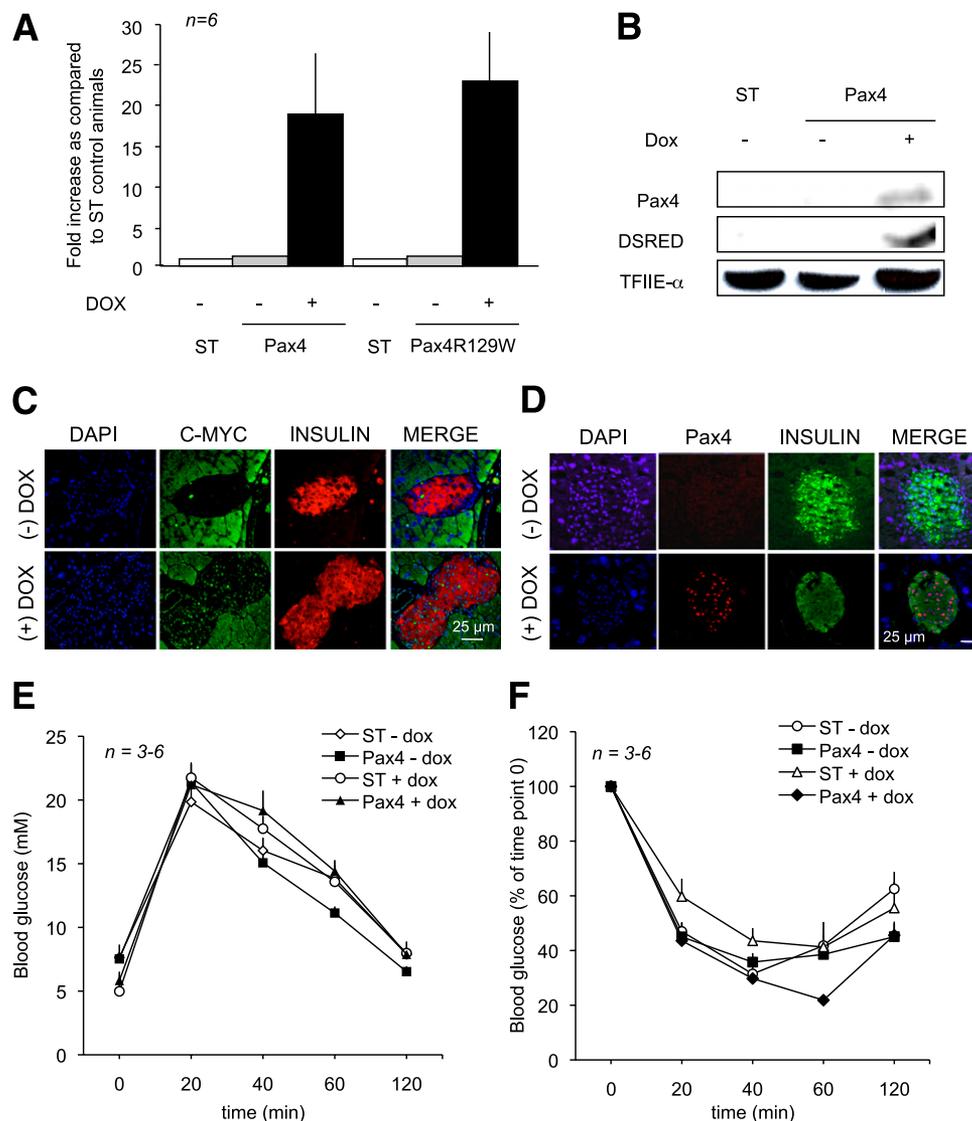


FIG. 1. Selective conditional overexpression of either Pax4 or Pax4R129W in mouse β -cells. **A:** Double transgenic animals bearing either the pTRE-Pax4 or Pax4R129W-myc-His-IRES-DsRedexpress construct along with the RIP-rtTA transgene (denoted as Pax4 or Pax4R129W) were provided with 1 g/L doxycycline (DOX) in drinking water for 1 month. Pax4 and Pax4R129W transcript levels were then evaluated by Q-RT-PCR. Relative mRNA levels were normalized to the transcript levels of the housekeeping genes cyclophilin and/or *rps29*. Data were calculated as fold change compared with ST animals. **B:** Doxycycline-induced Pax4 and DsRedexpress proteins were confirmed by Western blot analysis. Immunofluorescence analysis using antimyc (**C**) or anti-Pax4 (**D**) sera revealed specific expression of the Pax4 protein only in the pancreatic islet β -cells of animals that received doxycycline. Of note, the intense green staining within the islet in the c-myc (-) doxycycline image is nonspecific. **E:** Glucose tolerance tests were performed on control ST animals and Pax4 animals treated or not with doxycycline. **F:** Insulin tolerance tests were also performed on the same animals. Data are expressed as means \pm SEM from 3–6 animals per group. (A high-quality digital representation of this figure is available in the online issue.)

with a second high dose of STZ did not develop hyperglycemia, confirming Pax4-dependent protection even after two high doses of STZ (Fig. 2B). Pax4R129W animals challenged with a single dose of STZ developed an intermediate level of hyperglycemia, indicating that the diabetes-associated mutant is less efficient in protecting mice against stress-induced hyperglycemia (Fig. 2C). Immunohistochemistry combined with morphometry analysis revealed a clear reduction in insulin⁺ cells, with a concomitant increase in glucagon⁺ cells in STZ-injected ST animals compared with what was observed in untreated mice (Fig. 2D and Supplementary Fig. 2A and B). In contrast, islet morphology and cell composition in Pax4-overexpressing animals challenged with either one or two doses of STZ were similar to those of islets from control animals (Fig. 2D and Supplementary Fig. 2A and B). Islets from Pax4R129W mice

exhibited less insulin staining, suggesting a partial loss of β -cells correlating with intermediate hyperglycemia (Fig. 2D). These results show that overexpression of Pax4 protects β -cells against multiple STZ challenges and prevents development of hyperglycemia, whereas the mutant is less effective.

Pax4 but not Pax4R129W protects islets against cytokine-induced apoptosis. For further assessment of the protective action of Pax4, islets isolated from either Pax4 or Pax4R129W transgenic animals were treated with cytokines. Pax4 islets were protected against cytokine-induced cell death compared with ST control animals that displayed a threefold increase in cell death in the presence of cytokines (Fig. 3A). Islets isolated from Pax4R129W animals exhibited a similar degree of apoptosis compared with cytokine-treated ST animals (Fig. 3A).

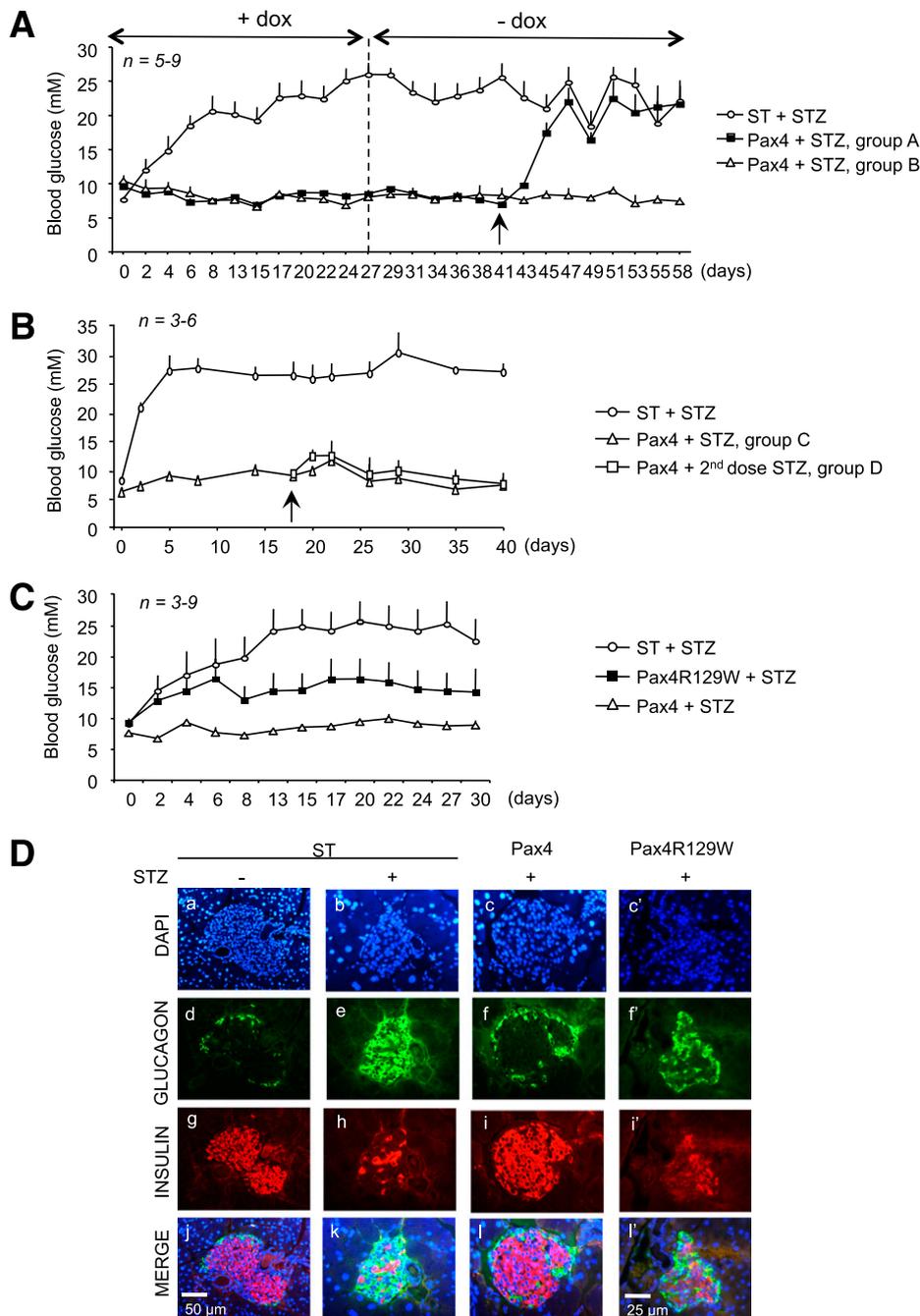


FIG. 2. Pax4-overexpressing animals are protected against STZ-induced hyperglycemia. **A:** A single high dose of STZ (200 mg/kg body wt) was injected in control (ST) and Pax4 animals treated with doxycycline (dox) (groups A and B). Blood glucose levels were measured three times per week for up to 58 days. All animals prior to STZ injection received doxycycline in drinking water for 1 month starting at weaning. For further assessment of the protective role of Pax4, doxycycline was removed from the drinking water at day 27. Blood glucose levels were followed for 2 weeks before a second injection of STZ on day 41 was administered to group A (arrow) while group B received only the vehicle. **B:** Alternatively, Pax4 animals maintained on doxycycline (group D) were challenged with a second high dose of STZ (arrow) and glycemia was monitored for an extra 22 days. Group C animals were sustained on doxycycline without an STZ rechallenge. **C:** Pax4, Pax4R129W, and ST animals were injected with STZ; glycemia was then monitored for 30 days. Data are expressed as the means \pm SEM from 3–9 animals per group. Statistical difference was tested by the nonparametric Mann-Whitney test (significance from $P < 0.05$). **D:** Immunohistochemical analysis of glucagon (green) and insulin (red) on representative pancreatic paraffin sections from doxycycline-treated ST, Pax4, and Pax4R129W mice challenged or not with STZ. Animals were killed on day 27, and paraffin sections were prepared from ST animals that received citrate buffer injection (a, d, g, and j) or challenged with STZ (b, e, h, and k) and from Pax4 (c, f, i, and l) or Pax4R129W (c', f', i', and l') animals injected with STZ injection. Bars, 50 μ m for a, d, g, and j and 25 μ m for remaining panels. (A high-quality digital representation of this figure is available in the online issue.)

Exogenous IL-1 β was shown to induce endogenous levels of IL-1 β and to lead to human islet β -cell death (17). Interestingly, IL-1 β transcript levels were suppressed by 50% in islets isolated from Pax4-overexpressing animals. In contrast, Pax4R129W mutant mice exhibited a twofold

increase in IL-1 β transcript levels compared with control untreated ST islets (Fig. 3B). A parallel modulation in transcript levels of the IL-1 β antagonist was observed in both transgenic animal models, whereas expression levels of the IL-1 β receptor remained unaltered (Fig. 3B).

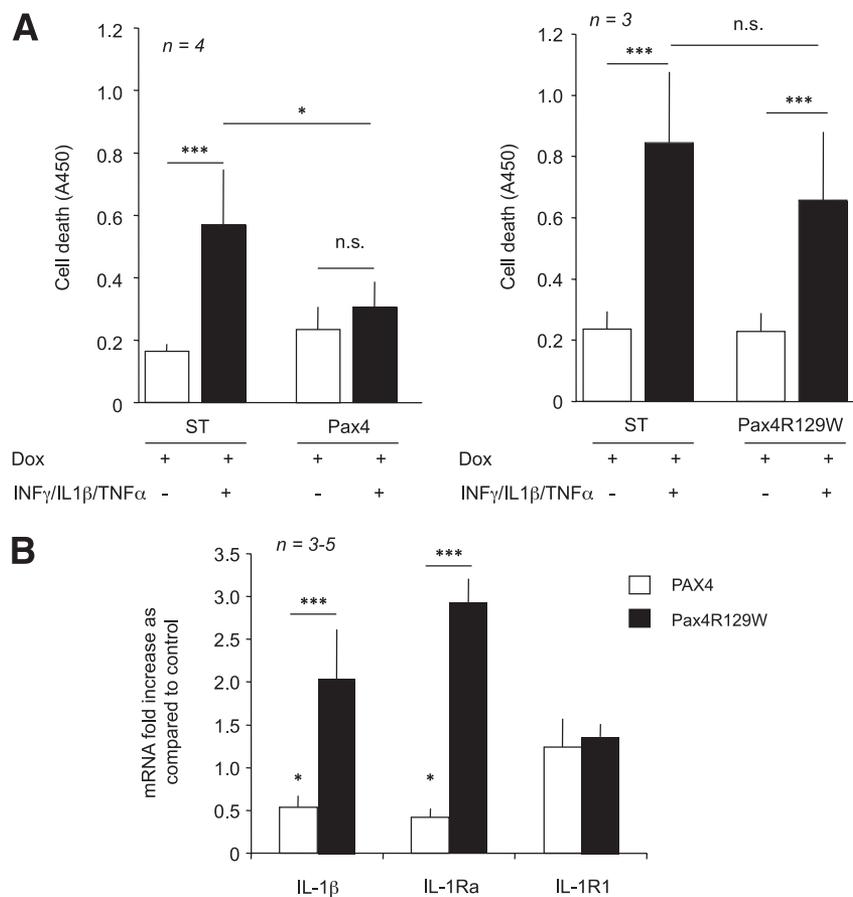


FIG. 3. Isolated islets from Pax4 transgenic mice are protected against cytokine-induced apoptosis. **A:** Freshly isolated islets from ST animals and double transgenic animals (Pax4, left panel, and Pax4R129W, right panel) were cultured with 1 μ g/L doxycycline for 48 h and subsequently challenged with a mix of IL-1 β , γ -interferon (INF γ), and TNF- α . Cell death was measured using a cell death detection ELISA kit. Results are expressed as means \pm SEM of three or four independent experiments relative to nontreated islets. **B:** IL-1 β , IL-1Ra, and IL-1R1 mRNA expression levels were measured by Q-RT-PCR in either Pax4- or Pax4R129W-overexpressing animals. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals that received doxycycline (dashed line) and expressed as means \pm SEM from 3–5 animals per group. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test. * $P < 0.05$ and *** $P < 0.001$.

Mitochondrial cytochrome C release is blunted in Pax4-overexpressing islets treated with cytokines.

A hallmark of cytokine-mediated cell death is the activation of the nuclear factor (NF)- κ B pathway, which leads to enhanced NOS2 and COX2 expression and the downstream increase in nitric oxide (NO) production, ultimately resulting in cytochrome C release from mitochondria (18). Consistent with this model, islets isolated from Pax4 mice and exposed to cytokines in the absence of doxycycline exhibited a 2.5-fold increase in cytochrome C release. In contrast, cytochrome C release was abrogated in doxycycline-treated Pax4 islets, whereas Pax4R129W-overexpressing islets displayed a threefold increase compared with noncytokine-treated islets (Fig. 4A and B). Interestingly, NOS2 transcript levels were only significantly elevated in Pax4R129W islets, whereas COX2 levels were augmented in both mutant and wild-type Pax4 islets (Fig. 4C). Despite differences in NOS2 expression, both Pax4 and Pax4R129W islets presented a significant increase in cytokine-induced nitrite production, an indicator of NO production (Fig. 4D). However, Pax4 islets displayed a trend toward a small decrease in nitrite production compared with non-doxycycline-treated islets.

Overexpression of Pax4 alters expression of MafA, GLUT2, insulin, and Bcl2. To further delineate the mechanism by which Pax4 protects β -cells from either STZ- or

cytokine-induced apoptosis, we determined mRNA expression levels of key β -cell markers and those of antiapoptotic genes in islets of Pax4- and Pax4R129W-overexpressing animals (Fig. 5A). Transcript levels of Pdx1 and Pax6 were not altered in Pax4- or Pax4R129W-overexpressing islets, whereas MafA, GLUT2, and insulin levels were decreased in both animal models. Immunohistochemistry confirmed decreased MafA and GLUT2 protein levels in islets of Pax4-expressing mice (Fig. 5B and C). Bcl-2 but not Bcl-xL expression levels were increased threefold specifically in Pax4 islets compared with those in control animals without doxycycline administration (Fig. 5A).

Because insulin and GLUT2 are target genes of MafA (19,20) and because a Pax4 consensus sequence was predicted in region 3 of the MafA promoter (21), we investigated whether Pax4 could directly regulate MafA transcription. Pax4 and Pax4R129W dose dependently inhibited a luciferase reporter construct driven by region 3 of the MafA promoter, reaching 50% inhibition at the highest dose (Fig. 5D). Repression was specific; Pax4 was unable to inhibit the control pFOX-Luc construct. However, Pax4 failed to interact with the putative binding site identified in this MafA region (MafAR3Pax4), whereas it displayed specific binding to the G3 element of the glucagon promoter (data not shown) (22).

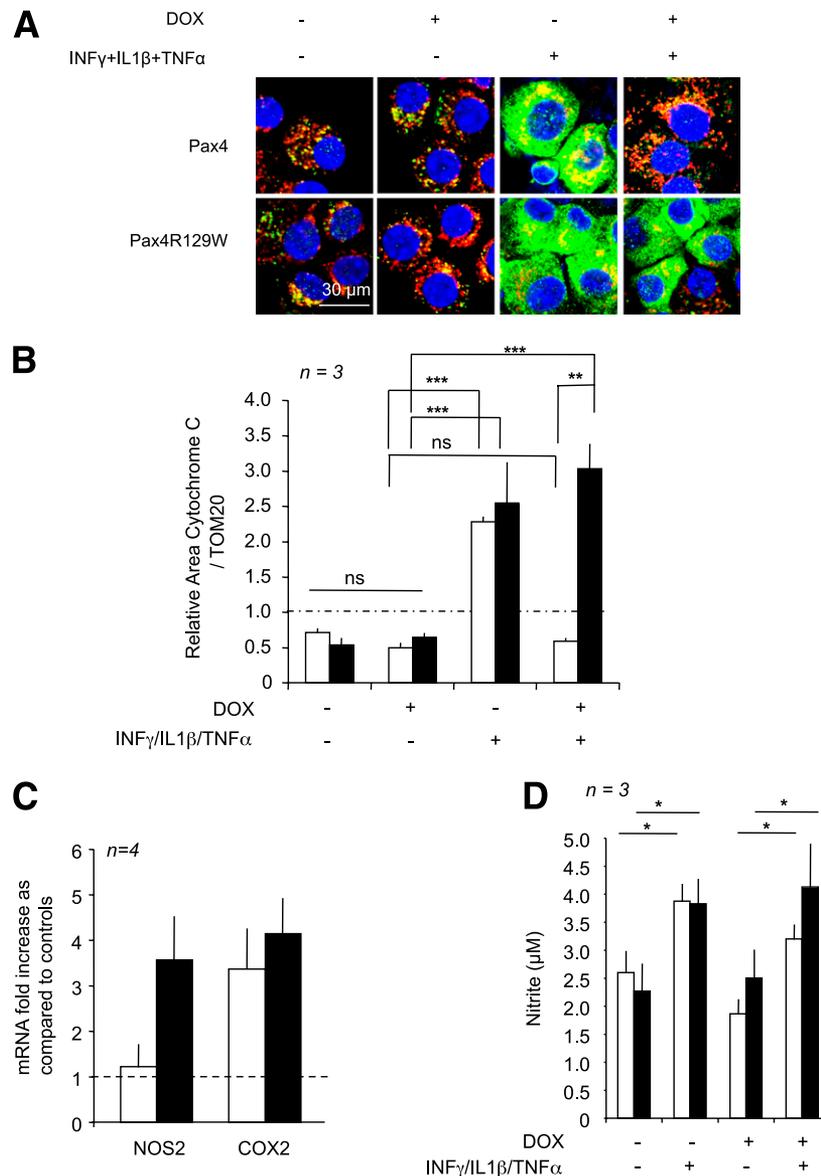


FIG. 4. Pax4 overexpression protects from cytokine-induced β -cell death by impeding mitochondrial cytochrome C release. **A:** Immunofluorescent localization of cytochrome C (green), TOM-20 (red), and DAPI (blue) within islet cells treated with or without cytokines in the presence or absence of either Pax4 (\square) or Pax4R129W (\blacksquare) expression. **B:** Quantification of cytochrome C release from the mitochondria. Metamorph software was used to measure the area of red (TOM-20) and green (cytochrome C) fluorescence. A total of 20 images, each containing 4–5 cells, were taken per condition and per mouse in three independent experiments. Total green fluorescence (cytochrome C) area was then divided by total red fluorescence (TOM-20) area. Values >1 indicate that cytochrome C is external to the mitochondria, and values ≤ 1 indicate that it is inside the organelle. **C:** Q-RT-PCR analysis of *nos2* and *cox2* transcript levels in islets of Pax4 and Pax4R129W transgenic animals treated with doxycycline. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals that received doxycycline (represented by dashed line) and expressed as the means \pm SEM from four animals per group. **D:** Nitrite release in culture media was estimated using the Griess reagent. Data were expressed as means \pm SEM from three independent experiments. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. INF γ , γ -interferon. (A high-quality digital representation of this figure is available in the online issue.)

GLUT2 heterozygous mice are protected against STZ-induced hyperglycemia. STZ is selectively taken up by β -cells through GLUT2 (23). Because GLUT2 levels were decreased in Pax4-overexpressing islets, we determined whether such inhibition would be sufficient to protect β -cells against STZ. To this end, the GLUT2 heterozygous mouse model was used in order to directly measure the impact of the glucose transporter in STZ-mediated cell death. Transcript levels of the transporter in STZ-treated mice were reduced by 60% in heterozygous GLUT2 mice (Fig. 6A). Similarly to Pax4 mice, these animals were refractory to STZ-induced β -cell apoptosis, whereas control wild-type littermates

were sensitive to the toxic agent (Fig. 6B). These results suggest that the protective effect of Pax4 against STZ is potentially partially conveyed by reduced GLUT2 expression. However, despite similarly lower levels of GLUT2, Pax4R129W mice are only partially protected against STZ, suggesting that alternative detrimental processes are associated with the mutant phenotype.

Transient overexpression of Pax4 improves total pancreatic insulin content after 4 months. Adenoviral-mediated overexpression of Pax4 in cultured rat islets was shown to hamper insulin secretion (11). Although this effect was not observed in Pax4 transgenic animals treated

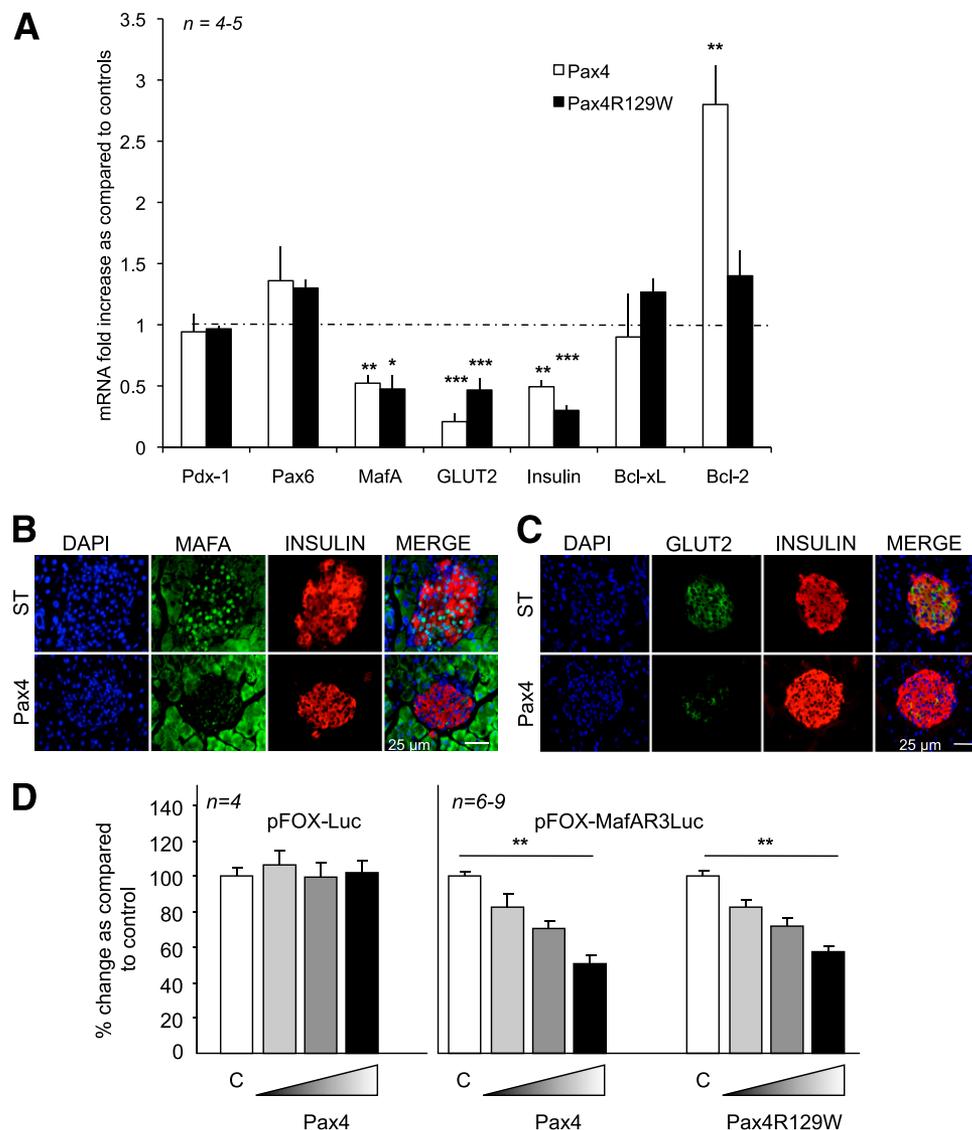


FIG. 5. Induction of Pax4 in islets suppresses MafA, insulin, and GLUT2 expression, whereas it increases Bcl-2. **A:** Q-RT-PCR was performed on islets isolated from doxycycline-treated Pax4 and Pax4R129W animals. Relative mRNA levels were normalized to the transcript levels of the housekeeping genes cyclophilin and/or *rps29*. Data were calculated as fold change compared with ST animals that received doxycycline (dashed line) and are expressed as means \pm SEM from 4–5 animals per group. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). **B:** Immunohistochemistry analysis of MAFA (B) and GLUT2 (C) was performed on pancreatic sections from Pax4-overexpressing animals and in control ST animals treated with doxycycline. **D:** Transient cotransfection studies using INS-1E cells were performed with increasing amounts of either Pax4 or PaxR129W expression vectors (0.25, 0.50, 0.75, and 1 mg) along with 500 ng of either pFOX-Luc or pFOX-MafAR3-Luc. Data are presented as fold change of basal luciferase activity and expressed as the means \pm SEM of three experiments (* $P < 0.05$). (A high-quality digital representation of this figure is available in the online issue.)

with doxycycline for 1 month, plasma insulin levels in response to glucose were greatly reduced in animals treated for 4 months (Fig. 7A). This was accompanied by glucose intolerance in these animals (Fig. 7B). Furthermore, morphological analysis of pancreas isolated from Pax4-expressing mice revealed altered islet architecture typified by an increased number of enlarged intercellular spaces, reminiscent of capillaries detected in control islets (Supplementary Fig. 3). To determine whether this effect was reversible, we removed doxycycline from the drinking water of 4-month-treated animals to repress Pax4 expression and reevaluated plasma insulin levels 1 month later. Consistent with our premise, the rise in plasma insulin levels in response to glucose was rescued in transgenic animals subsequent to removal of doxycycline (Fig. 7C). Total pancreatic insulin content was significantly

increased in Pax4 mice after removal of doxycycline (Fig. 7D).

Long-term (4 months) but not short-term (1 month) Pax4 overexpression increases islet β -cell replication. The increase in insulin content observed after doxycycline removal (Fig. 7D) suggests that long-term expression of Pax4 may induce β -cell replication. Consistent with this premise, islets isolated from Pax4 mice that underwent 4-month but not 1-month doxycycline treatment showed reduced glucose-induced plasma insulin levels (Fig. 7A) and exhibited increased BrdU labeling of β -cells compared with control ST islets (Fig. 8A). This was accompanied by an overall nonsignificant increase in the proportion of β -cells (Fig. 8B). A BrdU⁺/insulin⁻ cell subpopulation was also detected specifically in Pax4-overexpressing islets (Fig. 8A). These cells were also negative for glucagon and

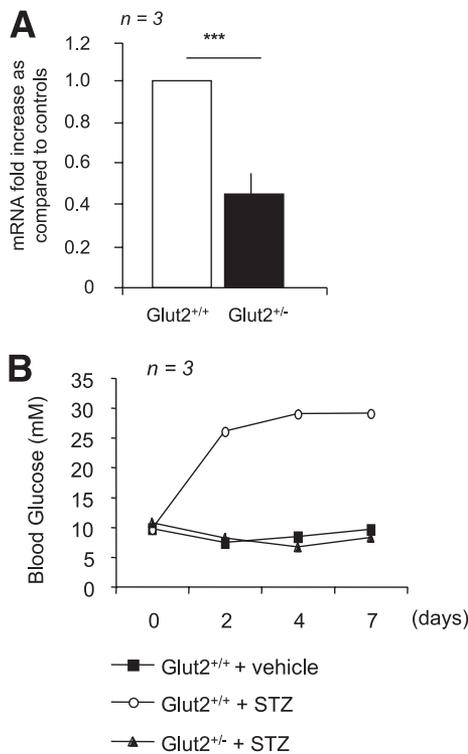


FIG. 6. GLUT2 heterozygous mice are protected against STZ-mediated hyperglycemia. **A:** GLUT2 diabetes mRNA expression levels were measured by Q-RT-PCR in isolated islets from GLUT2^{+/+} and GLUT2^{+/-} mice. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with GLUT2^{+/+} mice and expressed as means \pm SEM from three animals per group. Statistical difference was tested by unpaired *t* test ($***P < 0.001$). **B:** GLUT2^{+/+} and GLUT2^{+/-} male mice were injected with a single dose of STZ (200 mg/kg body wt), and glycemia was monitored for up to 7 days. Data are expressed as means \pm SEM from three animals per group. Statistical difference was tested by the nonparametric Mann-Whitney test.

somatostatin staining. Given that Pax4 overexpression impedes insulin transcription (Fig. 5A), we reasoned that these cells were likely β -cells with low levels of insulin. Therefore, Pdx1 was used as an alternative β -cell marker. Most BrdU⁺ cells were also Pdx1⁺, and the total percentage of these cells was equivalent to the sum of the insulin⁺ and insulin⁻ cell subpopulations that were BrdU⁺ (compare Fig. 8A and C). Despite a significant increase in overall proliferation (Fig. 8C), we did not detect an increase in the total population of Pdx1⁺ cells (Fig. 8D) or an increase in islet mass due to a low number of proliferating cells. Consistent with increased long-term cell replication, *c-myc* and *Cdk4* but not *Cdk2* or *-6* were increased in Pax4-overexpressing islets (Fig. 8E).

DISCUSSION

Our study highlights the physiological role of Pax4 *in vivo* as a regulator of mature islet β -cell survival and proliferation. Pax4 prevented development of hyperglycemia in animals exposed to environmental insults such as repeated STZ challenges and protected islets against cytokine-mediated apoptosis. The diabetes-associated R129W mutation was less efficient in protecting animals against STZ-induced hyperglycemia. The detrimental effect of the mutation was only revealed in the presence of harmful environmental cues highlighting the interplay between genetic determinants and the environment in the development

of hyperglycemia (24). Thus, in response to physiological demand or pathophysiological situations, Pax4 expression may be transiently induced to protect and to compensate β -cell mass, whereas the mutant variant would be unable to achieve this, resulting in the gradual loss of β -cells and ultimately diabetes. The regulation of Pax4 expression by environmental factors provides a likely explanation for the failure of GWAS to identify *Pax4* as a diabetic susceptibility gene as well as for discrepancies in different studies to validate the association of Pax4 polymorphisms with type 2 diabetes in various ethnic populations (7).

One mechanism by which Pax4, but not Pax4R129W, protects β -cells against STZ- and cytokine-induced cell death may, in part, be connected to the regulation of selective target genes of the NF- κ B signaling cascade (25). Indeed, we found that transcript levels of IL-1 β , the predominant cytokine implicated in the activation of the NF- κ B pathway, were decreased in Pax4-expressing islets, whereas they were increased in Pax4R129W islets. Consistent with NF- κ B activation, transcript levels of its target gene *nos2* were selectively higher in mutant animals, whereas levels of the target gene *cox2* were increased in both Pax4 and Pax4R129W. NOS2 is involved in NO production and downstream stimulation of apoptosis (25), whereas the COX2-generated metabolite prostaglandin E₂ was found to be antiapoptotic (26). As IL-1 β was shown to autostimulate expression levels of endogenous IL-1 β (17) and to induce β -cell death in human islets (27), Pax4-mediated inhibition of IL-1 β transcription with a concomitant increase in COX2 levels may confer increased resistance to apoptosis. In contrast, elevated basal levels of IL-1 β and NOS2 in Pax4R129W-overexpressing mice may sensitize islet cells to NO-induced apoptosis. Furthermore, because NO can also be released from islets exposed to STZ (28), we suggest that Pax4R129W mice are more prone to cell death than GLUT2 heterozygous mice because of an increased predisposition to NO-mediated apoptosis. Interestingly, the levels of the IL-1 β antagonist, IL-1Ra, were also increased in mutant islets, suggesting restoration of an IL-1 β -to-IL-1Ra ratio that prevents activation of the apoptotic program (29). Consistent with this model, several putative Pax4 binding sites were predicted in both the *il-1 β* and *nos2* gene promoters.

Despite differences in IL-1 β levels, both Pax4- and Pax4R129W-expressing islets exhibited similar levels of NO production upon exposure to cytokines. This indicates that the NF- κ B signaling pathway can still be fully activated upon strong exogenous stimulation and that the protection conveyed by Pax4 is relayed through additional gene product that blocks the NF- κ B apoptotic pathway. One such candidate gene could be *Bcl-2*, which regulates the mitochondrial response to proapoptotic signals, preventing the release of cytochrome C and the subsequent activation of the cell death program (30). Indeed, we found that Pax4- but not Pax4R129W-overexpressing islets exhibited increased expression of *Bcl-2* with blunted cytokine-mediated cytochrome C translocation. Corroborating our results, *Bcl-2* overexpression was shown to protect mouse and human islets against cytokine-induced apoptosis (31,32). In contrast to our previous study in rat islets overexpressing Pax4 (11), the increased expression of *Bcl-xL* in mouse islets was not detected, possibly as a result of species differences.

Overexpression of either Pax4 or Pax4R129W markedly perturbed expression of MafA and two of its target genes, *glut2* and *insulin*, which are key markers of mature

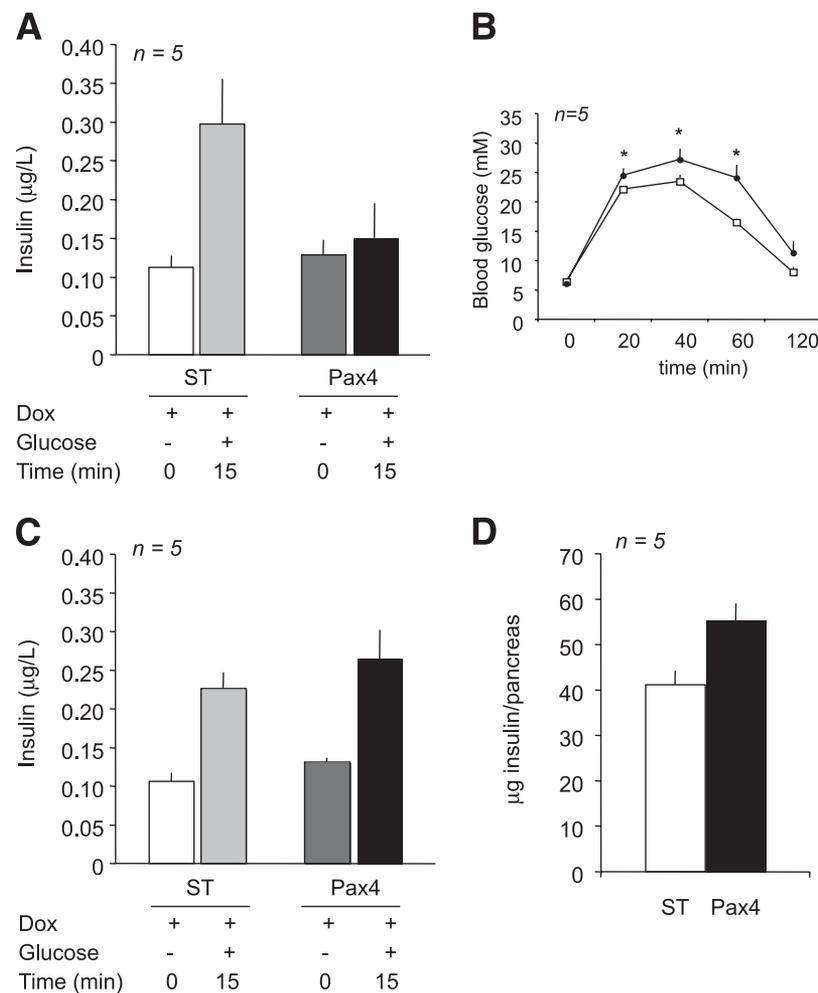


FIG. 7. Transient overexpression of Pax4 improves glucose tolerance and total pancreatic insulin content after 4 months. **A:** Plasma insulin levels, measured 15 min after an injection of 2 g glucose/kg body wt in Pax4-overexpressing animals and in ST animals that were maintained on doxycycline (Dox) for 4 months. **B:** Glucose tolerance tests were performed in the same animals (Pax4-overexpressing [●] and ST [□] animals that were maintained on doxycycline for 4 months). **C:** After doxycycline treatment was abolished for 1 month, plasma insulin levels were reevaluated as in **A**. **D:** Total pancreatic insulin content was measured as indicated in RESEARCH DESIGN AND METHODS in Pax4 animals that received doxycycline for 4 months and then were not treated for 1 month. Data are expressed as means \pm SEM from five animals per group. Statistical difference was tested by *t* test (* $P < 0.05$).

β -cells. Although both Pax4 proteins suppressed the activity of a luciferase construct harboring the MafA gene promoter region 3, we could not detect Pax4 binding to a putative binding site within this region. Preliminary ChIP assay results revealed no convincing interaction of Pax4 with region 3, suggesting an indirect effect of Pax4 on MafA expression. However, we have previously demonstrated that Pax4 impairs glucagon and insulin gene transcriptions through direct protein interaction with Pax6 (22). Given that Pax6 was recently shown to bind to the *mafa* gene promoter and to regulate transcription (33), we speculate that Pax4 may be inhibiting MafA expression by a mechanism similar to the one delineated for glucagon and insulin. Inhibition through protein-protein interaction, and not direct DNA binding activity, would explain the inhibitory effect observed with the weaker DNA-interacting mutant variant.

Long-term overexpression of Pax4 resulted in a blunted glucose-induced rise in plasma insulin levels—an effect reversible upon inhibition of Pax4. A recent study also reported impaired glucose-induced insulin secretion in an animal model in which Pax4 was constitutively

overexpressed in α -cells. These cells were converted to β -cells that remained functional for up to 4 weeks before an unexpected decline in insulin secretion occurred. Animals ultimately died of hyperglycemia (8). We would therefore like to propose that Pax4-dependent impaired insulin secretion is mediated by the long-term repression of the mature β -cell marker MafA and insulin.

Aberrant expression of *Pax* genes were shown to convert cells to a less differentiated state and to promote self-renewal and increasing survival (34). Consistent with this premise, a subpopulation of replicating Pdx1⁺/insulin⁻ cells was identified in Pax4-overexpressing islets. These cells were also negative for both glucagon and somatostatin, suggesting that they likely represent insulin-deprived β -cells resulting from repression of MafA and insulin transcription by Pax4. A similar β -cell subpopulation was recently characterized in vitro (35). These cells displayed impaired glucose-induced insulin secretion and were prone to proliferation in response to activin A (36). More importantly, MafA and GLUT2 transcript levels were lower in this subpopulation, whereas Pax4 levels were higher. Taken together, these results suggest that Pax4 under physiological

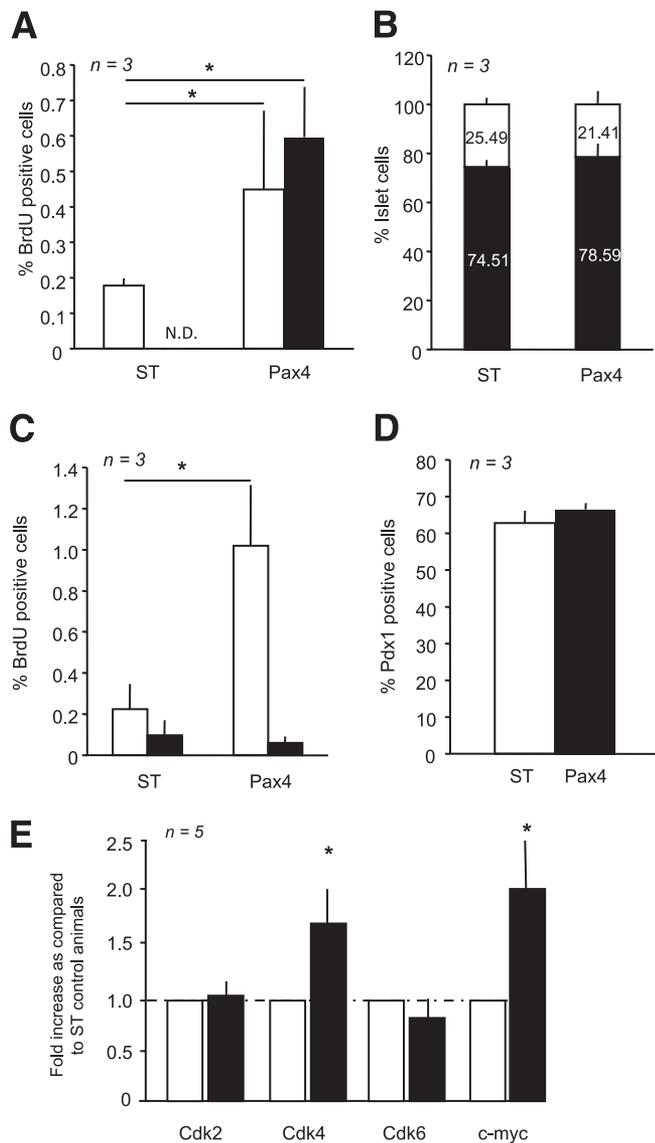


FIG. 8. Long-term expression of Pax4 increases β -cell proliferation. **A:** Pancreatic sections from 3 months' Pax4-overexpressing animals were coimmunostained for BrdU and insulin. Approximately 8,000 β -cells were counted from five different pancreatic sections separated 50 μ m from each other. \square , BrdU⁺/insulin⁺; \blacksquare , BrdU⁺/insulin⁻. ND, not detected. **B:** The proportion of insulin⁻ (\square) and insulin⁺ (\blacksquare) cells was counted on the slides from **A**. **C:** Pancreatic sections from 3 months' Pax4-overexpressing animals were coimmunostained for BrdU and Pdx-1. Cell counting was performed as in **A**. \square , BrdU⁺/Pdx-1⁺; \blacksquare , BrdU⁺/Pdx-1⁻. **D:** Total number of Pdx-1⁺ cells was counted on the slides from **B**. \square , BrdU⁺/Pdx-1⁺; \blacksquare , BrdU⁺/Pdx-1⁻. **E:** Transcript levels of proliferative genes were assessed by Q-RT-PCR on freshly isolated islets from Pax4-overexpressing animals (\blacksquare). Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals (\square) that received doxycycline (dashed line) and are expressed as means \pm SEM from five animals per group. Statistical difference was tested by *t* test (**P* < 0.05).

conditions is most likely expressed in a subpopulation of β -like cells prone to proliferation. Substantiating this hypothesis, Cdk4 and c-myc transcript levels were increased in Pax4-overexpressing islets. Adenovirus-mediated Cdk4 expression was shown to increase mouse islet cell proliferation (37), whereas its repression causes insulin deficiency and hyperglycemia (38). Similarly, β -cell-specific overexpression of c-myc caused downregulation of insulin

gene expression with a concomitant increase in proliferation and apoptosis (39). That a fraction of islet β -cells responds to proliferation would provide a mechanism by which a pool of mature cells can rapidly undergo regulated replication to compensate for lost cells while quiescent cells retain the ability to regulate blood glucose.

In summary, *in vivo* overexpression of Pax4 reveals an important role of the transcription factor in islet β -cell proliferation and survival. The detrimental impact of Pax4 mutations is only revealed upon environmental stresses, which reinforces the critical role of gene-environment interaction in the development of diabetes.

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