Postnatal beta cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1

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ABSTRACT

All forms of diabetes are characterized by a loss of functional beta cell mass, and strategies for expanding beta cell mass could have significant therapeutic benefit. We have recently identified the transcription factor Nkx6.1 as an essential maintenance factor of the functional beta cell state. In addition, Nkx6.1 has been proposed to control beta cell proliferation, but a role for Nkx6.1 in regulating beta cell mass has not been demonstrated. Here, we show that Nkx6.1 is required for postnatal beta cell mass expansion. Genetic inactivation of Nkx6.1 in newly-formed beta cells caused a drastic decrease in early postnatal beta cell proliferation, leading to reduced beta cell mass and glucose intolerance. Interestingly, Nkx6.1 was dispensable for prenatal beta cell proliferation. We found that Nkx6.1 regulates the expression of several beta cell maturation markers as well as expression of the nutrient sensors Glut2 and Glp1r. Manifestation of the beta cell mass defect at the transition to postnatal feeding suggests that Nkx6.1 could regulate beta cell growth by enabling beta cells to respond to nutrient-dependent proliferation signals, such as glucose and Glp1. Identification of beta cell-intrinsic regulators that connect nutrient-sensing and proliferation suggests new therapeutic targets for expanding functional beta cell mass.
INTRODUCTION

The establishment of sufficient beta cell mass depends on the rapid expansion of beta cell numbers during early postnatal life (1-5). The extent of this early postnatal beta cell growth is postulated to influence later susceptibility to type 2 diabetes (6). Postnatal beta cell mass expansion is driven by beta cell proliferation (7), which is controlled by the cell cycle regulators Cyclin D1 or Cyclin D2 (encoded by Ccnd1 and Ccnd2, respectively) (2; 3). It has been shown that beta cells are highly proliferative in the perinatal period and that this early proliferation is necessary to establish sufficient beta cell mass for maintaining glucose homeostasis (1-5). However, the cell extrinsic and intrinsic factors that drive beta cell proliferation and mass expansion during the perinatal period are still poorly defined.

Glucose has been identified as a systemic factor that stimulates beta cell proliferation (8; 9), and recent studies suggest that glucose is a significant driver of early postnatal beta cell proliferation (10). Furthermore, it has been shown that glucose metabolism in beta cells produces signals that increase Cyclin D2 expression and beta cell proliferation (11-13). Independent of glucose, beta cell proliferation is also stimulated by gut-derived hormone glucagon-like peptide 1 (Glp1), which is secreted by intestinal enteroendocrine cells in response to food intake (14; 15). Thus, there is an established link between feeding, increases in blood glucose levels, and beta cell proliferation. However, beta cells also exhibit significant proliferation during fetal life, when blood glucose concentrations are low and glucose has little effect on beta cell proliferation (16). The distinct mechanisms employed in prenatal and postnatal beta cells to regulate proliferation remain unclear.

The beta cell-restricted transcription factor Nkx6.1 is essential for maintaining the functional state of beta cells during adulthood (17). Both in vitro and in vivo experiments have suggested a role for Nkx6.1 in beta cell proliferation (17-19), but whether it is required for beta cell growth in vivo is unknown. To reveal a possible role for Nkx6.1 beta cell mass expansion, we inactivated Nkx6.1 in newly-formed beta cells of the embryo and examined the effects on beta cell proliferation and mass during the prenatal and postnatal period.
RESEARCH DESIGN AND METHODS

RIP-Cre (20), Nkx6.1\textsuperscript{flax} (21), Nkx6.1 null (22), and R26-YFP mice (23) have been described. RIP-Cre;Nkx6.1\textsuperscript{flax+};R26-YFP mice served as control mice in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of California.

Methods for tissue preparation, immunofluorescence staining, and terminal deoxynucleotidyl transferase dUTP nicked end labeling (TUNEL) have been previously described (21). The following primary antibodies were used: guinea pig anti-insulin (Dako), 1:2000; mouse anti-Nkx6.1, (BCBC #2023), 1:500; rabbit anti-Glp1r (S. Heller, Novo Nordisk), 1:2000; rat anti-GFP (C. Kioussi, Oregon State University), 1:1000; rabbit anti-Ki67 (Lab Vision), 1:500; rabbit anti-Ucn3 (M. Huising, UC Davis), 1:500; rabbit anti-MafA (Bethyl), 1:200; rabbit anti-Pdx1 (Abcam), 1:500. Staining with antibodies raised in mice was performed using the M.O.M. Kit (Vector Labs). When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 µg/ml. Primary antibodies were detected with donkey-raised secondary antibodies conjugated to Cy3, Cy5, or Alexa 488 (Jackson ImmunoResearch). Beta cell mass and marker\textsuperscript{+} area were determined as described (21). Images were captured on a Zeiss Axio Observer Z1 microscope with an Apotome module and processed with Zeiss Axiovision 4.8 software. All images were processed in accordance with Diabetes journal guidelines.

The qRT-PCR analysis was performed as previously described (17) on total RNA isolated from postnatal day 2 pancreata from individual mice. Primers used are as follows: Nkx6.1 (f-CTTCTGGCCCGGAGTATG; r-GGGTCTGGTGTTTCTCTTC), Ucn3 (f-GCTGTGCCCTCGACCT; r-TGGGCATCAGCATCGCT), Adh1 (f-GCAAAGCTGGGTGGGTATG; r-TACACACGGTCCTCTTC), Angptl7 (f-TGACTGTCTTCCCCTCCTAC; r-CAGAGCCACTCTTGTA), Dlk1 (f-CCACGGTGAGCTTCGAG; r-GGAAGGGTACTCTTGAGA), Gstm2 (f-ACACCCGCATACTACGG; r-TGCTGCCCAGAAACTCGAG), Zyx (f-TCCCACCGCAGGCTATAC; r-GGAGCTAAGGGTCTTCCA), Gapdh (f-CATGTCCAGATGACTCCACT; r-GGCCTACCCCCATTTGAGTGT).

Glucose tolerance tests and blood glucose measurements were performed as described (17). For glucose tolerance tests, a 1.5 g/kg body weight intraperitoneal injection of glucose was administered after overnight fasting.

All values are shown as mean ± standard error of the mean (SEM); p-values were calculated using a two-tailed student’s t-test in Microsoft Excel. P<0.05 was considered significant.
RESULTS

*Nkx6.1* inactivation in embryonic beta cells causes hyperglycemia and reduced beta cell mass.

To investigate the role of Nkx6.1 in perinatal beta cell development, we intercrossed mice to generate progeny carrying a Nkx6.1 null allele (*Nkx6.1*), a Nkx6.1 conditional loss of function allele (*Nkx6.1*fox), and the *rat insulin2-Cre* transgene (RIP-Cre). Additionally, the mice carried a conditional YFP reporter gene targeted to the *Rosa-26* locus (R26-YFP), resulting in heritable YFP expression upon RIP-Cre-mediated recombination of a translational stop signal. Thus, in RIP-Cre;Nkx6.1fox−/R26-YFP (hereafter referred to as *Nkx6.1∆β*) mice, YFP labels all cells in which Nkx6.1 has been inactivated (Figure 1A).

*Nkx6.1∆β* mice were born with the expected mendelian frequency (data not shown). Consistent with previous reports showing incomplete targeting of beta cells by the RIP-Cre transgene (20), most but not all beta cells were devoid of Nkx6.1 at birth (Figure 1B,C). At six weeks of age, *Nkx6.1∆β* mice exhibited significantly elevated blood glucose levels (Figure 1D) and impaired glucose tolerance after intraperitoneal injection of a glucose bolus (Figure 1E). To investigate whether Nkx6.1 deficiency affects postnatal beta cell growth, we examined beta cell mass in *Nkx6.1∆β* mice. Compared to littermate controls, six-week-old *Nkx6.1∆β* mice exhibited a 40% reduction in beta cell mass (1.26±0.05 mg in *Nkx6.1∆β* mice versus 2.13±0.29 mg in controls) (Figure 1F). Thus, Nkx6.1 is necessary to establish appropriate beta cell mass.

*Nkx6.1* is required for postnatal, but not prenatal beta cell mass expansion.

To determine when beta cell mass is first affected in *Nkx6.1∆β* mice, we measured the relative insulin+ area in *Nkx6.1∆β* mice immediately after birth. In contrast to six-week-old mice, beta cell mass in neonatal *Nkx6.1∆β* mice was indistinguishable from control mice (Figure 2A), showing that Nkx6.1 is required for postnatal expansion but not for establishing prenatal beta cell mass.

Because RIP-Cre-mediated recombination of the *Nkx6.1*fox allele is mosaic and did not delete Nkx6.1 in all beta cells (Figure 1C), both unrecombined Nkx6.1+ and recombined Nkx6.1-deficient beta cells can contribute to beta cell growth in *Nkx6.1∆β* mice. To investigate the contribution of recombined beta cells to postnatal beta cell mass expansion, we quantified the percentage of recombined beta cells in *Nkx6.1∆β* and control mice. In line with our observation that Nkx6.1 is dispensable for prenatal beta cell growth (Figure 2A), the percentage of recombined YFP+ beta cells was similar in newborn *Nkx6.1∆β* and control mice (73±1.4% in *Nkx6.1∆β* mice versus 76±9.5% in control mice) (Figure 2B-C”,H). Consistent with a slight decrease in overall beta cell mass in *Nkx6.1∆β* mice at postnatal (P) day 4 (Figure 2A), a reduction in the percentage of recombined beta cells was discernable in *Nkx6.1∆β* mice by P4 (Figure 2D-E”,H). At six weeks of age the reduction of YFP+ beta cells was highly significant (15±2.02% of beta cells in *Nkx6.1∆β* mice versus 83±1.72% in control mice) (Figure 2F-H). The decrease of YFP+ beta cells in *Nkx6.1∆β* mice was accompanied by an age-dependent increase in the percentage of beta cells expressing Nkx6.1 (Figure 2B-G”,I). Closely mirroring the reported 82% recombination efficiency of the RIP-Cre transgene (20), 27±1.41% of beta cells expressed Nkx6.1 in newborn *Nkx6.1∆β* mice (Figure 2I). This percentage increased significantly to 40±3.75% at P4 (Figure 2I). These findings indicate that a selective disadvantage becomes apparent for Nkx6.1-deficient beta cells shortly after birth.
Postnatal, but not prenatal beta cell proliferation depends on Nkx6.1.
We next investigated whether the postnatal beta cell growth defect in Nkx6.1Δβmice is caused by reduced beta cell proliferation and/or survival. First, we examined the possibility that Nkx6.1 deficiency causes increased beta cell apoptosis by performing TUNEL assays on pancreatic sections. Virtually no TUNEL+ beta cells were detected in either Nkx6.1Δβ or control mice at P4 (Figure 3A-C), indicating that apoptosis does not account for the negative selection of Nkx6.1-deficient beta cells. By contrast, analysis of beta cell proliferation by immunofluorescence staining for Ki67, insulin, and YFP in Nkx6.1Δβ mice at P4 revealed reduced numbers of Ki67+ beta cells (Figure 3F-H). Quantification of Ki67+YFP+ beta cells showed a 3-fold decrease in beta cell proliferation in four-day-old Nkx6.1Δβ compared to control mice (4.48±1.01% in Nkx6.1Δβ mice versus 13.00±1.58% in control mice) (Figure 3H). Consistent with our finding that Nkx6.1 inactivation does not affect prenatal beta cell growth (Figure 2A), the frequency of Ki67+ beta cells did not differ between Nkx6.1Δβ and control mice at P0 (2.67±1.14% in Nkx6.1Δβ mice versus 1.78±1.05% in control mice) (Figure 3D-E,H). Thus, Nkx6.1 is required for beta cell proliferation and expansion during early postnatal life but is dispensable prenatally. Furthermore, the effect of Nkx6.1 deletion on beta cell proliferation is cell autonomous, as revealed by comparing proliferation rates between recombined and unrecombined beta cells in Nkx6.1Δβ mice at P4 (4.48±1.01% YFP+insulin+ cells versus 11.0±0.93% YFP+insulin+ cells expressed Ki67) (Figure 3I).

Nkx6.1 deletion causes a cell-autonomous loss of markers for beta cell maturation and nutrient sensing.
To determine whether loss of Nkx6.1 affects other beta cell markers, we performed immunofluorescence staining for Pdx1 and MafA. While Pdx1 was unaffected, MafA was lost in recombined Nkx6.1-deficient beta cells (Figure 4A-F”). We further assessed whether Nkx6.1 regulates beta cell maturation markers. To this end, we selected genes found to be significantly changed between immature and mature postnatal beta cells (24), and performed qRT-PCR analysis on pancreata from control and Nkx6.1Δβ mice at P2, when beta cell mass is similar between Nkx6.1Δβ and control mice (Figure 2A). Of these genes, Ucn3, Adh1, Gstm2, and Zyx were expressed at significantly lower levels in Nkx6.1Δβ mice, while Angptl7 and Dlk1 were unchanged (Figure 4G-J”). These results suggest that Nkx6.1 regulates a subset of genes associated with beta cell maturation. Given the postnatal onset of the beta cell proliferation defect in Nkx6.1Δβ mice, we next investigated whether Nkx6.1-deficient beta cells are able to receive feeding-induced signals that stimulate beta cell proliferation. We analyzed the expression of glucose transporter 2 (Glut2) and the Glp1 receptor (Glp1r), which are known to have a role in the regulation of postnatal beta cell growth (14; 16). In accordance with Glut2 being a direct Nkx6.1 target gene (17), Nkx6.1Δβ mice exhibited a selective loss of Glut2 expression only in recombined beta cells (Figure 4K-M”). Similarly, recombined beta cells displayed a cell autonomous reduction in Glp1r expression (Figure 4N-P”). The cell autonomous role of Nkx6.1 in regulating beta cell proliferation, Glut2, and Glp1r expression argues against an Nkx6.1-dependent paracrine or systemic factor affecting beta cell proliferation in Nkx6.1Δβ mice. These findings demonstrate that Nkx6.1-deficient beta cells lack key sensors for extrinsic stimuli of postnatal beta cell growth.
DISCUSSION

The role of Nkx6.1 in beta cell proliferation has been controversial. While in vitro studies have suggested a direct role of Nkx6.1 in stimulating beta cell proliferation through the regulation of Cyclin gene expression (18), in vivo overexpression of Nkx6.1 in beta cells showed no effect on beta cell proliferation or mass (19). Moreover, we have recently reported that beta cell-specific inactivation of Nkx6.1 in adult mice has no overt effect on beta cell mass (17). However, due to the extremely low proliferation rate of beta cells in adult animals (1), the role of Nkx6.1 in beta cell mass expansion could not be rigorously tested in this model. By ablating Nkx6.1 in newly-formed beta cells of the embryo, we here show that postnatal beta cell proliferation and mass expansion depends on Nkx6.1 activity. We found that Nkx6.1-deficient beta cells begin to exhibit reduced proliferation between P0 and P4, which manifests in a measurable decrease in the contribution of Nkx6.1-deficient beta cells to beta cell mass as early as P4. We have previously reported that Nkx6.1 deficiency leads to a loss of beta cell identity and ultimately their conversion into delta cells (21). It is important to note that this fate conversion occurs later and is not yet observed at P4 (see Fig. 2E-Eʺ; all YFP⁺ cells express insulin). Thus, the reduced contribution of Nkx6.1-deficient beta cells to beta cell mass is caused by the proliferation defect and cannot be attributed to a beta-to-delta cell fate conversion.

Employing ChIP-seq analysis, we have recently shown that Nkx6.1 does not bind to Cyclin gene regulatory regions (17). Therefore, Nkx6.1 is likely an indirect regulator of beta cell proliferation. Consistent with this idea, our current work shows that prenatal beta cell proliferation is unaffected in Nkx6.1Δβ mice. Interestingly, we found that the onset of reduced beta cell proliferation in Nkx6.1Δβ mice coincides with birth and thus the beginning of food intake, suggesting that Nkx6.1 could enable beta cells to respond to nutrient-dependent inducers of beta cell proliferation. Supporting this notion, Nkx6.1-deleted beta cells fail to express two important nutrient sensors, Glut2 and Glp1r. At the transition from prenatal to postnatal life, glucose becomes an important stimulus of beta cell proliferation (16) and similar to Nkx6.1Δβ mice, Glut2-deficient mice exhibit reduced beta cell proliferation during the early postnatal period (25). Since Glp1 regulates beta cell proliferation independent of glucose (15), loss of Glut2 and Glp1r in Nkx6.1Δβ mice likely have additive effects on beta cell proliferation. In addition to regulating nutrient sensors, we found that Nkx6.1 also regulates several markers associated with postnatal beta cell maturation (24). It is still largely unclear whether and how these genes affect beta cell maturation, but the regulation of several of these genes by Nkx6.1 suggests a role for Nkx6.1 in beta cell maturation. Collectively, our results demonstrate that Nkx6.1 controls multiple relevant pathways for postnatal beta cell development.

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The authors have declared that no conflict of interest exists. M.S. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. B.L.T. and J.B. designed and performed experiments, analyzed data and prepared figures. B.L.T., J.B., and M.S. wrote the manuscript.
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FIGURE LEGENDS

Figure 1. Nkx6.1 deletion in newly-formed beta cells leads to glucose intolerance and reduced beta cell mass. (A) Schematic of alleles and transgenes utilized to inactivate Nkx6.1 in fetal beta cells. Rectangles: coding sequences; triangles: loxP sites; red rectangle: DsRed coding sequence. (B, C) Immunofluorescence staining for Nkx6.1 and insulin reveals loss of Nkx6.1 in most beta cells of Nkx6.1<sup>Δβ</sup> mice at postnatal day (P) 0. (D) Blood glucose levels in 6-week-old Nkx6.1<sup>Δβ</sup> mice fed ad libitum compared to control mice (n=6). (E) Intraperitoneal glucose tolerance test shows glucose intolerance in 6-week-old Nkx6.1<sup>Δβ</sup> mice as compared to control mice (n=6). (F) Quantification of beta cell mass reveals decreased beta cell mass in Nkx6.1<sup>Δβ</sup> mice at 6 weeks of age (n=3). Scale bars = 20 µm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean ± SEM. *p<0.05, **p<0.01.

Figure 2. Nkx6.1 is required for postnatal beta cell mass expansion. (A) Quantification of the insulin immunofluorescent area relative to total pancreatic area reveals no difference in beta cell mass between Nkx6.1<sup>Δβ</sup> and control mice at postnatal day (P) 0 and a slight but not significant decrease at P4 (n=3). (B-G′′′) Immunofluorescence staining for insulin, Nkx6.1, and YFP at P0 (B-C′′′), P4 (D-E′′′), and 6 weeks of age (F-G′′′). (H) Quantification of insulin<sup>+</sup> cells expressing YFP at P0, P4, and 6 weeks shows a progressive decrease of YFP<sup>+</sup> recombinant beta cells in Nkx6.1<sup>Δβ</sup> mice postnatally (n=3). (I) Quantification of insulin<sup>+</sup> cells expressing Nkx6.1 reveals a progressive increase of Nkx6.1-expressing unrecombined beta cells in Nkx6.1<sup>Δβ</sup> mice between P0 and 6 weeks (n=3). Scale bar = 20 µm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean ± SEM. *, p<0.05; **, p<0.01; ****, p<0.001.

Figure 3. Nkx6.1 is required for postnatal beta cell proliferation. (A-C) Beta cells are not apoptotic at postnatal day (P) 4 in Nkx6.1<sup>Δβ</sup> or control mice based on TUNEL combined with immunofluorescence staining for insulin and DAPI. TUNEL<sup>+</sup> cells in the pancreas are shown as a positive control (arrowheads) and TUNEL<sup>+</sup> insulin<sup>+</sup> cells were quantified. (D-G′′′′) Immunofluorescence staining for insulin, Ki67, and YFP at P0 and P4. (H) Quantification of the percentage of insulin<sup>+</sup>YFP<sup>+</sup> cells expressing Ki67 shows decreased beta cell proliferation in Nkx6.1<sup>Δβ</sup> mice at P4, but not at P0 (n=3). (I) Quantification of Ki67-expressing YFP<sup>+</sup> insulin<sup>+</sup> cells and YFP<sup>+</sup>insulin<sup>+</sup> cells in Nkx6.1<sup>Δβ</sup> mice at P4 reveals a selective decrease in proliferation of recombined compared to unrecombined beta cells within the same animal (n=3). Scale bar = 20 µm. Ins, insulin; YFP, yellow fluorescent protein. Data shown as mean ± SEM. *, p<0.05; **, p<0.01.

Figure 4. Nkx6.1 inactivation leads to a cell autonomous loss of beta cell maturation and nutrient sensing markers. Immunofluorescence staining for insulin, Pdx1, and YFP (A-C′′′′) or insulin, MafA, and YFP (D-F′′′′) shows Pdx1 but not MafA expression in recombined YFP<sup>+</sup>insulin<sup>+</sup> cells of Nkx6.1<sup>Δβ</sup> mice at postnatal day (P) 4. Unrecombined YFP<sup>+</sup>insulin<sup>+</sup> cells express Pdx1 and MafA in Nkx6.1<sup>Δβ</sup> mice. (G) qRT-PCR analysis of pancreata from Nkx6.1<sup>Δβ</sup> and control mice at P2 for genes associated with beta cell maturation (n=3). Immunofluorescence staining for insulin, Ucn3, and YFP (H-J′′′′), insulin, Glut2, and YFP (K-M′′′′), or insulin, Glp1r, and YFP (N-P′′′′) shows loss of Ucn3, Glut2, and Glp1r expression in recombined YFP<sup>+</sup>insulin<sup>+</sup> cells but not in unrecombined YFP insulin<sup>+</sup> cells of Nkx6.1<sup>Δβ</sup> mice at P4. For each marker, representative areas are shown in lower panels for Nkx6.1<sup>Δβ</sup> mice, as indicated by a dashed box.
in the merged middle panel. White arrowheads point to recombined $\text{YFP}^+\text{insulin}^+$ cells and blue arrowheads to unrecombined $\text{YFP}^+\text{insulin}^-$ cells. Scale bar $= 20 \, \mu\text{m}$. Ins, insulin; YFP, yellow fluorescent protein. Data shown as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$. 