Chapter 10

Mouse Models of Type 2 Diabetes Mellitus in Drug Discovery

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Abstract

Type 2 diabetes is a fast-growing epidemic in industrialized countries, associated with obesity, lack of physical exercise, aging, family history, and ethnic background. Diagnostic criteria are elevated fasting or postprandial blood glucose levels, a consequence of insulin resistance. Early intervention can help patients to revert the progression of the disease together with lifestyle changes or monotherapy. Systemic glucose toxicity can have devastating effects leading to pancreatic beta cell failure, blindness, nephropathy, and neuropathy, progressing to limb ulceration or even amputation. Existing treatments have numerous side effects and demonstrate variability in individual patient responsiveness. However, several emerging areas of discovery research are showing promises with the development of novel classes of antidiabetic drugs.

The mouse has proven to be a reliable model for discovering and validating new treatments for type 2 diabetes mellitus. We review here commonly used methods to measure endpoints relevant to glucose metabolism which show good translatability to the diagnostic of type 2 diabetes in humans: baseline fasting glucose and insulin, glucose tolerance test, insulin sensitivity index, and body type composition. Improvements on these clinical values are essential for the progression of a novel potential therapeutic molecule through a preclinical and clinical pipeline.

Key words Type 2 diabetes mellitus, Drug discovery, Glucose tolerance test, Insulin tolerance test, Insulin secretion, Insulin sensitivity, Diet-induced obesity, Leptin, Insulin, NEFA

Abbreviations

DEXA	Dual energy X-ray absorptiometry
DIO	Diet-induced obesity
D-PBS	Dulbecco's Phosphate Buffered Saline
ED_{50}	Dose providing 50 % efficacy
GSIS	Glucose-stimulated insulin secretion
GTT	Glucose tolerance test
i.p.	Intraperitoneal
i.v.	Intravenous
ITT	Insulin tolerance test
MRI	Magnetic Resonance Imaging
NEFA	Nonesterified fatty acid

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p.o.	per oral gavage
PD	Pharmacodynamics
PK	Pharmacokinetics
s.c.	Subcutaneous
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus

1 Introduction

The name Diabetes Mellitus, meaning "honey passing through," describes graphically the increased elimination of blood sugar in the urine of diabetic patients or glycosuria. The most common form of diabetes is Type 2 Diabetes Mellitus (T2DM), accounting for 90–95 % of all cases in the United States. It is characterized by insulin resistance, i.e., the inability of insulin to stimulate glucose uptake in target organs such as liver and fat (for review, [1]).

The prevalence of T2DM has steadily increased along with what is thought to be a key causative agent, obesity, in the past few decades. Other risk factors such as aging, lack of physical exercise, and genetic predisposition (family history and/or ethnicity) are also associated with the diseases development. The Center for Disease Control and Prevention estimates that in the United States of America, T2DM affects more than 10 % of the population over 45 years old, and perhaps of greater concern 3–4 % of the younger population [2].

1.1 Diagnostic

T2DM is diagnosed clinically using either of these two criteria: a fasting plasma glucose higher than 126 mg/dL or a 2 h postload glucose level exceeding 200 mg/dL in a glucose tolerance test (GTT). Because these clinical markers are susceptible to daily and other variations, continued monitoring of diabetic patients is performed using the glycated hemoglobin or the HbA_{1c} value to monitor the average plasma glucose concentration over prolonged periods of time [3]. However, HbA1c values are usually not reliable for patients who have undergone recent diet or treatments changes (within 6 weeks). For this reason, fasting glucose and GTT are most commonly used in preclinical animal models.

1.2 Disease Progression

In the early stages of the disease, the pancreas of T2DM patients increases insulin production to compensate for an elevated glycemic index. However, as the disease progresses, these increased plasma insulin levels become insufficient to restore a normal glycemia. Left untreated, this chronically elevated blood glucose becomes cytotoxic, contributing to pancreatic beta cell failure, causing patients to become insulin dependent. Hyperglycemia causes damage in capillaries and other internal tissues. Patients are at serious risks of suffering from retinopathy potentially leading to blindness, nephropathy, and neuropathy causing limb ulceration and even amputation in some cases.

1.3 Treatments

Lifestyle changes, a healthy diet, and an increase in physical activity can improve the symptoms of T2DM and sometimes revert serum glucose values to a normal level and even revert the signs onset of T2DM. Unfortunately, in the vast majority of cases, although it is most often insufficient pharmaceutical intervention is necessary to normalize the patient's glycemic index. Metformin (Glucophage), an agent known to reduce hepatic glucose output, is currently the first-line drug therapy recommended by the American Diabetes Association (ADA) and several other medical associations [4, 5]. As the disease progresses, management of the glycemic index with monotherapy becomes ineffective with T2DM patients requiring a second agent. Second-line drugs are grouped into several classes, each targeting a distinct mechanism of action, yet with respective associated adverse events. Insulin sensitizers such as thiazolidinodiones (TZD) act through PPAR-γ in adipocytes and have been associated with body weight gain. Despite some controversy over the safety of TZD, the FDA has approved to keep this drug on the market [6]. Another class of active drugs are the insulin secretagogues, such as sulfonylureas, act through potassium channels in pancreatic beta cells. They do, however, present some risks of hypoglycemia and may also cause body weight gain [7]. Alpha-glucosidase inhibitors, e.g., acarbose, prevent the digestion of complex carbohydrates, but can also cause several gastrointestinal side effects such as flatulence and bloating. Peptide analogs include injectable incretin mimetics, e.g., exenatide, a GLP-1 analog, and dipeptidyl peptidase-4 (DPP-4) inhibitors, can be used to prevent the degradation of GLP-1 [8]. While GLP-1 analogs induce weight loss, DPP-4 inhibitors are weight neutral. Besides stimulating insulin secretion, long-acting GLP-1 act through decreasing gastric emptying which may cause commonly observed nausea side effects. Amylin analogs also slow gastric emptying and suppress glucagon. Like incretins, the most frequent side effect of amylin analog is nausea. Glifozins, a class of renal glucose reabsorption inhibitors, or glycosurics, acting through SGLT2, constitute the newest class of antidiabetic drugs approved by the FDA [9]. They present little risk of hypoglycemia and can even induce mild weight loss. However, urinary tract infection is a common side effect of this latest class.

Despite a wide range of treatment options, a large proportion of diabetic patients eventually become insulin deficient and progress to the insulin-dependent stage of the disease, along with life-threatening diabetic complications.

Bariatric surgery such as sleeve gastrectomy or Roux-en-Y gastric bypass (RYGB) is highly successful in improving the metabolic profile of T2DM patients [10]. While part of its success may be due to the mechanical restriction of food intake, several lines of evidence points to the role of yet unknown additional factors to explain the rapid and long-lasting restoration of normoglycemia even prior to any major body weight loss. The invasive nature of

RYGB hinders its potential for large-scale use. However, it highlights the possible existence of novel potential targets that could be identified with continued discovery research.

1.4 Emerging Discovery Research

Genetic and genomic approaches have led to the identification of a large number of novel potential targets for the treatment of metabolic disorders. For example, genome-wide association studies (GWAS) in T2DM patients have pointed to approximately 75 susceptibility loci influencing the disease [11]. Genome wide expression analysis after bariatric surgery, fasting, or feeding with a high caloric diet has pointed to additional candidate genes for drug discovery [12]. Secretome screening, using large-scale in vivo overexpression in mice has led to the identification of additional potential targets [13].

Fibroblast Growth Factor 21 (FGF21), a secreted protein produced by the liver in response to PPAR-γ activation or fasting, has emerged as an important biomarker of T2DM. Long-acting FGF21 [14] or mimetic antibodies [15] show efficacy in improving the metabolic profile of mice and nonhuman primates. The potential of the FGF21-related molecules to treat T2DM continues to be explored with several ongoing clinical trials [16].

Emerging trends in discovery research over the past decade are highlighting the importance of the gut, the microbiota, muscle, brown fat, and the brain. Variants of FGF19 [17, 18], a secreted protein from the ileum, show promises in the treatment of diabetes and many liver diseases in preclinical models. With regard to the microbiota, fecal transplants into mice from obese and lean twins have demonstrated convincingly the role of gut bacteria in the control of the host energy balance [19]. Efforts have also been made to identify factors secreted by muscles affecting insulin resistance, with the hope of generating "exercise in a pill" form [20]. Finally, our understanding of the activation of brown fat, the so-called good fat, which increases energy expenditure by combustion of fatty acids into heat, has deepened greatly in recent years [21]. Previous attempts at modulating hypothalamic hormones in the brain to suppress appetite have resulted in little success, either due to lack of efficacy or adverse effects. Lorcaserin, a selective serotonin 5-HT-2c receptor agonist, produces modest body weight loss in obese patients and remains the only brain-mediated anorectic drug approved by the FDA to treat obesity, an underlying factor of T2DM [22]. New findings about the role of the reward system in food intake point to the importance of the ventral tegmental area (VTA), in addition to the hypothalamus, as a potential site of intervention [23]. Altogether, the exploration of these pathways offers ample promises for novel classes of therapeutics to treat diabetes.

1.5 Preclinical Models

Many genetic and diet-induced rodent preclinical models of T2DM exist, meeting three fundamental criteria for validation. First, they recapitulate the hallmarks of the disease in humans: elevated fasting glucose and glucose intolerance. Second, all existing T2DM

treatments in humans are equally effective at reversing diabetes symptoms in model rodents. Third, they have been mostly predictive of the translation potential of novel therapeutic molecules into clinical trial results. Although large mammals such as pigs and monkeys also develop obesity and insulin resistance as a result of aging and diet, their postprandial glycemic index tends to remain quite low as plasma insulin levels increase. Therefore, fasting glucose levels are used more commonly than GTT in those species. Mouse also has further key advantages over these species because of its small size, high fecundity, the availability of genetic tools to manipulate its genome, and its short generation time.

While symptoms used for a diagnostic of T2DM in humans are well reproduced in mice, differences in the progression of the disease exist. For example, amyloid formation resulting from the aggregation of islet amyloid polypeptide (IAPP), or amylin, has been linked to T2DM in humans. In contrast, IAPP in rodents is not amyloidogenic. In spite of this difference, several mouse models recapitulate the progression of beta-cell failure and pancreatic islets degeneration. Similarly, several models manifest severe symptoms of diabetic nephropathy, yet, none recapitulate all renal histological features seen in humans, e.g., a 50% decline in glomeruli filtration rate and greater than 10-fold increase in albuminuria.

The methods presented in this chapter cover most commonly used in vivo pharmacology protocols and focus on the glycemic index used to diagnose T2DM per se and other early stage symptoms, rather than subsequent complications of the disease (*see* **Note 1**).

Preclinical models for diabetes can roughly be divided in two types: genetic models and diet-induced obesity (DIO) models.

1.5.1 Genetic Models

Some of the most widely used genetic models of type 2 diabetes are B6.Cg-*Lepob*/J and BKS.Cg-*Dock7m* +/+ *Leprdb*/J [24]. These strains carry single gene spontaneous mutations in either the leptin (*Lepob*) or the leptin receptor (*Leprdb*) genes in an inbred C57BL/6J and a C57BLKS/J background, respectively.

Mice homozygous for the *Lepob* mutation are hyperphagic, gain weight becoming rapidly obese, and become hyperglycemic at a young age. In a C57BL/6J background, hyperglycemia is transient. Fasting glucose level decreases and insulin increases steadily. Mice become normoglycemic, yet hyperinsulinemic by 14 to 16 weeks of age, with the disease progressing similarly in males and females (*see* **Note 2**).

The BKS.Cg-*Dock7m* +/+ *Leprdb*/J strain models are more severe and show advanced stages of the disease. Mice become severely diabetic by 6 weeks of age, suffering pancreatic islet degeneration and renal complications, resulting in lethality sometimes seen as early as 16 to 20 weeks of age. Further, BKS.Cg-*Dock7m* +/+ *Leprdb*/J is considered one of the most robust model of diabetic nephropathy.

While these models are widely used, a concern with such genetic models is that mutations in the leptin gene and its receptor are rare occurrences in humans. Moreover, leptin administration in humans is ineffective in treating T2DM, except for a rare population of patients with mutations in their leptin or leptin receptor genes [25]. In fact, diabetic patients develop hyperleptinemia and leptin resistance. For those reasons, these mutant mouse strains, while convenient, may have shortcomings in modeling all physiological aspects of T2DM in humans.

1.5.2 Diet-Induced Obesity

Obesity is strongly associated with type 2 diabetes, as a high fat diet is a major cause of insulin resistance both in humans and several mouse strains. AKR/J, DBA/2J, and BTBR T+ tf/J strains are very responsive to high fat ("Western") diets, C57BL/6J can be considered a strain of intermediate susceptibility, while A/J and Balb/cJ mice are diet-induced "diabetes resistant" [26–28]. C57BL/6J males develop more insulin resistance in response to the DIO regimen than females, for reasons yet unclear [29].

C57BL/6J is one of the most commonly used mouse strains for diabetes studies. DIO in this strain causes hyperglycemia, hyperinsulinemia, and the development of a fatty liver. One shortcoming of this model, however, is that diet alone is insufficient for the symptoms to progress to a later stage disease, such as beta-cell degeneration and diabetic nephropathy. To mimic late stage T2DM symptoms observed in humans, DIO-C57BL/6J mice can be treated with low doses of streptozotocin (STZ). While mice treated with high doses of STZ are considered a model of type 1 diabetes, when the drug is administered at low doses to DIO mice, it mimics the partial loss of islet cells in the advanced stages of T2DM [30].

B6D2F1/J, a F1 hybrid between C57BL/6 and DBA/2J, has been increasingly used for a DIO model of T2DM [31]. While they have not been as thoroughly characterized as C57BL/6J mice, they develop insulin resistance as quickly. They are also easy to handle being in general gentle, and little less prone to fighting, allowing group housing over extended periods.

2 Materials

2.1 Mice

1. C57BL/6J males, 4 to 6 weeks old (The Jackson Laboratory, stock 000664).
2. Inventoried (DIO) C57BL/6J males, fed for 12 weeks with D12492i, 60 kcal% fat diet (Research Diets, Inc.) (Jackson Laboratory, stock 000664) (*see* **Note 3**).
3. BKS.Cg-*Dock7m* +/+ *Leprdb*/J males, 3 to 4 weeks old (The Jackson Laboratory, stock 000642) (*see* **Note 4**).
4. B6.Cg-Lep^{ob}/J, 3 to 4 weeks old males (The Jackson Laboratory stock 000632) (*see* **Note 5**).

2.2 Feeding and Dosing

1. 10 kcal% fat diet (standard diet) (Research Diets, D12450B).
2. 45 kcal% fat diet (Research Diets, D12451i) (*see* **Note 6**).
3. 60 kcal% fat diet (Research Diets, D12492i) (*see* **Note 6**).
4. 60 kcal% fat diet supplemented with rosiglitazone (Avandia®; GlaxoSmithKline), custom-order (Research Diets) (*see* **Note 7**).
5. Vehicles
   (a) Dulbecco's Phosphate-Buffered Saline (D-PBS), 1X without calcium and magnesium (Mediatech, 21-031-CM).
   (b) Sodium Chloride Injection Solution, Saline: NaCl 0.9%, 250 mL, Baxter IV solutions (VWR, 68000-342).
   (c) 1% hydroxypropyl methylcellulose (Alfa Caesar, 9004-65-3). Mix overnight using a magnetic stirrer.
6. Rosiglitazone (Avandia®; GlaxoSmithKline). To prepare a 1 mg/kg solution, put 50 mg rosiglitazone in a mortar and add 500 μL Tween-80 (Alfa Caesar, 9005-65-6). Mix using the pestle. Add 50 mL 1% methylcellulose and mix with a 10 mL pipette. The solution may remain cloudy. The solution can be stored at 4 °C for the duration of the experiment. Mix well before dosing. Dose per oral gavage (p.o) at 5 mg/kg (100 μL for a mouse of 20 g).
7. Metformin, 1,1-Dimethylbiguanide hydrochloride (Sigma, D5035). Prepare a 200 mg/10 mL metformin solution in D-PBS. Dose via intraperitoneal (i.p.) injection at 100 mg/kg (100 μL for a mouse of 20 g).
8. Exendin-4 (California Peptide Research, 507-77). Prepare a 1 mg/mL solution in D-PBS. Dilute in D-PBS 100-fold for dosing via i.p. injection at 10 μg/kg (200 μL for a mouse of 20 g).
9. Disposable Sterile Animal Feeding Needles, Popper & Sons, 20G × 1½ inch (VWR, 20068-666).
10. Tuberculin syringe with 27 G × ½ inch needle (Becton-Dickinson, 309623).

2.3 Glucose Tolerance, Insulin Tolerance, and Glucose-Stimulated Insulin Secretion Tests

1. Micro-hematocrit centrifuge: Autocrit™ Ultra-3 (Becton Dickinson, 420575).
2. Clean cages with water bottles.
3. Gibco® Glucose, 20% Solution (Thermo Fisher Scientific, cat# 19002013).
4. Human insulin: NovolinR, 100U/mL, 10 mL (NovoNordisk).
5. Tuberculin syringe with 27 G × ½ inch needle, Becton-Dickinson, Cat no. 309623.
6. Scalpel.
7. Mineral oil, Sigma, cat nr. M3516.
8. Paper towels.

9. Accu-chek Aviva glucometer, Roche Diagnostics (Battery: 3-volt lithium type CR 2032).
10. Accu-chek Aviva test strips (Roche Diagnostics).
11. Batteries, Eveready button cells, ECR2032, 3 V Lithium Cell (Sigma, B0653).
12. SurePrep™ capillary tubes, 75 mm, self-sealing, Becton Dickinson, cat nr. 420315 (*see* **Note 8**).
13. MicroWell 96-well polystyrene plates, round bottom (non-treated), sterile (Nunc, Sigma, P4241).
14. Small Wire Clipper.
15. 20–200 µL pipette with tips.
16. Alumina-Seal (Diversified Biotech, ALUM-1000).

2.4 Insulin ELISA

1. Insulin (Mouse) Ultrasensitive (Alpco Diagnostics, EIA 80-INSMSU-E10).
2. Titer Plate Shaker (Lab-line instruments, 5246).
3. EL406™ Combination Washer Dispenser (BioTek Instruments).
4. Microplate Spectrophotometer, SpectraMax Plus 384 (Molecular Devices).

2.5 NEFA

1. HR Series NEFA-HR(2) kit (Wako Chemicals GmbH, 994-75409), which include:
   (a). Color Reagent A (999-346691).
   (b). Solvent A (995-34791).
   (c). Color Reagent B (991-34891).
   (d). Solvent B (993-35191).
   (e). NEFA standard (oleic acid) solution (276-76491).
   (f). Control sera (410-00101 and 416-00202).
2. Clear flat bottom 96-well plates.
3. Polyester films for ELISA and Incubation (VWR, 60941-120).

2.6 Body Composition

1. EchoMRI-100™ (Echo Medical System, Houston, TX) (*see* **Note 9**)

2.7 Terminal Blood Collection and Tissue Collection for Histology

1. One cc syringes with 25G × 5/8 needle, Becton-Dickinson, cat nr. 305122.
2. Microtainer serum separator tubes (BD Diagnostics, 365956).
3. Nunc MicroWell 96-well polystyrene plates, Sigma, P4241. round bottom (nontreated), sterile.
4. Formalin solution, neutral buffered, 10%, Sigma, HT501128.
5. 50 mL conical tubes, VWR, Cat. Nr. 21008-178.
6. Standard dissection tools: scissors and forceps.

3 Methods

Circulating glucose and insulin levels are the key values for a diagnosis of type 2 diabetes. Obesity and elevated levels of nonesterified fatty acids (NEFA) are known to cause insulin resistance and diabetes. Comorbidity of T2DM and dyslipidemia are common in animal models and in clinical populations and therefore, cholesterol, triglycerides, inflammation markers, and blood pressure are often measured within the same experiments. However, for the purpose of this chapter, we will focus on values directly linked to T2DM.

Although the protocols for the genetic and DIO models are largely overlapping, they have a number of important differences, e.g., a high fat diet is required for C57BL/6J mice to develop symptoms of T2DM, while genetic models develop the disease spontaneously. When differences apply, they will be detailed below. Otherwise, the following protocols apply to both types of models.

3.1 Experimental Design

1. Choose a positive control. These need to be chosen depending on the mechanism of action being investigated: for example,
   (a) Insulin secretagogues: exendin-4 is a long-acting homolog of GLP-1, which induces insulin secretion and thereby lowers glucose.
   (b) Insulin resistance: rosiglitazone is an insulin sensitizer that targets PPAR-γ.
   (c) Hepatic glucose output: metformin is an orphan drug which lowers blood glucose by interfering with hepatic glucose output. Its mechanism of action is poorly understood.
2. Choose a neutral vehicle for the test therapeutic molecule(s) (*see* **Note 10**).
3. Choose a negative control. This will be the vehicle used to deliver the positive control and test compounds: e.g., exendin-4 is soluble in D-PBS; therefore, one group of mice injected with D-PBS alone will be included as a negative control.
4. Choose a range of doses to be injected to determine a pharmacokinetic/pharmacodynamic (PK/PD) relationship. Six different concentrations per compound are sufficient to establish an ED_{50} (dose providing 50% efficacy) value with an acceptable confidence interval. For protein therapeutics, this may range from 0.1 ng/kg to 10 μg/kg. For small molecules this can range from 1 ng/kg to 100 mg/kg.
5. Use 10 to 15 mice per group to obtain statistically significant results. For genetic models, where 80% of the mice will be

used (*see* preselection in Subheading 3.3), adjust the number of mice accordingly. For example, 100 B6.Cg-*Lepob*/J mice will be sufficient to test one compound at six doses that include a positive and a negative control, using 10 mice per group.

6. Choose the injection schedule. Some compounds, such as rosiglitazone are effective at lowering glucose levels after multiple injections over several days, but not in an acute, one-day injection setting. Other compounds such as Exendin-4 are active in an acute setting. When studying novel compounds, both types of study—acute and chronic dosing—should be performed to determine the properties of the molecule tested. The schedule of injections will depend on the pharmacokinetic properties of the test compounds.
7. Choose a mode of administration. Therapeutic compounds can be administered intraperitoneally (i.p.), orally (p.o.), subcutaneously (s.c.), or intravenously (i.v). Prolonged methods of administration also include osmotic pumps (e.g., Alzet mini-pumps). Alternatively, transgenic delivery of nucleic acids is useful for proof of concept studies: e.g., germ-line transgenic mice, viral expression, or systemic delivery of naked DNA via tail vein injection. The optimal mode of administration for a given compound will depend on its solubility and on whether the therapeutic is a small molecule, a protein, siRNA, or DNA. For example, rosiglitazone is not water soluble and its best modes of administration are via oral gavage or mixed in the diet (*see* **Note 11**).
8. Choose a method and a schedule for blood collection. Common survival blood collection methods for metabolic profiling are tail nick, tail snip, saphenous vein, submandibular (cheek), and retroorbital bleeding. With the tail nick or tail snip blood collection methods, 75 μL samples of blood can be collected up to four times in a one-day experiment, not exceeding a total of 250 μL, and are used as default blood collection procedures. This usually provides up to 30 μL serum samples and is sufficient to measure glucose, insulin, and other metabolic markers. In some cases, larger volumes are needed (up to 250 μL). Then, consider alternatives such as saphenous or submandibular vein collection or retroorbital bleeding (*see* **Note 12**). For terminal blood collection, exsanguination via cardiac puncture or via the vena cava and decapitation are commonly used. It is worth noting that CO_2 asphyxiation and many anesthesia methods can interfere significantly with baseline glucose and insulin measurements.

3.2 Animal Housing

1. Keep mice housed under standard specified pathogen-free conditions with food and water ad libitum and a 12:12 dark:light cycle.
2. Keep mice group housed if possible (*see* **Note 13**).

3. For C57BL/6J, feed mice with 60% fat diet. For genetic models, B6.Cg-Lep^{ob}/J and BKS.Cg-*Dock7m +/+ Leprdb*/J, feed mice with standard diet, D12450B.
4. Perform daily assessment of the bedding conditions. While this is a standard procedure in most vivaria, some strains of diabetic mice such as BKS.Cg-*Dock7m +/+ Leprdb*/J mice need additional attention because of related kidney problems, resulting in polyuria. Even with a HEPA-filtered-ventilated caging system, mice caging may need to be changed two to three times weekly as the diabetic condition worsens.
5. Identify individual mice with ear notches several days before performing an experiment as the stress from the procedure may have a transient effect on glucose levels.

3.3 Randomization and Preselection of Mouse Cohorts

Preselection of mouse cohorts based on their baseline glucose and body weight is particularly important for genetic models as baseline glucose can span a range of several hundred units even for mice of the same gender and age: e.g., 200 to 600 mg/dL (*see* **Note 14** and Fig. 1).

1. Fast mice for four hours and measure mouse body weight.
2. Measure baseline fasting glucose: Insert glucometer strip into glucometer. Using gloves, pick up mouse by the tail. Wrap its body in a paper towel restraining it loosely with one hand, leaving the tail exposed. With the other hand, cut approximately one mm off the tail tip with a scalpel or perform a nick of the tail vein on the side of the tail close to the tip. Put one drop of blood onto a glucometer strip and take reading.

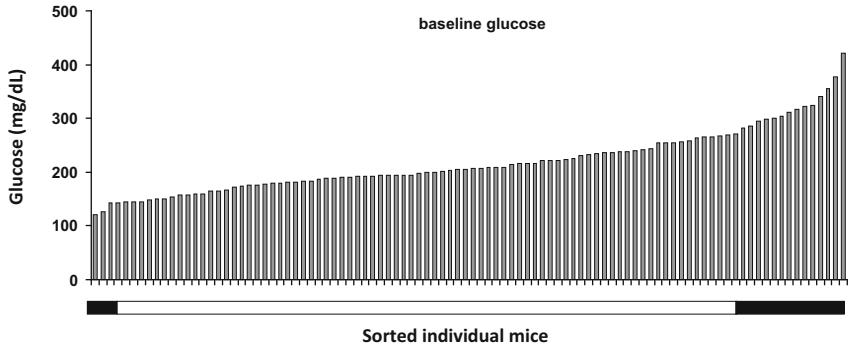

Fig. 1 Selection of mice based on baseline glucose value. Fasting glucose was measured on 100 mice and sorted in increasing order. Each bar represents the glucose value for one mouse. Values ranged from 115 to 430 mg/dL. Mice with glucose values higher than 275 and lower than 140 were excluded (mice over the *black rectangles*). Selection of 80 mice in the middle range was done in a semiquantitative manner (mice at either end of the glucose value range that seem to deviate from the mean the most substantially were discarded) and used for randomization (mice over the *open/white rectangle*)

3. Sort mice by body weight values. Exclude mice with outlier body weight values.
4. Sort glucose values in increasing order.
5. Select mice with mid-range values.
6. Distribute mice of similar baseline glucose values evenly among experimental groups. For example, for three groups, assign the group to mice in sequential order: e.g., A, B, C, C, B , A, A, B, C, C, B, A, etc.

3.4 Tail Snip Blood Collection for Multiple Measurements: Baseline Glucose, Insulin, and NEFA

1. Insert glucometer strip into glucometer.
2. Using gloves, pick up mouse by the tail and cut approximately one mm or less off the tail tip or perform a nick of the tail vein on the side of the tail close to the tip as before. If the tail tip was previously cut, remove the scab off the tail tip with the scalpel.
3. Put one drop of blood on glucometer strip and take reading.
4. Optional: Put a minute amount of mineral oil on index finger and thumb.
5. Rub the sides of the tail on each side with index finger and thumb from the base toward the tail tip. Too much pressure may damage the blood veins, the lighter pressure, the better. Collect the blood into a capillary tube. Repeat this step until the tube is full.
6. Spin the capillary tubes in a micro-hematocrit centrifuge at 10,000 rpm for 8 min.
7. Cut the capillary tubes at the separation between serum and red blood cells with the sharp edge of a file.
8. Use a pipette with a 200 μL tip to gently blow the serum out of the capillary tube into a 96-well plate.

3.5 Insulin ELISA

1. Reagent preparation: Dilute the enzyme conjugate concentrate (11×) with 10 parts of enzyme conjugate buffer. Dilute insulin controls with 0.6 mL of distilled water. Dilute wash buffer concentrate with 20 parts distilled water.
2. Designate wells for standards, controls, and unknown samples. Pipette 5 μL of each in their respective wells.
3. Add 75 μL of enzyme conjugate. Seal the plate with the polyester film provided. Place the plate on an orbital microplate shaker, shaking at 700–900 RPM for 2 h at room temperature.
4. Remove sealing film and wash the plate six times with wash buffer with a microplate washer.
5. Add 100 μL of substrate to each well. Reseal the plate with a polyester film and incubate for 30 min on an orbital microplate shaker at room temperature.

6. Remove sealing film and add 100 μL of stop solution to each well. Gently mix to stop the reaction, remove bubbles before reading with the microplate reader.
7. Program the location of the standards, controls, and unknown samples into an absorption plate reader, with the absorbance at 450 nm with a reference wavelength of 650 nm. Read the plate within 30 min following the addition of the stop solution (*see* **Note 15**).

3.6 NEFA (Nonesterified Free Fatty Acids)

Free fatty acids are elevated in the plasma of obese patients and are known to cause muscle and liver insulin resistance. The Wako HR series NEFA-HR(2) is an in vitro enzymatic colorimetric method assay for the quantitative determination of nonesterified fatty acids (NEFA) in serum. Perform the assay on serum collected from mice fasted for a period greater than 4 h but less than 16 h. Perform the test on samples immediately after collection, without freezing. Also note that hemolysis in the serum samples may interfere with the assay.

1. Prepare reagent solutions. Add 10 mL Solvent A into one vial of Color reagent A and mix gently. Add 20 mL Solvent B into one vial of color reagent B and mix gently. Solvent A and B solutions are stable at 2–10 °C for 5 days.
2. Prepare standard solutions. Stock solution is 1 mEq/L. The test is linear from 0.01 to 4.00 mEq/L. Carry out a serial dilution 1:1 of the standard with water to obtain concentrations of 0.5, 0.25, 0.125 mEq/L.
3. Add samples to 96-well plates in duplicates. Add 10 μL of standard stock solution in two of the wells, to obtain a reading of 2 mEq/L. Then add 5 μL of the 1, 0.5, 0.25, 0.125 mEq/L of the standard solutions in subsequent wells, respectively. Add 5 μL of water in two wells to serve as blanks. Add 5 μL of two control samples. Add 5 μL of unknown samples. If values greater than 2 mEq/L are expected, dilute the samples in PBS.
4. Add 50 μL of reagent A solution to each well. Add polyester film to seal the plate. Mix and incubate at 37 °C for 10 min.
5. Add 100 μL of reagent B solution to each well. Mix and incubate at 37 °C for 10 min. Seal the plate with a polyester film. Mix and incubate at 37 °C for 10 min.
6. Program the location of the standards, controls, and unknown samples into an absorption plate reader. Set the wavelength to 550 nm. Read the plate.

3.7 Glucose Tolerance Test (GTT)

GTTs can be conducted on mice fed ad libitum or following a fasting period. Because food intake in mice occurs mainly during the dark period of the dark–light cycle, we prefer to fast mice during daytime at the beginning of the light period, from 6:00 AM to 10:00

AM. This more closely mimics an overnight fast in humans. Fasting mice also reduces the range of baseline glucose readings and can reveal significant differences between experimental groups that would not reach significance in nonfasted animals.

Glucose can be administered by intraperitoneal injection (i.p.) or by oral gavage (p.o.). Injection is usually faster than gavage; however, it bypasses a potential effect from stimulating incretins in enteroendocrine cells.

1. To initiate a fasting period, transfer mice to clean cages without food but with water ad libitum.
2. Weigh mice. If mice are group housed, draw bars on tails with a black marker pen for quick identification during the test: e.g., zero, one, two, three, or four bars, respectively. Marks on the tail can be read faster than ear notches. These marks will fade after a few days.
3. Use a 20% (w/v) glucose solution in distilled water. Glucose is dosed at 2 g/kg (glucose/body weight) in a 10 mL/kg volume: e.g., 250 μL for a mouse of 25 g (*see* **Note 16**).
4. Preload one milliliter syringes with the glucose solution. Ensure that all air bubbles have been removed by tapping on the side of the syringes and expressing the air.
5. Shortly before the 4-h fasting is complete, take a measurement of the baseline glucose level. Insert a glucometer strip into a glucometer. Wrap the mouse in a paper towel and restrain loosely with one hand. With the other hand, cut approximately one mm off the tail tip with a scalpel or perform a nick of the tail vein on the side of the tail close to the tip. Put one drop of blood on glucometer strip and take reading. Record glucose value. The range of readable glucose values is 20–600. If the glucometer indicates the value "Hi," i.e., off scale, use 800 as a value (*see* **Note 17**).
6. Upon completion of the 4 h fast, inject mice with glucose, intraperitoneally, or by oral gavage.
7. Repeat blood glucose measurements at 20, 40, 60, and 90 min. Three persons can usually handle up to 120 mice—one dosed mice at 20 s intervals, and a second and third person taking blood measurements starting and 20 and 40 min after dosing, respectively.
8. Add food to mouse cages.

3.8 Glucose-Stimulated Insulin Secretion (GSIS)

Glucose-stimulated insulin secretion (GSIS) is central to normal control of metabolic fuel homeostasis, and its impairment is a key factor in beta-cell failure in T2DM. Some targets may show a phenotype only with GSIS and not in a GTT [32].

GSIS can be performed along with a GTT. The peak of insulin secretion occurs approximately at 7 to 8 min after glucose injection. Blood is typically collected at 7.5, 15, and 30 min after glucose administration. GSIS procedure is more time consuming than a standard GTT and fewer mice can be handled/person. For example, five to eight mice can be handled per person for a GSIS experiment (1 mouse per 1–2 min) compared to 40 mice per person in a standard GTT experiment (1 mouse per 20–25 s).

1. Weigh mice and transfer to clean cages.
2. Rub gently the sides of the tail on each side with index finger and thumb from the base toward the tail tip. Too much pressure may damage the blood veins, the lighter pressure, the better. Collect the blood into a capillary tube. Repeat this step until the tube is half-full (~40 μL). Optional: Use a drop of mineral oil on finger tips.
3. Administer glucose to mice (1 mg/kg in 10 mL/kg volume), $t = 0$.
4. At 7.5, 15, 30, and 60 min after glucose injection, repeat the blood collection procedure.
5. Spin the capillary tubes into a micro-hematocrit centrifuge at 10,000 rpm for 8 min.
6. Cut the capillary tubes at the separation between serum and red blood cells with the sharp edge of a file.
7. Use a pipette with a 200 μL tip to gently blow the serum out of the capillary tube into a 96-well plate.
8. Samples may be frozen at –20 °C for later measurements. Cover the plates with Alumi-Seal.
9. Measure levels of insulin according to the manufacturer's protocol.

3.9 Insulin Tolerance Test (ITT)

Insulin sensitivity can be measured directly by injecting insulin in mice and measuring its effect on circulating glucose levels. The dose of insulin to be administered ranges from 1 to 5 U/kg and is adjusted depending on the model system used. For example, lean C57BL/6J mice will have their baseline glucose reduced substantially at 1 U/kg, while mice fed on a high fat diet will often need to be treated with 1.5 or 2.0 U/kg, and ob/ob mice will sustain up to 5U/kg.

1. Provide access to food and water ad libitum (*see* **Note 18**).
2. Weigh mice.
3. Calculate injection volumes using a volume of 10 mL/kg.
4. Dilute insulin in saline to the desired concentration: 1 to 5 U/10 mL. Keep on ice during preparation. Mix gently and preload syringes.

5. Measure baseline glucose by tail tip or tail snip blood collection, according to procedure described above.
6. Inject mice i.p. with insulin.
7. Measure glucose 15, 30, 60, 90, and 120 min after insulin injection. If the glucometer indicates "Lo," mice should be injected immediately with glucose to prevent loss of consciousness due to hypoglycemia.

3.10 Insulin Sensitivity

Measuring glucose alone is not sufficient to evaluate the state of sugar metabolism. For example, as ob/ob mice age, they become normoglycemic yet they are severely insulin resistant. Further, their pancreatic islets become hypertrophic, and the levels of circulating insulin exceed 10 ng/mL, thereby compensating for the insulin resistance.

The "gold standard" for calculating an insulin sensitivity index is the hyperinsulinemic–euglycemic clamp. It measures the amount of glucose necessary to compensate for an increased insulin level without causing hypoglycemia. This method involves the catheterization of carotid arteries and jugular veins in rodents. It is particularly challenging in mice because of their small size and is not amenable to large-scale studies. Consequently, alternatives that correlate closely to clamp results are often used in determining insulin sensitivity indices. The direct measurement of insulin tolerance, insulin tolerance test (ITT), is often used. Alternatively, simple surrogate indexes for insulin sensitivity/resistance are available (e.g., QUICKI, HOMA, 1/insulin, Matusda index) that are derived from blood insulin and glucose concentrations under fasting conditions (steady state) or after an oral glucose load (dynamic).

The homeostasis model assessment-insulin resistance (HOMA-IR) can be calculated with the following formula:

HOMA-IR index = fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5 [33]

3.11 Body Composition Measurements

Chemical carcass analysis is considered the "gold standard" for accurate whole body composition analysis [34]. It is, however, terminal and time consuming. The adiposity index can also be measured by dissecting and weighing of fat depots in individual animals [35]. This method is also terminal and less accurate. The collection of visceral fat required can be particularly challenging as it is often spread throughout internal organs.

Two imaging systems, Dual Energy X-ray Absorptiometry scanning (DEXA) and Magnetic Resonance Imaging (MRI), allow for longitudinal studies of whole body composition. DEXA measures bone mineral density and content, fat content, and lean content in anesthetized mice. Echo MRI from Echo Medical System, Houston, TX, is used to measure whole body composition parameters such as total body fat, lean mass, body fluids, and total body

water in live mice without the need for anesthesia or sedation [36]. The MRI technology is more rapid, less than a minute to scan one mouse, than DEXA which takes about 5 min per mouse. In recent years, GE Medical Systems has discontinued the production and service support for the PIXImus which allowed for DEXA scanning in mice.

3.11.1 Magnetic Resonance Imaging

1. Prior to each run, calibrate the system using a calibrated standard provided by Echo Medical System.
2. Record mouse body weights.
3. Place each mouse into an appropriate size tube and place into the MRI machine.
4. EchoMRI software records spectra on each mouse.
5. The output information is expressed as lean tissue mass, fat mass, and free body fluids in grams.

3.12 Terminal Blood and Tissue Collection

Terminal blood collection is often performed upon completion of an experiment, as it is useful to perform measurements of additional metabolic markers on cohorts that showed compound efficacy. Immediately following blood collection tissue collection can also be performed at the same time from the same animal.

The cytoarchitecture of fat, liver, pancreas, and kidney is seriously affected by T2DM. As mentioned in the introduction, the pancreatic islets of Langerhans become hypertrophic in the early stages of the disease and subsequently undergo degeneration. Adipocytes in fat tissues become hypertrophic. Fatty livers can also be observed by gross morphology and the hepatocytes show accumulation of lipids at the histological level. Finally, in the later stages of the disease, glucotoxicity induces irreversible damage to kidney tubules. Consequently, those are the four main tissues to be examined in histology.

1. Prepare the syringes: Break the vacuum seal of 1 mL syringes by pulling the plunger slightly and push back. Fit the syringes with 25 G × 5/8 inch needles.
2. Before beginning the terminal collection procedures have all serum separation tubes for blood collection labeled and all tubes for tissue collection labeled and filled with 5 to 10 mL 10% formalin (3.7% formaldehyde). Organs from the same mouse can be pooled in the same tube as they can simply be embedded in a single block for histological analysis.
3. Euthanize the animal by CO_2 asphyxiation.
4. Put the animal on a paper towel on its back and palpate the sternum with a finger of one hand.
5. With the other hand, insert a needle slightly below and to the left of the sternum, with a mild inclination, aiming into the left cardiac ventricle (*see* **Note 19**).

6. Draw blood slowly so as not to collapse the ventricle. Usually 0.3 to 1.0 mL can be obtained in 10 to 30 s. Once the blood starts flowing in, the depth and the angle of the needle may need to be slightly adjusted to maintain the flow of blood into the needle.
7. To avoid hemolysis remove the needle from the syringe and push the blood into a serum separation tube. Do not exceed the volume indicator on the tube (frosted area).
8. Centrifuge for 10 min at 10,000 rpm in an Eppendorf centrifuge at 4 °C.
9. Transfer the supernatant (serum) to a clean tube or 96-well plate (*see* **Note 20**).
10. Make an incision through the skin and peritoneal membrane to open the abdomen.
11. Cut a lobe of the liver and put in formalin.
12. Collect examples of visceral fat; e.g., the adipose tissue surrounding the intestine.
13. Pull out the spleen and the pancreas which is loosely attached to the spleen and cut out both. Put both organs on a paper towel. Separate the pancreas and put in formalin.
14. Collect one kidney and add to the formalin tube.
15. Proceed using standard histology procedures for paraffin embedding and sectioning (*see* **Note 21**).
16. Stain sections with hematoxylin/eosin (*see* **Note 22**).

4 Notes

1. A large number of additional methods are commonly used in diabetes research to understand mechanisms of action of given drugs or pathways. A few examples are the use of a Comprehensive Laboratory Animal Monitoring System (C.L.A.M.S.) to measure oxygen consumption and thermogenesis, perfusion of pancreatic islets for in vitro studies, gastric emptying measurement protocols, and gastric bypass surgery. Many resources exist elsewhere to cover these methods.
2. In a C57BLKS/J background, the *Lepob* mutation express a phenotype closely related to that observed in the BKS.Cg-*Dock7m* +/+ *Leprdb*/J strain, highlighting the importance of the genetic background in these models.
3. Reported duration of feeding with a high fat diet varies greatly among published studies from a few to up to 20 weeks. Insulin resistance increases steadily during this period. Therefore, the period of high fat diet feeding is a compromise between time efficiency and the window necessary to see an effect.

4. Glucose increases rapidly in these mice and reaches 600 mg/dL by 6 to 8 weeks of age. Therefore, the youngest mice are best. If the availability of BKS.Cg-*Dock7m* +/+ *Leprdb*/J males is limited, females of the same age can be used.
5. B6.Cg-Lep^{ob}/J mice become normoglycemic as they age because of compensatory hyperinsulinemia. We have used these mice successfully up to 12 weeks for pharmacological studies.
6. The most commonly used diets are 45 kcal% fat diet (Research Diets, D12451i) and 60 kcal% fat diet (Research Diets, D12492i). The "i" in "D124xxi" refers to the irradiated form of the diet. Many vivaria use the irradiated form for sterility purposes, but this is not required. 60% fat diet induces a more severe insulin resistance. However, the high fat pellets crumble more easily, falling in the bedding and make it more difficult to perform accurate food intake measurements. Also, under these regimes more skin lesions can be observed, presumably because of increased subcutaneous fat, greasy fur from the diet, and fighting. Use of high fructose/sucrose diets is not commonly used in mice. While a high carbohydrate diet is efficient at inducing insulin resistance in rats, effects vary strongly between mouse strains. It has little effect on C57BL/6J mice (cf. Research Diets website).
7. Food intake can vary substantially by strain and diet within a range of 2 to 6 g per mouse daily. Therefore, the concentration of rosiglitazone in the food may need to be adjusted. Formulation is custom made by the vendor.
8. Mouse serum collection tubes or the Microvette® CB300 collection system (Kent Scientific) are suitable alternatives to the capillary collection method presented here.
9. The PIXImus™ densitometer from GE Medical Systems was removed from this edition, as this system has now been discontinued. Two systems for MRI in mice are commonly used, EchoMRI™ and the Minispec from Bruker. Both provide reliable body composition measurements. The loading of the mice in the Echo system allows the mice to remain horizontal during measurement regardless of their size—as opposed to vertical—and may cause less stress on the animal.
10. Most peptides and proteins are water soluble, and therefore D-PBS or saline can be used as a vehicle. Often however, limited information is available about the solubility properties of novel small molecules, and the choice of a nontoxic vehicle is more difficult. For example, 200 µL of a 5% ethanol solution is equivalent to one beer in humans and may affect behavior. A solution of 20% cyclodextrin has no known side effects in vivo, but in rare cases, some compounds are trapped in the solution and therefore mice have no exposure to the compound. Some

vehicles used for in vitro studies can be toxic in live mice. Some vehicles such as methylcellulose have no side effect when given p.o., but are toxic if administered i.v. Access to information about the pharmacokinetics properties of a test compound can help in the choice of a vehicle. Also, many institutions have internal guidelines determining the use of acceptable vehicles in pharmacological studies. If so, verify that the vehicle chosen is approved in the said guidelines.

11. When mixing a compound to the diet, pilot experiments should be conducted to verify that food intake is not affected by the change of palatability in the diet. This is counterintuitive, as a compound reducing food intake might be considered a benefit for a metabolic disease. However, unspecific effects on metabolism due to diet unpatability should be tested and excluded early on.
12. Retroorbital bleeding can cause blindness in animals and is increasingly discouraged by animal welfare committees. However, alternatives such as saphenous vein blood collection are cumbersome. Submandibular vein blood collection is rapid but causes glucose values to increase (unpublished data.)
13. Mice can be either group housed or single housed. Mice are often singly housed in DIO studies to avoid skin lesions resulting from cage mate aggression. Single housing also allows for individual food intake measurements. On the other hand, mice are social animals and being single housed increases their level of stress and decreases food intake. Additionally, housing space can be a limited resource in many vivariums, in which case group housing may be preferable. Although less commonly used, B6D2F1/J mice are responsive to DIO feeding, can be easily randomized and regrouped with noncage mates into adulthood without increased aggression behavior. In either case, singly- or group-housed, all mice from a given experimental cohort should be housed in the same manner.
14. Given the rapid progression of hyperinsulinemia, the range of body weight and baseline serum glucose value varies greatly. In addition, the date of birth used for shipment is usually a "bin" of the stated date of birth, and if often uses animals born 3 to 4 days before or after the stated date. On a 3-week-old mouse this can lead to significant weight differences. Limits are chosen somewhat arbitrarily to eliminate mice that deviate the most from the average values, while keeping enough mice for cohort size providing statistically significant values.
15. Values may differ slightly depending on the settings chosen in the template used for reading. The test is designed to use a "Blank" value rather than using "standard values = 0" in the template for the wells corresponding to the "zero standard."

The test is also designed to provide a linear correlation of absorbance and concentration when using log–log scales.

16. Lower glucose doses can be used (e.g., 1 g/kg) when mice are hyperglycemic and insulin resistant, and that many of the glucose values collected during a GTT will exceed the detection limit of the glucometer, 800 mg/dL.
17. Alternative glucose measurement methods are available. However, their relevance to glucose metabolism is of limited value considering that when glucose levels exceed 600 mg/dL, glucose is eliminated through urine rather than by glucose uptake in peripheral tissues.
18. GTT and ITT can be conducted either in fasted or nonfasted conditions. This will affect the baseline glucose values and the standard deviation but has little effect on glucose values later in the tests in comparison to the effects of glucose and insulin. Fasting causes an increase in appetite signals and other molecules involved in metabolism. Depending on the test molecule tested, this might interfere with the drug target and study results.
19. Right and left are defined from the mouse perspective, i.e., "left" refers to the mouse's left side.
20. The serum should be yellowish. A reddish color is indicative of erythrocytes lysis (hemolysis) which may interfere with clinical chemistry assays based on colorimetric values. If this happens, you may need to adjust the speed at which the blood is collected and processed or other steps that may cause sheer and red blood cell lysis.
21. All samples can be embedded in a single block for histological analysis as a multitissue block. Histology services are offered by many commercial providers, e.g., IDEXX, a veterinary service with locations worldwide.
22. Hematoxylin–eosin is sufficient to reveal pancreatic islet hyperplasia or degeneration, pancreatitis, liver steatosis, adipocyte hyperplasia, and diabetic nephropathies. Additional staining can be requested, such as immunostaining with anti-insulin or antiglucagon antibodies if additional information is needed about drugs mechanisms of action.

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