Original Article

Nkx2.2 Regulates β-Cell Function in the Mature Islet

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Nkx2.2 is a homeodomain transcription factor that is critical for pancreatic endocrine cell specification and differentiation in the developing mouse embryo. The purpose of this study was to determine whether Nkx2.2 is also required for the maintenance and function of the mature β-cell in the postnatal islet. We have demonstrated previously that a repressor derivative of Nkx2.2 can functionally substitute for endogenous Nkx2.2 to fully restore α- and immature β-cells in the embryonic islet; however, Nkx2.2 activator functions appear to be required to form a functional β-cell. In this study, we have created transgenic mouse lines to express the Nkx2.2-repressor derivative in the mature β-cell in the presence of endogenous Nkx2.2. The transgenic mice were assessed for β-cell function, overall islet structure, and expression of β-cell–specific markers. Using this transgenic approach, we have determined that the Nkx2.2-repressor derivative disrupts endogenous Nkx2.2 expression in adult mice and causes downregulation of the mature β-cell factors, MafA and Glut2. Consistently, the Nkx2.2-repressor mice display reduced insulin gene expression and pancreatic insulin content and impaired insulin secretion. At weaning, the male Nkx2.2-repressor mice are overtly diabetic and all Nkx2.2-repressor transgenic mice exhibit glucose intolerance. Furthermore, the loss of β-cell function in the Nkx2.2-repressor transgenic mice is associated with disrupted islet architecture. These studies indicate a previously undiscovered role for Nkx2.2 in the maintenance of mature β-cell function and the formation of normal islet structure. *Diabetes* 56:1999–2007, 2007

Over the past several years, numerous transcription factors essential for islet cell development and differentiation have been identified and characterized (reviewed in ref. 1). Several of these transcription factors have also been found to be required for the regulation of β-cell function in adults. Furthermore, five of the six genes that contribute to the etiology of maturity-onset diabetes of the young encode transcription factors, including Pdx1 (2–4) and NeuroD (5). There are many additional transcription factors that are essential for pancreas development, islet cell differentiation, and proper β-cell function that have yet to be linked to diabetes in humans including Ngn3, Pax4, Pax6, Nkx6.1, and Nkx2.2 (6). Each of these transcription factors plays a unique role in the development of the islet; however, very little is known about their functions in the mature β-cell.

Nkx2.2 is a homeodomain transcription factor that is essential for the differentiation of islet α- and β-cells. Nkx2.2 is expressed early in pancreatic progenitors beginning at embryonic day 9.5 (7) and is ultimately restricted to β-cells and a subset of α-cells and PP cells (8). In Nkx2.2 null mice, β-cells and the majority of α-cells fail to differentiate and are replaced by ghrelin-producing cells (8,9). Expression of Nkx2.2 is maintained in mature β-cells in the adult; however, because Nkx2.2 knockout mice die shortly after birth, the role of Nkx2.2 in mature β-cell function has not been uncovered.

Although Nkx2.2 is critical for the specification and differentiation of β-cells, the molecular aspects of this function are just beginning to be elucidated. In a previous study, we used an Nkx2.2-derivative approach in transgenic mice to test whether dominant Nkx2.2-activator (Nkx2.2hdVP16) or -repressor (Nkx2.2hdEnR) derivatives could substitute for endogenous Nkx2.2 activity in the developing embryonic islet (10,11). These studies demonstrated that Nkx2.2 predominantly functions as a transcriptional repressor during endocrine cell differentiation; the Nkx2.2hdEnR derivative is able to rescue the specification of α- and β-cells during embryogenesis in the absence of endogenous Nkx2.2. We also determined that although the repressor form of Nkx2.2 was able to functionally substitute for endogenous Nkx2.2 in early β-cell formation, mature β-cells were not fully restored and the Nkx2.2$^{-/-}$; Nkx2.2hdEnR mice died perinatally.

Based on these findings, we propose that Nkx2.2 functions normally as a repressor during embryonic pancreas development but may function as an activator in mature β-cells. If this hypothesis is correct, a dominant Nkx2.2hdEnR transgene expressed at physiological levels may disrupt endogenous Nkx2.2 function in the adult islet. To test this idea, we maintained the Nkx2.2hdEnR (repressor) transgene on an Nkx2.2 wild-type background and assessed β-cell function in the adult islet. This analysis revealed that in postnatal Nkx2.2-repressor mice there is a downregulation of endogenous *Nkx2.2* and *MafA*, two genes that have been proposed to be activated by Nkx2.2 (12,13). As a consequence of these gene expression changes, the Nkx2.2 transgenic mice exhibit reduced insulin production and impaired glucose-stimulated insulin secretion, implicating Nkx2.2 as a critical regulator of β-cell function in the adult. Furthermore, these studies demonstrated that the Nkx2.2 repressor disrupts islet morphology, suggesting an additional role for Nkx2.2 in the establishment of normal islet architecture in the mouse.

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RESEARCH DESIGN AND METHODS

Mice were maintained on a Swiss black (Taconic) background and housed in the University of Colorado Health Sciences Center animal resource center. The mice were fed standard rodent chow, and all animal procedures were performed according to University of Colorado Health Sciences Center institutional review board approval protocols. Pdx1:Nkx2.2hdEnR, Pdx1:Nkx2.2hdVP16, and Pdx1:Nkx2.2hd control transgenic mice were generated and genotyped as described previously (10).

Tolerance tests and insulin enzyme-linked immunosorbent assays. Mice were fasted for 14 h overnight, weighed, and injected intraperitoneally with 1 mg/g body weight glucose (45% solution; Sigma), 0.75 mU/g human insulin (Lilly), or 1 mg/g L-arginine (Sigma). Blood glucose concentrations were determined from tail vein blood samples using a Lifescan One Touch glucometer at 0, 15, 30, 60, 90, 120, and 180 min postinjection. Samples were collected in heparinized capillary tubes from the tail vein at 0, 5, 15, and 30 min after glucose or arginine injection to measure plasma insulin concentrations. To measure total pancreatic insulin content, the pancreas was harvested, and protein was extracted with acid ethanol and measured using the Bio-Rad DC Protein assay. The total pancreatic insulin content was normalized to total protein input. Total pancreatic and plasma insulin levels were measured using the Ultrasensitive rat insulin enzyme-linked immunosorbent assay kit with the mouse insulin standard (Crystal Chem).

Immunohistochemistry. Whole pancreata were harvested in 1× PBS and fixed overnight in 4% paraformaldehyde for frozen embedding (embryonic day 18.5) or 10% buffered formalin for paraffin processing (adult). Primary antibodies used were rabbit anti-glucagon (1:200; Phoenix Pharmaceuticals), rabbit anti-somatostatin (1:200; Phoenix), guinea pig anti-insulin (1:1,000; Linco Research), rabbit anti-Glut2 (1:100; B. Thorens, Lausanne, Switzerland), rabbit anti-Pdx1 (1:1,000; Chemicon), and rabbit anti-MafA (1:1,000; Bethyl Laboratories). Secondary antibodies used were conjugated to Texas red, Cy2, and Alexa 488 or 594 (1:800; Jackson ImmunoResearch and Molecular Probes). Images were obtained with a Leica DM5000 microscope and an Evolution MP color camera and ImagePro software from Media Cybernetics. Confocal images were obtained and processed on a Zeiss LSM510-Meta microscope.

Morphometrics. Every 10th 5-μm section from three wild-type littermates and three transgenic mice was collected and stained for insulin. The total number of islets on each section was counted, and total islet area was determined as described previously using ImagePro software (14). The area of endocrine cells (α-cells, β-cells, δ-cells, and PP cells) was determined per islet area.

Western blots. Nuclear extracts from pancreatic tissue were prepared as described previously (15), and 100 μg of total protein per lane was loaded. Antibodies used were mouse anti-engrailed (1:100; DSHB) or mouse anti-myc (1:600; Sigma) and horseradish peroxidase–conjugated anti-mouse (1:10,000; Jackson ImmunoResearch). Blots were developed using chemiluminescence (Pierce).

Quantitative real-time PCR. Total RNA was extracted from whole pancreas or isolated islets from 2- to 3-month-old mice and prepared using a Qiagen RNeasy kit. cDNAs were prepared with oligo(dT) primers and Superscript III (Invitrogen). Real-time PCR was performed using TaqMan probes for Nkx2.2 (probe: FAM-CCATTGACTCTGCC CCATCGCTCT-MGB; forward primer: CCT CCCCGAGTGGCAGAT; and reverse primer: GAGTTCTATCCTCTC CAAAA GTTCAAA), Nkx2.2hdEnR transgene (probe: FAM-CAGGGCCCCGGGCGA-NFQ; forward primer: GGCCGAGAAAGGTATGAATTCG A; and reverse primer: GGGC TGCAGCGATCCT), MafA (probe: CGGCGCACGCTCAAG AAC CG; forward primer: CATCCGACTGAAACAGAAG; and reverse primer: CTC GCTC CAGAATG), Pdx1, Nkx6.1, NeuroD, Glut2, Gck, Myt1, insulin, glucagon, and Gapdh (ABI Assays on Demand) on the ABI 7000 real-time PCR machine. A standard curve was prepared for each probe/primer set, and samples were normalized to Gapdh and quantified with ABI Prism software. Relative levels of expression were compared using the standard curve method (ABI Prism 7700 User Bulletin 2).

Statistical analysis. All values are expressed as means ± SEM. Statistical analysis was performed with a two-tailed Student's unpaired *t* test. For sample sizes <5, all *P* values were confirmed with a nonparametric Mann-Whitney test. Results were considered significant when $P < 0.05$.

RESULTS

Characterization of adult Pdx1:Nkx2.2hdEnR transgenic mice. We generated transgenic mice expressing the Nkx2.2-repressor derivative (Nkx2.2hd-EnR^{myc}) (11) under control of the Pdx1 promoter to characterize the molecular function of Nkx2.2 in islet cell specification (10). We established two stable transgenic lines (7414 and 7660) that accurately express the Nkx2.2hd-EnR^{myc} fusion protein in the islet and can partially rescue embryonic islet cell specification and differentiation in the absence of endogenous Nkx2.2 (10). In the adult islet, Pdx1 is also highly expressed in β-cells, allowing us to assess the effect of the Nkx2.2-repressor in the mature β-cell. In the presence of endogenous Nkx2.2, mice derived from both Nkx2.2-repressor lines survive for a normal lifespan and are fertile with weight and size comparable to those of transgene-negative littermates (hereafter referred to as wild-type littermates). Furthermore, in all assays tested, both lines of Nkx2.2-repressor mice display similar phenotypes. In adult pancreata from each transgenic line, a 50-kDa fusion protein can be detected by Western blot analysis using antibodies directed against either Engrailed or the Myc epitope tag; line 7414 expresses the transgene at higher levels than line 7660 (Fig. 1*C* and data not shown). To determine the relative expression levels of the Nkx2.2-repressor transgenes compared with those of endogenous Nkx2.2 in adult mice, we performed quantitative real-time PCR on mRNA extracted from adult pancreas. The expression level of the Nkx2.2hdEnR transgenic males is significantly lower than levels of endogenous Nkx2.2 (Fig. 1*E*), suggesting that the transgenes are expressed at levels that should not lead to off-target or non–Nkx2.2-related phenotypes (see below and DISCUSSION).

We generated two additional founder males (7546 and 7566), which give rise to pups that express the transgene; however, these pups are growth retarded, appear dehydrated, and do not survive longer than 10 days postnatally. As seen in Fig. 1, these two males express the Nkx2.2-repressor fusion protein at higher levels than those for line 7414, and it appears that higher transgene expression may be contributing to the early lethality of the 7546 and 7566 offspring. We have also established two additional transgenic lines (5631 and 5635) that express a Nkx2.2 homeodomain myc tag fusion protein (Pdx1:Nkx2.2hd^{myc}) (Fig. 1) to demonstrate that the Nkx2.2 homeodomain derivatives do not function as dominant negative proteins (10). These mice are phenotypically normal in all assays tested (Figs. 2, 5, and 6 and data not shown).

Male Nkx2.2-repressor transgenic mice are hyperglycemic and glucose intolerant. To determine whether the Nkx2.2-repressor protein had effects on mature β-cell function, blood glucose levels were measured in fed Nkx2.2-repressor mice. Before weaning, the mice did not exhibit any overt phenotypes. By 6 weeks of age, male Nkx2.2-repressor transgenic mice exhibited severely elevated blood glucose concentrations compared with wild-type mice; whereas female Nkx2.2-repressor mice, as well as Nkx2.2hd^{myc} control and Nkx2.2-activator mice (10) did not exhibit elevated blood glucose concentrations (Table 1). Furthermore, the female mice did not become hyperglycemic with age (data not shown). To more accurately assess the degree of pancreatic dysfunction in the Nkx2.2-repressor adult mice, we performed an intraperitoneal glucose tolerance test in fasted mice aged between 2 and 3 months. Fasting blood glucose levels were significantly elevated in male transgenic mice at all ages tested (2–6 months) (Table 1). Upon glucose challenge, male transgenic mice exhibit significantly greater blood glucose concentrations at each time point tested compared with the wild-type controls (Fig. 2*A*). The impaired glucose tolerance worsened as the Nkx2.2-repressor transgenic mice aged; blood glucose concentrations peaked at 34

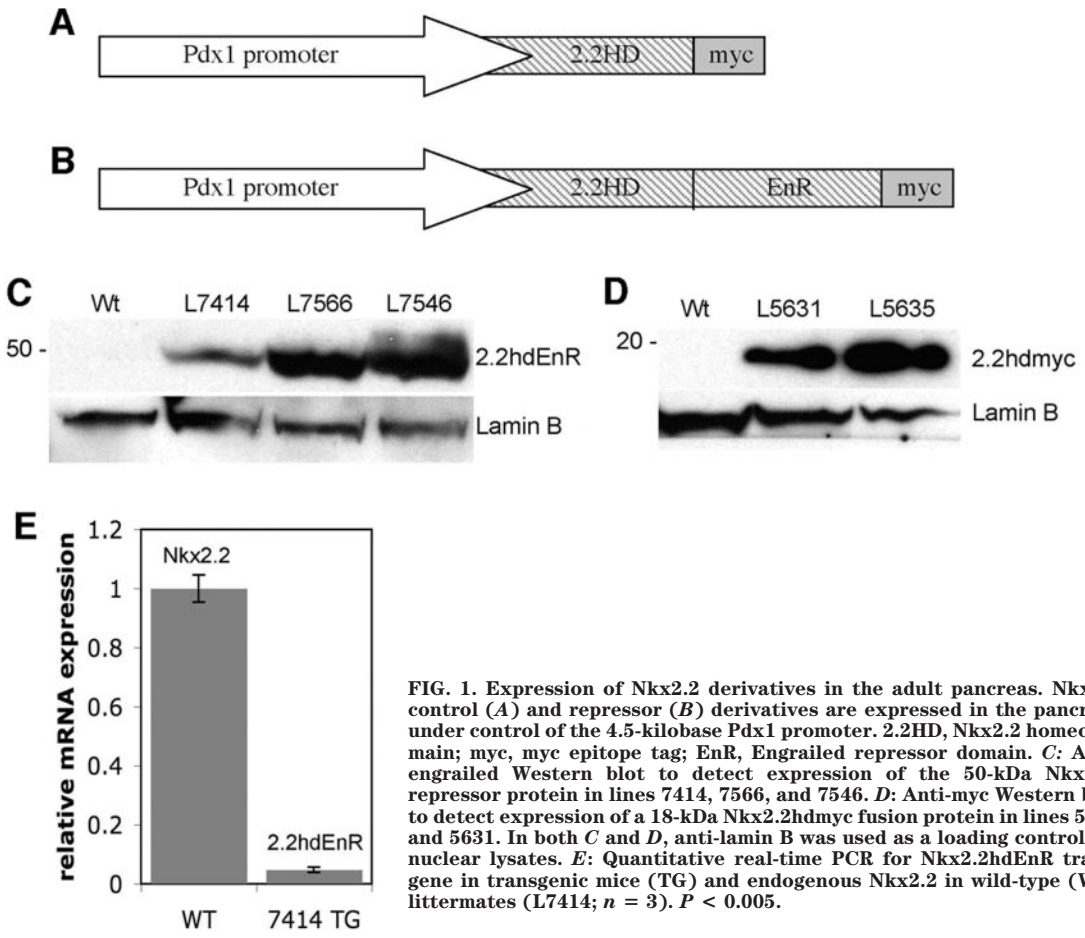

FIG. 1. Expression of Nkx2.2 derivatives in the adult pancreas. Nkx2.2 control (*A*) and repressor (*B*) derivatives are expressed in the pancreas under control of the 4.5-kilobase Pdx1 promoter. 2.2HD, Nkx2.2 homeodomain; myc, myc epitope tag; EnR, Engrailed repressor domain. *C*: Anti-engrailed Western blot to detect expression of the 50-kDa Nkx2.2-repressor protein in lines 7414, 7566, and 7546. *D*: Anti-myc Western blot to detect expression of a 18-kDa Nkx2.2hdmyc fusion protein in lines 5635 and 5631. In both *C* and *D*, anti-lamin B was used as a loading control for nuclear lysates. *E*: Quantitative real-time PCR for Nkx2.2hdEnR transgene in transgenic mice (TG) and endogenous Nkx2.2 in wild-type (WT) littermates (L7414; $n = 3$). $P < 0.005$.

mmol/l at 30 min in male transgenic mice aged 5–6 months (data not shown). Female transgenic mice do not have significantly elevated fasting blood glucose levels compared with wild-type female controls but do show significantly increased glucose levels at 15 min after a glucose challenge (Fig. 2*B*). Unlike in the Nkx2.2-repressor males, the ability to clear glucose does not worsen with age in female mice. Additionally, neither Nkx2.2hd^{myc} control mice nor Nkx2.2-activator mice demonstrate impaired glucose tolerance (Fig. 2*A*).

Nkx2.2-repressor transgenic mice have reduced pancreatic insulin content and impaired insulin secretion. Nkx2.2 has been implicated in direct regulation of insulin transcription (16). To determine whether the glucose intolerance and diabetic phenotypes observed in the Nkx2.2-repressor transgenic mice were associated with a loss of insulin gene transcription, we assessed insulin mRNA levels in the Nkx2.2-repressor islets using quantitative real-time PCR analysis. These studies demonstrate that insulin mRNA is moderately decreased in Nkx2.2-repressor islets compared with islets from wild-type littermates (Fig. 2*D*). In addition, the total pancreatic insulin content was reduced ~2-fold in Nkx2.2-repressor mice compared with wild-type littermates (Table 2). To determine whether the male Nkx2.2-repressor mice also exhibited defects in insulin secretion, we assessed their plasma insulin levels in response to an intraperitoneal glucose tolerance test. The Nkx2.2-repressor transgenic mice were unable to mount an insulin secretory response upon glucose challenge (Fig. 2*C*). Female Nkx2.2-repressor mice also had impaired insulin secretion in response to glucose stimuli (data not shown).

To determine whether the failure of male Nkx2.2-repressor mice to secrete insulin was due to impaired insulin release, we tested the Nkx2.2-repressor mice's responsiveness to arginine, a secretogogue that promotes insulin secretion via mechanisms independent of glucose metabolism (18). Upon arginine stimulation, male transgenic animals have impaired glucose clearance and significantly reduced insulin secretion compared with wild-type littermate controls (Fig. 2*E* and *F*). These results suggest that the β-cells of the Nkx2.2-repressor mice appear to have multiple defects in the insulin secretion pathway.

Nkx2.2-repressor transgenic mice are not insulin resistant. Although peripheral insulin resistance would not be expected in the Nkx2.2-repressor mice because the Nkx2.2hdEnR transgene is expressed specifically in β-cells, we wanted to eliminate the possibility that insulin resistance was contributing to the observed glucose intolerance. We examined insulin sensitivity directly by measuring blood glucose levels of the diabetic male and female Nkx2.2-repressor mice in response to an insulin challenge. Although the fasting blood glucose concentrations begin and end much higher for the male transgenic animals, the rates of glucose disposal in male and female Nkx2.2-repressor mice are very similar to those in wild-type mice (Fig. 3). These results indicate that the male and female Nkx2.2-repressor mice were not significantly insulin resistant, despite the males having abnormally high glucose levels.

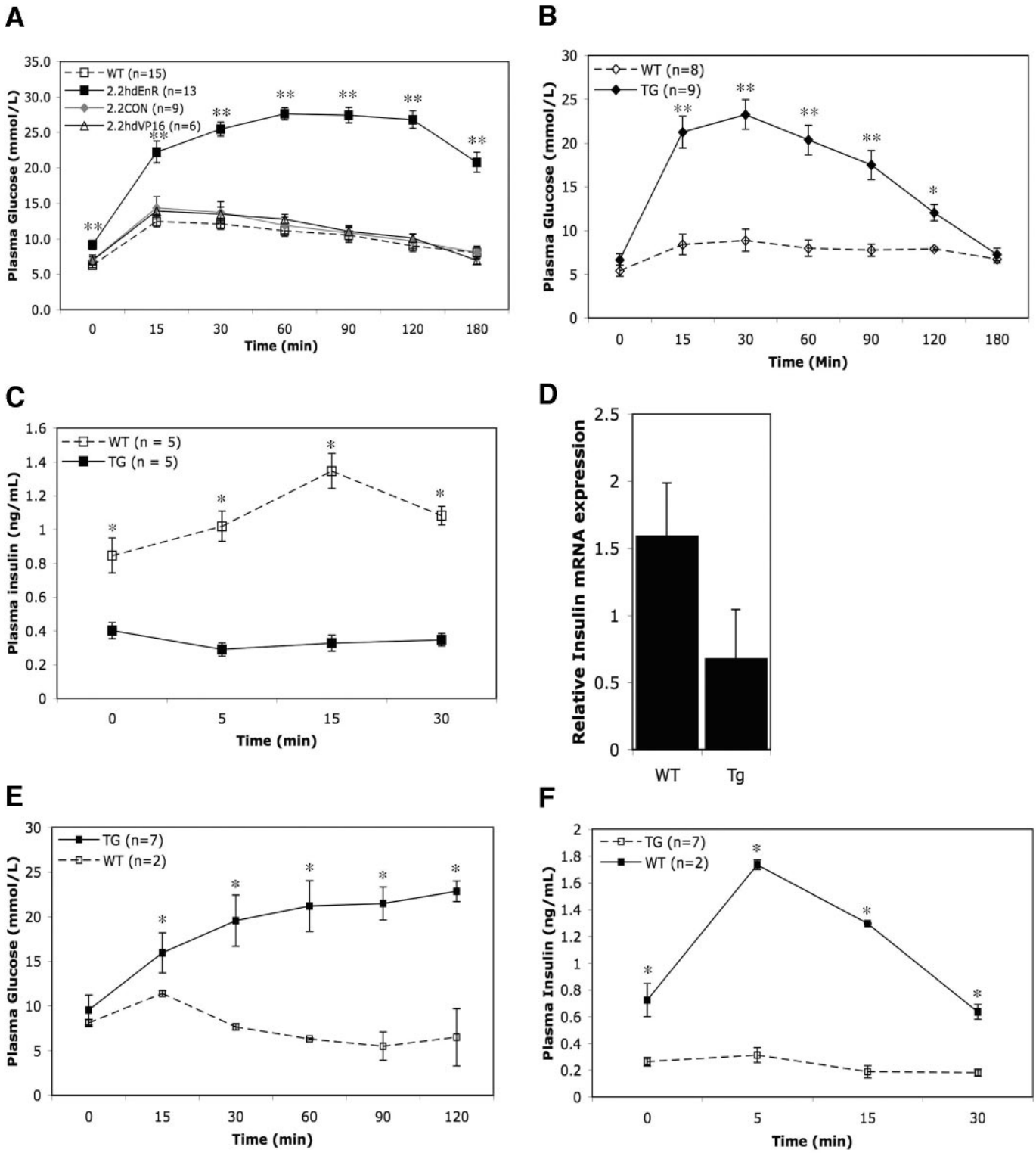

FIG. 2. Nkx2.2-repressor transgenic mice are glucose intolerant with impaired insulin secretion. Two- to 3-month-old mice were fasted overnight and injected with glucose (*A*, *B*, and *C*) or arginine (*E* and *F*) at *t* = 0. Blood glucose levels were measured and tail vein blood collected for insulin enzyme-linked immunosorbent assays. Plasma glucose or plasma insulin concentrations are indicated. *A*, *C*, *E*, and *F*: Male mice: □, wild-type (WT); ■, Nkx2.2-repressor transgenic (TG) (L7414); ◇, control transgenic (L5635 and L5631); △, Nkx2.2-activator transgenic (L7319 and L7318). *B*: Female mice: ◇, wild-type; ◆, transgenic (L7414). **P* < 0.005; ** *P* < 0.0001. *D*: Quantitative real-time PCR on RNA isolated from whole pancreata for insulin mRNA indicated insulin mRNA is decreased in Nkx2.2-repressor transgenic mice.

Nkx2.2 repressor interferes with the expression of endogenous Nkx2.2 and its downstream target, MafA. To begin to elucidate the underlying molecular mechanisms that are disrupted by expression of the Nkx2.2-repressor transgene in the β-cell, we analyzed the expression status of genes thought to be activated by Nkx2.2. Although Nkx2.2 appears to function as a repressor during embryonic islet and spinal cord development (10,11), additional studies have suggested that Nkx2.2 activates its own promoter and the *MafA* gene in mature

TABLE 1
Fed and fasting blood glucose concentrations of Nkx2.2 transgenic mice

	Blood glucose concentrations (mmol/l)	
	Fed	Fasted
Males		
Nkx2.2hdEnR transgenic	18.2 ± 0.9 (24)*	9.5 ± 0.8 (15)†
Wild type	8.4 ± 0.3 (23)	6.1 ± 0.4 (11)
Nkx2.2hd-control transgenic	9.4 ± 0.5 (15)	7.0 ± 0.7 (9)
Nkx2.2.hdVP16 transgenic	9.1 ± 0.5 (12)	6.9 ± 0.5 (6)
Females		
Nkx2.2hdEnR transgenic	8.5 ± 0.4 (20)	5.6 ± 0.6 (9)
Wild type	8.4 ± 0.7 (6)	6.6 ± 0.5 (13)

Data are means ± SEM (n). Blood glucose concentrations were measured between 4 weeks and 24 weeks of age and are shown as the average of all animals ±SEM. A minimum of two readings for each animal was taken. *$P < 0.005$; †$P < 0.0001$.

β-cells (12,13). To assess the regulatory effect of the dominant Nkx2.2 repressor on these two putative Nkx2.2 targets, we used real-time PCR to quantify endogenous Nkx2.2 and MafA mRNA levels in isolated islets. Interestingly, both endogenous Nkx2.2 and MafA are significantly downregulated in the presence of the Nkx2.2-repressor transgene (Fig. 4). This indicates that, even at low expression levels, the Nkx2.2-repressor transgene is able to dominantly repress Nkx2.2 in in vivo targets in the presence of endogenous Nkx2.2. Furthermore, it is likely that the loss of endogenous Nkx2.2 is contributing to the hyperglycemia and impaired GSIS phenotypes. The Nkx2.2-repressor protein does not appear to be nonspecifically affecting targets of the closely related pancreas transcription factor Nkx6.1, as the expression of known Nkx6.1 targets, including Nkx6.1, glucagon, and Myt1 (19–21) are not significantly altered, as assessed by real-time PCR (Fig. 4).

Functional β-cells fail to form in the Nkx2.2-repressor transgenic mice. Expression of the Nkx2.2-repressor transgene results in the disruption of normal β-cell function in the adult. This phenotype is in marked contrast to that observed in Nkx2.2-null mice, in which α- and β-cells fail to differentiate appropriately, and the mice die at birth, suggesting that we have not merely created a dominant negative Nkx2.2 protein. This observation is also consistent with our previous studies, which provide evidence that Nkx2.2 normally functions as a repressor during islet cell specification, but Nkx2.2-activator functions may be required to allow β-cell maturation and function (10).

TABLE 2
Insulin concentrations of male Nkx2.2-repressor transgenic mice

	Plasma insulin concentrations (ng/ml)		Total pancreatic content (μg/mg)
	Fed	Fasted	
Wild type	1.4 ± 0.2 (8)	0.6 ± 0.09 (12)	5.98 ± 0.9 (8)
Nkx2.2hd-EnR	1.3 ± 0.2 (10)	0.3 ± 0.03 (15)*	3.00 ± 0.4 (8)*

Data are means ± SEM (n). Tail vein blood was collected in fed and fasted mice for plasma insulin concentrations. The pancreas was harvested in fed mice to determine total pancreatic insulin content. Insulin concentrations were determined by insulin enzyme-linked immunosorbent assay. The relative pancreatic content represents the ratio of insulin and total protein concentrations (17). *$P < 0.005$.

These findings suggest that expression of the Nkx2.2 repressor in the presence of endogenous Nkx2.2 would not disrupt islet cell specification in the embryo but may cause later β-cell differentiation defects. To determine when and how the Nkx2.2 repressor might be affecting pancreatic formation and function, we performed histological analysis to assess exocrine and endocrine cell formation in the Nkx2.2-repressor transgenic mice. We did not observe a difference in the size or weight of the whole pancreatic organ, and mRNA and protein levels of the exocrine enzyme amylase are unchanged (data not shown). As shown in Fig. 5, overall islet morphology, size, and number in the Nkx2.2-repressor transgenic mice are also indistinguishable from those in the wild-type littermates and the Nkx2.2hd^{myc} controls. Standard morphometric analysis of adult islets also indicated that there was no change in total islet cell mass of the transgenic animals (data not shown). To determine the status of the individual islet cell subpopulations, we used immunofluorescence to quantify the respective islet cell populations. Unlike the Nkx2.2 null mice, morphometric analysis of the individual endocrine cell types indicated that each of the islet cell populations was present in their expected relative ratios (Fig. 5*F* and *G*; data not shown). Furthermore, there was no significant difference in the numbers of β-cells or the other endocrine cell types in Nkx2.2-repressor mice (Fig. 5 and data not shown). We further assessed the differentiation state of β-cells by looking at four β-cell markers: Pdx1, MafA, Gck, and Glut2. Pdx1 is coexpressed with all insulin-positive cells and Pdx1 mRNA expression is unchanged (Figs. 4 and 6*B*). However, consistent with the observed decrease in MafA mRNA expression, the number of insulin-positive cells that coexpress MafA is significantly reduced (Fig. 6*E*). Additionally, Glut2 mRNA, but not Gck mRNA, is downregulated in the adult islet (Fig. 4). Glut2 protein expression is also significantly reduced in neonatal and adult islets (Fig. 6*G–J*). This indicates that the Nkx2.2-repressor transgene does not interfere with islet cell specification during pancreogenesis but does disrupt the activation of genes such as *MafA* and *Glut2* that are required for terminal β-cell differentiation and/or β-cell function. These results are consistent with our previous finding that Nkx2.2 normally functions as a repressor during islet cell differentiation but may function predominantly as an activator during β-cell maturation and maintenance.

Islet architecture is disrupted in Nkx2.2-repressor transgenic mice. Surprisingly, despite the fact that the individual islet subtypes were present in their normal numbers and ratios, the α-cells, δ-cells, and PP-cells were mislocalized from the periphery of the islet and scattered throughout the islet core in both male and female Nkx2.2-repressor mice (Fig. 5*B*, *C*, *F*, and *G* and data not shown). Islets of adult wild-type littermates and control transgenic mice (Nkx2.2hd^{myc}) have normal islet architecture (Fig. 5*B–D*, *I*, and *J*). To determine whether the disruption of islet architecture was initiated at the onset of islet formation, we examined islet structure in perinatal mice. Even at the earliest time point of islet assembly, the Nkx2.2-repressor mice failed to form characteristic islet organization (Fig. 5*H*). Additionally, we often observed abnormal clustering of glucagon-positive cells in the perinatal transgenic animals (Fig. 5*H*, arrowheads). This abnormal islet architecture is similar to that observed in the adult MafA-null mice, suggesting that the reduction of MafA may be contributing to this phenotype. However, a disruption of

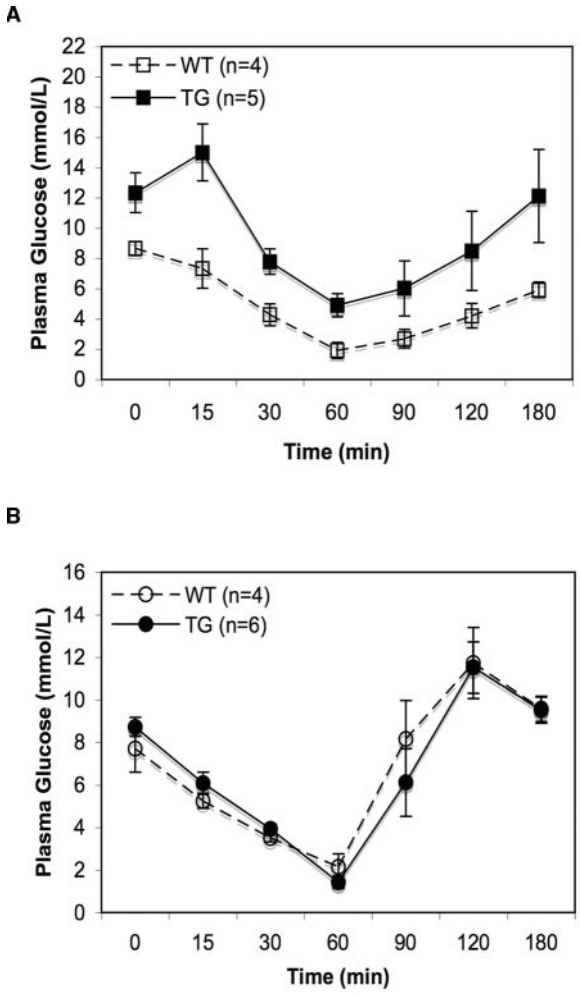

FIG. 3. Neither male nor female Nkx2.2-repressor transgenic mice are insulin resistant. To determine insulin tolerance, 2- to 3-month-old mice were fasted overnight and injected with insulin at $t = 0$ and blood glucose levels measured. Plasma glucose concentrations are indicated. *A*: Male mice: □, wild-type (WT); ■, transgenic (TG) (L7414). *B*: Female mice: ○, wild-type; ●, transgenic (L7414).

islet organization is not observed in the neonatal MafA-null mice, indicating that the Nkx2.2 repressor interferes with organization of islet architecture through additional regulatory pathways.

DISCUSSION

The data presented in this study suggest a critical role for Nkx2.2 in maintaining mature β-cell function and forming correct islet architecture. Consistent with our previous findings that Nkx2.2 functions as a repressor during embryonic islet cell specification (10), embryonic pancreas development and differentiation proceed normally in the Nkx2.2-repressor mice until birth, when the Nkx2.2-repressor derivative interferes with the expression and function of endogenous Nkx2.2, leading to a reduction in MafA, Glut2, and insulin gene expression in mature β-cells. Furthermore, the islets of the Nkx2.2-repressor mice fail to cluster properly, and disrupted islet architecture persists throughout adulthood. In adult Nkx2.2-repressor mice, β-cells display impaired insulin secretion, suggesting that Nkx2.2 is also an important regulator of mature β-cell function. We are currently exploring whether the defects observed in the perinatal islets contribute to the functional defects that are manifest later in the adult β-cell.

In this analysis we used a dominant repressor derivative of Nkx2.2 to assess the function of Nkx2.2 in the mature islet. Although there are limitations associated with this approach and the transgene does not contain regions of the Nkx2.2 protein that may contribute to the specificity of DNA binding, we have been able to demonstrate that the Nkx2.2hdEnR transgene is able to functionally substitute for endogenous Nkx2.2 in the embryo (10). Furthermore, we have determined that the observed adult β-cell phenotypes are not caused by inordinately high levels of transgene expression that could lead to transcriptional squelching effects or misregulation of gene targets of the closely related pancreatic transcription factor Nkx6.1; however, we cannot definitively rule out the possibility that other nonspecific off-target binding of the transgene is occurring. Finally, we have shown that the Nkx2.2 repressor is able to interfere with the expression of two known endogenous Nkx2.2 targets, Nkx2.2 itself and MafA, suggesting that the dominant transgene is functioning to block endogenous Nkx2.2 activity in the adult islet. It is possible that this reduction of Nkx2.2 in the islet is a major contributing factor to the disruption of normal β-cell function.

We observed two β-cell defects related to the expression of the Nkx2.2 repressor: a reduction of insulin content and a defect in insulin secretion. Insulin mRNA levels are moderately reduced, and insulin protein content is decreased in the adult islet of Nkx2.2-repressor mice by ~2- to 3-fold. These findings are consistent with previous in vitro studies, which suggested that Nkx2.2 regulates insulin transcription directly (16); however, we also observed a reduction of MafA, which could contribute to the decrease in insulin gene expression. We did not detect changes in other known regulators of insulin transcription, including Pdx1, NeuroD, and Islet1 (Fig. 4), suggesting that the defect is primarily associated with transcriptional targets of Nkx2.2. Interestingly the decrease in circulating insulin upon glucose challenge in the Nkx2.2-repressor males is much more severe than was expected from the moderate reduction of islet insulin content; therefore, there may be additional defects associated with the insulin secretion pathway. The decreased expression of Glut2 probably contributes to the insulin secretion defects.

Our discovery that the Nkx2.2hdEnR transgene does not disrupt embryonic pancreas development but does interfere with adult β-cell functions strengthens our hypothesis that Nkx2.2 functions normally as a repressor during embryonic development but as an activator in the mature β-cell. This observation is consistent with our previous findings that the Nkx2.2 repressor can replace endogenous Nkx2.2 in the embryonic islet to rescue the immature β-cell population but is not sufficient to fully rescue the mature β-cell. An activator function for Nkx2.2 in the adult islet also supports findings by others that Nkx2.2 directly activates both its own promoter and the recently discovered insulin regulatory protein MafA (12,13). It is not surprising that Nkx2.2 has complex regulatory activities in the development and maintenance of the different islet cell types. The *Drosophila* ortholog of Nkx2.2, vnd, functions as either an activator or repressor, depending on its interaction with regulatory cofactors (22). The identification of Nkx2.2 islet cofactors and additional downstream targets will be instrumental in understanding how Nkx2.2 carries out its essential roles in islet cell development and function.

A novel finding of this study is that the Nkx2.2-repressor

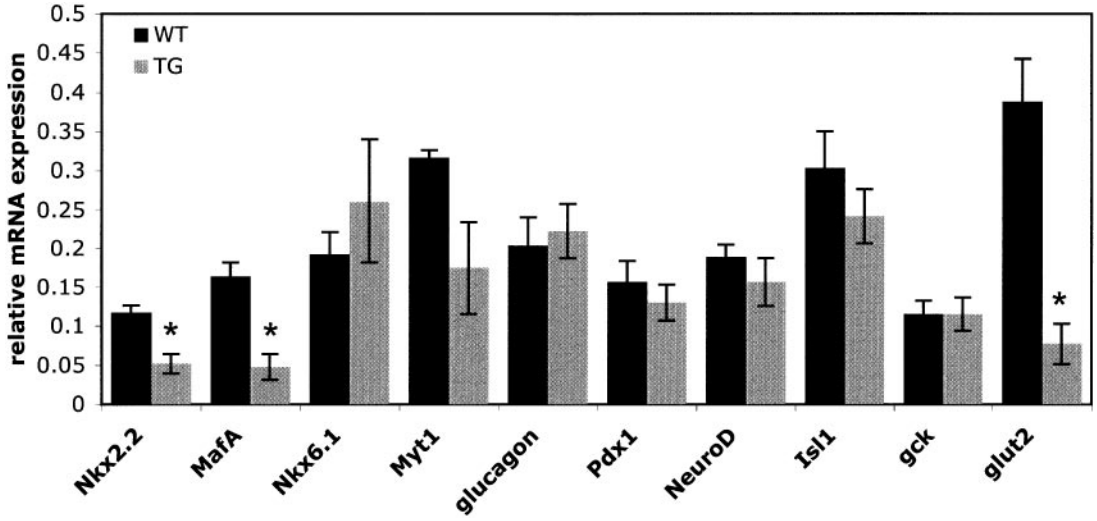

FIG. 4. Real-time PCR demonstrates that Nkx2.2, MafA and Glut2 are downregulated in Nkx2.2hdEnR islets. Real-time PCR for select islet genes was performed using total RNA from isolated islets of wild-type (■) and Nkx2.2-repressor (▨) mice (n = 4 each; islets were pooled from two mice for each sample). Nkx2.2, MafA, and Glut2 are significantly decreased in Nkx2.2-repressor islets. *$P < 0.005$. All other genes are not significantly changed. Error bars represent SEM of four individual samples for each genotype.

mice display altered islet morphology, and this defect is manifest at the onset of islet formation. We have been unable to identify the underlying cause of the altered islet architecture; islet cell ratios are normal, and there are no significant alterations in expression of many of the islet cell adhesion molecules, including the integrins (β1, α3, α5, or α6), cadherins 1 and 2, Ncam, α- and β-catenins, occludin, or members of matrix metalloproteinase family (data not shown). Of note, islet architecture is also disrupted in several other transcription factor mutation models, including MafA, and in each case the contributing cause of this phenotype has yet to be ascertained (4,23–25). At this point, we cannot distinguish whether the defects in β-cell function are due directly to inappropriate Nkx2.2-repressor activity or are a consequence of the disrupted islet architecture.

We have demonstrated that Nkx2.2 functions in the mature islet to influence the formation of the characteristic islet architecture and to maintain normal β-cell functions, including optimal expression of the insulin genes and glucose-stimulated insulin secretion. Interestingly, the β-cell defects observed in the Nkx2.2-repressor transgenic

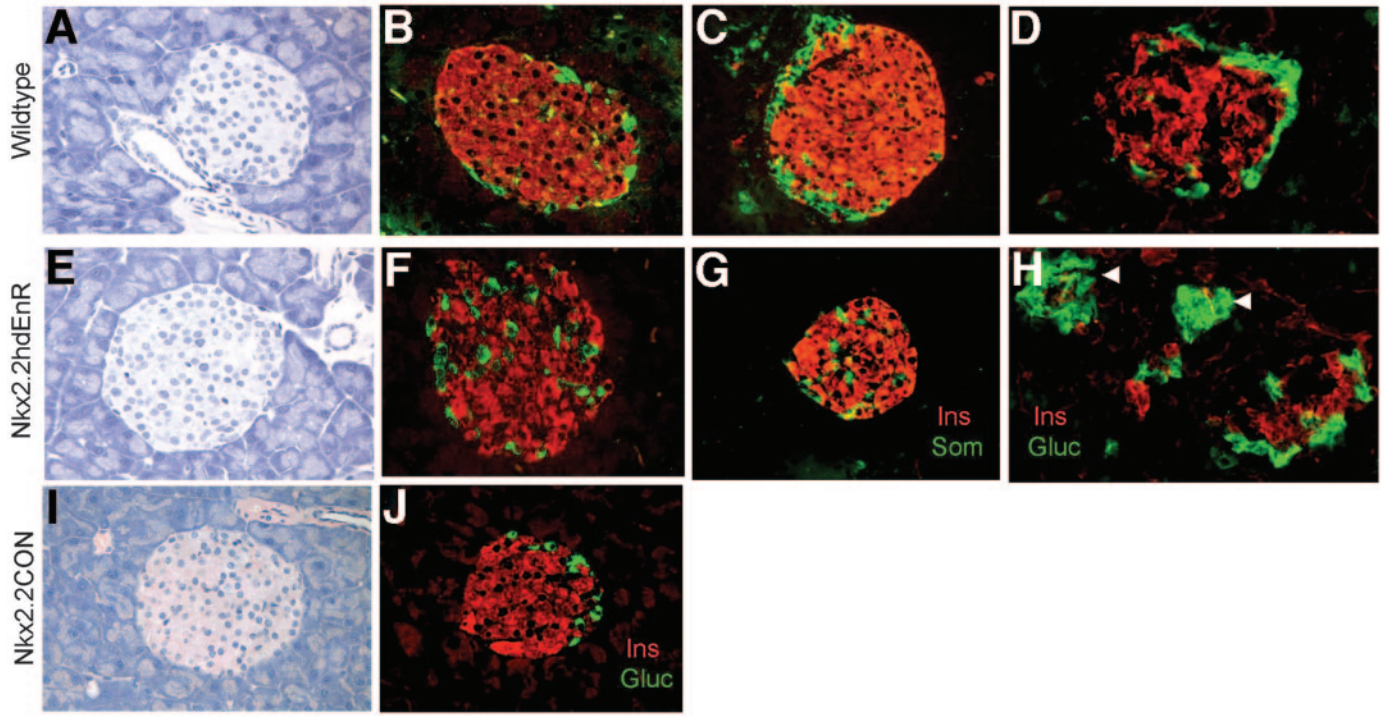

FIG. 5. Islet architecture is disrupted in Nkx2.2-repressor transgenic mice. Hematoxylin and eosin staining of wild-type (*A*), Nkx2.2-repressor (L7414) (*E*) or Nkx2.2 control (L5635) (*I*) islets demonstrates that transgenic islets are of normal size and morphology. Hormone immunostaining for insulin (red in all panels) and glucagon (green in *B*, *D*, *F*, *H*, and *J*) or somatostatin (green in *C* and *G*) show that islet organization is disrupted in the Nkx2.2-repressor mice (*F*, *G*, and *H*) in adult islets (*B*, *C*, *F*, *G*, and *J*) and in embryonic day 18.5 islets (*D* and H) compared with wild-type (*B–D*) or Nkx2.2-control islets (*J*). Magnification ×20.

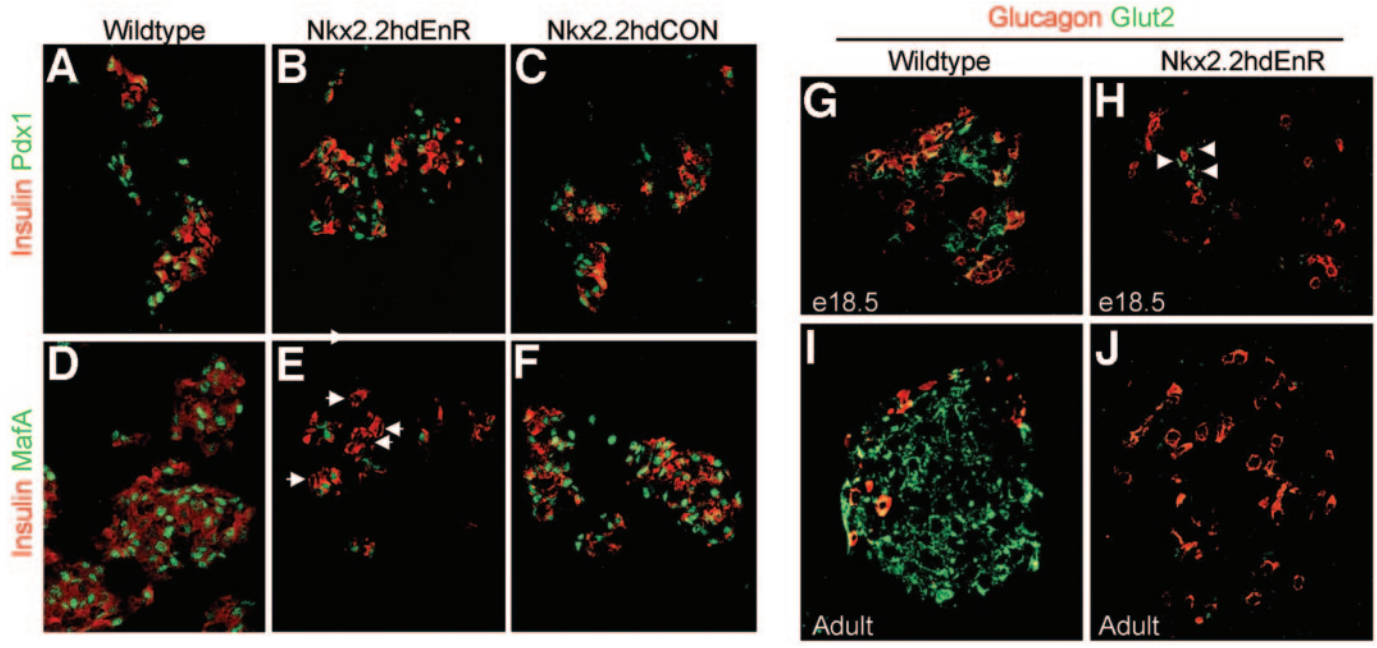

FIG. 6. Markers of terminally differentiated β-cells are reduced in Nkx2.2hdEnR mice. *A–F*: Confocal images of insulin staining (red) with Pdx1 (green) (*A–C*) or MafA (green) (*D–F*) demonstrates all insulin-positive cells coexpress Pdx1, but only ~50% of insulin-positive cells coexpress MafA in Nkx2.2-repressor islets (*B* and *E*) compared with wild-type islets (*A* and *D*) or Nkx2.2hdmyc control islets (*C* and *F*) at embryonic day 18.5. *E*: Arrows indicate MafA-negative cells in Nkx2.2-repressor islets. *G–J*: Confocal images of glucagon (red) and glut2 (green) staining in neonatal and adult islets indicate that glut2 is significantly reduced at embryonic day 18.5 and lost in adult islets of Nkx2.2-repressor mice. Arrowheads indicate glut2-positive cells (*H*).

mice are similar to defects associated with several maturity-onset diabetes of the young transcription factors. Additional studies with hypomorphic and conditional Nkx2.2 alleles are ongoing to determine the mechanism by which Nkx2.2 may contribute to β-cell dysfunction.

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