β-Cell Proliferation, but not Neogenesis, Following 60% Partial Pancreatectomy is Impaired in the Absence of FoxM1

Amanda Ackermann Misfeldt, B.S.2,4, Robert H. Costa, Ph.D.5, Maureen Gannon, Ph.D.1,2,3,4

1Department of Medicine, Division of Diabetes, Endocrinology, and Metabolism, Vanderbilt University Medical Center, Nashville, Tennessee
2Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee
3Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, Tennessee
4Program in Developmental Biology, Vanderbilt University Medical Center, Nashville, Tennessee
5Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, Illinois

Corresponding Author:
Maureen Gannon, Ph.D.
Vanderbilt University Medical Center
Division of Diabetes, Endocrinology and Metabolism
2215-B Garland Ave, 7425C MRBIV, Nashville, TN 37232-0475
maureen.gannon@vanderbilt.edu

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ABSTRACT

Objective: This study was designed to determine whether the transcription factor FoxM1 was required for regeneration of β-cell mass via proliferation and/or neogenesis in the adult after 60% partial pancreatectomy (PPx).

Research Design and Methods: Adult mice with a pancreas-wide deletion of Foxm1 (Foxm1<sup>flox/flox</sup>;Pdx1-Cre [FoxM1<sup>Δpanc</sup>]) and their Control littermates (Foxm1<sup>flox/flox</sup>) were subjected to PPx or a Sham operation, after which islet expression of Foxm1 and several target genes, β-cell mass, proliferation, β-cell size, islet size, islet density, and Neurogenin3 expression were analyzed.

Results: In Control mice, PPx stimulated β-cell proliferation and neogenesis and up-regulated Foxm1 and several of its known targets (Plk1, Cenp-a, Birc5/Survivin, Ccnb1) in islets. Within 1 week post-PPx, Control mice underwent significant regeneration of β-cell mass, and average islet size within the regenerating lobe was similar to that after a sham operation. However, FoxM1<sup>Δpanc</sup> mice exhibited specific impairments in β-cell mass regeneration and islet growth after PPx, with reduced proliferation of α and β-cells but no impairments in acinar or ductal cell proliferation. Interestingly, FoxM1 was not required for proliferation of β-cells within small endocrine cell clusters located in the regenerating portion of the pancreas, but was specifically required for proliferation of β-cells within larger islets. Additionally, FoxM1 was not required for β-cell neogenesis following PPx.

Conclusions: Our results indicate that FoxM1 is partially required for increased β-cell proliferation, but not β-cell neogenesis, stimulated by PPx. Furthermore, FoxM1 seems to be dispensable for proliferation of β-cells following neogenesis but is required for proliferation of pre-existing β-cells.
As type I diabetes and type II diabetes result from absolute or relative deficiencies in β-cell number, respectively, understanding how β-cell number is determined and can be manipulated may lead to new therapeutic options. While transplantation of cadaveric islets remains promising, additional sources of islets or β-cells are required to make this treatment more widely available (1). Diabetic patients would also benefit from in vivo manipulation of their β-cell population. β-cells proliferate slowly after birth (2), but increased proliferation can be stimulated under various conditions, including obesity, pregnancy, and growth factor over-expression (reviewed in [3]). In the adult mouse, β-cell replication is the primary mechanism by which new β-cells are formed (4,5). However, a recent study in mice has conclusively shown that new β-cells are generated from facultative adult progenitor cells (neogenesis) in response to pancreatic injury, in a manner reminiscent of embryonic endocrine cell differentiation (6).

We previously showed that the forkhead box transcription factor FoxM1 regulates postnatal β-cell proliferation (9). In other tissues, FoxM1 transactivates genes that coordinate the G1/S and G2/M transitions, karyokinesis, and cytokinesis (10,11). For example, the FoxM1 targets Cell division cycle 25 homolog A (Cdc25A) and Cdc25B dephosphorylate and activate Cyclin-dependent kinase 2 (Cdk2) and Cdk1, respectively (12). Cdk1 activity also depends on its binding to Cyclin B1 (CcnB1), another FoxM1 target (12,13). Additional targets include S-phase kinase-associated protein 2 (Skp2) and Cdk subunit 1 (Cks1), components of the Skp1-Cullin1-F box (SCF) ubiquitin ligase complex that mediates degradation of the Cdk inhibitors p21\(^{\text{Cip1}}\), p27\(^{\text{Kip1}}\), and p57\(^{\text{Kip2}}\) (14). FoxM1 also directly activates transcription of Polo-like kinase 1 (Plk1) (15), Aurora B kinase, Centromere protein A (Cenp-A), Cenp-B, and Survivin (Birc5) (14). Plk1 is necessary for centrosome duplication and, with Aurora B kinase, mediates attachment of the microtubule spindles to kinetochores. Survivin regulates Aurora B kinase centromere localization, while Cenp-A and -B are involved in kinetochore assembly.

Although all proliferating cells express FoxM1, certain cell types are more profoundly affected than others by Foxm1 deletion. Foxm1\(^{−/−}\) mice die during late embryogenesis due to hepatic and cardiac defects (16). Additionally, pancreas-wide Foxm1 deletion (Foxm1\(^{\text{flox/flox}}\); Pdx1\(^{5.5\text{kb-Cre}}\) [FoxM1\(^{\text{Δ} \text{panc}}\]) does not affect embryonic pancreas or β-cell development but does impair postnatal β-cell mass expansion via reduced β-cell proliferation, resulting in progressive diabetes in male mice (9). These outcomes mirror those reported for widespread deletion of other cell cycle regulators (Cdk4, Cyclin D), with endocrine cell-specific effects causing diabetes (17-19). Female FoxM1\(^{\text{Δ} \text{panc}}\) mice remain glucose tolerant despite significantly reduced β-cell mass and thus are the focus of this study, allowing for analysis of β-cell proliferation and regeneration without the confounding effects of hyperglycemia.

To determine whether FoxM1 is required for β-cell regeneration, we performed 60% partial pancreatectomy (PPx) on adult female FoxM1\(^{\text{Δ} \text{panc}}\) and Control (Foxm1\(^{\text{flox/flox}}\)) littermates. Here, we show that Foxm1 and some of its transcriptional targets were up-regulated within islets following PPx during the period of peak regeneration, and that endocrine cell proliferation and regeneration of β-cell mass following PPx were specifically impaired in FoxM1\(^{\text{Δ} \text{panc}}\) mice.
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Furthermore, we present evidence that loss of FoxM1 did not impair islet neogenesis but did impair islet growth after PPx.

RESEARCH DESIGN AND METHODS

Mice. Foxm1\textsuperscript{flox/flox} mice on a mixed background (129SvJ, C57Bl/6) were described previously (12). Briefly, loxP sequences flank exons 4-7, yielding a null allele after Cre-mediated recombination. Pdx1\textsuperscript{5.5kb-Cre} mice (on a mixed ICR, CBA, C57Bl/6 background) were generously provided by Guoqiang Gu (Vanderbilt University) (20) and express Cre recombinase throughout the Pdx1 domain, including the entire pancreatic epithelium, by embryonic day (e) 10.5 (21,22). Foxm1 and Cre were genotyped as described (9,20). Rosa26-FoxM1 transgenic (Tg) mice (on a mixed FVB/N, C57Bl/6 background) were previously described (23) and genotyped as in (24). For embryonic analyses, the morning of vaginal plug was considered e0.5. All mice received food and drink \textit{ad libitum} and were on a 12 hour light-dark cycle. All mouse studies were performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee guidelines under the supervision of the Division of Animal Care.

60% Partial Pancreatectomy (PPx) and Intraperitoneal Glucose Tolerance Test (IPGTT). Eight week old female mice were anesthetized using isoflurane. A midline abdominal incision allowed exteriorization of the splenic lobe of the pancreas (between the gastro-duodenal junction and the spleen). 60% PPx was performed by gently denuding the pancreatic tissue from the splenic lobe using cotton-tipped swabs soaked in 0.9% saline, leaving the main pancreatic duct and splenic artery intact. For Sham operations, the splenic lobe was isolated using cotton-tipped swabs and gently rubbed with a moistened gloved finger for 1 minute. Abdominal muscles were sutured using 5-0 vicryl (Ethicon), and the skin was stapled using 9.0 mm staples (Reflex Skin Closure Systems). 0.1 mg/kg buprenorphine-HCl in normal saline was injected subcutaneously for analgesia, and 0.8 mg/ml bromo-deoxyuridine (BrdU; Sigma-Aldrich) was administered in the drinking water in a light-safe bottle and was replaced on the 4\textsuperscript{th} day. Mice were sacrificed 1 week post-operation by cervical dislocation, and the splenic and duodenal lobes of the pancreas were harvested and analyzed separately. One day prior to and 7 days after the operation, IPGTTs were performed as described previously (9). See Fig. 1 for experimental timeline.

RNA Isolation and Real-Time Quantitative RT-PCR (qRT-PCR). RNA from whole pancreas or freshly-isolated islets was extracted and DNase-treated using the RNAqueous and TURBO DNA-free kits (Ambion). RNA was assessed using the ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent). cDNA was generated from 20 ng RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad) with iQ Real-Time SYBR Green PCR Supermix (Bio-Rad) and the primer sets in Supplemental Table S1. Results were quantitated using the ΔΔCt method.

Tissue Preparation and Histology. Pancreatic tissue was fixed in 4\% paraformaldehyde for 2-4 hours at 4°C, processed for paraffin embedding, and sectioned at 5 μm. Primary antibodies were incubated overnight at 4°C: guinea pig anti-insulin or anti-glucagon (both 1:1,000; Linco), rabbit anti-cytokeratin (1:1,000; DakoCytomation), rat anti-BrdU (1:400; Accurate Chemical & Scientific), rabbit anti-phospho-histone H3 (1:250; Upstate Cell Signaling Solutions), and mouse anti-Neurogenin3 (Ngn3; 1:100; Developmental Studies Hybridoma Bank, The University of Iowa). Secondary antibodies were incubated for 1 hour at room
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BrdU labeling was previously described (9). Phospho-histone H3 labeling of sections 125 μm apart (7-9 sections per sample) required microwave antigen retrieval in 10 mM sodium citrate, pH 6.5 followed by 0.2% Triton-X-100 in PBS.

Islet, β-Cell, and Nuclear Size Analyses. Using images collected for β-cell proliferation, the cross-sectional area of each insulin+ cell grouping was circumscribed and measured using MetaMorph. Average cross-sectional β-cell size per islet equaled the total area of the islet divided by the number of β-cell nuclei within it. β-cell nuclear size was measured by circumscribing the DAPI+ cross-sectional area within insulin+ cells.

Statistical Analyses. Data were analyzed by unpaired t-test or two-way ANOVA with Bonferroni’s post-tests, as appropriate, using GraphPad Prism v5.01. P<0.05 was considered significant. Specific analyses performed and sample sizes are included in the figure legends.

RESULTS

60% PPx up-regulated Foxm1 and select targets within islets while maintaining normoglycemia. FoxM1 mRNA and protein are relatively low in adult islets (data not shown and [9]), but it was unknown whether up-regulation occurs upon stimulation of endocrine cell proliferation. Indeed, Foxm1 expression was up-regulated 2-fold in Control islets 6 days following PPx versus Sham (Fig. 2), suggesting that FoxM1 plays a role in the regenerative response of endocrine cells. Importantly, Foxm1 up-regulation corresponded with the peak of β-cell proliferation following PPx (25).

Additionally, we observed significant up-regulation of known FoxM1 targets, including Plk1, Cenp-a, Birc5/Survivin, and Ccnb1 (Fig. 2). Other targets, including Skp2, Cks1, and Cdc25b, displayed a trend of up-regulation following PPx versus Sham but did not reach significance. A similar trend was observed...
for Ccna2 but not for Ccnd2, both of which have been linked to β-cell proliferation (17,18,26), but neither of which is a known FoxM1 target.

To determine whether FoxM1 is necessary for endocrine cell regeneration following pancreatic injury, FoxM1Δpanc and Control female littermates underwent PPx or Sham operation at 8 weeks of age. Previous reports showed that 60% PPx does not induce hyperglycemia (25), unlike 90% PPx (27). IPGTTs performed 7 days following 60% PPx or Sham confirmed that Control and FoxM1Δpanc female mice remained glucose tolerant (Fig. 3).

**Regeneration of β-cell mass within the splenic lobe was specifically impaired in FoxM1Δpanc mice.** Similar to FoxM1Δpanc male mice (9), FoxM1Δpanc female mice also exhibited ~50% reduced β-cell mass at 9 weeks of age compared to littermate Controls (data not shown), although FoxM1Δpanc female mice remained normoglycemic. This ~50% reduction in β-cell mass was evident in the splenic, but not duodenal, lobe and was unchanged after a Sham operation (Fig. 4B,C). Total pancreatic weight and weight of the individual lobes were not significantly different between Control and FoxM1Δpanc mice (Fig. 4A, data not shown), indicating a specific defect in the endocrine compartment of the pancreas. Consistent with previous findings (28), a significant majority of β-cell mass was located within the splenic versus duodenal lobe of Control mice after a Sham operation (P<0.01).

PPx eliminated the majority of splenic lobe tissue, and within 7 days a significant and equivalent amount of tissue regenerated in both FoxM1Δpanc and Control mice (Fig. 4A, Supplemental Fig. S1A-D), suggesting that overall pancreas regeneration during this time period was not impaired by Foxm1 deletion. β-cell mass regeneration within the splenic lobe, however, was impaired in FoxM1Δpanc mice, as β-cell mass in these mice did not significantly increase within 7 days following PPx, in contrast to Control littermates (Fig. 4B).

**β-cell proliferation was reduced in FoxM1Δpanc mice after 60% PPx.** Because β-cells are replenished primarily by self-replication (4), and FoxM1 is important for postnatal β-cell proliferation (9), it seemed likely that reduced β-cell proliferation contributed to impaired β-cell mass regeneration in FoxM1Δpanc mice. Indeed, PPx dramatically stimulated β-cell proliferation in both pancreatic lobes of Control mice, and these effects were significantly blunted in FoxM1Δpanc mice (Fig. 5A-F). Moreover, β-cell proliferation within the duodenal lobe of FoxM1Δpanc mice did not significantly increase after PPx. β-cell proliferation in the splenic lobe of FoxM1Δpanc mice did increase significantly following PPx versus Sham, despite Foxm1 deletion (Fig. 5E) due to increased proliferation within small endocrine cell clusters (8 or fewer insulin+ cells) and definitive islets (9 or more insulin+ cells) (Fig. 5G,I). However, β-cell proliferation within definitive islets was significantly reduced in FoxM1Δpanc versus Control mice (Fig. 5J). Because there was no significant impairment in β-cell proliferation in small endocrine cell clusters in FoxM1Δpanc mice, we hypothesized that these clusters represented newly-formed β-cells that did not require FoxM1 for proliferation. This hypothesis was supported by the finding that embryonic β-cell proliferation did not require FoxM1 (Supplemental Fig. S2A,B). Additionally, proliferation of β-cells within small endocrine cell clusters after PPx was specifically stimulated in the regenerating splenic (Fig. 5G), but not duodenal (Fig. 5H), lobe. We hypothesize that, just as perinatal β-cells can proliferate in the absence of FoxM1, newly-differentiated β-cells in the adult can undergo several rounds of FoxM1-independent
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PPx stimulated β-cell proliferation within definitive islets of Control mice to the same extent (7-fold) in both the splenic and duodenal lobes versus Sham (Fig. 5I,J). Proliferation in the splenic lobe of FoxM1Δpanc mice increased only 5-fold, while the increase within the duodenal lobe did not reach significance, suggesting that proliferation of pre-existing β-cells was impaired in the absence of FoxM1. The increased BrdU incorporation observed within definitive islets in the splenic lobe of FoxM1Δpanc mice 7 days after PPx versus Sham (Fig. 5I) may represent proliferation that occurred at an earlier stage of new islet development. These results are summarized in Table 1.

α-cells similarly required FoxM1 for PPx-stimulated proliferation (Fig. 5K). In contrast, acinar and ductal cell proliferation were not impaired in FoxM1Δpanc mice (Supplemental Fig. S3A,B), revealing that FoxM1 is specifically required for endocrine cell proliferation.

FoxM1Δpanc mice exhibited increased β-cell and nucleus size. Sham-operated FoxM1Δpanc mice exhibited larger average β-cell size in both pancreatic lobes (Fig. 6A,B) versus Control littermates, due in part to increased β-cell nucleus size (Fig. 6C,D). However, hypertrophy also contributed, likely as a compensatory measure due to reduced β-cell number. PPx did not alter β-cell size in Control mice. However, average β-cell size was significantly reduced specifically within the splenic lobe of FoxM1Δpanc mice after PPx, likely due to new β-cell formation.

Islet size in FoxM1Δpanc mice was reduced following 60% PPx. Although PPx reduced total β-cell number within the splenic lobe, islets present within this lobe 7 days after PPx were of the same average size as after Sham-operation in Control mice (Fig. 7A). However, FoxM1Δpanc islets were significantly smaller 7 days after PPx compared to Sham. Similarly, the average islet size within the duodenal lobe of Control mice trended toward an increase following PPx versus Sham, but no expansion was observed in FoxM1Δpanc mice (Fig. 7B). These data suggest that Foxm1 deletion impairs islet growth after PPx. Of note, islets within the splenic lobe of Control mice after Sham operation averaged 34% larger than islets within the duodenal lobe (P<0.05), which may be due to a greater proportion of small endocrine cell clusters versus definitive islets normally found in the duodenal versus splenic lobe (P<0.05) (Fig. 7C,D). Interestingly, after PPx FoxM1Δpanc mice exhibited an increased proportion of small endocrine cell clusters versus definitive islets within their splenic lobe in comparison to Control mice (Fig. 7C), which likely contributed to their reduced average islet size. No differences were observed in the proportion of small endocrine cell clusters between Control and FoxM1Δpanc mice after Sham operation or within the duodenal lobe under any condition (Fig. 7D).

β-cell neogenesis following 60% PPx was not impaired in FoxM1Δpanc mice. The average number of insulin+ cell groupings in the splenic lobe of both FoxM1Δpanc and Control mice trended toward an increase 7 days after PPx versus Day 0 (Fig. 7E) while no differences were observed in the duodenal lobes of either genotype (Fig. 7F). Insulin+ cells were observed within foci of regeneration in the splenic lobes of both groups of mice (Supplemental Fig. 1B,D), suggesting that generation of new islets, or neogenesis, occurred specifically in the splenic lobe after PPx and was not impaired in FoxM1Δpanc mice. These findings were supported by observation of Ngn3, a marker of endocrine progenitor cells (29), in ductal epithelial cells in both Control (Fig. 7G) and FoxM1Δpanc (Fig. 7H) mice following PPx. Importantly, Ngn3 expression was only observed in ductules within the regenerating portion of the splenic lobe, but not within
differentiated pancreas tissue. No difference in the number of Ngn3⁺ ductal epithelial cells was detected between Control and FoxM1Δpanc mice following PPx.

**Foxm1 over-expression did not enhance β-cell mass regeneration following 60% PPx.** To determine whether Foxm1 over-expression affects pancreas and β-cell regeneration following PPx, we utilized Rosa26-FoxM1 Tg mice that constitutively and ubiquitously over-express FoxM1 (23). Despite FoxM1 over-expression within islets (Supplemental Fig. S4A), Tg mice exhibited no significant differences in glucose tolerance (Supplemental Fig. S4B) nor β-cell mass following PPx (Supplemental Fig. S4C) compared to wild-type (WT) littersates. Furthermore, we observed no differences in pancreatic tissue weight (Supplemental Fig. S4D), average islet size (Supplemental Fig. S4E), or islet density (Supplemental Fig. S4F) between Tg and WT mice in either lobe.

**DISCUSSION**

FoxM1 is highly expressed within pancreatic endocrine cells during embryogenesis and is down-regulated with age (9), corresponding with reduced β-cell proliferation. We previously showed that mice with Foxm1 deleted throughout the pancreatic epithelium (FoxM1Δpanc) exhibit impaired growth and maintenance of postnatal β-cell mass secondary to reduced postnatal β-cell proliferation, associated with increased nuclear-localized p27Kipl and premature cellular senescence (9). Several of FoxM1’s direct transcriptional targets inhibit p27Kipl by promoting its ubiquitination and subsequent degradation (Skp2, Cks1) (14) or its nuclear export (KIS) (30). Here we show that FoxM1Δpanc mice have impaired β-cell mass regeneration following PPx, due to reduced β-cell proliferation, but that neogenesis proceeds normally.

60% PPx is a normoglycemic pancreatic injury model that stimulates regeneration by differentiation and proliferation, and hyperplasia of pre-existing tissue. We found that Foxm1 and some of its targets were up-regulated within islets following PPx during a period of enhanced α and β-cell proliferation, and we hypothesized that FoxM1 was required for injury-stimulated endocrine cell proliferation. Indeed, we found that FoxM1Δpanc mice exhibited impaired α and β-cell proliferation and impaired β-cell mass regeneration following PPx. PPx stimulated β-cell proliferation in both the splenic (regenerating) and duodenal (expanding) lobes of Control mice, but not in the duodenal lobe of FoxM1Δpanc mice. Thus, hyperplasia of pre-existing β-cells after injury requires FoxM1.

Interestingly, PPx-stimulated β-cell proliferation was blunted but not completely inhibited within the splenic lobe of FoxM1Δpanc mice, due at least in part to impaired proliferation in definitive islets, but not small endocrine cell clusters, in the absence of FoxM1. These data suggest that another pathway(s), which may mimic that utilized during embryonic pancreas development, compensates for loss of FoxM1 during regeneration, as FoxM1 was also not required for embryonic β-cell proliferation. Thus, pre-existing β-cells seemingly exhibit a stronger requirement for FoxM1 than do new β-cells formed by neogenesis. Although it is currently unclear which factors are required for β-cell proliferation during embryogenesis and following islet neogenesis, our studies support the hypothesis that neogenesis in the adult is similar to embryonic β-cell development, both with regard to re-expression of Ngn3 and initial FoxM1-independent proliferation.

Because BrdU was administered over 7 days, we may have underestimated the effects of Foxm1 deletion on endocrine cell proliferation. Previous studies have shown that Foxm1⁺⁺ cells can undergo multiple rounds of DNA synthesis without karyo-
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cytokinesis (endoreduplication), resulting in polyploid nuclei (16). We found evidence of polyploid β-cells and exocrine cells in FoxM1Δpanc mice. These cells would have been counted as proliferating cells in our analyses, but would not have actually resulted in increased cell number. This phenomenon could partially explain the increased BrdU incorporation in β-cells without significant β-cell mass regeneration within the splenic lobe of FoxM1Δpanc mice after PPx compared to Sham, although it most likely would not be restricted to one lobe.

Additionally, incomplete Foxm1 recombination could have resulted in mosaic expression, allowing some β-cells to continue proliferating. However, we think this unlikely because we previously showed that Pdx1^5.5 kb-Cre recombined the Rosa26-LacZ reporter allele within all endodermally-derived pancreatic cells and yielded a significant reduction in FoxM1 protein by e15.5 (9). We also found that Foxm1 transcripts were significantly reduced in FoxM1Δpanc neonatal pancreas (Supplemental Fig. S5A) and even more dramatically in adult islets (Supplemental Fig. S5B). These tissues contain non-endodermally-derived mesenchymal, endothelial, and neuronal cells, which would not undergo Foxm1 recombination, and thus likely account for remaining transcript expression.

Proliferation of α and β-cells, but not acinar or ductal cells, was reduced in FoxM1Δpanc mice versus Control littermates following PPx, suggesting that FoxM1 is required specifically within endocrine cells. These data concur with other studies showing that endocrine cells are particularly susceptible to perturbations in cell cycle regulation (17-19,31). The current study utilized qRT-PCR on islet RNA to assess changes in Foxm1 expression following PPx. Unfortunately, antibodies to detect FoxM1 protein in mouse tissue do not currently exist, precluding precise identification of the cell population(s) in which FoxM1 expression or localization changes with time and after PPx. Such studies will allow for better understanding of cell type-specific requirements for FoxM1 and may help to explain the differing effects of FoxM1 absence that we observe between endocrine and exocrine cells following PPx. However, our expression analyses identified several potentially important regulators of pancreatic endocrine cell proliferation and regeneration that are themselves regulated by FoxM1 (Plk1, Cenp-a, Birc5/Survivin, Ccnb1).

We also presented evidence of β-cell neogenesis following 60% PPx, including Ngn3^+ duct cells, insulin^+ cells within regenerating tissue, and increased islet density. These findings were equivalent between Control and FoxM1Δpanc mice, revealing that β-cell neogenesis does not require FoxM1. Peshavaria et al. (25) showed increased small endocrine cell clusters by 2 days post-PPx, which normalized by 7 days. Our data are consistent with this finding, as Control mice at 7 days post-PPx exhibited a normal proportion of small endocrine cell clusters. However, FoxM1Δpanc mice had an increased proportion of small endocrine cell clusters at 7 days post-PPx, indicating that formation of these clusters is not impaired in the absence of FoxM1, but significant growth is. These data support our hypothesis that initial proliferation of newly-differentiated β-cells does not require FoxM1, but these cells later switch to a FoxM1-dependent state. Unfortunately, current technology precludes real-time monitoring of β-cell proliferation and islet growth. However, this study highlights the importance of β-cell proliferation as a mechanism of β-cell mass regeneration, as, our data show that neogenesis, without adequate proliferation, is not sufficient to regenerate a significant amount of β-cell mass after 60% PPx.

As β-cell regeneration after partial duct ligation mimics embryonic β-cell
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differentiation with Ngn3 re-expression (6), it is not surprising that similar processes occur following 60% PPx, although perhaps to a lesser extent. Previous studies asserting the lack of reactivation of the embryonic program, and thus neogenesis, following partial pancreatectomy were performed in a manner that likely precluded true regeneration, with surgical excision of the splenic lobe and pancreatic duct (5,32), while other studies did not analyze the splenic lobe specifically (4), possibly missing the contribution of neogenesis and regeneration to new β-cell formation. The current study removed tissue from the splenic lobe, leaving the main pancreatic duct intact. This lobe was analyzed separately, and only specific foci of regeneration exhibited evidence of neogenesis. Thus, this study does not contradict but adds to the current knowledge regarding the potential for pancreas regeneration and β-cell neogenesis.

FOXM1 over-expression did not affect β-cell regeneration following PPx, suggesting that FoxM1 is not sufficient to induce β-cell proliferation, emphasizing that the β-cell cell cycle is under tight negative regulatory control, even after injury. As reviewed (33), β-cells express all of the cell cycle “brakes” studied thus far, but only some “accelerators”. Thus, it may be more difficult than initially anticipated to stimulate β-cell proliferation in vivo or in vitro as a therapeutic option for diabetic patients. Because FoxM1 positively regulates cell cycle accelerators and negatively regulates cell cycle brakes, it is of particular interest. However, better understanding of post-translational and inhibitory regulators is necessary to successfully manipulate FoxM1 activity in β-cells.

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**Figure Legends**

**FIG. 1. Experimental timeline for 60% PPx.** Either PPx or a Sham operation was performed on 8 week old female mice, after which BrdU was administered in the drinking water for 1 week. IPGTT was performed 1 day prior to and 7 days following the operation, and the mice were sacrificed on day 7.
FIG. 2. Expression levels of Foxm1 mRNA and some of its targets were up-regulated following 60% PPx. Real-time qRT-PCR was performed on RNA extracted from isolated islets 6 days following either PPx or Sham. Error bars represent standard error of the mean (SEM). Unpaired t-tests were used to measure significance. *P < 0.05, **P < 0.01. n=4-6 per group.
FIG. 3. Glucose tolerance was maintained following 60% PPx. No significant differences in blood glucose levels were observed in either Control or FoxM1<sup>Δpanc</sup> mice following PPx compared to Sham at any time point during IPGTT. Error bars represent SEM. Two-way ANOVA with Bonferroni’s post-tests was used to measure significance. n=5-7 per group.
FIG. 4. Regeneration of β-cell mass, but not gross pancreatic tissue, was impaired in FoxM1Δpanc mice. (A) Wet weight of the pancreatic splenic lobe was measured immediately following (Day 0) or 7 days after PPx or Sham. The majority of tissue was removed by PPx, and a moderate amount of regeneration occurred within 7 days in both Control and FoxM1Δpanc mice. (B) β-cell mass, which takes into account tissue wet weight, was significantly reduced within the splenic lobe of FoxM1Δpanc pancreata after a Sham operation, and regeneration of β-cell mass was not observed in FoxM1Δpanc mice following PPx. (C) β-cell mass within the duodenal lobe of FoxM1Δpanc pancreata was only significantly reduced in comparison to Control pancreata following PPx. Error bars represent SEM. Two-way ANOVA with Bonferroni’s post-tests, comparing Sham Day 7 versus PPx Day 0 and PPx Day 0 versus PPx Day 7, was used to measure significance. ns = not significant. n=4-6 per group.
Figure 4

A

Impaired β-Cell Proliferation Without FoxM1

B

C

Ducal Lobe

β-Cell Mass (mg)

0.0 0.2 0.4 0.6

Sham Day 7  PPx Day 7

ns  P<0.05
FIG. 5. Proliferation of endocrine cells was impaired in FoxM1Δpanc mice following 60% PPx. BrdU incorporation (red) over 7 days was used as a marker of cell proliferation. Double-labeling with insulin (green) revealed low, basal levels of β-cell proliferation within the splenic lobes following a Sham operation in both Control (A) and FoxM1Δpanc (C) mice. PPx enhanced BrdU incorporation into all pancreatic cell types within the splenic lobe of Control mice (B), but incorporation into endocrine cells was reduced in FoxM1Δpanc mice (D). β-cell proliferation was quantified for both the splenic (E) and duodenal (F) pancreatic lobes. β-cell proliferation within small endocrine cell clusters (<8 β-cells) after PPx versus Sham was enhanced in the splenic lobes of FoxM1Δpanc and Control mice (G), but was not altered in the duodenal lobes (H). β-cell proliferation within definitive islets (≥9 β-cells) after PPx was enhanced in the splenic lobe of Control mice, and this effect was partially inhibited in FoxM1Δpanc mice (I). PPx also significantly stimulated β-cell proliferation within definitive islets in the duodenal lobe of Control, but not FoxM1Δpanc, mice (J). α-cell proliferation within the splenic lobe of FoxM1Δpanc pancreata (K) was also impaired in comparison to Control pancreata following PPx. Error bars represent SEM. Two-way ANOVA with Bonferroni’s post-tests was used to measure significance. ns = not significant. n=4-5 per group for β-cell proliferation, n=3-4 per group for α-cell proliferation.
Figure 5

Sham Day 7  PPx Day 7

Control

Insulin BrdU (DAPI)

400X

FoxM1^panc

E

P<0.01

P<0.001

P<0.05


Splenic Lobe % BrdU β-Cells

P<0.001  P<0.01

Sham 7 Days  PPx 7 Days

F

P<0.001  P<0.01

Sham 7 Days  PPx 7 Days

ns

Duodenal Lobe % BrdU β-Cells
Figure 5

G

H

I

J

K

Impaired \( \beta \)-Cell Proliferation Without FoxM1
**FIG. 6.** FoxM1Δpanc mice exhibited increased β-cell and nucleus size. After a Sham operation, FoxM1Δpanc mice exhibited increased β-cell size in both the splenic (A) and duodenal (B) pancreatic lobes. Increased β-cell size was also observed after PPx in the splenic lobe of FoxM1Δpanc mice versus Controls but was significantly reduced compared to after a Sham operation. The average β-cell size in Control pancreata was unchanged by PPx. FoxM1Δpanc mice also exhibited increased β-cell nucleus size in the splenic (C) but not duodenal (D) lobe, which was unchanged by PPx. Error bars represent SEM. Two-way ANOVA with Bonferroni’s post-tests was used to measure significance. n=4-5 per group.

Figure 6
FIG. 7. FoxM1Δpanc mice exhibited impaired islet growth but no impairment in islet neogenesis after 60% PPx. There was no significant difference in average islet size, measured as the cross-sectional area of each insulin+ cell grouping, between Control and FoxM1Δpanc mice after a Sham operation or PPx in either the splenic (A) or duodenal (B) pancreatic lobes. Following PPx, the average islet size in the splenic lobe of Control pancreata was similar to that observed in Sham-operated mice, but FoxM1Δpanc islets were significantly reduced in size. Within the duodenal lobe, Control mice trended toward increased average islet size after PPx, but no increase was observed in FoxM1Δpanc mice. FoxM1Δpanc mice specifically exhibited an increased proportion of small insulin+ cell clusters (1-8 β-cells) within the splenic lobe after PPx (C); no differences were observed within the duodenal lobe (D). The number of insulin+ cell groupings per section was reduced within the splenic lobe of FoxM1Δpanc mice compared to Control littermates after a Sham operation (E), but at Day 0 and Day 7 following PPx, the number of insulin+ cell groupings per section was equivalent between FoxM1Δpanc and Control mice. 7 days after PPx, there was a trend in both groups of mice to show increased numbers of insulin+ cell groupings per section within the splenic lobe, but no differences between any groups of mice were observed in the duodenal lobe (F). Ngn3 expression was detected only after PPx within cytokeratin+ (CK+) ductal epithelial cells located within foci of regeneration in the splenic lobes of both Control (G) and FoxM1Δpanc mice (H). Error bars represent SEM. Two-way ANOVA with Bonferroni’s post-tests was used to measure significance. n=4-5 per group.
Figure 7

A

B

C

D

Impaired β-Cell Proliferation Without FoxM1
Impaired β-Cell Proliferation Without FoxM1

**Figure 7**

- **E**: Graph showing the average number of insulin+ cell groupings/section across different conditions. The y-axis represents the number of cells, and the x-axis represents different days and conditions (Sham Day 7, PPx Day 0, PPx Day 7). The graph includes bars for control and FoxM1 expression conditions, with statistical significance indicated by P-values.<br>

- **F**: Similar graph for the duodenal lobe, comparing Sham Day 7 and PPx Day 7 conditions for control and FoxM1 expression.<br>

- **G** and **H**: Images of the splenic lobe showing staining for cells and DAPI. The images are labeled as Control and FoxM1 expression conditions, respectively, with a magnification of 400X.
TABLE 1. Summary of β-cell proliferation results for Control and \( \text{FoxM1}^{\text{Δpanc}} \) mice after Sham versus PPx.

|                      | β-Cell Proliferation in Clusters | β-Cell Proliferation in Islets |
|----------------------|----------------------------------|--------------------------------|
| **Control PPx vs. Sham** |                                  |                                |
| Splenic Lobe         | ↑                                | ↑                              |
| Duodenal Lobe        | ↔                                | ↑                              |
| **FoxM1^{Δpanc} PPx vs. Sham** |                                  |                                |
| Splenic Lobe         | ↑                                | ↑                              |
| Duodenal Lobe        | ↔                                | ↔                              |
| **FoxM1^{Δpanc} Sham vs. Control Sham** |                  |                                |
| Splenic Lobe         | ↔                                | ↔                              |
| Duodenal Lobe        | ↔                                | ↔                              |
| **FoxM1^{Δpanc} PPx vs. Control PPx** |                  |                                |
| Splenic Lobe         | ↔                                | ↓                              |
| Duodenal Lobe        | ↔                                | ↓                              |