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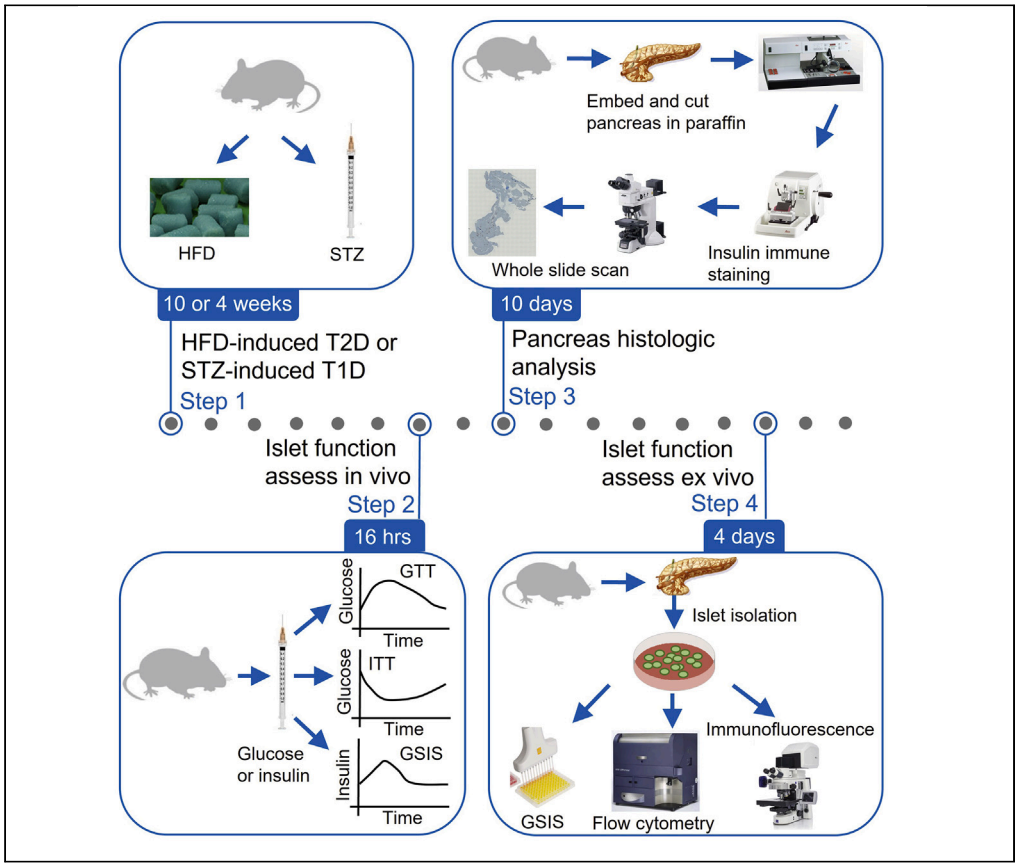
Protocol for *in vivo* and *ex vivo* assessment of hyperglycemia and islet function in diabetic mice



Mouse hyperglycemia model and islet function assessment are essential in diabetes research. Here, we provide a protocol to evaluate glucose homeostasis and islet functions in diabetic mice and isolated islets. We describe steps for establishing type 1 and 2 diabetes, glucose tolerance test, insulin tolerance test, glucose stimulated insulin secretion (GSIS) assay, and histological analysis for islet number and insulin expression *in vivo*. We then detail islet isolation, islet GSIS, β-cell proliferation, apoptosis, and programming assays *ex vivo*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for the establishment of type 1 and 2 diabetes in C57BL/6J mice

In vivo evaluation of islet function using GTT, ITT, GSIS, and histological analysis

Steps for islet isolation from mouse pancreas and islet GSIS

Ex vivo functional assessment of β-cell proliferation, apoptosis, and programming

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Protocol for *in vivo* and *ex vivo* assessment of hyperglycemia and islet function in diabetic mice

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SUMMARY

Mouse hyperglycemia model and islet function assessment are essential in diabetes research. Here, we provide a protocol to evaluate glucose homeostasis and islet functions in diabetic mice and isolated islets. We describe steps for establishing type 1 and 2 diabetes, glucose tolerance test, insulin tolerance test, glucose stimulated insulin secretion (GSIS) assay, and histological analysis for islet number and insulin expression *in vivo*. We then detail islet isolation, islet GSIS, β-cell proliferation, apoptosis, and programming assays *ex vivo*.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022).[1]

BEFORE YOU BEGIN

Type 1 (T1D) and type 2 diabetes (T2D) are prevalent all over the world in the past decades and characterized by substantial or partial reduction of β-cell function. β-cell dysfunction and mass loss lead to insufficient insulin production and worsening of glycemic control.[2–4] It has crucial importance for treatment and prevention of T1D and T2D to recover or reprogram β-cell functional mass. Thus, establishment of mouse hyperglycemia model and assessment of islet function are essential in diabetes research. This protocol is provided based on our previous work on interleukin 18 (IL18) signaling supporting of islet β-cell development, proliferation, and insulin secretion.[1] Compared with our prior published work, here we provide more detailed information of mouse and islet model to assess glucose homeostasis and islet function *in vivo* and *ex vivo*. The preparation of streptozotocin (STZ)-induced T1D and high fat diet (HFD)-induced T2D mouse models for metabolic analyses were elaborated in this protocol. STZ exerts type 1 diabetogenic effects by selective destructing pancreatic islet β-cells, resulting in insulin deficiency and hyperglycemia. HFD-induced insulin resistance increases the workload of insulin-secreting β cells in pancreatic islets and leads to hyperglycemia and T2D.

Institutional permissions

Diabetes is more prevalent in male than in female, and male are more susceptible to the development of diabetes.[5] Thus, male C57BL/6J mice were used in this protocol and housed under a 12-h light/dark cycle at 22°C–25°C, and the animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were preapproved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee (protocol# 2016N000442). Before attempting this protocol, the use of animals and tissue received ethical approval.

HFD-induced insulin resistance

Timing: 10 weeks

To induce insulin resistance, 6-week-old male mice were fed on a 60% high fat diet (HFD) for 10 weeks to develop insulin resistance. Total body weight was monitored weekly. Glucose tolerance test (GTT), and insulin tolerance test (ITT) were performed to confirm the diet-induced insulin resistance after 10 weeks HFD feeding.

1. Separate two groups (n = 12/each) of 5-week-old C57BL/6J male mice and accommodate them in housing cages (4 mice per cage) for 1 week.
2. Feed one group of six-week-old mice with HFD (60 kcal% Fat, D12492, Research Diets Inc., New Brunswick, NJ), another group with chow diet (CD) (D12450B, Research Diets Inc.) for 10 weeks.
3. Monitor the body weight of each mouse weekly.
4. Perform GTT after 10 weeks of HFD feeding.
5. House mice in original cage for another 2 days to ensure blood glucose returned to basal level.
6. Perform ITT after 2 days of recovery.
7. House mice in original cage for another 2 days to ensure blood glucose returned to basal level.
8. Perform GSIS after 2 days of recovery.
9. After 2 days, fast mice overnight (8 h), euthanize mice by CO_2, and collect the plasma and pancreas.

Streptozotocin (STZ)-induced hyperglycemia

Timing: 4 weeks

A single high dose STZ (150 mg/kg) was given by i.p. injection to induce β-cell depletion and hyperglycemia in 12-week-old male mice as previous report.[6] Total body weight, and blood glucose level were monitored weekly. Mice with non-fasting blood glucose >250 mg/dL were considered hyperglycemia. Glucose tolerance test (GTT) was performed to confirm hyperglycemia. Refer to troubleshooting 1.

10. Dissolve STZ in freshly prepared sodium citrate buffer, pH 4.5, at a concentration of 10 mg/mL.
11. Separate two groups (n = 12) of 12-week-old C57BL/6J male mice, fast mice for 4–6 h prior STZ injection.
12. Intraperitoneal inject of 150 mg/kg STZ or vehicle.
13. Monitor mouse body weight, blood glucose and blood insulin levels weekly for 4 weeks.
14. Perform GTT after 4 weeks STZ treatment.
15. House mice in original cage for another 2 days to ensure blood glucose returned to basal level.
16. Perform GSIS after 2 days of recovery.
17. After 2 days, fast mice overnight (8 h), euthanize mice by CO_2, and collect the plasma and pancreas.

Isolate pancreatic islets

Timing: 22 h

Pancreatic islets were isolated from 12–16 weeks old male mice. Islets were incubated in RPMI-1640 containing 10% fetal bovine serum (FBS) for further assay. Refer to troubleshooting 2.

18. Sacrifice mice by CO_2 inhalation and immediately process for pancreatic perfusion with 3 mL of 1.4 mg/mL fresh type-IV collagenase dissolved in HBSS.
19. Inject collagenase solution by inserting a 30 G1/2 needle into the common bile duct through the joint site of the hepatic duct under a dissecting microscope (Figure 1).

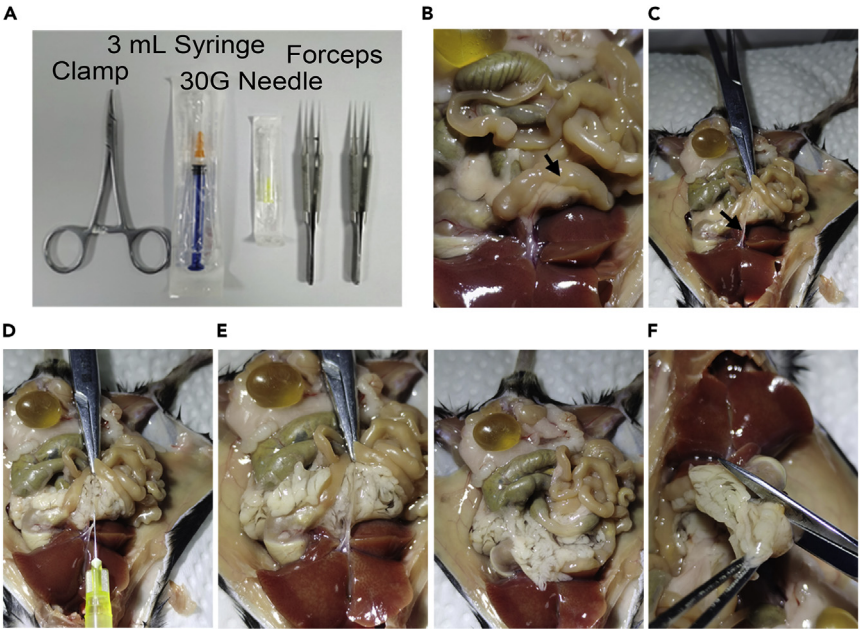

Figure 1. Steps of pancreas perfusion

(A) Surgical tools used for pancreas perfusion.

(B and C) Arrow (B) and clamp (C) bile duct before it connects with ampulla of vater.

(D) Inject collagenase into the bile duct.

(E) Images of a fully perfused pancreas.

(F) Cut the perfused pancreas.

20. After complete distension, digest the pancreas tissue by incubation at 37°C for 17 min in 5 mL digestion solution.
21. Shake digested tissue vigorously and purify islets using 1 mm nylon mesh.
22. Wash islets twice in RPMI-1640 medium and centrifuge islets at 200 × *g* for 3 min.
23. Resuspend islets in a mixture of lymphocyte separation medium and RPMI-1640 medium (2:1).
24. Centrifuge islets at 800 × *g* for 25 min without brake.
25. Collect islets from the interface between the medium and lymphocyte separation medium and resuspend them in RPMI-1640 medium.
26. Hand-pick islets under a dissecting microscope.
27. After washing with PBS, culture islets in RPMI-1640 medium with 10% FBS overnight (8 h).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-glucagon (1:200)	eBioscience	Cat# 14-9743-82; RRID: AB_2572972
Rat monoclonal anti-insulin (1:200)	R&D Systems	Cat# MAB 1417; RRID: AB_2126533
Rabbit monoclonal anti-insulin (1:500)	Cell Signaling Technology	Cat# 3014; RRID: AB_2126503
Rabbit polyclonal anti-Ki67 (1:100)	Novus Biologicals	Cat# NB600-1209; RRID: AB_10001641
Rabbit polyclonal anti-cleaved- caspase 3 (1:200)	Cell Signaling Technology	Cat# 9661; RRID: AB_10001641
Mouse monoclonal anti-BrdU (1:100)	Invitrogen	Cat# MA3-071; RRID: AB_10986341
Biotin-conjugated goat anti-rabbit secondary antibody (1:200)	Vector Laboratories	Cat# BA-1000; RRID: AB_2313606
HRP-streptavidin (1:500)	Dako	Cat# P039701-2; RRID: N/A
Alexa Fluor 555-labeled goat anti-mouse secondary antibody (1:500)	Thermo Fisher Scientific	Cat# A-21422; RRID: AB_141822
Alexa Fluor 555-labeled goat anti-rabbit secondary antibody (1:500)	Thermo Fisher Scientific	Cat# A-21428; RRID: AB_141784
Alexa Fluor 488-labeled Donkey anti-Rat secondary antibody (1:300)	Thermo Fisher Scientific	Cat# A-48270; RRID: AB_2896336

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 647 anti-insulin (1:100)	BD Pharmingen	Cat# 565689; RRID: AB_2739331
PE anti-glucagon (1:100)	BD Pharmingen	Cat# 565860; RRID: AB_2739382
DAPI (1:12.5)	Invitrogen	Cat# R37606; RRID: N/A
Chemicals, peptides, and recombinant proteins		
Fetal bovine serum	Gibco	Cat# 16140071
Normal goat serum blocking solution	Vectorlabs	Cat# S-1000
HBSS	GIBCO	Cat# 14175095
10% neutral buffered formalin	Richard-Allan Scientific	Cat# 5701
Sodium Citrate	Sigma-Aldrich	Cat# PHR1416
Saline	Sigma-Aldrich	Cat# S8776
10× PBS	Boston BioProducts	Cat# BM220
Adenosine dialdehyde (Adox)	Sigma-Aldrich	Cat# A7154
AEC chromogenic agent	DAKO	Cat# K3464
Fluorescence Mounting Medium	DAKO	Cat# S3023
High fat diet	Research Diets Inc.	Cat# D12492
Chow diet (CD)	Research Diets Inc.	Cat# D12450B
Streptozotocin (STZ)	Sigma-Aldrich	Cat# S0130; CAS: 18883-66-4
D-glucose	Sigma-Aldrich	Cat# G7021; CAS: 50-99-7
Insulin	NOVOLIN	Cat# NDC0169-1833-11; CAS: 11061-68-0
Type-IV collagenase	Worthington Biochemical Co	Cat# LS004188
Lymphocyte separation medium	MP Biomedicals	Cat# 0850494
Accutase	GIBCO	Cat# A1110501
Fc block	eBioscience	Cat# 16-0161-82
Trizol reagent	Invitrogen	Cat# 15596018
SYBR green dye	Bio-Rad	Cat# 1725125
L-Glutamine	Gibco	Cat# 21051024
Penicillin-streptomycin solution	Gibco	Cat# 15140122
Normal goat serum	Vector Laboratories	Cat# S-1000
RPMI 1640	Thermo Fisher Scientific	Cat# 11879020
Ethanol	Fisher Scientific	Cat# S25309B
Acetone	Fisher Scientific	Cat# A18-4
Xylene	Fisher Scientific	Cat# X3S-4
Chloroform	Fisher Scientific	Cat# C298-500
Critical commercial assays		
Insulin ultra-sensitive kit	Crystal Chem	Cat# 90080
Intracellular staining buffer	eBioscience	Cat# 88-8823-88
Experimental models: Organisms/strains		
Male 6-week-old C57BL/6J mice	Jackson Laboratory	Cat# 000664
Male 12-week-old C57BL/6J mice	Jackson Laboratory	Cat# 000664
Male 12–16 weeks old C57BL/6J mice	Jackson Laboratory	Cat# 000664
Other		
1 mm nylon mesh	Thomas Scientific	Cat# 146479
Eppendorf tubes	Denville scientific inc.	Cat# C2170
Cytospin	Thermo Scientific	Cat# A78300003
24-well plate	Costar	Cat# 3526
3 mL syringe	BD Microlance	Cat# 309657
Forceps	Jzsf	Cat# WA30010
Clamp	Jzsf	Cat# J31010
30 G 1/2 needle	BD Microlance	Cat# 305106
Software and algorithms		
Image-Pro Plus	Media Cybernetics	https://www.mediacy.com/imageproplus
FlowJo	BD Biosciences	https://www.flowjo.com/
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Excel	Microsoft Office	https://www.office.com/
Adobe Photoshop CS3	Adobe	https://www.adobe.com/

Note: PPE recommended when handling antibodies, biological samples, chemicals, peptides, recombinant proteins, critical commercial assays, and experimental models.

MATERIALS AND EQUIPMENT

Islet Culture Medium (store at 4°C for 3 months)		
Reagent	Final concentration	Volume for 500 mL
RPMI 1640 medium		440 mL
Fetal bovine serum (FBS)	10%	50 mL
Glutamine	2 mM	5 mL
Penicillin-streptomycin solution	1%	5 mL

RPMI Medium for Insulin Secretion Assay (store at 4°C for 3 months)		
Reagent	Final concentration	Volume for 500 mL
RPMI 1640 medium		440 mL
Fetal bovine serum (FBS)	10%	50 mL
Glutamine	2 mM	5 mL
Penicillin-streptomycin Solution	1%	5 mL
D-Glucose	2.8 mM or 16.7 mM	0.252 g or 1.5 g

FACS Buffer (store at 4°C for 1 month)		
Reagent	Final concentration	Volume for 500 mL
10 × PBS	1 ×	50 mL
Fetal bovine serum (FBS)	2.5%	12.5 mL
Double distilled water		437.5 mL

IHC Block buffer (store at 4°C for 1 month)		
Reagent	Final concentration	Volume for 500 mL
10 × PBS	1 ×	50 mL
Normal Goat Serum	5%	25 mL
Double distilled water		425 mL

Glucose Solution for GTT (store at 4°C for 1 week)		
Reagent	Final concentration	Volume/Amount for 500 mL
Saline		500 mL
D-glucose	0.05 g/mL	25 g

Glucose Solution for GSIS (store at 4°C for 1 week)		
Reagent	Final concentration	Volume/Amount for 500 mL
Saline		500 mL
D-glucose	0.15 g/mL	75 g

Glucose Solution for ITT (store at 4°C for 1 week)		
Reagent	Final concentration	Volume/Amount for 500 mL
Saline		495 mL
Insulin solution	0.8 U/mL	5 mL

Sodium Citrate Buffer, pH 4.5 (store at 4°C for 1 month)		
Reagent	Final concentration	Volume/Amount for 500 mL
Sodium Citrate	100 mM	14.7 g
Double distilled water		500 mL

STZ Solution (prepare it fresh every time)		
Reagent	Final concentration	Volume/Amount for 500 mL
Sodium Citrate Buffer		500 mL
Streptozotocin (STZ)	10 mg/mL	5 g

STEP-BY-STEP METHOD DETAILS

This protocol allows studies of islet proliferation, apoptosis, programming, and insulin secretion including GTT, ITT, and GSIS *in vivo* and histological and flow cytometry analyses *ex vivo*.

Glucose tolerance test

Timing: 8 h

This section describes how to measure the fasting blood glucose level from tail veins at different time point after D-glucose injection.

1. Fast mice for 6 h with free to access autoclaved water.
2. Prepare 0.05 g/mL glucose solution in saline.
3. Label the mice with ear tag or marker pen.
4. Scale mice body weight and measure the fasting blood glucose level from tail veins using a blood glucose meter in sequence.
5. Calculate the D-glucose volume (1 g/kg bodyweight) for each mouse according to the body weight.
6. I.p. inject D-glucose to mice in sequence and start timing.
7. Measure blood glucose level at 15, 30, 45, 60, 90, and 120 min after injection.

Insulin tolerance test

Timing: 8 h

This section describes how to measure the fasting blood glucose level from tail veins at different time point after insulin injection.

8. Fast mice for 6 h with free to access autoclaved water.
9. Dilute insulin solution in saline for 100× to 0.8 U/mL.
10. Label mice with ear tag or marker pen.
11. Scale mice body weight and measure the fasting blood glucose level from tail veins using a blood glucose meter in sequence.
12. Calculate the insulin volume (1.5 U/kg bodyweight) for each mouse according to the body weight.
13. I.p. inject insulin to mice in sequence and start timing.
14. Measure blood glucose level at 15, 30, 45, 60, 90, and 120 min after injection.

Note: We recommended a prior test to confirm whether the mice are insulin sensitive with a lower insulin dose at 0.5–1 U/kg.

Glucose-stimulated insulin secretion (GSIS) assay

Timing: 24 h

This section describes how to measure the fasting blood insulin level from tail veins at different time point after D-glucose injection. Refer to troubleshooting 3.

15. Fast mice for 6 h with free to access autoclaved water.
16. Prepare 0.15 g/mL glucose solution in saline to increase plasma insulin level rapidly.
17. Label the mice with ear tag or marker pen.
18. Scale mice body weight and calculate the D-glucose volume (3 g/kg bodyweight) for each mouse according to the body weight.
19. I.p. inject D-glucose to mice in sequence and start timing.
20. Measure blood glucose level and collect blood samples from tail-tip bleeding at 0, 2, 5, and 15 min (first phase insulin secretion) after glucose injection.
21. Centrifuge the blood by 5,000 × *g* for 15 min at 4°C, collect the plasma, and store at −80°C for later use.
22. Determine plasma insulin levels by insulin ultra-sensitive kit according to the manufacturer's instruction (https://www.crystalchem.com/media/catalog/product/9/0/90080c.pdf).

Pause point: If measurements of insulin level will be done on another day, store plasma at −80°C.

⚠ CRITICAL: Pancreatic β-cells secrete insulin in a biphasic manner at first and second phase. First phase insulin secretion occurs within 10 min of glucose stimulation, while second phase insulin secretion is occurring 25–30 min after glucose stimulation. Loss of first phase insulin secretion is an independent predictor of T2D onset. Don't collect blood samples from tail-tip bleeding more than 15 min after glucose injection.[7]

Pancreas histologic analysis for islet number and insulin expression

Timing: 5 days

This section describes how to perform histologic analysis for islet function by measuring insulin expression, total islet number, and islet size distribution in pancreas. Refer to troubleshooting 4.

23. Collect mouse pancreas tissues without saline perfusion.
24. Fix pancreas tissues in 10% neutral phosphate buffered formalin for 24 h at room temperature (22°C).
25. Dehydrate pancreas tissues through graded increasing ethanol (50, 70, 95, and 100%) 2 h each at room temperature (22°C), acetone, and chloroform 30 min each at 60°C.
26. Embed pancreas tissues in paraffin.
27. Slice serial pancreatic sections at 6 μm in thickness and place on histological glass slide.
28. Deparaffinize pancreatic paraffin sections in xylene at room temperature (22°C).
29. Rehydrate sections in a graded decreasing ethanol series (100, 95, 70, and 50%) 5 min each and then in water for 5 min at room temperature (22°C).
30. Block sections with 5% normal goat serum for 30 min.
31. Incubate sections with rabbit anti-insulin in 1:500 overnight (8 h) at 4°C.
32. Incubate sections with biotin-conjugated goat anti-rabbit in 1:200 for 45 min at room temperature (22°C).
33. Incubate sections with HRP-streptavidin in 1:500 for 30 min at room temperature (22°C).
34. Detect insulin positive area with AEC chromogenic agent for 1 min at room temperature (22°C).
35. Counterstain slides with hematoxylin for 10 s at room temperature (22°C).

36. Scan whole pancreatic tissue sections with the Olympus VS120 Whole Slide Scanner.
37. Detect insulin staining intensity with Image-Pro Plus and present as positive area.
38. Quantify total islet number per pancreas.
39. Quantify each islet area in μm^2 by identifying islet area with Image-Pro Plus and calculating with scale bar in whole section picture.
40. Quantify islet numbers of different size groups including <1,000 μm^2/islet, 1,000–5,000 μm^2/islet, 5,000–15,000 μm^2/islet, >15,000 μm^2/islet.

Pause point: If the staining of insulin will be done on another day, put the pancreatic paraffin slide storage box and store it at room temperature (22°C).

Note: Xylene or toluene are also accessible methods for paraffin embedding of the pancreas and can be used to replace acetone and chloroform.

Islet insulin secretion assay *ex vivo*

Timing: 36 h

This section describes how to measure insulin secretion at different time point after D-glucose stimulation in cultured islets.

41. Hand-pick up 25 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.
42. Culture islets in medium containing 2.8 mM (low glucose) or 16.7 mM (high glucose) glucose for 0.5, 1, or 1.5 h.
43. Collect culture medium from each group.
44. Determine media insulin levels by insulin ultra-sensitive kit according to the manufacturer's instruction (https://www.crystalchem.com/media/catalog/product/9/0/90080c.pdf) and normalize to per mg of islets protein.

Pause point: If measurements of insulin level will be done on another day, store media and islets at −80°C.

Ki67 expression for islet proliferation assay *ex vivo*

Timing: 60 h

This section describes how to measure islet proliferation by immunofluorescent co-localization staining. Refer to troubleshooting 5.

45. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.
46. Culture islets in medium containing 16.7 mM glucose for 24 h.
47. Centrifuge and resuspend islets in 200 μL PBS.
48. Place slides and filters into slots with the cardboard filters appropriately.
49. Quickly move 200 μL of each sample into the appropriate wells of the cytospin.
50. Cytospin each group of islets to slides by 2,000 rpm for 5 min.
51. Remove the filters from their slides carefully and wait for air dry.
52. Fix slides with cold acetone for 5 min.
53. Wash slides in PBS for 3 times after air dry.
54. Block islet sections with 5% normal goat serum for 30 min.
55. Immunofluorescent co-localization stain islet sections with 5% normal goat serum containing rabbit anti-Ki67 (1:100) and rat anti-insulin (1:500) antibodies overnight (8 h) at 4°C.

56. Wash slides in PBS for 3 times.
57. Incubate sections in 5% normal goat serum containing Alex Fluor 555-labeled goat anti-rabbit (1:300) and Alex Fluor 488-labeled donkey anti-rat (1:500) secondary antibodies for 30 min.
58. Wash slides in PBS for 3 times.
59. Incubate sections in 0.4 μM DAPI for 10 min and cover slide with coverslips in mounting medium.
60. Capture images with Olympus confocal microscopy.

Pause point: If the staining of Ki67 will be done on another day, store the slide at −80°C after fixation.

Flow cytometry detection of BrdU pulse labeling for islet proliferation assay

Timing: 60 h

This section describes how to detect islet proliferation by flow cytometry staining of freshly prepared islets cell after BrdU pulse.

61. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.
62. Pulse isolated WT islets with 5 μg/mL BrdU for 2 h.
63. Disperse islets with 1 mL fresh accutase for 10 min at 37°C incubator with 5% CO_2.
64. Wash islet cells in PBS once.
65. Block islet cells with Fc block (1:100) in FACS buffer for 20 min.
66. Wash islet cells in PBS once.
67. Label islet cells with Alexa Fluor 647 anti-insulin (1 μg/mL) and FITC anti-BrdU (1 μg/mL) or isotypes for 30 min in intracellular staining buffer according to the manufacturer's instruction (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.cn/TFS-Assets%2FLSG%2Fmanuals%2F88-8823.pdf).
68. Flow cytometry analysis using a FACS Analyzer LSR Fortessa (BD Biosciences) or other Flow Cytometer.

Immunofluorescent staining detection of BrdU pulse labeling for islet proliferation assay

Timing: 60 h

This section describes how to measure islet proliferation by immunofluorescent co-localization staining after BrdU pulse.

69. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.
70. Pulse isolated WT islets with 5 μg/mL BrdU for 2 h.
71. Cytospin each group of islets to slides following the steps 47–51.
72. Wash, fix, and block sections following the steps 52–54.
73. Immunofluorescent co-localization stain islet sections with 5% normal goat serum containing mouse anti-BrdU (1:100) and rat anti-insulin (1:500) antibodies overnight (8 h) at 4°C.
74. Wash slides in PBS for 3 times.
75. Incubate sections in 5% normal goat serum containing Alex Fluor 555-labeled goat anti-mouse (1:300) and Alex Fluor 488-labeled donkey anti-rat (1:500) secondary antibodies for 30 min.
76. Wash and cover slides, and capture images following the steps 58–60.

Pause point: If the staining of BrdU will be done on another day, store the slide at −80°C after fixation.

STZ-induced apoptosis assay *in vitro*

Timing: 60 h

This section describes how to measure STZ-induced islets apoptosis by immunofluorescent co-localization staining.

77. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation at 37°C incubator.
78. Stimulate islets with 50 mM STZ for 30 min.
79. Cytospin each group of islets to slides following the steps 47–51.
80. Wash, fix, and block sections following the steps 52–54.
81. Immunofluorescent co-localization stain islet sections with 5% normal goat serum containing rabbit anti-Cleaved caspase 3 (1:200) and rat anti-insulin (1:500) antibodies overnight (8 h) at 4°C.
82. Wash slides in PBS for 3 times.
83. Incubate sections in 5% normal goat serum containing Alex Fluor 555-labeled goat anti-rabbit (1:300) and Alex Fluor 488-labeled donkey anti-rat (1:500) secondary antibodies for 30 min at room temperature (22°C).
84. Wash and cover slides, and capture images following the steps 58–60.

Pause point: If the staining of Cleaved caspase 3 will be done on another day, store the slide at −80°C after fixation.

Flow cytometry detection of islet β-cell programming assay

Timing: 4 days

This section describes how to detect islet β-cell programming by flow cytometry staining of freshly prepared islets cell.

85. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.
86. Culture islets in medium containing 50 μM adenosine dialdehyde (Adox) for 72 h at 37°C incubator with 5% CO_2.
87. Disperse, wash, and block islets cells following the steps 63–66.
88. Stain islets with Alexa Fluor 647 anti-insulin (1 μg/mL) and PE anti-glucagon (1 μg/mL) or isotypes for 30 min at 4°C with intracellular staining buffer according to the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.cn/TFS-Assets%2FLSG%2Fmanuals%2F88-8823.pdf).
89. Flow cytometry analysis using a FACS Analyzer LSR.
90. Quantify Glucagon$^+$, Ins$^+$, and Glucagon$^+$Ins$^+$cells in each group of islets.

Immunofluorescent staining detection of Islet β-cell programming assay

Timing: 5 days

This section describes how to detect islet β-cell programming by immunofluorescent co-localization staining.

91. Hand-pick up 40 similar size islets from each group and culture islets in RPMI 1640 medium with 10% FBS overnight (8 h) after isolation.

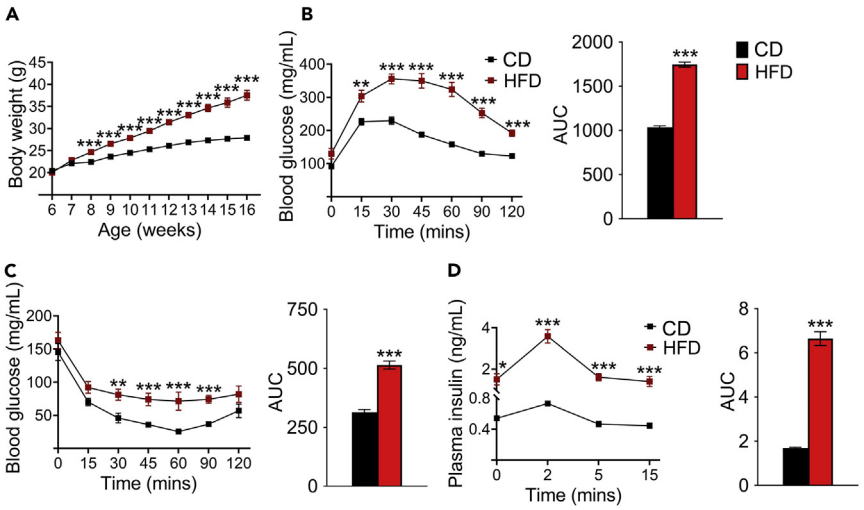

Figure 2. Total body weight and glucose homeostasis in high fat diet (HFD)-fed and chow diet (CD)-fed mice

(A–D) Bodyweight gain (A), glucose tolerance test (GTT) with area under curve (AUC) (B), insulin tolerance test (ITT) with AUC (C), and glucose stimulated insulin secretion (GSIS) with AUC (D) in HFD-fed and CD-fed mice. n = 8. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. Non-parametric Mann-Whitney *U* test.

92. Culture islets in medium containing 50 μM adenosine dialdehyde (Adox) for 72 h at 37°C incubator with 5% CO_2.
93. Cytospin each group of islets to slides following the steps 47–51.
94. Wash, fix, and block sections following the steps 52–54.
95. Immunofluorescent co-localization stain islet sections with 5% normal goat serum containing mouse anti-Glucagon (1:200) and rat anti-insulin (1:500) antibodies overnight (8 h) at 4°C.
96. Wash slides in PBS for 3 times.
97. Incubate sections in 5% normal goat serum containing Alex Fluor 555-labeled goat anti-mouse (1:300) and Alex Fluor 488-labeled donkey anti-rat (1:500) secondary antibodies for 30 min.
98. Wash and cover slides, and capture images following the steps 58–60.

Pause point: If the staining will be done on another day, store the slide at −80°C after fixation.

EXPECTED OUTCOMES

Islet function assessment *in vivo*

Six-week-old wild-type (WT) mice were fed with a chow diet (CD) or HFD diet to induce insulin resistance. After 10 weeks, total body weight was plotted as body weight *vs.* age. Plasma glucose levels obtained from GTT, or ITT were plotted as glucose level *vs.* glucose or insulin administration time. Plasma insulin levels obtained from GSIS were plotted as insulin level *vs.* glucose administration time. CD-fed mice that had normal body weight gain (Figure 2A), glucose tolerance (Figure 2B), insulin tolerance (Figure 2C), and insulin secretion (Figure 2D) showed typical shaped curves. GTT demonstrated that mice on CD were able to elevate their blood glucose to the highest level around 200 mg/dL and normalize blood glucose level by the end of the measured timepoints (Figure 2B). ITT demonstrated that mice on CD were able to reduce their blood glucose to the lowest level around 25 mg/dL and normalize blood glucose level by the end of the measured timepoints (Figure 2C). GSIS demonstrated that mice on CD were able to increase their blood glucose to the highest level around 0.8 ng/mL and normalize blood insulin level by the end of the measured timepoints (Figure 2D). Total area under curve (AUC) was calculated according to GTT, ITT, and GSIS curves. Mice gained more body weight after HFD feeding (Figure 2A). HFD led to defects with insulin signaling that resulted in insufficient glucose uptake by insulin-responsive tissues. The islets secreted high level of compensatory insulin to promote glucose uptake.[8] Glucose and insulin

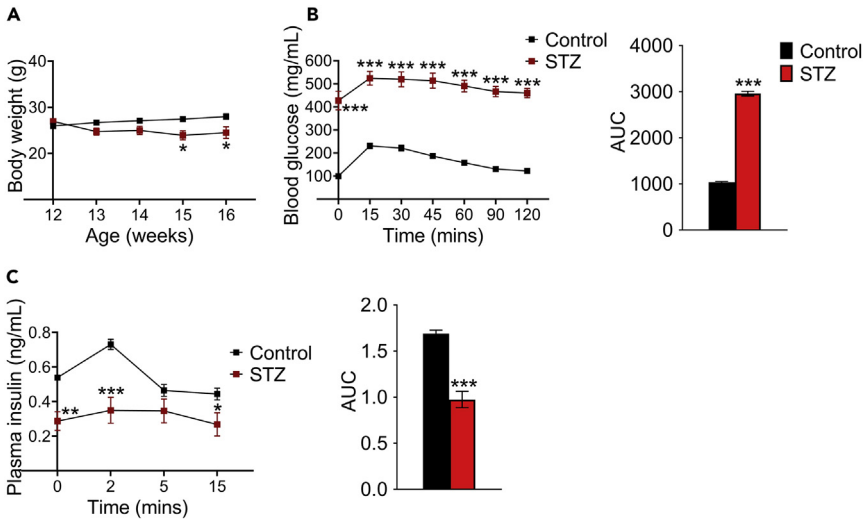

Figure 3. Total body weight and glucose homeostasis in streptozotocin (STZ)-induced hyperglycemic and control mice

(A–C) Bodyweight gain (A), glucose tolerance test (GTT) with area under curve (AUC) (B), and glucose stimulated insulin secretion (GSIS) with AUC (C) in STZ-induced hyperglycemic and control mice. n = 8. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. Non-parametric Mann-Whitney *U* test.

tolerance were worse after HFD-induced insulin resistance (Figures 2B and 2C). Mice secreted high levels of insulin sustainedly and result in hyperinsulinemia (Figure 2D).

Twelve-week-old WT mice were i.p. injected with one dose of 150 mg/kg STZ or vehicle to induce hyperglycemia. After 4 weeks, total body weight was plotted as body weight *vs.* age. Plasma glucose levels obtained from GTT were plotted as glucose level *vs.* glucose or insulin administration time. Plasma insulin levels obtained from GSIS were plotted as insulin level *vs.* glucose administration time. Vehicle-injected mice that had normal body weight gain (Figure 3A), glucose tolerance (Figure 3B), and insulin secretion (Figure 3C) also showed typical curves. Total area under curve (AUC) was calculated according to GTT and GSIS curves. STZ eliminates β-cell mass in mice with pancreatic islet inflammation and a subsequent lack of insulin production.[9] Mice gained less body weight after STZ treatment (Figure 3A). Glucose tolerance and insulin secretion response to glucose were worse after STZ-induced hyperglycemia (Figures 3B and 3C).

Pancreas histological analysis

To further assess islet insulin secretory function, mouse pancreatic paraffin sections were immunohistochemical stained with insulin antibody. The β-cell mass was assessed as total islet count, insulin positive area, and islet numbers in different size groups. Total islets were plotted as islet number per pancreas. Insulin positive area was plotted as percentage of insulin positive area in total pancreas area. A HFD triggers mild β cell proliferation and islet mass expansion and increases islet size and β-cell mass.[10] The number of islets per mm^2 was reduced in each size categories of people with T1D compared to non-diabetic subjects.[11] STZ cause β cell loss and decrease islet size. To test islet size distribution, islet number in each size group was plotted as islet number in 4 size groups, <1,000 μm^2, 1,000–5,000 μm^2, 5,000–15,000 μm^2, >15,000 μm^2 as previous report.[12] Pancreas with insulin immunohistochemical staining of LFD-fed mice was shown in Figure 4A. HFD did not affect total islets number (Figure 4B), but increased insulin positive area (Figure 4C), and islet number in 1,000–5,000 μm^2, 5,000–15,000 μm^2, >15,000 μm^2 groups, and decreased islet number in <1,000 μm^2 size (Figure 4D). Pancreas with insulin immunohistochemical staining of vehicle-treated control mice was shown in Figure 5A. STZ decreased total islets number (Figure 5B), insulin positive area (Figure 5C), and islet number in <1,000 μm^2 size (Figure 5D).

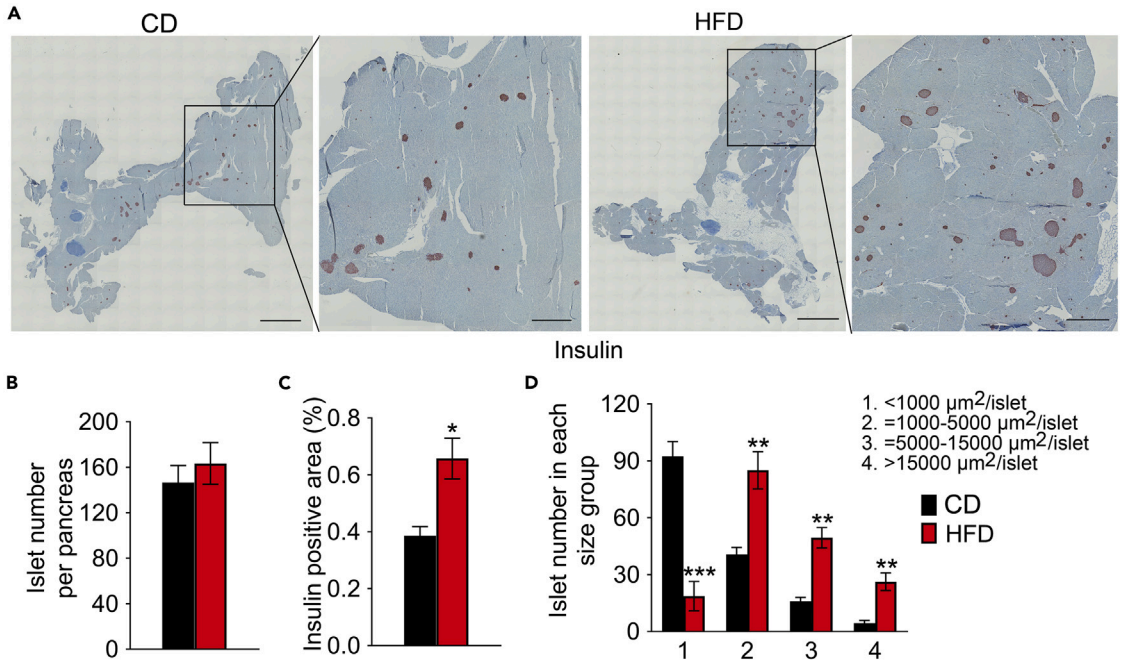

Figure 4. Insulin immunostaining in pancreas from high fat diet (HFD)-fed and chow diet (CD)-fed mice

(A–D) Insulin immunostaining (A), total islet number per pancreas (B), insulin-positive area on pancreas sections (C), and islet number in different size groups per pancreas area (D) in HFD-fed and CD-fed mice. n = 8. Scale: 2.3 mm, inset: 300 μm. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. Non-parametric Mann-Whitney *U* test.

Islet function assessment *ex vivo*

Islet isolation from pancreas provides a method to study *ex vivo* islet insulin secretion, proliferation, apoptosis, and reprogramming. To confirm the GSIS *in vivo*, GSIS was assessed *ex vivo* under different glucose level and time conditions. The typical results of insulin secretion in response to glucose and time were shown in Figure 6A. Insulin level in islet culture medium was elevated significantly after high glucose treatment (Figure 6A). Ki67 is expressed throughout the active cell cycle (G1, S, G2, and M phases) except for the resting phase (G0), while bromodeoxyuridine (BrdU) can be incorporated only during the S face. Using two staining methods to detect β cell proliferation could obtain stronger and more credible data. Immunofluorescent co-localization staining with Ki67 and insulin antibodies revealed β-cell proliferation in islets. Ki67-positive red area merged with DAPI blue in nuclei will be purple (Figure 6B). BrdU pulse labeling further characterized islet cell proliferation. Immunofluorescent co-localization staining with BrdU and insulin antibody revealed islet β-cell proliferation after BrdU pulse labeling (Figure 6C). BrdU-positive red area merged with DAPI blue in nuclei was purple (Figure 6C). Flow cytometry assay with BrdU and insulin conjugated antibodies identified BrdU-positive β cells (Figure 6D). STZ induced islet apoptosis *ex vivo*.[13] Immunofluorescent co-localization staining with cleaved-caspase 3 and insulin antibodies revealed β-cell apoptosis in islets. Cleaved-caspase 3-positive red area merged with insulin positive green area was orange (Figure 6E). Adenosine dialdehyde (Adox) as a β-cell development activator promoted β-cell reprogramming.[14] Immunofluorescent co-localization staining with insulin and glucagon antibodies detected α cells and β cells in islets. Glucagon-positive red area merged with insulin-positive green area contained Glucagon$^+$Insulin$^+$ orange cells (Figure 6F). Flow cytometry assay with glucagon and insulin conjugated antibodies identified Glucagon$^+$, Insulin$^+$, and Glucagon$^+$Insulin$^+$ cell (Figure 6G). Adox enhanced islet insulin expression from glucagon-positive cells (Figure 6F) and the number of Insulin$^+$ β cells (Figure 6G). Glucagon$^+$Insulin$^+$ cells, as shown in Figure 6F, were intermediate state cells that express both α-cell and β-cell markers. It is necessary to present more evidence to confirm β-cell reprogramming activation and α-to-β-cell transformation, such as expression of transcription factors PDX1, MAFA and PAX4.[15]

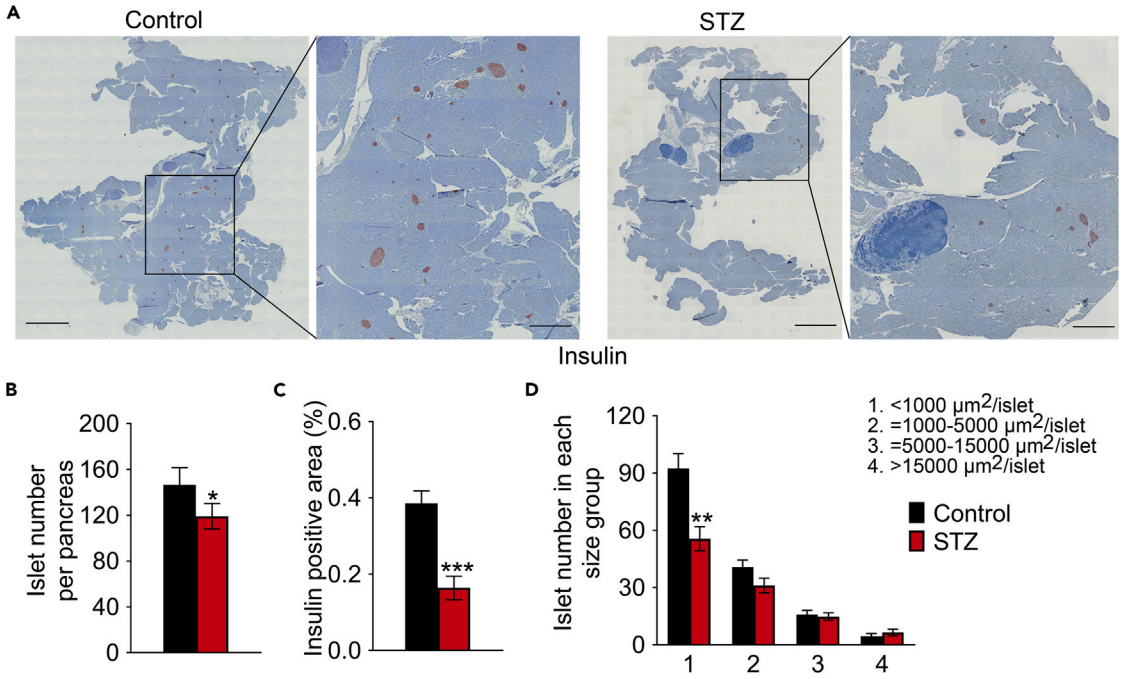

Figure 5. Insulin immunostaining in pancreas of streptozotocin (STZ)-induced hyperglycemic and control mice

(A–D) Insulin immunostaining (A), total islet number per pancreas (B), insulin-positive area on pancreas sections (C), and islet number in different size groups per pancreas area (D) in STZ-induced hyperglycemic and control mice. n = 8. Scale: 2.3 mm, inset: 300 μm. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001. Non-parametric Mann-Whitney *U* test.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were expressed as mean ± SEM. We used non-parametric Mann-Whitney *U* test followed by Bonferroni correction to compare two-group data that did not pass the normality test. GraphPad Prism 8 version was used for analysis and $p < 0.05$ was considered significant.

LIMITATIONS

Further tests such as hyperglycemic clamps and measurement of glycolysis and mitochondrial function during glucose stimulated insulin secretion are required to gain further evaluating of islet function. Besides immunofluorescent staining and flow cytometry assay, more techniques such as real-time PCR and western blot are used to test β-cell proliferation and apoptosis.

TROUBLESHOOTING

Problem 1

The mice died after 150 mg/kg STZ injection at "Streptozotocin (STZ)-induced hyperglycemia".

Potential solution

Mice have different sensitivities to STZ dependent on their backgrounds and genotypes. Try to decrease STZ concentration to 120–140 mg/kg or take a prior test with a lower dose to make sure whether the mice are STZ sensitive.

Problem 2

The production of islet isolation was low at " Isolate pancreatic islets".

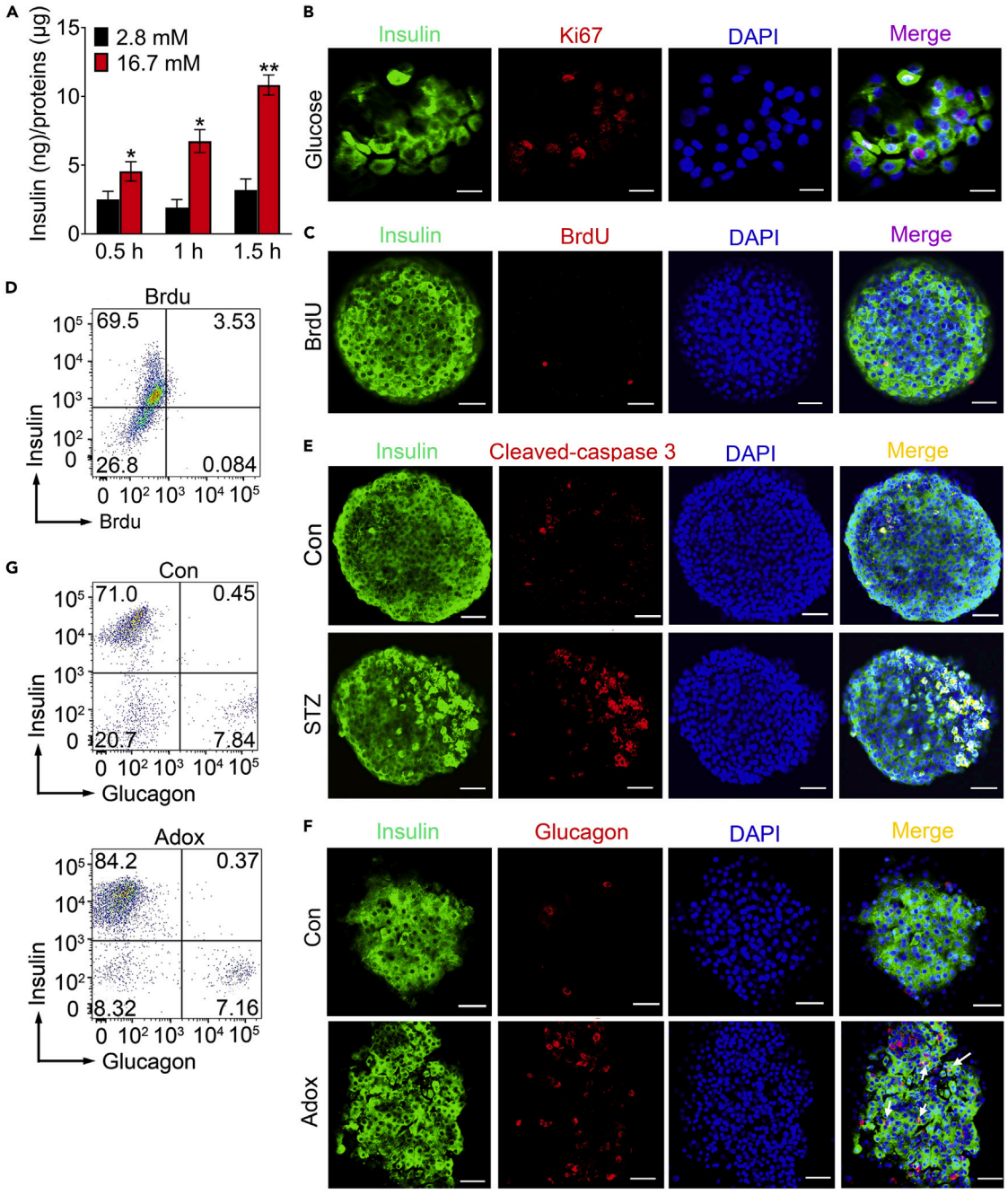

Figure 6. Islet insulin secretion and β-cell function analysis

(A) Glucose stimulated insulin secretion (GSIS) *in vitro*. Insulin level in culture medium per μg islets from indicated islets cultured in 2.8 mM or 16.7 mM glucose for indicated time.

(B) Immunofluorescent-double staining of insulin (green) and Ki67 (red) in islets cultured in high glucose for 24 h, scale: 12.5 μm.

(C and D) Immunofluorescent-double staining of insulin (green) and BrdU (red) and quantification (C), and representative flow cytometry (FACS) images (D) of $BrdU^+$ $insulin^+$ cells in WT islets pulsed with BrdU for 2 h, scale: 25 μm.

(E) Immunofluorescent-double staining of insulin (green) and cleaved caspase-3 (red) and quantification of apoptotic β cells in STZ-treated or control wild type (WT) islets, scale: 25 μm.

(F) Immunofluorescent-double staining of insulin (green) and glucagon (red) and quantification of WT islets treated with or without 50 μM Adox, scale: 25 μm.

(G) Representative FACS images and quantification of glucagon-positive α cells and insulin-positive β cells in WT islets treated with or without Adox. n = 4. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, Non-parametric Mann-Whitney *U* test.

Potential solution

Make sure to let the pancreas be fully perfused, and the collagenase flow slowly to prevent the duct from rupturing. Pancreas tissue digestion time should be exactly 17 min. Digestion for more than 17 min may disperse islet cells and directly decrease islet production. After digestion, digested tissue should be shaken vigorously by hand but not vortex. Vortex will let the digested pancreas into a mass without islet separation.

Problem 3

The blood collection from tail-tip bleeding was difficult to achieve at step 20.

Potential solution

Try to collect blood samples from retro-orbital instead of tail-tip.

Problem 4

Pancreas section was difficult to cut. Paraffin pancreas tissue was easy to crack during cutting at step 27.

Potential solution

Pancreas on an embedding box should be flat and unfold. Make sure to let the pancreas be fully fixed in 10% formalin and fully dehydrated. Cutting should be slow and consecutive.

Problem 5

Immunofluorescent staining didn't work well on cytospin islets at step 55.

Potential solution

Try to use islet embedded paraffin sections instead of cytospin islets.[16]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xian Zhang (zhangxian@hfut.edu.cn).

Materials availability

No new materials are generated in this protocol.

Data and code availability

Data reported in this paper are shared by the lead contact upon request. This manuscript does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Writing—original draft, H.C., X.Z.; Writing—review and editing, G.P.S., J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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