Figures and figure supplements

Adrb2 controls glucose homeostasis by developmental regulation of pancreatic islet vasculature

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Figure 1. Loss of Adrb2 in neonatal β-cells results in glucose intolerance and impaired insulin secretion in female mice. (A) Adult (2-month-old) female Adrb2 cKO mice have elevated fasting blood glucose and are glucose intolerant. Means ± SEM for n = 6 control and seven mutant female mice.

Figure 1 continued on next page
*p<0.05, ***p<0.001, t-test. (B) Area under the curve (AUC) for glucose tolerance. *p<0.05, t-test. (C) Glucose tolerance is unaffected in male Adrb2 cKO mice at 2 months. Means ± SEM for n = 6 control and nine mutant mice for glucose tolerance. (D) Area under the curve (AUC) for glucose tolerance in males. (E, F) Glucose-stimulated insulin secretion (GSIS) in vivo is reduced in female but not male Adrb2 cKO mice. Means ± SEM for n = 6 control and eight mutant female mice; n = 5 control and six mutant male mice *p<0.05, t-test. (G) Decreased basal insulin secretion and GSIS in isolated adult female Adrb2 cKO islets. Means ± SEM from n = 4 control and six mutant mice. **p<0.01, ****p<0.0001, two-way ANOVA with Bonferroni's post-test. (H) Islet Adrb2 expression declines postnatally and is significantly lower in adult males and females compared to neonatal stages. For female islets, p<0.01, t-test for P60 compared to P6. For male islets, p<0.0001 for P60 compared to P6 (one sample t-test since male P6 values were normalized to 1). Adrb2 levels are higher in female islets compared to males at all timepoints assessed. *p<0.05, **p<0.01, t-test. Adrb2 expression in P2, P6, and P60 islets was assessed by qRT-PCR analyses and data were normalized to 18S rRNA. Results are means ±SEM and expressed as fold-change relative to P6 male islets for n = 3–5 mice/sex/age. (I) Neonatal β-cell-specific Adrb2 deletion elicits glucose intolerance in mice. Neonatal Adrb2 i-cKO mice were injected with TMX or vehicle on the day of birth and 1 day later (P0–P1), and glucose tolerance was tested when mice were 2 months old. Means ± SEM for n = 7 vehicle and 5 TMX-injected Adrb2 i-cKO mice. *p<0.05, **p<0.01, ***p<0.001, t-test. (J) AUC for glucose tolerance. *p<0.05, t-test. (K) Neonatal β-cell-specific Adrb2 deletion results in impaired GSIS. Means ± SEM for n = 4 vehicle and 4 TMX-injected Adrb2 i-cKO mice. *p<0.05, **p<0.01, t-test.

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Figure 1—figure supplement 1. Adrb2 expression, insulin sensitivity and islet morphology in adult Adrb2 cKO mice, and effects of adult β-cell-specific Adrb2 deletion on glucose tolerance and insulin secretion. (A) Adrb2 transcript levels are significantly decreased in P6 Adrb2 cKO pancreas, while other
adrenergic receptors are unaltered. Transcript levels were normalized to 18S RNA. Results are means ±SEM and expressed as fold-change relative to control Adrb2\textsuperscript{f/f} values. \(****p<0.0001\), one sample t-test. (B) Adrb2 mRNA is significantly reduced in both female and male Adrb2 cKO adult (2 month) islets compared to same-sex controls. Note that in controls, Adrb2 expression is higher in female islets compared to males. \(n = 6\) control and 6–8 mutant mice. \(****p<0.0001\), one-sample t-test. (C, D) Normal insulin sensitivity in male (C) and female (D) Adrb2 cKO mice. Means ± SEM for \(n = 3\) female control mice, four male control, six mutant females and six mutant male mice. \(**p<0.01\), \(****p<0.0001\), one-sample t-test. (E) Pancreatic Adrb2 loss does not affect adult islet organization, but results in increased insulin immunoreactivity. Scale bar, 50 µm. (F) Increased insulin content in adult (2 month) Adrb2 cKO islets. \(n = 5–7\) mice/genotype. Normal insulin sensitivity in female (C) and male (D) Adrb2 cKO mice. Means ± SEM for \(n = 3\) female control mice, four male control, six mutant females and six mutant male mice. \(**p<0.01\), \(****p<0.0001\), one-sample t-test. (G) Normal endocrine cell numbers in adult Adrb2 cKO islets. Means ± SEM from \(n = 4\) mice/genotype, one-way ANOVA with Tukey’s post-test. (H) Adrb2 cKO mice have smaller islets relative to controls. Means ± SEM from \(n = 3\) control and four mutant mice, **p<0.01, t-tests. (I, J) Adrb2 loss does not affect β-cell proliferation in adult islets. Scale bar, 50 µm. Quantification of EdU/insulin-double positive cells from \(n = 3\) mice/genotype. **p<0.01, t-test. (K) Adrb2 is enriched in neonatal β-cells. β-cells were isolated by FACS purification from MIP-GFP mice at postnatal day 6 (P6). \(n = 9\) β-cell samples and four non-β-cell samples, \(**p<0.01\), one sample t-test. (L) Adult β-cell Adrb2 loss does not affect glucose tolerance. Mature Adrb2 i-cKO mice were injected with tamoxifen (TMX) or corn oil (vehicle) at 5–6 weeks of age and glucose tolerance was tested 4 weeks later. Means ± SEM for \(n = 5\) vehicle and 7 TMX-injected mice. (M) Area under the curve (AUC) for glucose tolerance. (N) Normal GSIS with adult β-cell Adrb2 deletion. Means ± SEM for \(n = 6\) control and 5 TMX-injected mice. (O) Adult β-cell Adrb2 loss does not affect GSIS in Adrb2 i-cKO islets. Means ± SEM for \(n = 3\) mice per condition. **p<0.01, two-way ANOVA with Bonferroni’s post-test.

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Figure 2. Adb2 suppresses insulin expression and islet vasculature in neonatal mice. (A) Adb2 loss increases insulin immunoreactivity in neonatal (P6) islets, although islet cyto-architecture is unaffected. Scale bar, 25 μm. (B) Enhanced Ins2 transcript levels in neonatal Adb2 cKO pancreata. Ins2 levels

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were assessed by qRT-PCR and normalized to 18S RNA. Results are means ±SEM for n = 4 mice per genotype for the P0 and P2 time points, and 10 mice per genotype for P6. *p<0.05, n.s. not significant, one-sample t-test. (C) Transmission electron microscopy reveals an increase in insulin granules in β-cells from P6 Adrb2 cKO mice. β-cells outlined in dashed lines. Scale bar, 2 μm. (D) Quantification of cytoplasmic insulin granule density. Means for n = 3 mice per genotype. *p<0.05, t-test. (E) Adrb2 cKO mice have excess intra-islet vasculature, using PECAM1 immunostaining. Islets are outlined in dashed lines. Scale bar, 50 μm. (F) Quantification of total vessel length (mm) per islet area (mm²). Means ± SEM for n = 3 mice per genotype. **p<0.01, t-test. (G) Transmission electron microscopy shows disruptions in endothelial morphologies and vascular basement membrane in Adrb2 cKO islets. Fenestrae (arrows) are reduced, while caveolae (arrowheads) are increased in Adrb2 cKO islets. Basement Membrane (B.M) is expanded in mutants. Scale bar, 500 nm. (H) Quantification of fenestrae and (I) caveolae density. Means for n = 3 mice per genotype, *p<0.05, **p<0.01, t-test. (J) Vegfa transcript levels are significantly increased in the Adrb2 cKO pancreas starting at P2. Vegfa levels were assessed by qRT-PCR analysis and normalized to 18S RNA. Results are means ±SEM expressed as fold-change relative to age-matched control Adrb2f/f values. n = 3 P0 per genotype, 3 control and four mutant P2, and 5 P6 mice per genotype **p<0.01, n.s. not significant, one-sample t-test. (K) Increased Vegfa mRNA in purified β-cells from P6 Adrb2 cKO mice. Vegfa levels were assessed by qRT-PCR analysis and normalized to 18S RNA. Means ± SEM and expressed as fold-change relative to control values. n = 5 control and seven mutant mice. *p<0.05, one sample t-test. (L) Vegfa transcript levels (normalized to 18S rRNA) are unchanged in non-β-cells from Adrb2 cKO mice. Means ± SEM and expressed as fold-change relative to control values. n = 4 control and six mutant mice. n.s. not significant, one sample t-test.

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Figure 2—figure supplement 1. Islet morphology and vasculature in neonatal Adrb2 cKO mice. (A) Normal endocrine cell numbers in P6 Adrb2 cKO islets. Means ± SEM from n = 6 control and four mutant mice, one-way ANOVA with Tukey’s post-test. (B) Neonatal (P6) Adrb2 cKO mice have smaller-
sized islets relative to controls. Means ± SEM from n = 6 control and four mutant mice, *p<0.05, ****p<0.0001 one-way ANOVA with Tukey’s post-test. (C) β-cell proliferation is unchanged in P6 Adrb2 cKO islets. Scale bar, 50 µm. (D) Quantification of EdU/insulin double-positive cells from n = 3 mice/genotype. (E) Insulin content is increased in neonatal (P6) Adrb2 cKO islets. Means ± SEM from n = 3 control and five mutant mice, *p<0.05, t-test. (F) Ins2 transcript levels are unchanged by stimulation with Adrb2 agonists, Salbutamol (SALB), Epinephrine (EPI), or Norepinephrine (NE) in MIN6 cells. MIN6 cells were treated with each agonist (10 µM) for 16 hr. Ins2 levels were assessed by qRT-PCR analysis and normalized to 18S RNA. Means ± SEM and expressed as fold-change relative to control values. n = 3 independent experiments for SALB and NE, four independent experiments for EPI. (G) Vascular basement membrane proteins, Collagen IV and Laminin-411/511, are increased in Adrb2 cKO islets at P6. Scale bar, 25 µm. (H) Sympathetic innervation, assessed by TH immunoreactivity, is unaltered in the Adrb2 cKO pancreas. Scale bar, 50 µm. (I) Quantification of TH immunofluorescence. Means from n = 3 control and five mutant mice. (J) Vegfa transcript levels are significantly decreased by Salbutamol (SALB), or epinephrine (EPI), but not norepinephrine (NE), in MIN6 cells. MIN6 cells were treated with each agonist (10 µM) for 16 hr. Means ± SEM and expressed as fold-change relative to control values. n = 3 independent experiments for SALB and NE, four independent experiments for EPI. *p<0.05, **p<0.01, one-sample t-test. (K) Vegfa transcript levels are higher in neonatal (P6) male pancreata compared to females. Adrb2 loss does not affect Vegfa expression in male pancreata. Vegfa levels were assessed by qRT-PCR analysis and normalized to 18S rRNA. Means ± SEM for n = 4 female and 4–5 male mice per genotype. *p<0.05 one-sample t-test. (L) Enhanced PECAM1 immunoreactivity in P6 male islets compared to females. PECAM1 expression is similar in male Adrb2 cKO and control islets. Scale bar, 50 µm. (M) Quantification of total vessel length (mm) per islet area (mm²) in P6 male islets. Means ± SEM for n = 3 mice per genotype. *p<0.05, t-test compared to control female values (dashed line), n.s. not significant.

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Figure 3. Loss of Adrb2 perturbs islet calcium responses and exocytosis. (A) Adrb2 is required for KCl-induced insulin secretion. Means ± SEM for n = 4 control and five mutant mice. *p<0.05, ****p<0.001, two-way ANOVA with Bonferroni's post-test. (B) Adrb2 is necessary for glucose-induced surface
localization of insulin granules. Docked insulin granules in β-cells (within 50 nm of plasma membrane) are indicated by arrowheads. Scale bar, 1 μm. (C) Quantification of docked insulin granules per micron of plasma membrane. Means ± SEM from n = 3 mice each per genotype/per condition. **p<0.01, n.s. not significant; two-way ANOVA with Bonferroni’s post-test. (D) Loss of Adrb2 loss impairs islet calcium responses induced by high glucose or KCl. Scale bar, 50 μm. (E) Quantification of normalized Fluo-4 intensities over time. Means ± SEM for n = 3 female mice per genotype, 10 cells analyzed per animal. (F) Decreased expression of transcripts involved in calcium signaling and insulin exocytosis in isolated islets from adult female Adrb2 cKO mice. Adrb2 loss also results in aberrant expression of genes involved in β-cell maturation including Neurod1, Npy, and Slc2a2. Transcript levels were assessed by qRT-PCR and data normalized to 18S RNA. Means ± SEM and expressed as fold-change relative to control female Adrb2+/+ values. n = 6 control and 3 – 5 mutants. *p<0.05, one sample t-test compared to normalized female control values.

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Figure 3—figure supplement 1. Altered expression of Glut2 and NPY in Adrb2 cKO islets. (A) Adult (1.5 – 2 month-old) Adrb2 cKO islets have diminished Glut2 and increased NPY expression. Scale bar, 50 μm. DOI: https://doi.org/10.7554/eLife.39689.011
Figure 4. Developmental VEGF blockade rescues defects in islet vasculature, insulin secretion, and glucose tolerance in Adrb2 cKO mice. (A) VEGFR2 antibody injections rescue islet hyper-vascularization in neonatal Adrb2 cKO mice. Islets are outlined in dashed lines. Scale bar, 25 µm. (B) Area under the curve (x10^3 mg • min/dL) for blood glucose (mg/dL) over time (minutes). (C) Fold change in transcript levels for Pecam1, Merge, and Insulin. (D) Glucose Tolerance. (E) AUC for control + veh., cKO + veh., and cKO + AB groups. (F) Islet insulin secretion normalized over basal. (G) Fold change in transcript levels for Cacna1c, Snap25, Polo, Rphp1al, Neurod1, and Npy. Figure 4 continued on next page.
Quantification of total vessel length (mm) per islet area (mm$^2$). Means ± SEM from three control and Adrb2 cKO + antibody injected, and 4 Adrb2 cKO + antibody injected mice. *p<0.05, one-way ANOVA with Tukey's post-test. (C) Ins2 levels are normalized in VEGFR2 antibody-injected Adrb2 cKO neonates. Means ± SEM from 12 control (Adrb2$f/f$) + vehicle injected, 7 Adrb2 cKO + vehicle injected, and 5 Adrb2 cKO + antibody injected mice. *p<0.05, **p<0.01, one-way ANOVA with Tukey's post-test. (D) Neonatal administration of VEGFR2 blocking antibody (P0–P6) rescues glucose intolerance in adult female Adrb2 cKO mice. Means ± SEM for n = 10 control + vehicle, five mutant + vehicle, and six mutant + antibody injected mice. *p<0.05, **p<0.01 Adrb2 cKO + vehicle significantly different from control Adrb2$f/f$ mice, and #p<0.05, ##p<0.01 Adrb2 cKO + vehicle significantly different from Adrb2 cKO + antibody injected mice, one-way ANOVA with Tukey's post-test. (E) Area under the curve (AUC) for glucose tolerance. ***p<0.001, n.s. not significant, one-way ANOVA with Tukey's post-test. (F) Rescue of adult islet GSIS in Adrb2 cKO mice injected with VEGFR2 antibody during the first week of birth. Means ± SEM for n = 4 control + vehicle, three mutant + vehicle, and four mutant + antibody injected mice. *p<0.05, n.s. not significant, two-way ANOVA with Bonferroni's post-test. (G) Developmental VEGFR2 blockade restores expression of genes involved in calcium signaling, exocytosis, and $\beta$-cell maturation in adult Adrb2 cKO mice. Transcript levels were assessed by qRT-PCR analysis and normalized to 18S RNA. Means ± SEM and expressed as fold-change relative to control Adrb2$f/f$ + vehicle values. n = 3 control + vehicle, 4 – 6 mutant + vehicle, and three mutant + antibody injected mice. *p<0.05, **p<0.01, one-sample t-test and t-test where Adrb2 cKO values are significantly different from both control and cKO + AB values.

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Figure 4—figure supplement 1. VEGFR2 neutralizing antibody treatment does not affect Vegfa levels in Adrb2 cKO mice, but restores Glut2 and NPY expression. (A) Vegfa transcript levels remain significantly increased in P6 Adrb2 cKO pups injected with VEGFR2 neutralizing antibody. Means ± SEM and expressed as fold change relative to vehicle injected control (Adrb2<sup>f/f</sup>) values for n = 6 control, 3 Adrb2 cKO per condition; **p<0.01, ***p<0.001, one-way ANOVA with Tukey’s post-test. (B) Islet Glut2 and NPY immunoreactivity in Adrb2 cKO mice injected with VEGFR2 neutralizing antibody are similar to control tissues. Scale bar, 50 μm.

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Adrb2 regulates adult islet function by controlling bi-directional signaling between β-cells and islet vasculature during development. During islet development, β-cell-Adrb2 receptors suppress VEGF-A expression to limit excessive growth of intra-islet endothelial cells. Endothelial cells, in turn, are critical for precise regulation of insulin gene expression in neighboring β-cells, via producing vascular basement membrane proteins, and likely for transcriptional regulation of key components of insulin exocytosis. Loss of Adrb2 disrupts bi-directional signaling between islet β-cells and endothelial cells during development, resulting in islet hyper-vascularization, aberrant insulin expression, and impaired glucose-stimulated insulin secretion in adult islets.

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