Supporting Information

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SI Materials and Methods

Construction of a Polycistronic Viral Vector Containing Three β-Cell Transcription Factors. Construction of the polycistronic construct *Ad-PNM* was described in ref. 1. Properties of the 2A sequence are described in ref. 2. Construction of the *Ad-EGFP-PNM* version was done by ligating a *EGFP-2A* fragment in front of the *Pdx1* gene as a Sal/Xho fragment. Recombinant adenoviruses were produced by electroporating the linearized shuttle vector into BJ5183 bacterial cells using the pAdEasy adenoviral vector system (Agilent Technologies), according to the manufacturer's instructions, and purified by CsCl banding to obtain a titer of 10^{11} pfu/mL. To confirm that the proteins were made and cleaved efficiently, Western blots were performed. Briefly, lysates were prepared from cells expressing the PNM cassette and run on a 10% (wt/vol) SDS/PAGE, transferred to a PVDF membrane, and incubated with polyclonal rabbit PDX1, NGN3, and MAFA antibodies. Rabbit HRP-conjugated secondary antibody was used to detect the translated proteins.

Induction of Diabetes and Delivery of Transcription Factors. All animal experiments were approved by the institutional animal care and use committee of the University of Minnesota. Diabetes was induced in mice with an i.p. injection of streptozotocin (STZ) (Sigma) at a dose of 120 mg/kg body weight. One or two injections of STZ were given depending on the responsiveness of the mice to the drug. Blood glucose was measured with an Accu-Chek glucose meter (Roche). Mice showing a blood glucose levels in the range from 360 to 600 mg/dL over at least 7 d were considered as diabetic and were used for treatment with *Ad-PNM* or *Ad-EGFP-PNM*. The standard dose used for the NOD-SCID mice was 150 μL of 10^{10} pfu/mL (i.e., 1.5×10^9 pfu/mouse), injected into the tail vein.

For assessment of any liver damage, blood was drawn 7 d after *Ad-PNM* delivery and was allowed to clot for 30 min at room temperature. It was then spun at $700 \times g$ for 20 min at 4 °C. The serum was collected and was sent to the pathological laboratory at the University of Minnesota Veterinary College for analysis of the bilirubin and the liver enzymes.

Blood Glucose Monitoring and Glucose Tolerance Test. The blood glucose levels in the treated mice were monitored every alternate day or once every week as needed, along with measurements of body weight. A glucose tolerance test was performed after 4 wk in the responding mice. They were made to fast for 16 hr followed by an i.p. injection of a glucose solution (2 g/kg body weight). Blood glucose levels were measured at 0, 30, 60, and 120 min after glucose administration. Also serum was collected from the mice at the indicated time points and insulin protein was measured using an Ultrasensitive ELISA kit for mouse (Alpco).

Immunohistochemistry. Livers were either fixed in 4% (wt/vol) paraformaldehyde made in PBS, pH 7.4, or in 10% (vol/vol) formalin buffered with PBS (Fischer) for 2 h or overnight at room temperature and were either frozen in optimal cutting temperature compound (OCT) or embedded in paraffin. Sections of 5–7 μm were cut and paraffin-embedded sections were subject to antigen retrieval with sodium citrate, pH 6 for 20 min using a vegetable steamer. Sections were incubated with respective primary antibody after permeabilization with 1% Triton X-100 for 20 min, blocked in 10% (vol/vol) goat or sheep serum, and incubated with the appropriate primary antibody overnight at 4 °C. Then they were washed in PBS and incubated with Alexa Fluor conjugated secondary antibody for 1 hr to visualize the required immunofluorescence staining.

The list of primary antibodies used is given in Table S1.

Electron Microscopy. Livers of *Ad-PNM*-treated mice were fixed in 2.5% (vol/vol) glutaraldehyde and 0.1 M sodium cacodylate, pH 7.4 for 2 h at room temperature and then overnight at 4 °C. For transmission electron microscopy, samples were further fixed with 1% osmium tetroxide/0.1 M sodium cacodylate buffer, dehydrated, embedded in Epon resin, and sectioned at 1 μm. Ectopic ductal structures were localized with Toluidine Blue staining and ultrathin sections of 60 nm were cut from the same region on a Leica U6 Ultracut-S microtome, picked up onto copper grids, stained with 0.2% lead citrate for 5 min, and examined in a JEOL 1200EX transmission electron microscope.

ELISA. Insulin was extracted from the liver tissues of the responding mice by an acid-ethanol (1:37) method containing (1:10) protease inhibitor mixture (Sigma). The cells were lysed and left on a rotator overnight at 4 °C. The mixture was centrifuged at $15{,}700 \times g$ for 10 min to separate supernatant and pellet, and the pellet was dissolved in 100 mM NaOH. The total protein in supernatant and dissolved pellet was measured using the Protein Measurement kit (Pierce). The insulin levels in the supernatant and dissolved pellet were measured using an Ultrasensitive ELISA kit for mouse (Alpco) according to the manufacturer's instructions.

Collagenase Perfusion of Mouse Liver and Collection of Cells. Isolation of cells from the liver was performed following systemic perfusion of a collagenase solution through the left ventricle according to ref. 3. An incision was made in the right atrium to ensure a route to overflow. Perfusion was then started by passing Earles balanced salt solution (EBSS) without calcium and magnesium, supplemented with 0.5 mM EGTA and 10 mM Hepes (pH 7.4) for 10 min at 10 mL/min. This was followed by EBSS with calcium and magnesium supplemented with 10 mM Hepes (pH 7.4) for 10 more min. Collagenase (Roche) dissolved at 0.25–0.5 mg/mL (depending on the individual lot) in the same solution was next perfused for another 20 min at 5 mL/min. Following perfusion, the whole liver was removed and teased with forceps to suspend the cells in DMEM with 10% (vol/vol) FBS. The cell suspension was then filtered through a 70-μm mesh and centrifuged three times for 3 min at $250 \times g$ to pellet all of the cell types. The pellet was resuspended in the same medium and cell viability was determined using a Trypan Blue dye exclusion test. These preparations contain all liver cell types, not just hepatocytes.

Newport Green Staining, Cell Sorting, Glucose Sensitivity. Labeling with Newport Green diacetate, (NG-Ac; Invitrogen) was done on isolated liver cells from mice that had responded to *Ad-PMN*. Cells were washed twice with PBS and then incubated for 30 min at 37 °C with PBS containing 1–10 μM NG-Ac plus 1 μL/mL Pluronic F127 (Sigma) to aid penetration of the dye. After washing in PBS with 5% (vol/vol) FCS, the cells were resuspended and the single-cell suspension was subjected to fluorescence microscopy and FACS analysis.

Newport Green-stained cells were resuspended at 2×10^6 cells per milliliter in wash buffer [PBS with 5% (vol/vol) FCS and 0.1% 7-AAD; Sigma]. This was fractionated with a FACS Aria flow cytometer. 7-AAD$^+$ cells were rejected and Newport Green-labeled live cells were selected. The negative control used to set the background level of fluorescence were liver cells from

an untreated diabetic mouse stained in the same way. Fig. S7 *A* and *B* show the two steps with side scatter on the vertical axis. The Newport Green$^+$ cells were collected in Krebs-Ringer buffer and incubated for 2 h at 37 °C. This was followed by 1-h incubation in Krebs-Ringer buffer containing 2.2 mM glucose, or 20 mM glucose, and 0.5 mmol/L isobutylmethylxanthine (IBMX) (Sigma). The cells were separated from the buffer following the incubation and lysed in acid-ethanol [0.18 M HCl/35% (vol/vol) ethanol] containing 1/10 protease mixture inhibitor (Sigma).

Quantitative Reverse Transcription PCR (qRT-PCR). RNA isolation and cDNA synthesis from the Newport Green$^+$ and Newport Green$^-$ negative cells were performed by using a Qiagen RNeasy micro kit, SuperScript II, and Oligo-dT or random primers (Invitrogen) according to the manufacturer's instructions. The gene expression pattern between Newport Green$^+$ and Newport Green$^-$ cells was then compared by performing qPCR using Prime Time qPCR probes from IDT for mouse *Albumin*, *C/ebpα*, *Insulin 1* (*Ins1*), *Insulin 2* (*Ins2*), *Sur1*, *Kir6.2*, *Slc30a8*, *Gck*, *Glut2*, *Pdx1*, *Gcg*, *Sst*, *Ppy*, *Isl1*, *Mnx1*, *FoxA2*, *Sox17*, *Sox9*, *GATA4*, *Fgf10*, *NeuroD1*, *Rfx6*, *Nkx2.2*, *Pax4*, *MafA*, *Ngn3*, *Pdx1*, *Ngn3 UTR*, *Nkx6.1*, *Arx*, and *Amy*. qRT-PCR conditions were 95 °C for 30 s initial denaturation, 95 °C for 10 s denaturation, 60 °C for 10 s annealing, 60 °C for 30 s extension, and overall 40 cycles were performed. Primers used for qRT-PCR are listed in Table S2.

Islet Isolation. The common bile duct was cannulated posterior to the branching point. Using a 30-gauge butterfly needle and a 3-mL syringe, distension solution containing HBSS supplemented with 10 mg/mL insulin, 1% heparin, and 5 mg/2.5 mL collagenase was injected through the duct until the pancreas was fully distended. The pancreas was then taken out and incubated at 37 °C with the remaining distension solution. After 10 min it was vortexed and digested for an additional 6 min. Stop solution was then added containing FBS and it was centrifuged for 2 min at 100 × *g* at 8 °C. The pellet was resuspended in the stop solution and the mixture passed through the 850-μm strainer. This solution was then centrifuged for 3 min at 250 × *g* at 8 °C. The pellet was resuspended in FBS and layered on a gradient maker containing OptiPrep Separation medium (Bioexpress) and gradient stock solution (Cellgro). It was then spun at 700 × *g* for 5 min. The upper fraction containing the layer of islets was collected. It was resuspended in Krebs-Ringer buffer and, using an islet picker and dissecting microscope, the islets were removed from any remaining nonislet tissue. The islets were finally resuspended in fresh Krebs-Ringer buffer for the assays.

Labeling of SOX9$^+$ Cells. *Sox9-CreERT2; mT/mG* double heterozygous animals were generated by mating homozygous *Sox9-CreERT2* males with *mT/mG*, a double-fluorescent Cre-reporter mouse that expresses membrane-targeted tandem dimer Tomato (mT) before Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision (4). Tamoxifen (Sigma) was dissolved in sunflower seed oil at a concentration of 10 mg/mL and injected intraperitoneally to pregnant mice at a dose of 3 mg. The stage of pregnancy was day 15 (embryonic day E15.5) to label the *Sox9*$^+$ cells of the ductal plates. Five-week-old postnatal *Sox9CreERT2; mT/mG* mice were made diabetic with streptozotocin at a dose of 120 mg/mL. Mice having a blood glucose level above 400 mg/dL were injected with *Ad-PNM* and cryosections were prepared of the livers. These were analyzed for GFP fluorescence and for SOX9 and insulin by immunostaining. Because the mice also contain the tdTomato label, far red conjugates were used for the secondary antibodies.

1. Akinci E, Banga A, Greder LV, Dutton JR, Slack JMW (2012) Reprogramming of pancreatic exocrine cells towards a beta (β) cell character using Pdx1, Ngn3 and MafA. *Biochem J* 442:539–550.
2. de Felipe P (2004) Skipping the co-expression problem: The new 2A "CHYSEL" technology. *Genet Vaccines Ther* 2:13.
3. Ouji Y, et al. (2010) Isolation and characterization of murine hepatocytes following collagenase infusion into left ventricle of heart. *J Biosci Bioeng* 110:487–490.
4. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo LQ (2007) A global double-fluorescent Cre reporter mouse. *Genesis* 45:593–605.

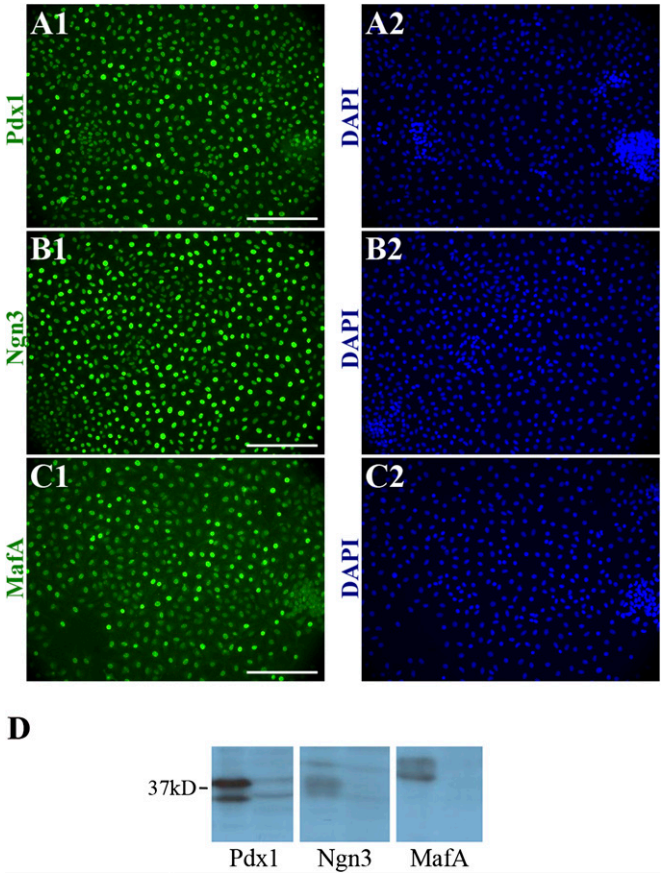

Fig. S1. Expression of PDX1, NGN3, and MAFA after transduction of AR42j-B13 cells with *Ad-PNM*. (*A–C*) AR42jB13 cells transduced with *Ad-PNM* expressed all three genes 3 d after delivery. (*D*) Western blots showing the expression of the three genes as individual cleaved peptides. These blots were performed on mES cells containing the same three-gene cassette in Dox-inducible form. In each case the *Left* lane shows the induced protein and the *Right* lane is uninduced.

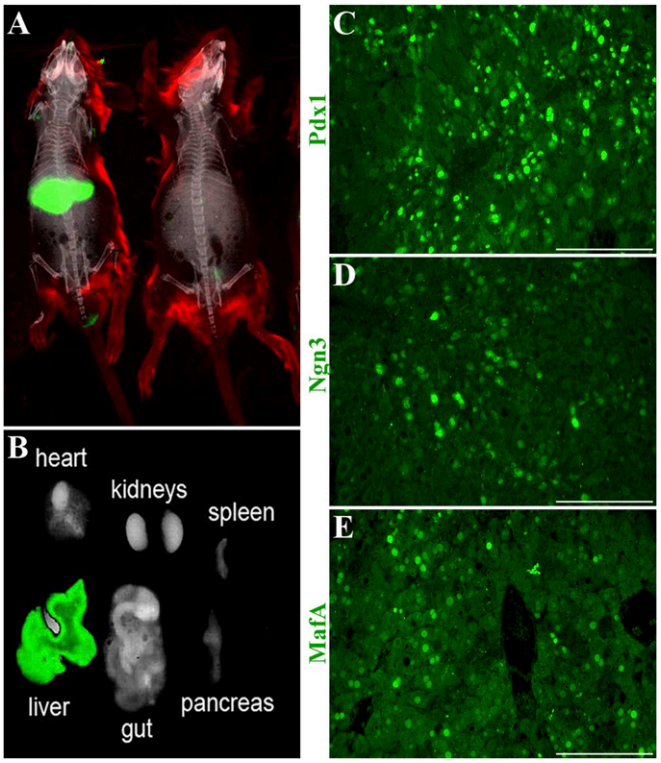

Fig. S2. Adenoviral delivery of the β-cell transcription factors by tail vein injection is confined to the liver. (*A*) In vivo imaging of a mouse dosed with *Ad-EGFP-PNM* shows presence of GFP in the liver. (*B*) No GFP expression is visible in the kidney, pancreas, or heart. (*C–E*) Expression of PDX1, NGN3, and MAFA in the liver 1 wk after delivery of *Ad-PNM*. (Scale bars, 100 μm.)

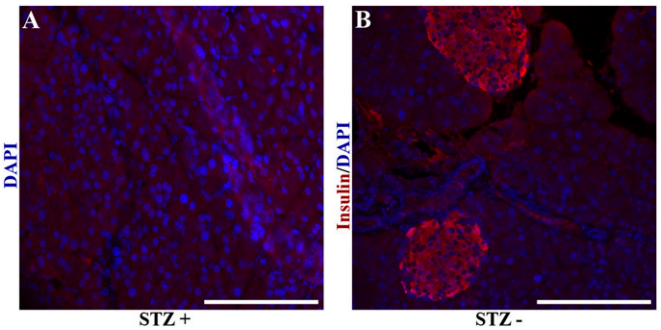

Fig. S3. Nonregeneration of islets in STZ-treated mouse liver. Pancreases were taken out 12 wk after *Ad-PNM* delivery. Random 10-μm sections were taken from throughout the pancreas, stained for insulin, and examined. Images shown are typical of the overall appearance. Immunostaining pictures are representative of at least three independent experiments. (Scale bars, 100 μm.)

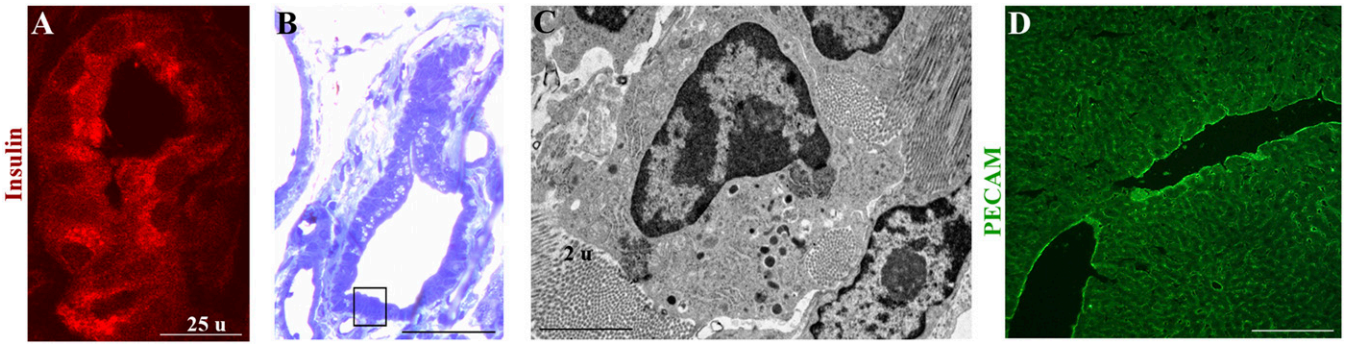

Fig. S4. Granular characteristics of the ductal insulin$^+$ cells. (*A*) Insulin staining of an ectopic duct at higher magnification shows the granular nature of the insulin. (*B*) Toluidine blue staining was done to localize ectopic ductal structures for electron microscopy. (*C*) TEM shows the presence of dense core granules in the cytoplasm of the insulin$^+$ ducts (different view from Fig. 2*E*). (*D*) PECAM staining on control liver of diabetic mouse showing specificity for blood vessels. Immunostaining pictures are representative of at least three independent experiments. (Scale bars, 100 μm.)

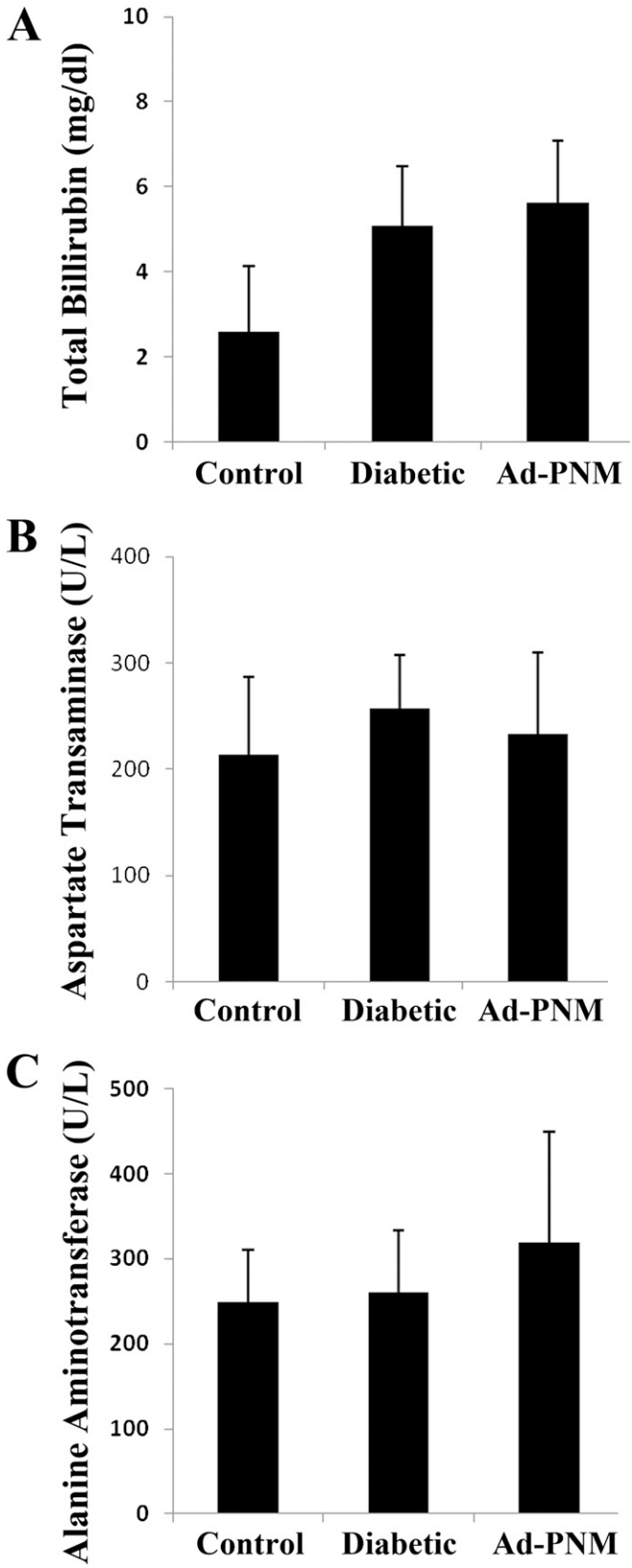

Fig. S5. Liver damage assessment after treatment with *Ad-PNM*. (*A*) Total bilirubin. (*B*) Aspartate transaminase. (*C*) Alanine aminotransferase. Blood samples were taken 7d after administration of *Ad-PNM*. Results are presented as mean ± SE (n = 3).

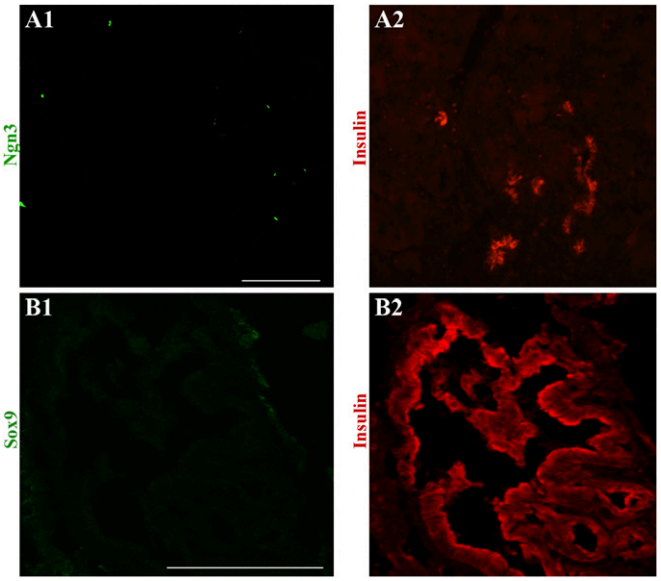

Fig. S6. (*A*) Absence of NGN3 expression in mouse liver 4 wk after *Ad-PNM* delivery. (*B*) Absence of SOX9 expression in mouse liver 4 wk after *Ad-PNM* delivery. Immunostaining pictures are representative of at least three independent experiments. (Scale bars, 100 μm.)

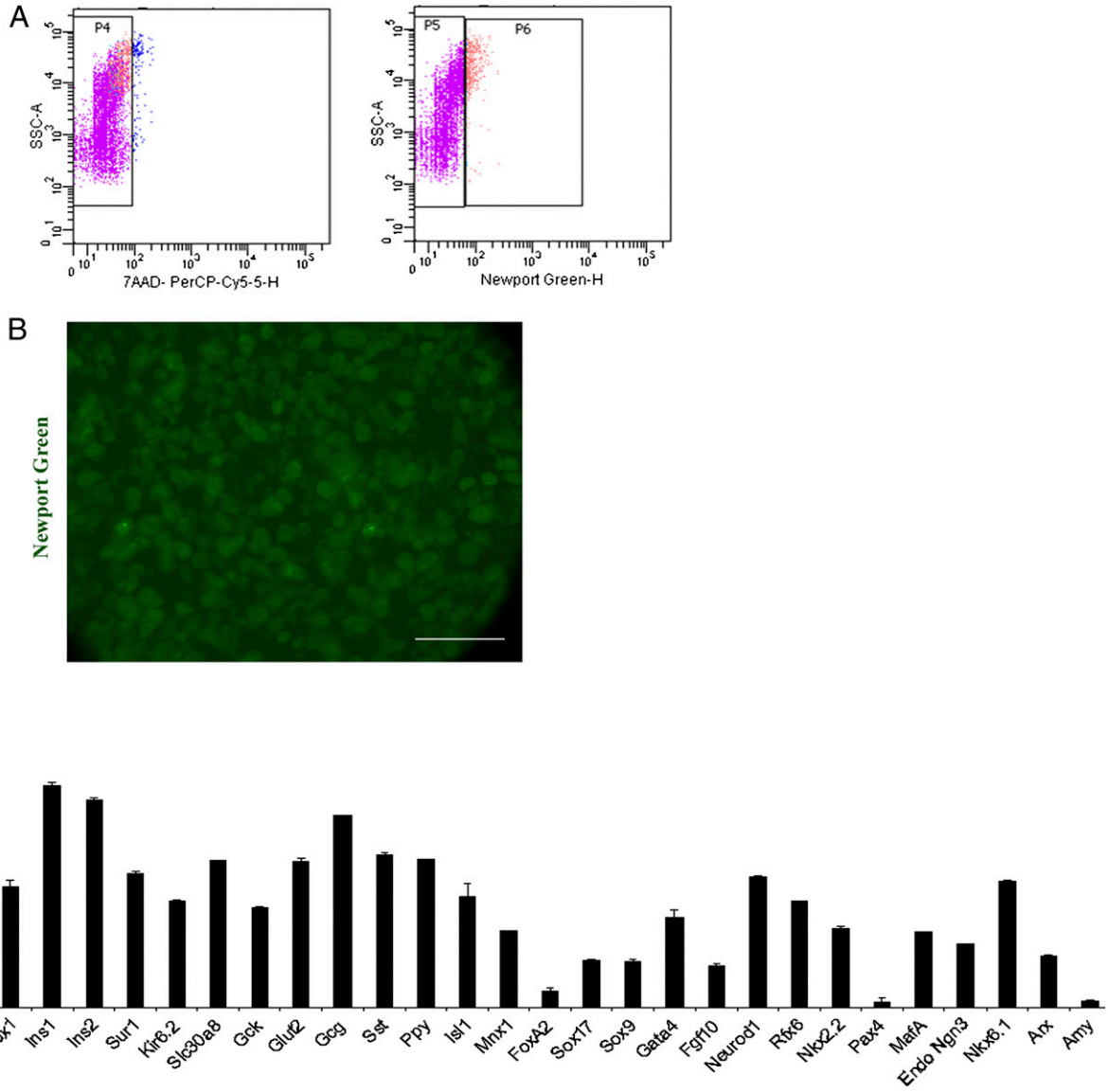

Fig. S7. FACS analysis of livers. (*A*) Cells were stained with Newport Green and also with 7-AAD to exclude any dead cells. The live cells, fraction P4, are divided into Newport Green negative (P5) and positive (P6) fractions. Dead cells made up 4–5% of the total, and Newport Green^{+} cells 6–10%. Data are representative of at least three independent experiments. (*B*) Absence of Newport Green staining in cells from liver of diabetic mouse given *Ad-EGFP*, 20 weeks after vector administration. (*C*) qRT-PCR of control mouse islets.

Table S1. Antibodies used

Protein of interest	Species	Source	Concentration
Insulin	Guinea pig	Sigma	1:200
Pdx1	Rabbit	Millipore	1:1,000
Ngn3	Rabbit	Santa Cruz	1:50
MafA	Rabbit	Santa Cruz	1:50
C-Peptide	Rabbit	Cell Signaling	1:100
SOX9	Rabbit	Millipore	1:1,000
Isl1	Mouse	HLBI	1:500
SOX17	Rabbit	Millipore	1:100
Rfx6	Rabbit	Sigma	1:100
E-CAD	Mouse	BD Biosciences	1:250
CK19	Goat	Santa Cruz	1:100
Albumin	Goat	Bethyl Lab	1:100
Glucagon	Rabbit	Dako	1:100
Somatostatin	Rabbit	Dako	1:100
CD31/PECAM	Rat	BD Pharmingen	1:100

Table S2. Primers used

	Primer 1	Primer 2
MafA	GCC AAC TTC TCG TAT TTC TCC T	CAC ATT CTG GAG AGC GAG AAG
Sur1	GAA TGA TGA CAG CTG CTC CA	TCT TCT TAT GCC CAA ACC TCT G
Albumin	CAG TCA GCC AGT TCA CCA TAG	TGT GTT GCC GAT GAG TCT G
Cebpa	GTC AAC TCC AGC ACC TTC T	ACA AGA ACA GCA ACG AGT ACC
GAPDH	GTG GAG TCA TAC TGG AAC ATG TAG	AAT GGT GAA GGT CGG TGT G
Glucagon	CAG CAT GCC TCT CAA ATT CAT C	ACA TTC ACC AGC GAC TAC AG
Somatostatin	GGC ATC ATT CTC TGT CTG GTT	AGA CTC CGT CAG TTT CTG C
Ppy	GCT GGA CCT GTA CTC TCC TA	TTG GCT TGA TTC CCT GCT C
Amylase	TCT CTG TGT TGG AAA ATG AAA TCT	CCT GGA GAC ATA AAG GCA GTT
Insulin 1	GCC ATG TTG AAA CAA TGA CCT G	GCC AAA CAG CAA AGT CCA G
Insulin 2	CAT GGG TGT GTA GAA GAA GCC	TTT GTC AAG CAG CAC CTT TG
Pdx1	GCA GTA CGG GTC CTC TTG T	GAT GAA ATC CAC CAA AGC TCA C
SOX17	GGG GAA ATA GGA AGG CTG AA	GAA CCT CCA GTA AGC CAG AT
Pax4	AGAAGCTGAAATGGGAAGCA	GGGGACTGTGCAGAGATGAT
Nkx2-2	CTT ATC CAA TCG CTC CAC CTT	TCC AGA ACC ATC GCT ACA AG
Nkx6-1	TCC GAG TCC TGC TTC TTC T	CAC GCT TGG CCT ATT CTC TG
Rfx6	CAA TAA ATG CCT CCA CTG TAG C	TCT TAC CAT ATC CAA CAG CAC A
Isl1	GCC TGT AAA CCA CCA TCA TGT	GTG CAA GGA CAA GAA ACG C
Fgf10	CTT TTG AGC CAT AGA GTT TCC C	CCA AGA ATG AAG ACT GTC CGT
FoxA2	GTA TGT GTT CAT GCC ATT CAT CC	GCC AGC GAG TTA AAG TAT GC
Mnx1	GCT GCG TTT CCA TTT CAT TCG	CAG TTC AAG CTC AAC AAG TAC C
NeuroD1	ACA CTC ATC TGT CCA GCT TG	AGA TCG TCA CTA TTC AGA ACC TTT
Arx	TGT GGG CTG TCT CAG GA	GGT CTG AGC ACT TTT CTA GGA G
Gata4	CTC CTT CCG CAT TGC AAG A	CCA ACT GCC AGA CTA CCA C
Glut2	CTCTTCCAACGTGGTCCCTA	GAGCCCCTCGTAGGTTTTTC
Kir 6.2	TTGGAAGGCGTGGTAGAAAC	CCCCATAGAATCTCGTCAGC
SOX9	CCTCACTACAGCCCCTCCTA	TCTGATGGTCAGCGTAGTCG
Glucokinase	ATAAGCCAGTGGTGGAGTGG	CCAACACAGGTGTGAAGTGG