Vascular Endothelial Growth Factor-A and Islet Vascularization Are Necessary in Developing, but Not Adult, Pancreatic Islets

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Pancreatic islets are highly vascularized mini-organs, and vascular endothelial growth factor (VEGF)-A is a critical factor in the development of islet vascularization. To investigate the role of VEGF-A and endothelial cells (ECs) in adult islets, we used complementary genetic approaches to temporally inactivate VEGF-A in developing mouse pancreatic and islet progenitor cells or in adult β-cells. Inactivation of VEGF-A early in development dramatically reduced pancreatic and islet vascularization, leading to reduced β-cell proliferation in both developing and adult islets and, ultimately, reduced β-cell mass and impaired glucose clearance. When VEGF-A was inactivated in adult β-cells, islet vascularization was reduced twofold. Surprisingly, even after 3 months of reduced islet vascularization, islet architecture and β-cell gene expression, mass, and function were preserved with only a minimal abnormality in glucose clearance. These data show that normal pancreatic VEGF-A expression is critical for the recruitment of ECs and the subsequent stimulation of endocrine cell proliferation during islet development. In contrast, although VEGF-A is required for maintaining the specialized vasculature observed in normal adult islets, adult β-cells can adapt and survive long-term reductions in islet vascularity. These results indicate that VEGF-A and islet vascularization have a lesser role in adult islet function and β-cell mass. ***Diabetes*** **62:4154–4164, 2013**

The pancreatic islets are endocrine mini-organs with a specialized vasculature. Islets are highly vascularized, with a dense network of capillaries that are thicker and more tortuous than vessels of the exocrine tissue (1). While islets occupy only a small volume of the pancreas, they receive a disproportionally greater fraction of pancreatic blood flow (2,3). Ultrastructurally, islets have a fenestrated endothelium, which allows for the rapid exchange of nutrients and hormones between endocrine cells and the bloodstream (1,4,5). This highly vascularized state leads to a greater partial oxygen pressure in islets than in exocrine tissue (6). The polyhedral β-cells appear to have multiple faces contacting blood vessels, and hypoxia impairs glucose-stimulated insulin secretion (7,8). Furthermore, the islet vasculature and the ECs near or in the developing pancreas and islet provide critically important instructive signals necessary for islet formation and β-cell differentiation (9,10).

Much work to understand the mechanisms directing normal islet vascularization has focused on the role of islet-derived angiogenic factors. Islet endocrine cells produce multiple factors from the VEGF, angiopoietin, and ephrin families, with VEGF-A being the predominant regulator of islet angiogenesis and vascularization. When VEGF-A is inactivated either in the early pancreas (5) or in newly formed β-cells (1), the intraislet capillary plexus fails to fully mature, resulting in substantial defects in insulin secretion and glucose intolerance. In contrast, overexpression of VEGF-A in developing pancreata (11) or β-cells (12) is detrimental to endocrine cell differentiation and islet formation. Therefore, VEGF-A expression must be precisely controlled in the developing pancreas for proper islet development and long-term glucose homeostasis.

While existing genetic mouse models demonstrated a role for VEGF-A and ECs in islet formation, the precise role of VEGF-A in adult islets is unclear. Prior studies inactivated VEGF-A during embryogenesis, thus making it difficult to identify which phenotypes resulted from developmental defects and which reflected the role of VEGF-A and ECs in adult islets. In an alternate approach, VEGF signaling inhibitors administered to adult mice demonstrated the importance of VEGF-A in maintaining the islet vascular density and permeability (13). However, the effects of VEGF inhibitors on the vasculature of multiple tissues prevented a full understanding of the role of ECs in established islets.

To investigate the role of VEGF-A and ECs in adult islet function, we used complementary genetic approaches to temporally inactivate VEGF-A in developing pancreatic and islet progenitors or in adult β-cells using a tamoxifen (Tm)-inducible Cre-loxP system. We found that adult pancreatic β-cells tolerated a significant and prolonged reduction in intraislet capillary density and still maintained relatively normal function. By comparison, inactivation of VEGF-A in early pancreas development resulted in hypovascularized islets with a sustained reduction in β-cell proliferation and mass. These data indicate that VEGF-A plays distinctive roles in developing and adult pancreatic islets.

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Received 15 January 2013 and accepted 19 July 2013.

DOI: 10.2337/db13-0071

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-0071/-/DC1.

R.B.R. and M.B. contributed equally to this article.

See accompanying articles, pp. 4004, 4144, and 4165.

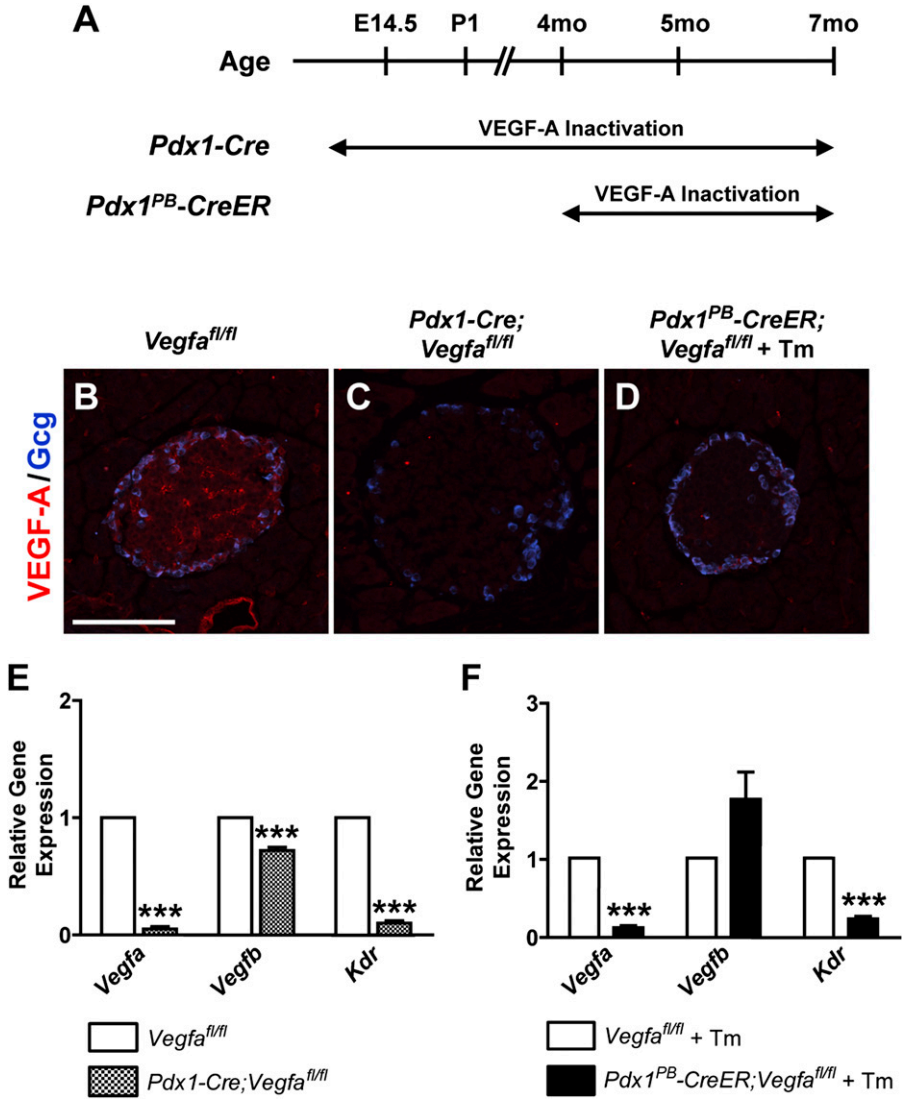

FIG. 1. The inducible *Pdx1-CreER;Vegfa*$^{fl/fl}$ line is an effective tool for VEGF-A inactivation in adult pancreatic islets. *A*: Time line of VEGF-A inactivation in *Pdx1-Cre;Vegfa*$^{fl/fl}$ and *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice. Effects of early VEGF-A inactivation in *Pdx1-Cre;Vegfa*$^{fl/fl}$ line were analyzed at e14.5, P1 and 2 months (mo) and 6 months of age. For inactivation of VEGF-A in adult islets, 4-month-old *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice and *Vegfa*$^{fl/fl}$ controls were treated with three injections of Tm or corn oil vehicle (Veh), and analysis was performed 3 months after Tm treatment unless stated otherwise. *B–D*: Representative images of double labeling for VEGF-A (red) and glucagon (Gcg) (blue) in pancreatic tissue from 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ and 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice and age-matched controls (n = 3–4 mice/genotype). Scale bar in *B* is 100 μm and applies to *C* and *D*. *E* and *F*: Relative mRNA expression measured by quantitative RT-PCR of *Vegfa*, *Vegfb*, and *Kdr* (VEGF receptor 2) in islets from 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ and 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice. *E*: In *Pdx1-Cre;Vegfa*$^{fl/fl}$, values are 0.05 ± 0.02 for *Vegfa* (n = 3), 0.72 ± 0.03 for *Vegfb* (n = 3), and 0.1 ± 0.02 for *Vegfr2* (n = 3). For all three genes, ***P < 0.001 *Pdx1-Cre;Vegfa*$^{fl/fl}$ vs. *Vegfa*$^{fl/fl}$. *F*: In *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$, values are 0.126 ± 0.0275 for *Vegfa* (n = 3; ***P < 0.0001 vs. *Vegfa*$^{fl/fl}$ control), 1.771 ± 0.349 for *Vegfb* (n = 3; P > 0.05 vs. control), and 0.241 ± 0.0323 for *Vegfr2* (n = 3; ***P = 0.0002 vs. control).

RESEARCH DESIGN AND METHODS

Mouse models were generated by breeding male hemizygous transgenic *Pdx1-Cre* mice (Mouse Genome Informatics [MGI] nomenclature: *Tg(Pdx1-cre)*$^{89.1Dam}$) (14) or *Pdx1*^{PB}*-CreER* mice (MGI nomenclature: *Tg(Pdx1-cre/ERT)*^{1Mga}) (15) with female mice expressing a conditional VEGF-A allele (*Vegfa*$^{fl/fl}$; MGI nomenclature: *Vegfa*^{tm2Gne}) (16). In all experiments, Cre-positive mice were compared with their respective age-matched *Vegfa*$^{fl/fl}$ littermates. PCR genotyping was performed on tail biopsies with primers described (14,16,17). Before all terminal procedures, mice were anesthetized with a solution of 90 mg/kg ketamine and 10 mg/kg xylazine (Henry Schein, Melville, NY). Animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Tm (cat. no. T5648; Sigma) administration was performed as previously described (18). Tm-treated mice were housed separately to prevent cross-contamination (19).

Islet isolation, VEGF-A ELISA, and quantitative RT-PCR. Islets were isolated by collagenase P digestion of the pancreas (20) and handpicked under microscopic guidance to nearly 100% purity. Quantification of islet VEGF-A production was performed by ELISA (R&D Systems, Minneapolis, MN) (1). Quantitative RT-PCR analysis on total cellular RNA from isolated islets was performed using *Rn18s* as housekeeping gene and the primer-probe sets (Applied Biosystems, Foster City, CA) listed in Supplementary Table 1 (21,22).

Tissue collection, immunohistochemistry, and imaging. Embryonic, neonatal, and adult pancreata were processed and imaged as previously described (12,20). Cryosections and whole mounts were labeled by immunohistochemistry as previously described (20) using antibodies and working dilutions listed in Supplementary Table 2. Transmission electron microscopy (TEM) was performed on pancreata from *Vegfa*$^{fl/fl}$ and *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice 1 month after Tm treatment as previously described (1).

Glucose tolerance testing, islet function, and pancreatic hormone content. Glucose tolerance testing (2 g/kg of body wt i.p.) was performed after a 14- to 16-h fast as previously described (1). Islet function was studied in a dynamic cell perifusion system (1,23). Insulin concentration in perifusate fractions and insulin content in pancreatic extracts were determined by radioimmunoassay (cat. no. RI-13K; Millipore) (24). Hyperglycemic clamps were

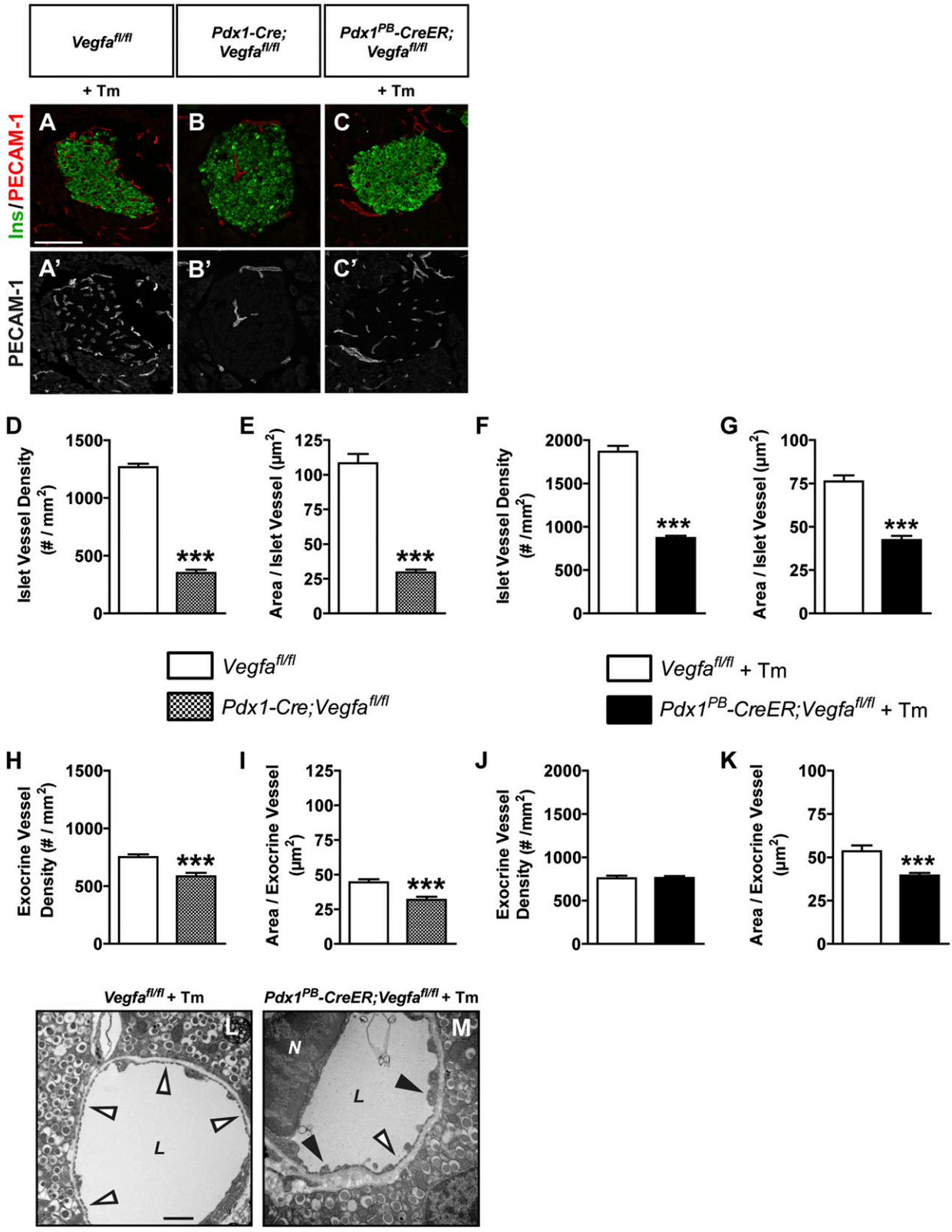

FIG. 2. VEGF-A is required to form and maintain the intraislet vasculature. The vasculature in 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ and 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice and their respective controls was assessed by integrated morphometry. *A–C*: Pancreatic sections labeled for insulin (Ins, green) and vascular marker PECAM-1 (red). Grayscale images of PECAM-1 labeling are displayed in corresponding panels *A'–C'*. Scale bar in *A* is 250 μm and applies to *B* and *C*. *D–G*: Islet vessel density and area per islet vessel in VEGF-A–deficient islets, ***$P < 0.0001$. *D*: Values are 1,267 ± 30 vessels/mm^2 in *Vegfa*$^{fl/fl}$ mice ($n = 4$) and 350 ± 27 vessels/mm^2 in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice ($n = 4$). *E*: Values are 108 ± 7 μm^2 in *Vegfa*$^{fl/fl}$ mice ($n = 4$) and 30 ± 2 μm^2 in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice ($n = 4$). *F*: Values are 1,868 ± 66 vessels/mm^2 in Tm-treated *Vegfa*$^{fl/fl}$ mice ($n = 4$) and 870 ± 27 vessels/mm^2 in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice ($n = 9$). *G*: Values are 76 ± 3 μm^2 in Tm-treated *Vegfa*$^{fl/fl}$ mice ($n = 4$) and 42 ± 2 μm^2 in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice ($n = 9$). Similar vascular changes in *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ islets were also observed 1 month after Tm treatment. *H–K*: Exocrine vessel density and area per exocrine vessel. *H*: Values are 750 ± 24 vessels/mm^2 in *Vegfa*$^{fl/fl}$ mice and

performed in conscious chronically catheterized (carotid artery and jugular vein) mice by the Vanderbilt Mouse Metabolic and Phenotyping Center according to established protocols (25).

Morphometric analysis. In adult pancreatic sections, analyses of vessel density and size were performed with MetaMorph software (Universal Imaging, Downington, PA) as previously described (1). In whole mount embryonic pancreata, the ratio of platelet endothelial cell adhesion molecule (PECAM-1$^+$) vessel area to area of Pdx1$^+$ pancreatic epithelium was calculated on every third optical section of z-stacks obtained by confocal microscopy and averaged for each pancreas.

Pancreatic β-cell area measurements were based on whole-section imaging with either a tiling (Olympus BX41) or scanning (ScanScope FL; Aperio Technologies, Vista, CA) approach. For β-cell area, four pancreatic sections (at least 200 μm apart) were immunolabeled for insulin/amylase or alternatively islet hormones. Sections were counterstained with the nuclear label DAPI. Analysis of β-cell area was performed using MetaMorph and ImageScope software (Aperio). β-Cell proliferation was assessed by either pH3 or BrdU labeling (2,000–3,000 β-cells analyzed/tissue). BrdU (Sigma, St. Louis, MO) was administered at 0.8 mg/mL in drinking water for 7 days prior to tissue collection. For these analyses, $n \geq 3$ mice were used per group.

Statistical analysis. Statistical analyses were performed with Prism software (GraphPad Software, La Jolla, CA). Comparisons between two groups were assessed by Student *t* test. Data are reported as means ± SEM. Statistical significance was assigned when $P < 0.05$.

RESULTS

Genetic models of VEGF-A inactivation in developing pancreas and adult islets. To investigate the role of VEGF-A and ECs in adult islet function, we used complementary genetic Cre-loxP approaches to temporally inactivate VEGF-A during pancreas and islet development (*Pdx1-Cre*) (14) or in adult islets (*Pdx1^{PB}-CreER*) (15) (Fig. 1*A*). For studies of VEGF-A inactivation in adult islets, we determined a Tm dose effectively reducing VEGF-A expression with minimal toxicity (3 × 1 mg Tm), which resulted in a 91.4% reduction in islet VEGF-A secretion in vitro (18) (Supplementary Fig. 1).

Compared with pancreatic acinar tissue, islet endocrine cells from adult vehicle-treated and Tm-treated *Vegfa$^{fl/fl}$* mice and from vehicle-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice expressed high levels of VEGF-A (Fig. 1 and data not shown). In contrast, reduced VEGF-A immunoreactivity was observed throughout the pancreas of *Pdx1-Cre;Vegfa$^{fl/fl}$* mice (Fig. 1*C*). Reduced VEGF-A immunoreactivity was also observed in β-cells from adult *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice both 1 and 3 months after Tm treatment, though VEGF-A was still expressed by α-cells (Fig. 1*D* and data not shown). Isolated islets from adult *Pdx1-Cre;Vegfa$^{fl/fl}$* mice and Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice showed a significant reduction in *Vegfa* and *Kdr* (VEGF-A receptor 2) mRNA compared with their respective *Vegfa$^{fl/fl}$* controls (Fig. 1*E* and *F*). There were no compensatory changes in other VEGF family members (Fig. 1*E* and *F* and data not shown).

VEGF-A inactivation in developing pancreas and adult islets reduces islet vascularity. To investigate effects of VEGF-A inactivation in the developing pancreas and adult islets, we assessed the pancreatic and islet vasculature by immunohistochemistry and morphometry (Fig. 2*A–C*). Morphometric analysis of islets from adult *Pdx1-Cre;Vegfa$^{fl/fl}$* mice showed a 72% reduction in islet vessel density (Fig. 2*D*). The islet vessel density was reduced 53% in Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice (Fig. 2*F* and data not shown). Similarly, the islet vessel size/branching was reduced 73% in adult *Pdx1-Cre;Vegfa$^{fl/fl}$* mice and 45% in Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice (Fig. 2*E* and *G*). Similar vascular changes in *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* islet were also observed 1 month after Tm treatment (data not shown). Exocrine vessel density and size were also reduced in *Pdx1-Cre;Vegfa$^{fl/fl}$* mice (Fig. 2*H* and *I*). In contrast, exocrine vessel density was unchanged in *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice, though exocrine vessel size was slightly reduced (Fig. 2*J* and *K*).

We next examined how reduced VEGF-A expression affected capillary permeability in adult *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* islets by TEM. As previously described (1,5), ECs lining capillaries in Tm-treated *Vegfa$^{fl/fl}$* controls contained highly fenestrated endothelium (Fig. 2*L*). The islet capillaries in Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice displayed a wide range in the degree of fenestrated endothelium. Fenestrations were clearly less prevalent (open arrowheads in Fig. 2*M* and Supplementary Fig. 2*A–D*), although they still could be readily found in some capillaries of VEGF-A–deficient islets. In contrast to VEGF-A inactivation during development (1,5), the abundance of caveolae in intraislet capillaries of Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice was more variable between individual capillaries (Supplementary Fig. 2*A–D*).

Timing of VEGF-A inactivation has differential effects on pancreatic and β-cell mass. Early pancreas-wide inactivation of VEGF-A not only led to severely reduced islet vascularization but also resulted in a 43% reduction in overall pancreatic tissue mass (Fig. 3*A*). This change in pancreatic mass was accompanied by a 42% reduction in pancreatic insulin content (Fig. 3*C*) and a 36% reduction in the islet area (even when normalized to the pancreatic area [Fig. 3*E*]). In contrast, pancreas size, pancreatic insulin content, and β-cell area per pancreas were unchanged in Tm-treated adult *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice (Fig. 3*B*, *D*, and *F*). Although the timing of VEGF-A inactivation in *Pdx1-Cre;Vegfa$^{fl/fl}$* and Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice differentially affected β-cell mass, islet morphology was indistinguishable between the two mutants and their respective controls (Fig. 3*G–I*).

VEGF-A–dependent maintenance of islet vasculature is required for normal glucose homeostasis but not for insulin secretion alone. We showed previously that islet vasculature is important for insulin delivery into the peripheral circulation (1). For examination of the impact of reduced adult islet vasculature on islet function and glucose homeostasis, mice from each line were challenged with a bolus of glucose. Six-month-old male *Pdx1-Cre;Vegfa$^{fl/fl}$* mice, which have greatly reduced islet vascular supply and β-cell mass, showed severe glucose intolerance (Fig. 4*A*). In contrast, Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* males had only mildly impaired glucose tolerance both 1 month and 3 months after VEGF-A inactivation (at ages 5 and 7 months [Fig. 4*B–D*]). In female mice, this phenotype only manifested 3 months after VEGF-A inactivation and was much less pronounced (Supplementary Fig. 3).

584 ± 31 vessels/mm^2 in *Pdx1-Cre;Vegfa$^{fl/fl}$* mice ($n = 4$; ***$P < 0.0001$). *I*: Values are 44 ± 2 μm^2 in *Vegfa$^{fl/fl}$* mice and 32 ± 2 μm^2 in *Pdx1-Cre;Vegfa$^{fl/fl}$* mice ($n = 4$; ***$P < 0.0001$). *J*: Values are 758 ± 30 vessels/mm^2 in Tm-treated *Vegfa$^{fl/fl}$* mice and 761 ± 21 vessels/mm^2 in Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice ($n = 4$; $P > 0.05$). *K*: Values are 54 ± 3 μm^2 in Tm-treated *Vegfa$^{fl/fl}$* mice and 39 ± 2 μm^2 in Tm-treated *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice ($n = 9$; ***$P < 0.001$). *L* and *M*: Transmission electron micrographs of islet capillaries in 7-month-old Tm-treated *Vegfa$^{fl/fl}$* and *Pdx1^{PB}-CreER;Vegfa$^{fl/fl}$* mice. Images were acquired at ×15,000 magnification. Scale bar in *L* is 100 nm and applies to *M*. L, capillary lumen; N, endothelial cell nucleus. Open arrowheads denote fenestrations, and closed arrowheads denote caveolae.

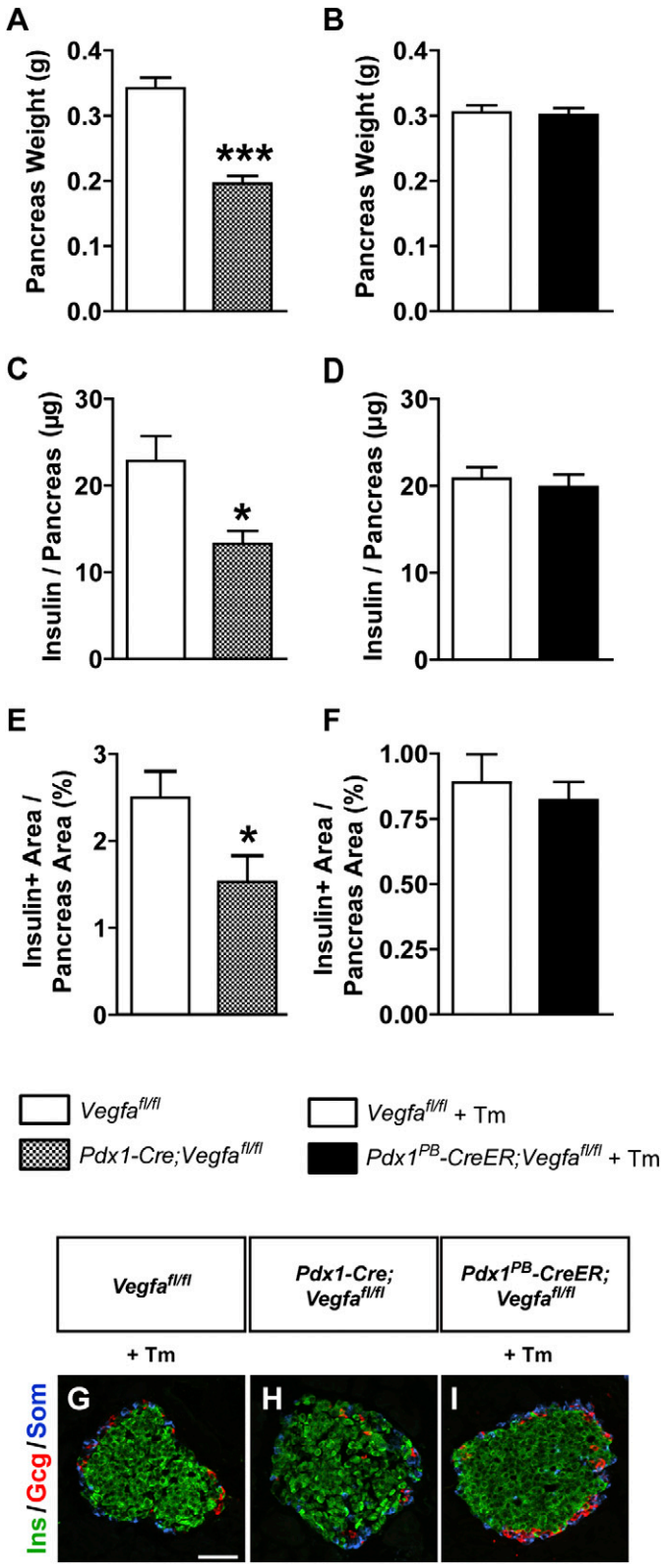

FIG. 3. Timing of VEGF-A inactivation has different effects on pancreatic and β-cell mass. *A* and *B*: Pancreatic weight is reduced in 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ (0.342 ± 0.016 g in *Vegfa*$^{fl/fl}$ vs. 0.196 ± 0.012 g in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice; n = 14–16/genotype; ***$P < 0.0001$) but is unchanged in 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice (0.305 ± 0.011 g in *Vegfa*$^{fl/fl}$ vs. 0.301 ± 0.011 g in *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice; n = 21–29/genotype; $P > 0.05$). *C*: Six-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice have reduced pancreatic insulin content (22.9 ± 2.8 µg in *Vegfa*$^{fl/fl}$ mice vs. 13.3 ± 1.5 µg in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice; n = 9–10/genotype; *$P < 0.05$). *D*: Pancreatic insulin content is unchanged in 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice (20.8 ± 1.3 µg in Tm-treated *Vegfa*$^{fl/fl}$ mice vs. 19.9 ± 1.4 µg in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice; n = 8–10/genotype; $P > 0.05$). *E*: Six-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice have reduced pancreatic β-cell area (2.50 ± 0.31% in *Vegfa*$^{fl/fl}$ mice vs. 1.53 ± 0.30% in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice; n = 3/genotype; *$P < 0.05$). *F*: Seven-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice have normal pancreatic β-cell area (0.89 ± 0.11% in Tm-treated *Vegfa*$^{fl/fl}$ mice vs. 0.82 ± 0.071% in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice; n = 3/genotype; $P > 0.05$). *G–I*: Pancreatic islets in 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice and 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice have normal architecture. Triple labeling for insulin (Ins) (green), glucagon (Gcg) (red), and somatostatin (Som) (blue). Scale bar in *G* is 50 µm and applies to *H* and *I*.

For further investigation of the milder changes in glucose tolerance in *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice, an in vivo analysis of insulin secretion by a hyperglycemic clamp experiment was performed. It demonstrated that insulin release into systemic circulation was slightly delayed in mice with hypovascularized *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ islets compared with *Vegfa*$^{fl/fl}$ controls (Supplementary Fig. 4). These data were consistent with the greater peak glucose and subsequently delayed glucose clearance during the glucose tolerance test of *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice (Fig. 4*B–D*).

Next, we sought to determine whether changes in islet vascularization had direct effects on β-cells. Reduced oxygenation, as a consequence of reduced islet vascularization, could lead to increased expression of hypoxia-inducible factor (Hif)1α and altered glucose sensing in β-cells (26,27). In *Pdx1-Cre;Vegfa*$^{fl/fl}$ islets with 72% capillary loss, Hif1α showed only a modest β-cell nuclear enrichment, and islets maintained normal *Gck* and *Glut2* expression (Supplementary Fig. 5), but Hif1α was undetectable in β-cells of Tm-induced *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ islets (which maintained 50% of their vascular supply), suggesting that islets have a relatively high capacity to adapt to reduced vascularization.

Islets from both *Pdx1-Cre;Vegfa*$^{fl/fl}$ and Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice had normal *Ins2* and *Mafa* gene expression, though *Pdx1* mRNA levels in *Pdx1-Cre;Vegfa*$^{fl/fl}$ islets were slightly reduced (Fig. 5*A* and *B*). Additionally, β-cells in both models of VEGF-A inactivation displayed normal nuclear expression of two principal regulators of insulin gene transcription, pancreatic and duodenal homeobox 1 (PDX1) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) (data not shown). β-Cells of Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice had normal morphology by TEM with densely packed insulin secretory granules displaying their characteristic halos (Fig. 5*C* and *D*). Furthermore, a functional analysis of islets isolated from Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice by an in vitro perifusion assay showed that reducing islet vasculature in adult islets does not affect β-cell insulin secretion (Fig. 5*E*).

Taken together, these data suggest that the insulin secretory defect in islets from both *Pdx1-Cre;Vegfa*$^{fl/fl}$ and Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice is mediated by impaired vascular delivery of insulin into the peripheral circulation. The defect in glucose clearance in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice was most likely exacerbated due to the combined reduction in both islet vasculature and β-cell mass. A caveat of these studies is that Cre recombinase activity has been detected in the brain (and specifically within the hypothalamus) in both our Cre driver lines (28).

How does VEGF-A inactivation in developing pancreas lead to reduced β-cell mass? Prior studies using the *Rip-Cre;Vegfa*$^{fl/fl}$ model to inactivate VEGF-A in developing islets showed that in the setting where islet

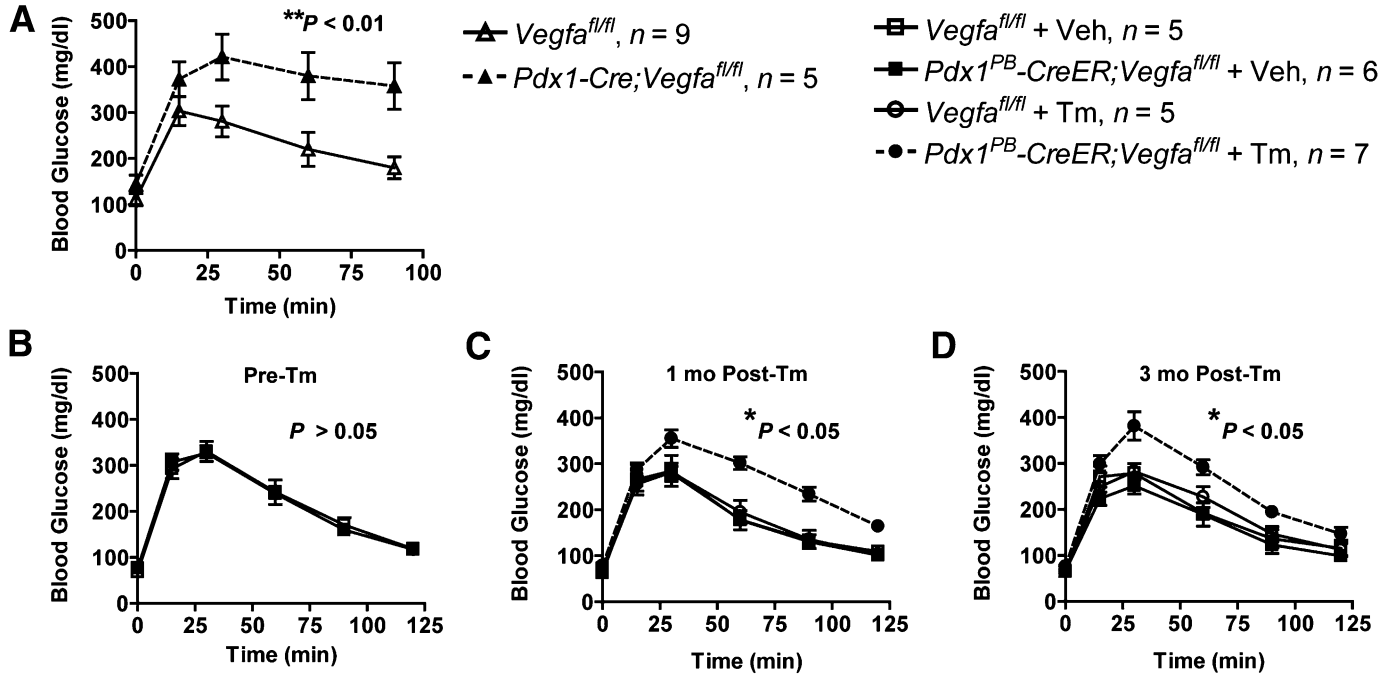

FIG. 4. Timing of VEGF-A inactivation differentially affects glucose tolerance. *A*: Glucose tolerance testing was performed in *Vegfa*$^{fl/fl}$ and *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice at 6 months of age. *B–D*: Glucose tolerance testing was performed in *Vegfa*$^{fl/fl}$ and *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice before Tm treatment (at 4 months of age [*B*]) and 1 month (*C*) and 3 months (*D*) after Tm or vehicle (Veh) treatment. In all experiments, male mice were fasted for 16 h before injection of 2 g/kg i.p. glucose. Data were analyzed by one-way ANOVA of the area under the curve. mo, month.

vasculature is reduced by 50%, mice maintained normal pancreatic insulin content and β-cell mass (1). In contrast, the current studies with *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice demonstrate that the earlier and more extensive loss of VEGF-A and islet vasculature (72%) reduces pancreatic insulin content and β-cell mass but not *Ins2* gene transcription. To address whether the reduction in β-cells was caused by reduced endocrine cell genesis, we first analyzed *Pdx1-Cre;Vegfa*$^{fl/fl}$ pancreata at the onset of secondary transition (Fig. 6*A–G*). VEGF-A inactivation with *Pdx1-Cre* line resulted in a substantially reduced pancreatic vascularization at e14.5 (Fig. 6*A–C*). Although the density of PECAM-1$^+$ vascular structures was significantly decreased in *Pdx1-Cre;Vegfa*$^{fl/fl}$ pancreata compared with their controls, there was no difference in the organization of carboxypeptidase A1$^+$ tip cells and SOX9$^+$ trunk progenitors, suggesting no defect in tip-trunk compartmentalization. Furthermore, the density of neurogenin 3$^+$ endocrine progenitors (Fig. 6*F* and *G*) and PAX6$^+$ endocrine precursors (data not shown) per E-cadherin$^+$ pancreatic epithelium was similar in *Pdx1-Cre;Vegfa*$^{fl/fl}$ and *Vegfa*$^{fl/fl}$ pancreata, suggesting that the process of endocrine cell specification is not perturbed by pancreatic hypovascularization.

At postnatal day 1 (P1), *Pdx1-Cre;Vegfa*$^{fl/fl}$ islets were severely depleted of ECs (Fig. 6*H* and *I*) but had a normal mantle-core arrangement of endocrine cells (Fig. 6*J* and K). However, severe islet hypovascularization was associated with a 31% reduction in pancreatic insulin content (Fig. 6*P*) and a 50% reduction in β-cell proliferation (Fig. 5*L*, *M*, and *Q*). β-Cells in *Pdx1-Cre;Vegfa*$^{fl/fl}$ islets continued to proliferate at lower rate throughout postnatal life, as measured by BrdU labeling in 2-month-old mice (Fig. 6*N*, *O*, and *R*). By contrast, β-cell proliferation was unchanged in 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice compared to *Vegfa*$^{fl/fl}$ controls (Supplementary Fig. 6). Collectively, these data indicate that pancreatic vasculature modulated by VEGF-A signaling is necessary for growth of both the exocrine and endocrine pancreas and that the extent and timing of VEGF-A inactivation differentially impact β-cell mass.

DISCUSSION

VEGF-A is critical for EC recruitment during islet development and is responsible for the highly vascularized state of pancreatic islets. Using complementary genetic models to temporally control VEGF-A inactivation, we show: *1*) that loss of VEGF-A expression during early pancreas development impairs pancreatic and islet vascularization and leads to a sustained reduction in β-cell proliferation, resulting in reduced pancreatic and β-cell mass and impaired glucose clearance; 2) that loss of VEGF-A expression in adult β-cells reduces islet vascularization and the number of intraislet ECs; and 3) that despite a 50% reduction in adult islet vasculature, adult islets are surprisingly capable of maintaining islet morphology, gene expression, and β-cell mass with only minimal impairment in glucose homeostasis. These results indicate that VEGF-A and the islet vasculature it regulates have a different role during pancreatic and islet development than in the adult islet (Fig. 7) and that the adult islet and β-cell have considerable capacity to survive and function when blood flow, oxygenation, and intraislet ECs are markedly reduced.

VEGF-A signaling during development is crucial for pancreas and islet growth and for islet vascularization. The close relationship between islet cells and the vasculature begins in early pancreatic development, when ECs are required for the initiation of pancreas bud formation and insulin gene induction (9,10). Pancreatic vascularization is also important in differentiation and development of the

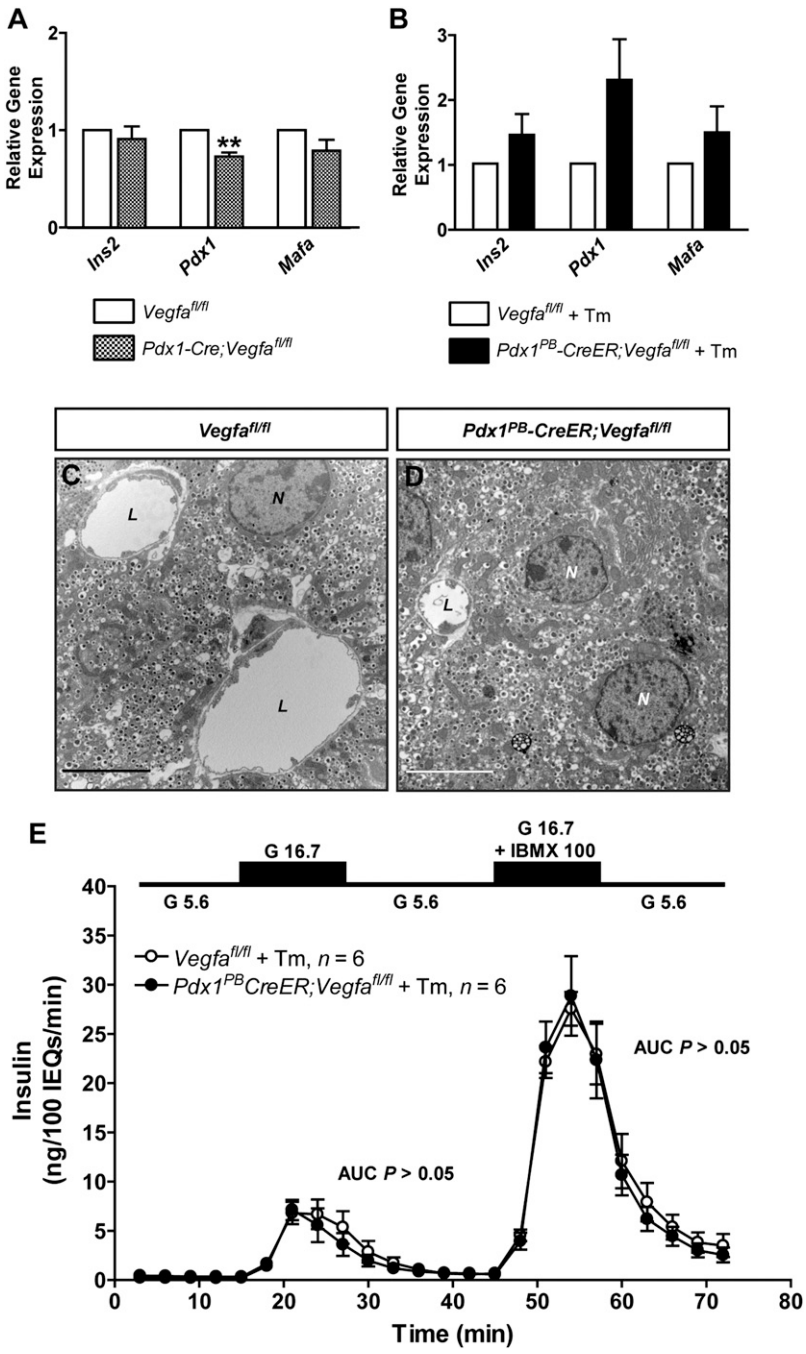

FIG. 5. VEGF-A inactivation in adult islets has no effect on β-cell gene expression, β-cell secretory granule morphology, or insulin secretion in vitro. *A* and *B*: Relative gene expression of *Ins2*, *Pdx1*, and *Mafa* in islets from 6-month-old *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice (*A*) and 7-month-old Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice (*B*), evaluated by quantitative RT-PCR. *A*: Values are 0.91 ± 0.13 for *Ins2* (n = 4; P > 0.05 vs. control), 0.73 ± 0.04 for *Pdx1* (n = 4; **P < 0.01 vs. control), and 0.79 ± 0.11 for *Mafa* (n = 4; P > 0.05 vs. control). *B*: Values are 1.46 ± 0.32 for *Ins2*, 2.31 ± 0.62 for *Pdx1*, and 1.50 ± 0.40 for *Mafa* (n = 3/genotype). For all three genes, P > 0.05 vs. control. *C* and *D*: Transmission electron micrographs of islets from Tm-treated *Vegfa*$^{fl/fl}$ (*C*) and *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ (*D*) mice revealed normal insulin secretory granule morphology and density. Images were acquired at ×5,600 magnification. Scale bars are 2 μm; L, capillary lumen; N, nucleus. *E*: Perifusion of islets isolated from Tm-treated mice. The effluent was collected at 3-min intervals using an automatic fraction collector. Areas under the curve (AUC) for islet insulin secretory responses to the 16.7 mmol/L glucose (G 16.7) and 16.7 mmol/L glucose + 100 μmol/L isobutylmethylxanthine (G 16.7 + IBMX 100) stimuli were not significantly different in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice (n = 6) vs. Tm-treated *Vegfa*$^{fl/fl}$ controls (n = 6). IEQs, islet equivalents.

exocrine pancreas (29). The assembly of the islet vasculature and the aggregation of developing islet cell clusters into an islet occur concomitantly in the later stages of development (E16-P1 in the mouse) (1). The data presented here provide new insights into these coordinated and integrated processes by showing that VEGF-A inactivation using a *Pdx1-Cre* leads to reduced β-cell mass at birth and that the β-cell mass is never recovered. These results are quite different from when VEGF-A is inactivated slightly later in development with an *Ins-Cre* where β-cell mass is not affected, but delivery of insulin into the systemic circulation is impaired by the reduced islet vascularization (1). The reduced β-cell mass in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice is caused not by reduced islet endocrine cell genesis but, instead, by a reduction in β-cell

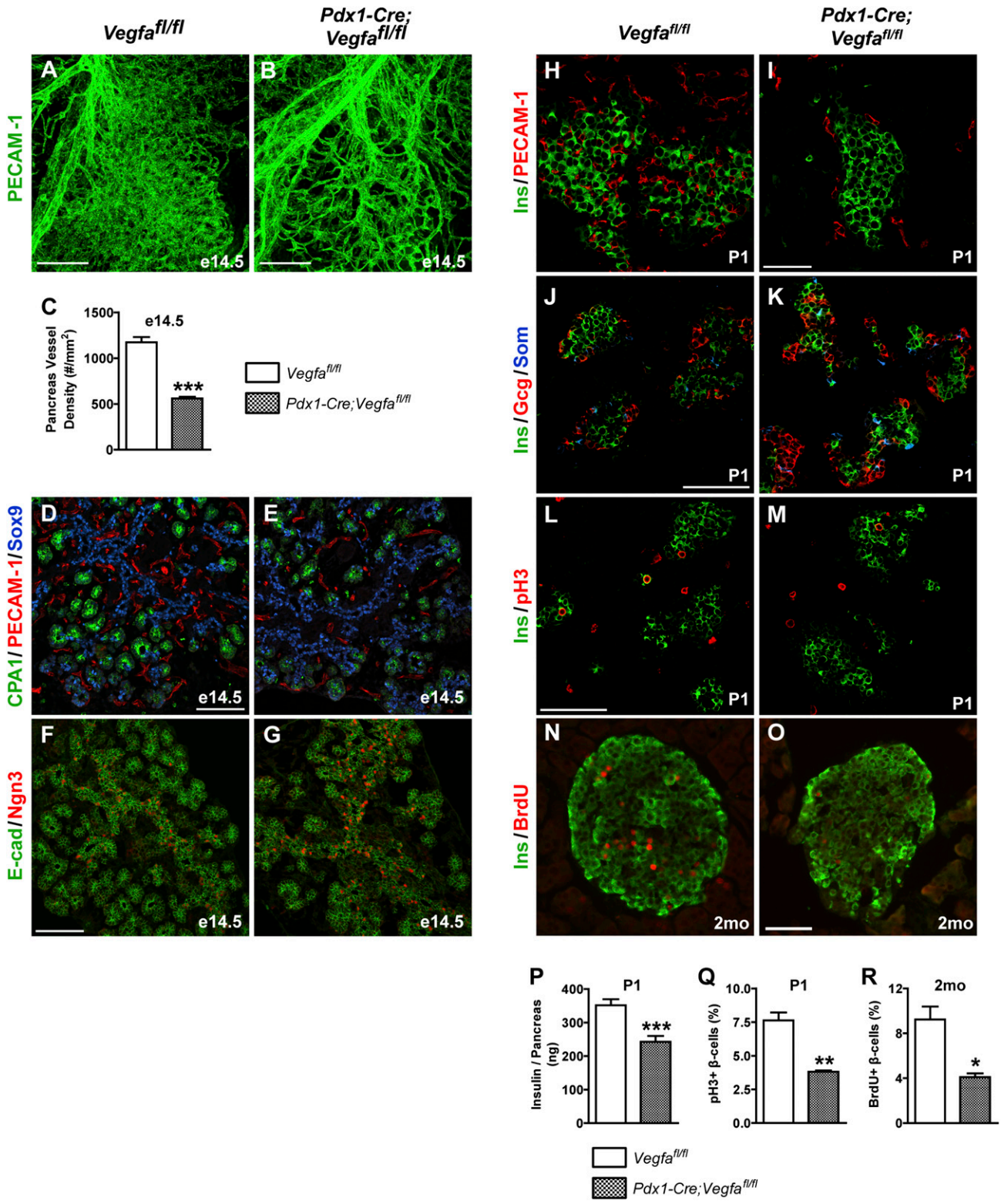

FIG. 6. Inactivation of VEGF-A in the developing pancreas reduces postnatal β-cell proliferation. *A* and *B*: Whole-mount immunohistochemistry labeling of *Vegfa*$^{fl/fl}$ (*A*) and *Pdx1-Cre;Vegfa*$^{fl/fl}$ pancreata (*B*) at e14.5 for vascular marker PECAM-1. *C*: Pancreatic vessel density in embryonic pancreas; 1,174 ± 57 vessels/mm^2 in *Vegfa*$^{fl/fl}$ vs. 561 ± 17 vessels/mm^2 in *Pdx1-Cre;Vegfa*$^{fl/fl}$ pancreata (n = 5, ***P < 0.001). *D* and *E*: Embryonic pancreas at e14.5, immunolabeled for carboxypeptidase A1 (CPA1) (green), SOX9 (blue), and PECAM-1 (red). *F* and *G*: Embryonic pancreas at e14.5, immunolabeled for E-cadherin (E-cad) (green) and neurogenin 3 (Ngn3) (red). *H–M*: Pancreatic sections from *Vegfa*$^{fl/fl}$ and *Pdx1-Cre; Vegfa*$^{fl/fl}$ mice at postnatal day 1 (P1) were labeled for insulin (Ins) (green) and PECAM-1 (red) in *H* and *I*; insulin (green), glucagon (Gcg) (red), and somatostatin (Som) (blue) in *J* and *K*; and insulin (green) and phosphorylated histone H3 (pH3) (red) in *L* and *M*. *N* and *O*: Pancreatic sections from 2-month-old *Vegfa*$^{fl/fl}$ and *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice were labeled for insulin (green) and BrdU (red). *P*: Pancreatic insulin content in *Vegfa*$^{fl/fl}$ and *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice at P1; 352 ± 18 ng in *Vegfa*$^{fl/fl}$ vs. 243 ± 17 ng in *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice (n = 13–16/genotype, ***P = 0.0002). *Q*: β-Cell proliferation at P1, as measured by staining for pH3; 7.63 ± 0.59% in *Vegfa*$^{fl/fl}$ vs. 3.81 ± 0.09% in *Pdx1-Cre;Vegfa*$^{fl/fl}$ pancreata (n = 3, **P = 0.0031). *R*: β-Cell proliferation in 2-month-old mice as measured by BrdU labeling; 9.24 ± 1.15% in *Vegfa*$^{fl/fl}$ and 4.10% ± 0.33% in *Pdx1-Cre; Vegfa*$^{fl/fl}$ pancreata (n = 3, *P = 0.0373). mo, month.

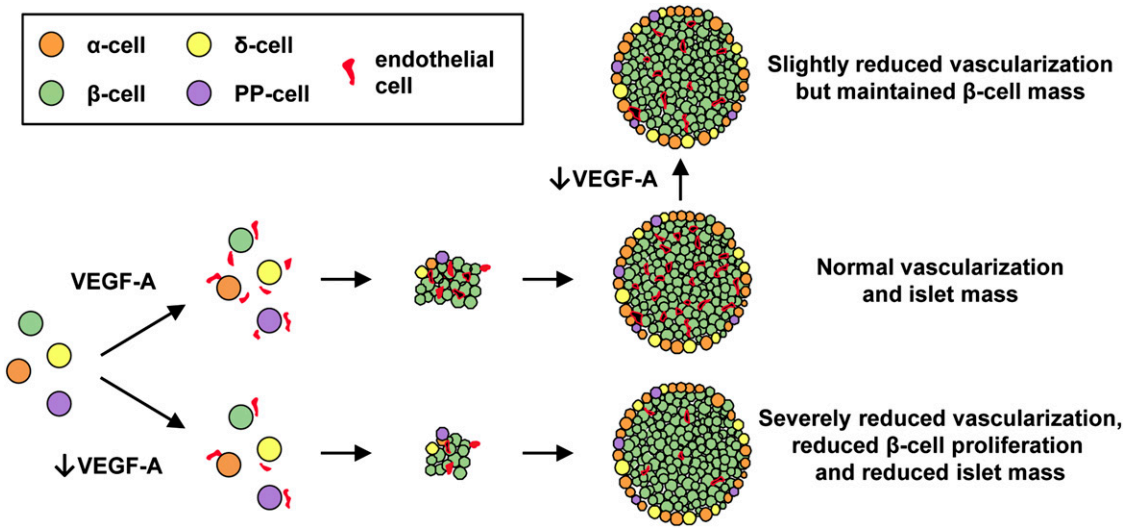

FIG. 7. Model of different roles for VEGF-A during development and in adult islets. Developing islet endocrine cell progenitors recruit ECs as they are coalescing into islet cell clusters through a VEGF-A–dependent process (*left side*). When VEGF-A was reduced early in development, fewer ECs were recruited, resulting in hypovascularized islets and reduced β-cell proliferation and mass. When VEGF-A was reduced in adult islets (*upper right*), vascularization was reduced, but β-cell proliferation and mass were unchanged. PP, pancreatic polypeptide.

proliferation. This reduced β-cell proliferation was noted at P1 and continued in adult islets. Since this reduction in β-cell proliferation was not seen when the Tm-based system was used to inactivate VEGF-A in adult β-cells, we conclude: *1*) that either β-cells present in the developmental window between VEGF-A inactivation with the *Pdx1-Cre* and *Ins-Cre* respond differently to EC signals promoting proliferation or ECs during this brief developmental window produce different signals than those in later development and in the adult; *2*) that if β-cells do not receive this proliferative stimulus from ECs during this developmental window, their subsequent ability to proliferate is compromised; and *3*) that more extensive loss in islet vasculature with *Pdx1-Cre* vs. *Ins-Cre* leads to reduced β-cell proliferation. In addition, the early inactivation of VEGF-A with the *Pdx1-Cre* could affect developing islets by reducing the number of growth factor–producing ECs adjacent to developing islets and limiting delivery of nutrients through a reduced blood supply. Some of the current results with the *Pdx1-Cre;Vegfa*$^{fl/fl}$ mice differ from a previous report (5), in which the pancreatic islet area was not reduced, but glucose tolerance was impaired. In the current report, more detailed analyses demonstrated that both pancreatic and β-cell mass were reduced, with β-cell mass being assessed by independent measurements of insulin content, β-cell area, and β-cell mass.

Neither VEGF-A signaling nor islet vascularization is required for maintenance of β-cell mass and function. Because inactivation of VEGF-A during pancreas and islet development had such a major effect on islet β-cell mass and because islet oxygenation and blood flow are thought to be critical for insulin secretion, we predicted that inactivation of VEGF-A in adult β-cells would lead to reduced β-cell mass and markedly impaired insulin secretion. Although we were able to induce a substantial and prolonged reduction in islet VEGF-A production, the β-cell mass and function were remarkably similar to controls and the mice had only slight changes in glucose metabolism in vivo. In addition, islets from Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice had no change in expression of the β-cell genes *Ins2*, *Pdx1*, or *Mafa* and no difference in pancreatic insulin content or β-cell area. We did not observe a reduction in pancreatic β-cell area or proliferation even 3 months after VEGF-A inactivation, suggesting that a 50% reduction of islet vasculature does not impair basal β-cell survival.

The main defect observed in *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice was slightly impaired glucose tolerance after VEGF-A inactivation. This suggests that there is either a delay in the glucose stimulus reaching the β-cells or a delay in insulin release into the bloodstream. Because β-cells can secrete insulin into the interstitium and directly into capillaries (30), it is likely that the insulin produced in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice has a longer path to traverse before finding the bloodstream. Indeed, perifusion of islets from Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice showed no alterations in glucose-stimulated insulin secretion in vitro, while the hyperglycemic clamp data demonstrated a slight delay in glucose-stimulated insulin secretion in vivo. Importantly, *Pdx*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice were able to attain the insulin secretion seen in the control mice at the end of the clamp, consistent with the minimal difference in blood glucose levels at the end of the glucose tolerance test. Previous studies showed that adult *Rip-Cre;Vegfa*$^{fl/fl}$ mice had more severe glucose intolerance and reduced insulin secretion, even though they display a reduction in intraislet vessel density similar to that in Tm-treated *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice and normal β-cell mass (1). However, *Rip-Cre;Vegfa*$^{fl/fl}$ mice also show dramatic changes in intraislet capillary ultrastructure, displaying few fenestrations and increased caveolae (1), similar to intraislet capillaries in mice treated with VEGF signaling inhibitors (13) and those reported by Lammert et al. (5). In contrast, *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice display a mix in capillary ultrastructure, with the preservation of many fenestrations. It is possible that non–β endocrine cells and β-cells in which VEGF-A was not inactivated are able to partially compensate for the lack of VEGF-A production by β-cells in *Pdx1*^{PB}*-CreER;Vegfa*$^{fl/fl}$ mice and that this lesser reduction in capillary permeability may account for the very mild glucose intolerance seen in this model. Our metabolic data contrast with data from mice treated with a VEGF receptor inhibitor, in which fasting blood glucose and glucose tolerance were improved (13). Although the inhibitor

led to a reduction in pancreatic islet vascular density similar to that in our model, it also likely affected the vasculature of multiple other tissues.

Implications for islet adaptation, type 2 diabetes, and islet transplantation. These findings have implications for islet-related physiology and pathophysiology. For example, in insulin-resistant states such as obesity, pregnancy, and puberty, the β-cell must respond by increasing insulin biosynthesis and output, and one would predict that both the delivery of nutrients and oxygen and the efflux of insulin from the islet require adaptations in blood flow. The current results indicate that there is likely sufficient redundancy in islet blood flow so that islet vascularization would not limit the β-cell's response to such challenges. We have noted that islet blood flow increases in hyperplastic islets, but this is the result of intraislet vessel dilatation and not an increase in the number of intraislet vessels (31). In these expanded islets with dilated vasculature, β-cells are a greater distance from a capillary and thus may have an islet/capillary relationship similar to that seen in islets with VEGF-A inactivated in adult mice.

Decreased vessel density and oxygen tension (32,33) in islet grafts may contribute to islet death or dysfunction in the early transplantation period. Since islets after transplantation are avascular, the reduction in islet blood flow would be greater than in Tm-induced reduction in islet vascularization, so efforts to hasten islet revascularization are still warranted. However, the current results do suggest that it may not be necessary for transplanted islets to achieve the highly vascularized state of islets within the pancreas. In fact, Olsson and Carlsson (34) reported recently that many islets in the pancreas are exposed to low oxygenation and are only recruited when necessary, so perhaps β-cells do not require a high oxygen tension and can function when vascularization is reduced. Thus, additional investigation is required before the relationship of islet vascularization and islet function in adult islets is clearly defined.

Finally, insufficient VEGF-A signaling to ECs and reduced EC signals to developing islet cells at critical stages of development not only reduced pancreatic and β-cell mass but also reduced basal β-cell proliferation in adult islets, suggesting that the embryonic effect persists. Thus, it is possible that reduced VEGF signaling during development could lead to a reduced ability to expand β-cell mass in response to challenges such as insulin resistance.

ACKNOWLEDGMENTS

This work was supported by a Merit Review Award from the VA Research Service (BX000666), by National Institutes of Health (NIH) grants (DK69603, DK68764, DK89572, DK66636, DK63439, DK72473, and DK89538), by grants from JDRF International, by the Vanderbilt Mouse Metabolic Phenotyping Center (NIH grant DK59637), and by the Vanderbilt Diabetes Research and Training Center (DRTC) (DK20593). Islet isolation was performed in collaboration with the Vanderbilt Islet Procurement and Analysis Core (supported by the Vanderbilt DRTC). Tissue sections were acquired in part by the Vanderbilt Translational Pathology Shared Resource. Imaging was performed with support from the Vanderbilt University Medical Center Cell Imaging Shared Resource (NIH grants CA68485, DK20593, DK58404, HD15052, DK59637, and EY08126).

No potential conflicts of interest relevant to this article were reported.

R.B.R. conceived and designed the experiments, performed the experiments, analyzed data, and wrote the manuscript. M.B. conceived and designed the experiments, analyzed data, and wrote the manuscript. A.S. performed the experiments. F.C.P. performed the experiments and reviewed and edited the manuscript. G.P. performed the experiments and reviewed and edited the manuscript. Q.C. and G.L.H. performed the experiments. J.K. conceived and designed the experiments and performed the experiments. C.S.T. performed the experiments and reviewed and edited the manuscript. C.D. conceived and designed the experiments, performed the experiments, analyzed data, and reviewed and edited the manuscript. O.P.M. conceived and designed the experiments, analyzed data, and reviewed and edited the manuscript. A.C.P. conceived and designed the experiments, analyzed data, and wrote the manuscript. A.C.P. is the guarantor of this work and, as such, had 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.

Parts of this study were presented in abstract form at the 66th Scientific Sessions of the American Diabetes Association, Washington, D.C., 9–13 June 2006, and at the 69th Scientific Sessions of the American Diabetes Association, New Orleans, Louisiana, 5–9 June 2009.

The authors thank Guoqiang Gu and Douglas A. Melton (Harvard University) for the *Pdx1-Cre* mice, Maureen Gannon and Christopher V.E. Wright (Vanderbilt University) for the $Pdx1^{PB}$*-CreER* mice, and Napoleone Ferrara for the $Vegfa^{loxP}$ mice. The authors are grateful to Christopher V.E. Wright, Roland Stein, and Guoqiang Gu for providing antibodies (Vanderbilt University).

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