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TITLE

Fat-covered islet transplantation using epididymal white adipose tissue

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KEYWORDS

islet transplantation, white adipose tissue, epididymal fat pad, intraperitoneal, mouse, experimental model

SUMMARY

This fat-covered islet transplantation method is suitable for the detection of engrafted islets in the intraperitoneal cavity. Notably, it does not require the use of biobinding agents or suturing.

ABSTRACT:

Islet transplantation is a cellular replacement therapy for severe diabetes mellitus. The intraperitoneal cavity is typically the transplant site for this procedure. However, intraperitoneal islet transplantation has some limitations, including poor transplant efficacy, difficult graft detection ability, and a lack of graftectomy capability for post-transplant analysis. In this paper, “fat-covered islet transplantation”, an intraperitoneal islet transplantation method that utilizes epididymal white adipose tissue, is used to assess the therapeutic effects of bioengineered islets. The simplicity of the method lies in the seeding of islets onto epididymal white adipose tissue and using the tissue to cover the islets. While this method can be categorized as an intraperitoneal islet transplantation technique, it shares characteristics with intra-adipose tissue islet transplantation. The fat-covered islet transplantation method demonstrates more robust therapeutic effects than intra-adipose tissue islet transplantation, however, including the improvement of blood glucose and plasma insulin levels and the potential for graft removal. We recommend the adoption of this method for assessing the

mechanisms of islet engraftment into white adipose tissue and the therapeutic effects of bioengineered islets.

INTRODUCTION:

Islet transplantation is a cellular replacement therapy for patients with severe diabetes mellitus. Recent reports have shown that rates of insulin-independence at three years after transplantation improve up to 44%[1] and that approximately 80% of recipients who receive more than 600,000 total islet equivalents achieve insulin independence[2]. Furthermore, in the most recent Collaborative Islet Transplant Registry report, it was revealed that fasting blood glucose levels were maintained at 60-140 mg/dL for over a period of 5 years in over 70% of patients who underwent islet transplant alone. The study also determined that around 90% of the patients who received islet transplant alone or islet transplantation after kidney transplant did not develop any severe hypoglycemic events for over 5 years[3].

Although the clinical outcomes of this treatment have been improving, some limitations must still be addressed, including the necessity of establishing an optimal transplant site. The liver is a typical transplant site for clinical islet transplantation because it is the largest organ that can accommodate a high volume of islets. However, in some patients the liver is unavailable (e.g., due to portal hypertension, hepatitis, and/or cirrhosis[4]) and therefore other sites, including the renal subcapsular space[5,6], omental pouch[7-10], mesentery[11], gastrointestinal tract[12], skeletal muscle[13], subcutaneous tissue[13], bone marrow[14], and spleen[15-17], have been considered as alternative transplant sites.

Although intraperitoneal islet transplantation can be performed easily under local anesthesia, making the intraperitoneal cavity an appealing site for clinical islet transplantation, upon transplant, the islets are dispersed throughout the entire intraperitoneal cavity, making islet engraftment detection and successful engraftment confirmation difficult. Therefore, the intraperitoneal cavity is not widely recognized as an ideal clinical transplant site. Instead, it is frequently utilized as a control model for preclinical studies to investigate the effectiveness of transplanted encapsulated[18] and bioengineered islets[19]. However, an exact comparison between bioengineered and control islets is difficult to achieve due to the challenges in performing an accurate engraftment assessment.

In contrast, the use of intraperitoneal white adipose tissue in the omental pouch[8], mesentery, and other extrahepatic locations has been well reported[10,20-23] and many of the studies investigating the function of bioengineered islets transplanted using white adipose tissue were able to report promising therapeutic outcomes[20,24-26]. As the use of epididymal adipose tissue facilitates the detection of transplanted islets, the “fat-covered islet transplantation method”, utilizing epididymal adipose tissue, was developed to overcome the limitations of intraperitoneal islet transplantation. In this paper, fat-covered islet transplantation using epididymal adipose tissue is described.

PROTOCOL:

The following procedure is performed in three steps. The first step includes the induction of diabetes in the recipient mice and the isolation of donor islets. The second step involves the preparation of islets before transplantation. In the third step, islet transplantation onto epididymal adipose tissue and covering of the islets using the adipose tissue is performed. After that, the therapeutic effects were assessed. The handling of the mice and the experimental procedures performed in this study comply with the ''Principles of Laboratory Animal Care'' (Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication 86-23, 1985) and the experimental protocol was approved by the Animal Care and Use Committee of Fukuoka University (approval number: 186018).

1. Surgical preparation

1.1. Induction of diabetes: Induce diabetes in 20-25 g body weight, 8-12-week-old recipient male mice through intravenous injection of streptozotocin (180 mg/kg body weight). Mice with blood glucose levels exceeding 400 mg/dL are considered to be diabetic. Use diabetic mice within 1 week after diabetes induction before excessive atrophy of the epididymal white adipose tissue for covering islets.

1.2. Islet isolation: Perform murine islet isolation one day before transplantation following Gotoh's method[27] for islet isolation.

1.2.1. In brief, digest pancreatic tissue using collagenase solution. Isolate islets by density gradient centrifugation using an appropriate cell separation solution. Then culture islets overnight in an incubator at 22 °C and 5% CO_2 (culture at <37 °C has been reported to prevent islet death[28-31]).

2. Preparation of islets for transplantation

2.1. Gather the appropriate instruments and materials as indicated in **Figure 1A**.

2.2. As digestive enzymes such as amylase and lipase may result in injury to the isolated and transplanted islets and a loss of islets can occur from being trapped in contaminating fibrous tissues within the culture dish, prior to transplantation, use forceps to handpick any extra-islet components from the pancreas, including acinar and fibrous tissues (**Figure 1B**), under a dissecting microscope. After picking, use a cell strainer to filter out single acinar cells.

2.3. Transfer the filtered islets to a new culture dish containing any appropriate culture medium or buffer solution (e.g., DMEM with low glucose, RPMI1640, CMRL1066, or HBSS) supplemented with bovine serum or albumin to prevent islet attachment to plastic and swirl the dish to position the islets in the center of the dish (**Figure 1C**). Using a P200 micropipette and the microscope, pick the individual islets into an appropriate collection tube (**Figure 1D**).

2.4. Place a new, 40 μm cell strainer on top of a 50 mL plastic tube (**Figure 1E left and center**) and wash the filter with fresh medium (**Figure 1E right**).

2.5. Use a 1000 µL pipette to add the islets to the strainer to separate the islets and single acinar cells (**Figures 1F1 and 1F2**).

NOTE: The purified islets on cell strainer will be approximately 100% pure.

2.6. Use forceps to invert the strainer on a new 60- or 100-mm sized non-treated culture dish containing culture medium or an appropriate buffer solution supplemented with bovine serum or albumin (**Figures 1F3 and 1F4**). Use fresh medium/buffer to flush the islets into a new culture dish. Then add enough medium/buffer to the culture dish to reach a total volume of approximately 20 mL.

2.7. Count the islets under a microscope and divide the number of islets equally between individual 1.5 mL plastic centrifuge tubes according to the number of donor animals (**Figure 1G**). For example, two hundred, 100-200 µm islet equivalents (IEQ) from two mice would be added to each of two tubes.

2.8. Centrifuge the islets at 2100 x g for 1 minute at room temperature and discard the supernatant. Around 20-30 µL of residual solution will typically remain in the tube (Figure 1H).

3. Islet transplantation onto epididymal adipose tissue and covering with epididymal white adipose tissue

3.1. Before the surgery, collect an anesthesia machine for small animals, stereo microscope, light source, 50-200 µL micropipette with 200 µL micropipette tips, cotton swabs, a 4-0 suturing set, and disinfected surgical instruments (**Figure 2A**). Disinfect Cooper scissors, ophthalmic scissors, Pean forceps, tweezers, and needle holders with an approximately 1% povidone-iodine solution (**Figure 2A**). Use cotton swabs for mobilization of the epididymal white adipose tissue and for hemostasis in cases of bleeding. Use a micropipette with a 50-200 µL tips for islet transplantation.

3.2. Deliver anesthesia to the diabetic recipient mouse using an inhaled anesthetic agent (2% isoflurane in oxygen). Then place the mouse in the supine position (**Figure 2B left**) and remove the hair from the abdomen to prevent infection using hair clippers and/or depilatory cream. Use 70% ethanol or a povidone-iodine solution to disinfect the abdomen and the inguinal region (**Figure 2B right**).

3.3. Incise the skin at the lower median area (**Figure 2C left**). A skin incision that is approximately 2-3 cm in length is recommended. Clamp the left abdominal wall with the Pean forceps and pull the tissue to the left side of the mouse to secure the surgical field (**Figure 2C right**). After laparotomy, decrease the percentage of isoflurane to 1.0–1.5% for anesthesia maintenance.

3.4. Use a cotton swab to mobilize the small and large intestine to the right side of the

mouse (i.e., left side of the operator). The left epididymal white adipose tissue in the abdominal cavity are located in the left inguinal area. Mobilize the epididymal white adipose tissue and the left testis to outside of the abdomen (**Figure 2D left**) and stretch out the tissue (**2D right**).

3.5. Use a P200 micropipette equipped with a 200 µL pipette tip to collect the entire volume of islets from one 1.5 mL tube with gentle pipetting (**Figure 2E left**), taking care that no islets are left in the tube upon collection. Allow the collected islets to settle to the tip of the pipette by gravity (**Figure 2E right**).

3.6. Place the micropipette tip lightly onto the distended adipose tissue. Taking care to prevent excessive flushing of the medium/buffer in the tip, carefully seed the islets onto the tissue (**Figure 2F left**). After seeding, confirm a correct placement of the islets under a dissecting microscope (**Figure 2F right**).

3.7. Cover the islets with the epididymal white adipose tissue (**Figure 2G**). The use of sutures or biobinding agents is not needed.

3.8. Place the left testis under the epididymal white adipose tissue and return the tissues to the intraperitoneal cavity (**Figure 2H**). Close the skin in two layers (peritoneum, then muscle and skin) using a 4-0 suture (any sutures such as nylon, silk, or absorbable sutures can be used) (**Figure 2I**). Then place the mouse under a heat lamp with monitoring until full recumbency.

4. Monitoring after Islet transplantation (Summary)

4.1. Assess the therapeutic effects of islet transplantation by monitoring blood glucose, glucose tolerance test and histological assessment at postoperative day (POD) 28.

4.1.1. Monitor the blood glucose, including the measurements of blood glucose at glucose tolerance test, using a small glucose meter.

4.1.2. Collect the blood samples (a little microliters) from tail vein. Regarding histological assessment, murine insulin (for detecting engrafted islets) and von Willebrand factor (for detection of vessels, which is an evidence for islet engraftment) were detected in transplanted islets in the recovered epididymal adipose tissue by immunohistochemistry.

REPRESENTATIVE RESULTS:

To compare the transplant efficacy of fat-covered islet transplantation to that after intraperitoneal islet transplantation, the same number of islets was implanted onto the peritoneum at the left paracolic space of control recipient diabetic animals. The blood glucose levels of mice with fat-covered islet transplantation were observed to gradually and significantly decrease compared to intraperitoneal islet transplanted mice ($p = 0.0023$; **Figure 3A**). One month after transplantation, the blood glucose in mice with fat-covered islet transplantation was maintained at lower levels than that observed in intraperitoneal islet transplanted mice as assessed by intraperitoneal glucose tolerance testing ($p = 0.0046$; **Figure**

3B). Furthermore, as we have previously, reported that plasma insulin levels also improved after fat-covered islet transplantation. Re-elevation of blood glucose levels was also confirmed. These data demonstrate that intraperitoneal fat-covered islet transplantation using 200 IEQs can significantly improve the diabetic conditions of recipient mice.

Histological examination was also performed to assess islet engraftment into the epididymal white adipose tissue. In fat-covered islet transplant recipient animals, hematoxylin-eosin staining reveals the presence of islets within the epididymal white adipose tissue (**Figure 3C, top image**). In addition, fluorescence-conjugated antibody staining of insulin-positive islets facilitated the detection of von Willebrand factor-positive microvessels within the epididymal white adipose tissue of all of the recipient mice (n = 6; **Figure 3C**). In contrast, in intraperitoneal islet transplanted mice, no engrafted islets were observed in either the epididymal white adipose tissue or the abdominal wall (data not shown).

FIGURE LEGENDS:

Figure 1. Preparation of islets for transplantation onto epididymal adipose tissue and covering with epididymal white adipose tissue. (**A**) Preparation of instruments: a. pipette aid, b. 10 mL pipette tips, c. 50 mL plastic tubes, d. 15 mL plastic tubes, e. 50-200 µL (left) and 200-1000 µL (right) micropipettes, f. 1.5 mL plastic centrifuge tubes, g. 200 and 1000 µL micropipette tips, h. medium or buffer containing albumin or fetal bovine serum (i.e., DMEM with low glucose containing 10% fetal bovine serum and 100 U/mL penicillin + 100 U/mL streptomycin solution), and i. 40 µm cell strainer. (**B**) Isolated islets with acinar (left: indicated by arrow) and fibrous tissues (right). Scale bar = 200 µm. (**C**) Collected islets in the plastic tube. Left, dispersed islets in culture dish. Center, islets are collected in the center of the culture dish by swirling. Right, collected islets in center of dish. Scale bar = 200 µm. (**D**) Collected islets (left) are transferred into 15 mL plastic tubes (right). (**E**) The 40 µm cell strainer is set on top of the 50 mL plastic tube (left and center). Prepared medium/buffer added to other 50 mL plastic tube for flushing islets on the cell strainer into a new culture dish (right). (**F)** 1. Islets collected using a 200-1000 µL micropipette. 2. Islets poured into the cell strainer. 3 and 4. Medium/buffer used to flush islets onto strainer into new culture dish. (**G**) Islets divided into 1.5 mL plastic centrifuge tube according to number of recipient mice. Here, two hundred 100-200 µm islet equivalent (IEQ) were divided equally into each tube. (**H**) Islets divided equally in 1.5 mL tubes before centrifugation (left). Islets centrifuged to collect at tube bottom (center). 20-30 µL of supernatant remain in tube after discarding excess solution (right).

Figure 2. The procedure of islet transplantation onto epididymal adipose tissue and covering using epididymal white adipose tissue. (**A**) Preparation of instruments: a. anesthesia machine for small animals, b. stereo microscope, c. light source, d. disinfected surgical instruments, e. divided islets in 1.5 mL plastic tubes, f. 50-200 µL micropipettes, g. 200 µL micropipette tips, and h. 4-0 sutures. (**B**) Diabetic recipient mouse in a supine position under general anesthesia of 2% isoflurane (left). The abdomen and the inguinal region are disinfected using 70% ethanol and covered by a paper lab wipe (right). (**C**) Skin is incised at lower median position (left). Left abdominal wall is clamped by Pean forceps and pulled to left side of mouse to secure surgical field (right). (**D**) The left epididymal white adipose tissue and the left testis are mobilized

outside of the abdomen (left) and distended (right). Scale bar = 1 cm. (**E**) Islets in 1.5 mL plastic tubes are completely collected using a micropipette with 200 µL pipette tip (left). Collected islets (indicated by arrow) allowed to completely sink by gravity to pipette tip (right). (**F**) Micropipette tip lightly placed onto the distended adipose tissue (left). Islet seeding (dotted circle) onto the tissue confirmed by dissecting microscope (right). Scale bar = 1 cm. (**G**) Islets are covered with epididymal white adipose tissue. (**H**) Left testis and epididymal white adipose tissue returned to intraperitoneal cavity. (**I**) Image after abdomen closure.

Figure 3. The therapeutic effect of fat-covered islet transplantation. (**A**) Blood glucose level. Blue line: fat-covered islet transplantation (n = 6); Orange line: intraperitoneal islet transplantation (n = 6) Statistical analysis was performed using repeated measures analysis of variance and a significant difference was defined as $p < 0.05$. (**B**) Blood glucose level from glucose tolerance test a month after transplantation. Blue line: fat-covered islet transplantation (n = 6); Orange line: intraperitoneal islet transplantation (n = 6). Statistical analysis was performed using repeated measures analysis of variance and a significant difference was defined as $p < 0.05$. (**C**) Histological image of engrafted islets one month after transplantation. Top image: hematoxylin-eosin staining; Bottom image: immunohistostaining for murine insulin (green) and von Willebrand factor (vWF: red, indicated by white arrow). Scale bar = 100 µm.

TABLE OF MATERIALS:

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DISCUSSION:

The fat-covered islet transplantation method incorporates techniques from two different transplant techniques: intraperitoneal islet transplantation and intra-adipose tissue islet transplantation. As the surface membrane of epididymal white adipose tissue is considered to be the white adipose tissue that is covered by the peritoneum and that is attached to the epididymis, the fat-covered islet transplantation method can be anatomically categorized as a type of intraperitoneal islet transplantation. The technique by which the islets are delivered to the recipient animal, however, are more similar to those utilized in intra-adipose tissue islet transplantation. Our data shows that the therapeutic effect of the fat-covered islet transplantation method is superior to that of intraperitoneal islet transplantation. Our previous study also showed that the transplant efficacy of this method is nearly equal to that of renal subcapsular islet transplantation, a method of islet transplantation with a supreme transplant efficacy[23]. It is speculated that the usefulness of this method may be due to the adhesion molecules present within white adipose tissues and adipocytokines and which are thought to contribute to islet engraftment[10,23].

The size of the epididymal white adipose tissue allows a large volume of islets to be accommodated. In contrast, the rodent greater omentum is too small to easily contain and access many islets. The only physical limitation of epididymal white adipose tissue as a potential experimental islet transplantation site is that it does not provide a portal circulation of insulin[10].

The method is unique because the islets are covered with adipose tissues, instead of being

directly infused into the tissue. Intra-adipose tissue transplanted islets may suffer from the influence of lipotoxicity, as the impaired insulin function in diabetic animals can lead to excess free fatty acids[32]. This method also does not require biobonding agents or suturing to prevent implanted islet loss. As observed in **Figure 3C**, islets remain successfully engrafted within the adipose tissue up to 1 month after transplant and blood glucose level maintenance has been confirmed after graftectomy[23]. This phenomenon may be due to the ability of adipose tissue to trap the islets, which become difficult to peel off.

The advantages of this method are that it is technically easy to perform, allows a metabolic and histologic assessment of the therapeutic effects of the islets, and facilitates the evaluation of islet gene and/or protein expression after graftectomy. Compared to previous studies, the efficacy of this method is not inferior to that of islet transplantation into epididymal white adipose tissue[10,33-36]. It is important to note that a precise seeding of the islets onto the epididymal white adipose tissue without islets loss is necessary for a successful engraftment. To ensure success, the entire volume of islets must be aspirated gently into the micropipette tip from the 1.5 mL plastic tube, as rough and rapid pipetting may result in islet adhesion to the walls of the plastic tube, making dispensing difficult. Islet adhesion is a major reason for inadequate islet seeding. After allowing the islets to settle at the tip of the micropipette tip, the islets must be implanted onto the epididymal adipose tissue without flushing. It is important to minimize depression of the plunger button when aspirating and dispensing the islets to prevent excessive medium/buffer flushing of the adipose tissue. Therefore, it is essential to wait until the islets have aggregated completely at the tip of the micropipette tip before depressing the plunger button of the micropipette for implantation. It is sufficient to place the tip lightly onto the adipose tissue and to confirm the seeding of islets onto the tissue by light microscopy.

In conclusion, this method is very simple, despite requiring several critical steps for its success. We hope for the wide adoption of this method to further aid in the facilitation of islet engraftment onto white adipose tissue and for further evaluation of the therapeutic effects of bioengineered islets.

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DISCLOSURES:

We have no conflict of interests.

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Figure

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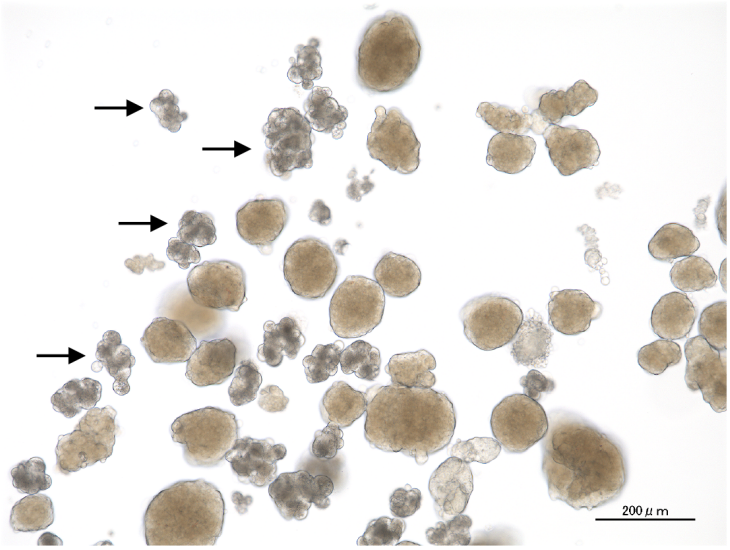
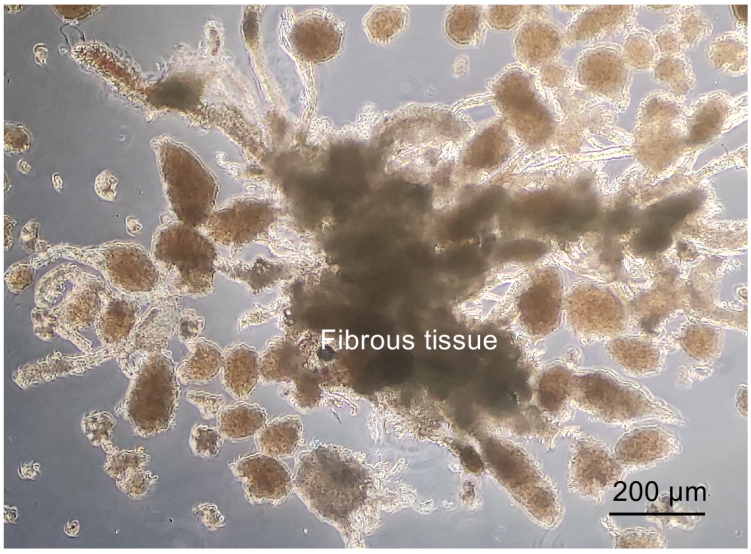
Figure 1

A

B





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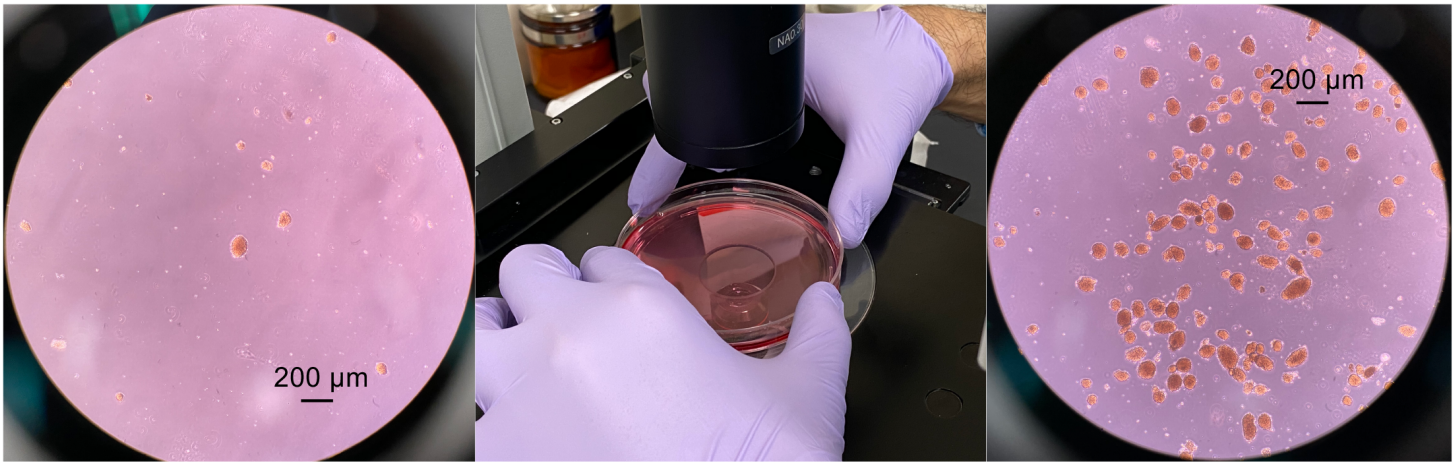

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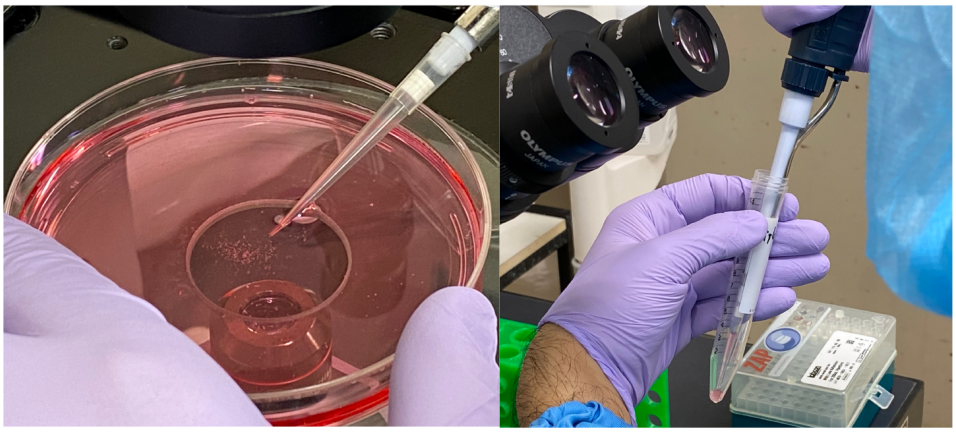

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Figure

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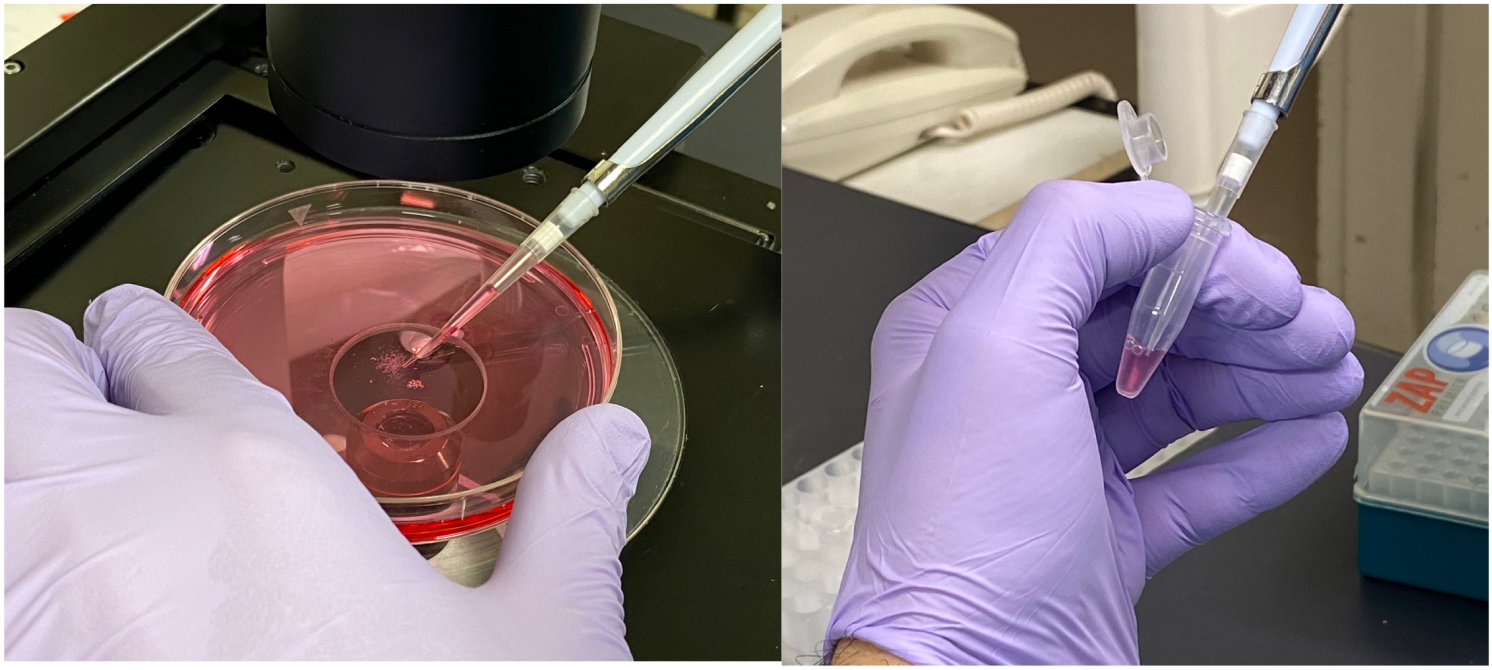
Figure 1 (Continue)

G



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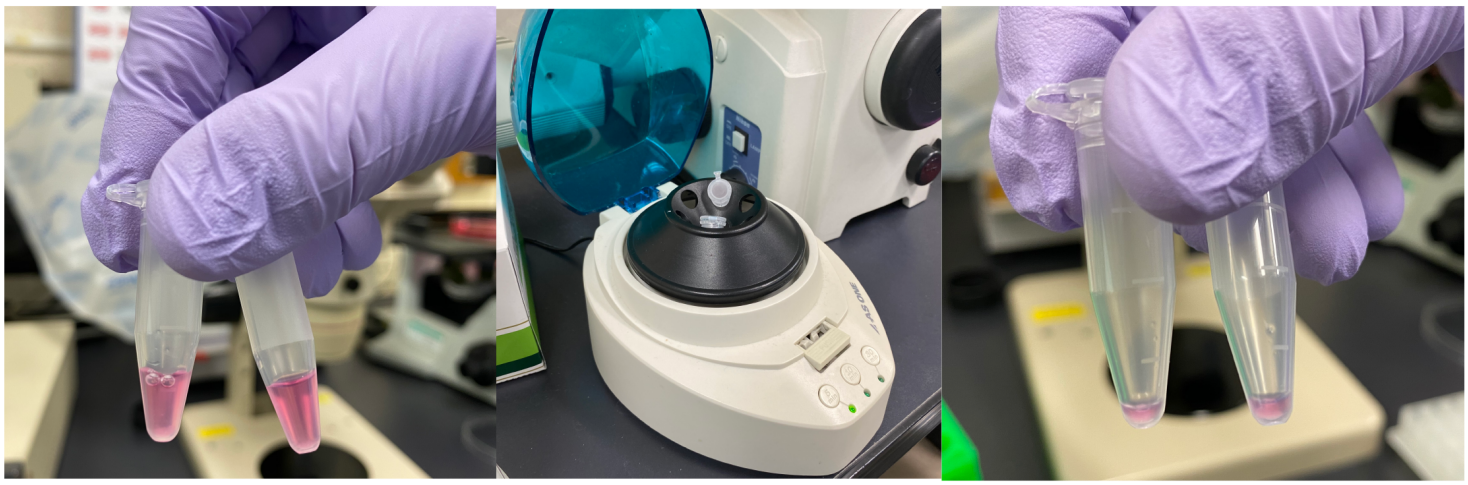
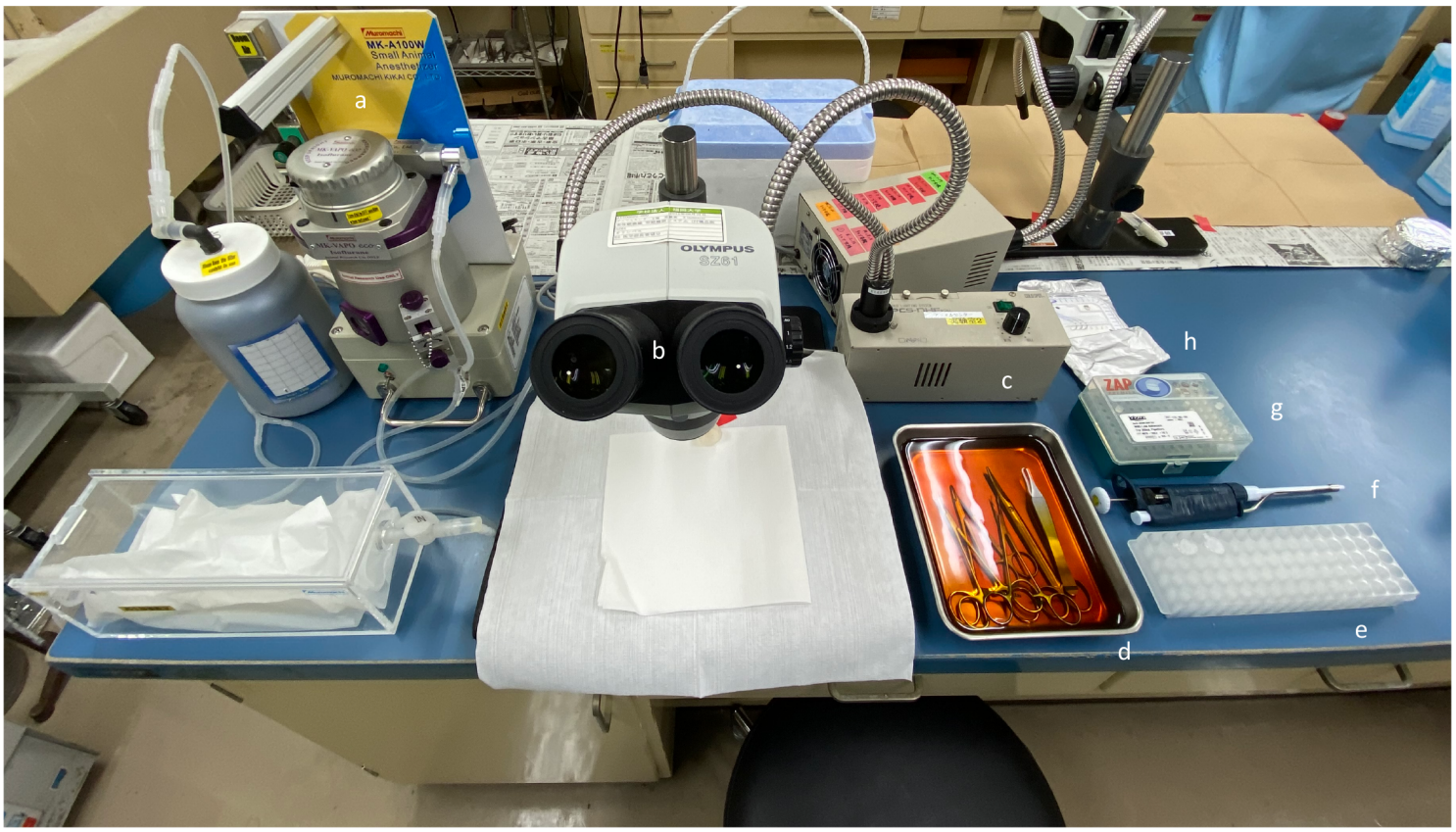


Figure

Click here to access/download;Figure;Fat-covered method resubmission Figure 2-1.pdf

Figure 2

A



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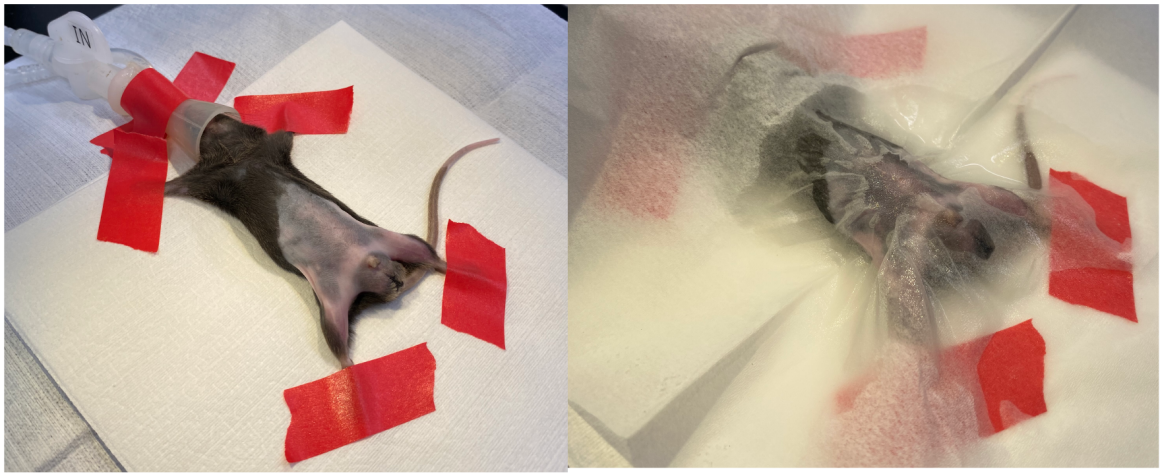

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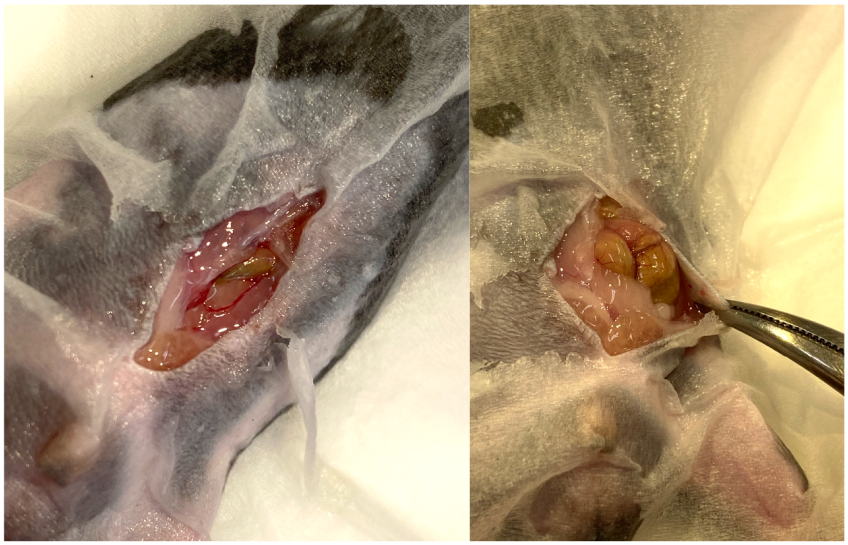

Figure

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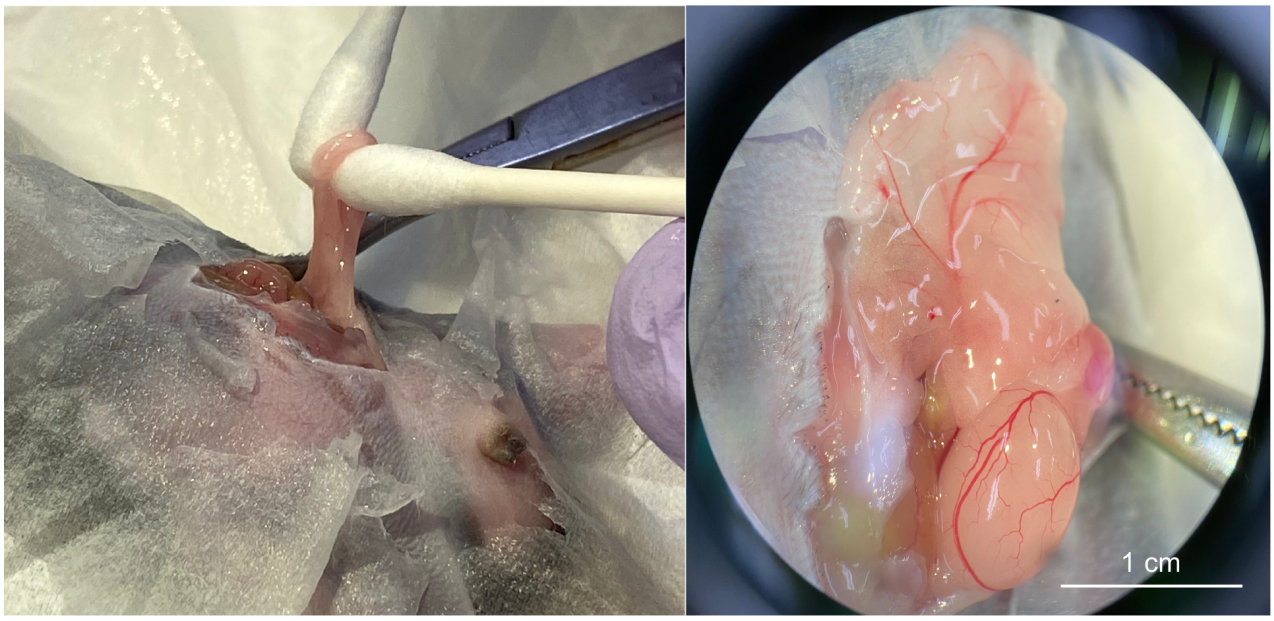
Figure 2 (Continue)

D



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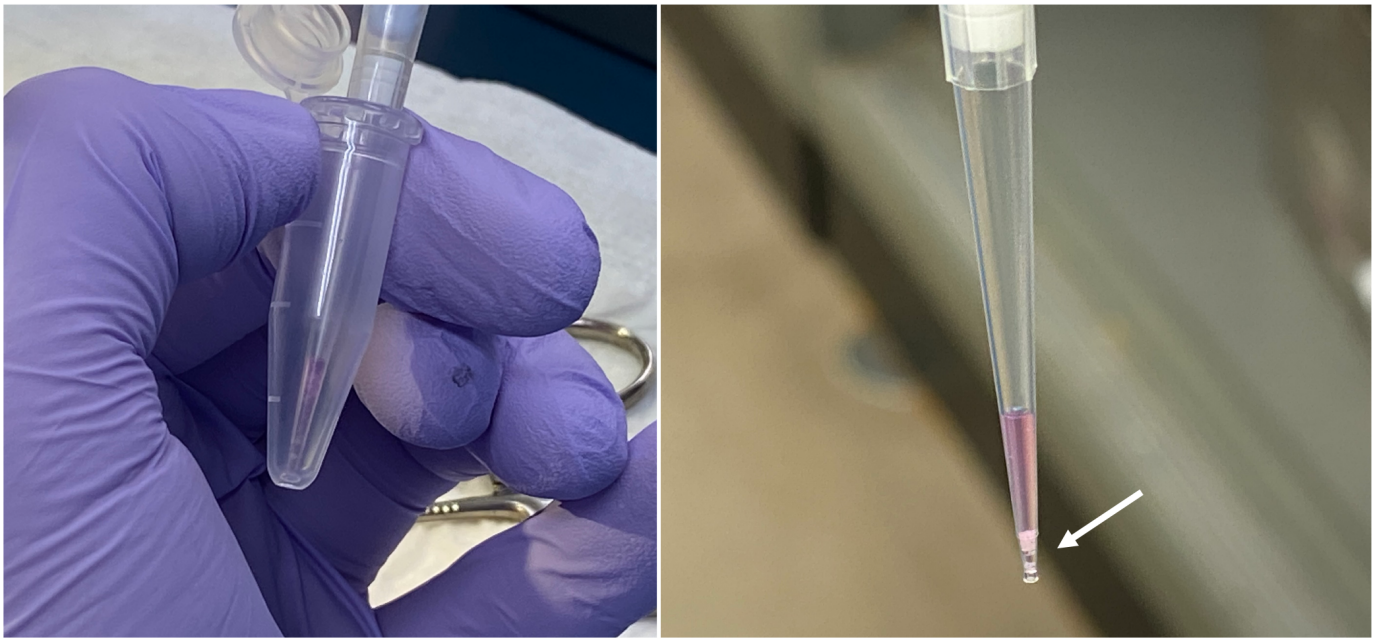

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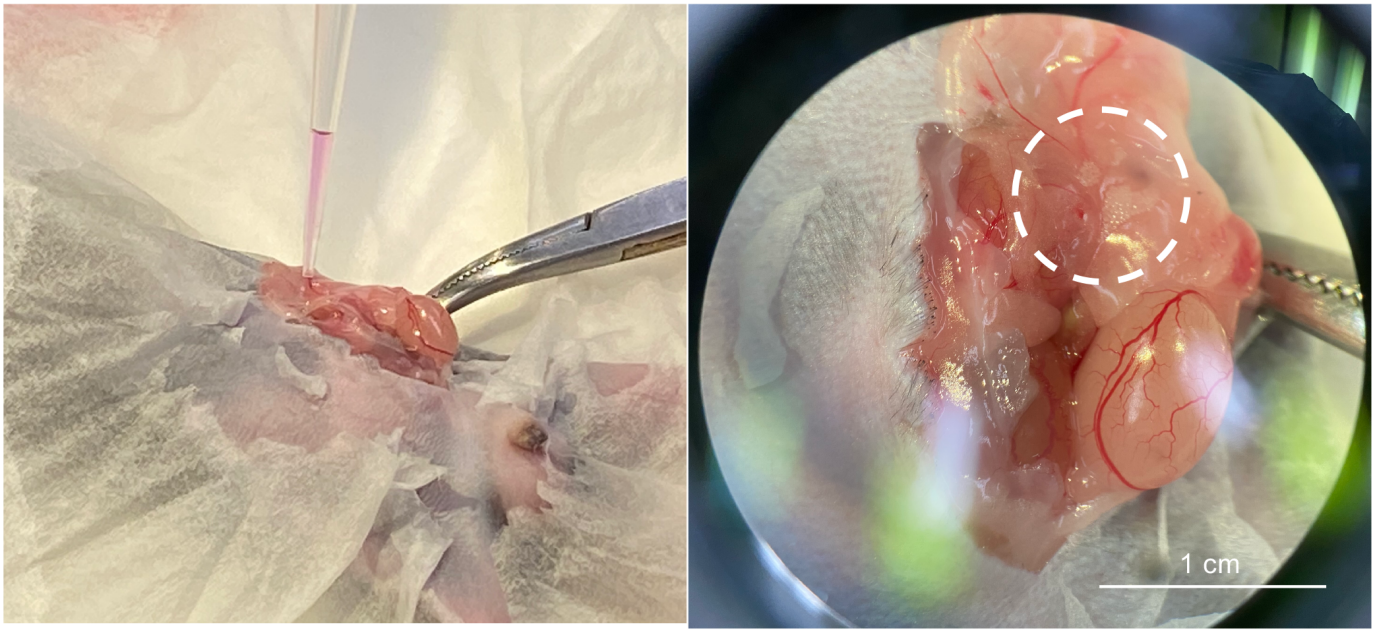

Figure

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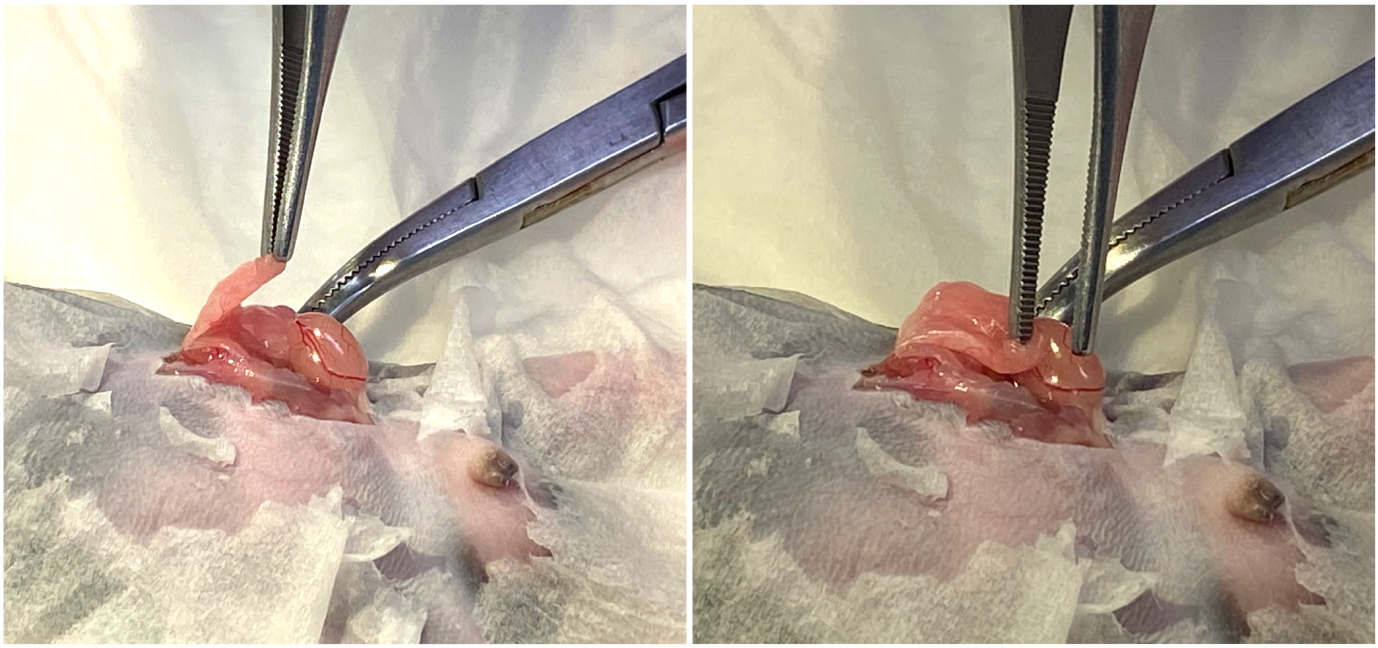
Figure 2 (Continue)

G



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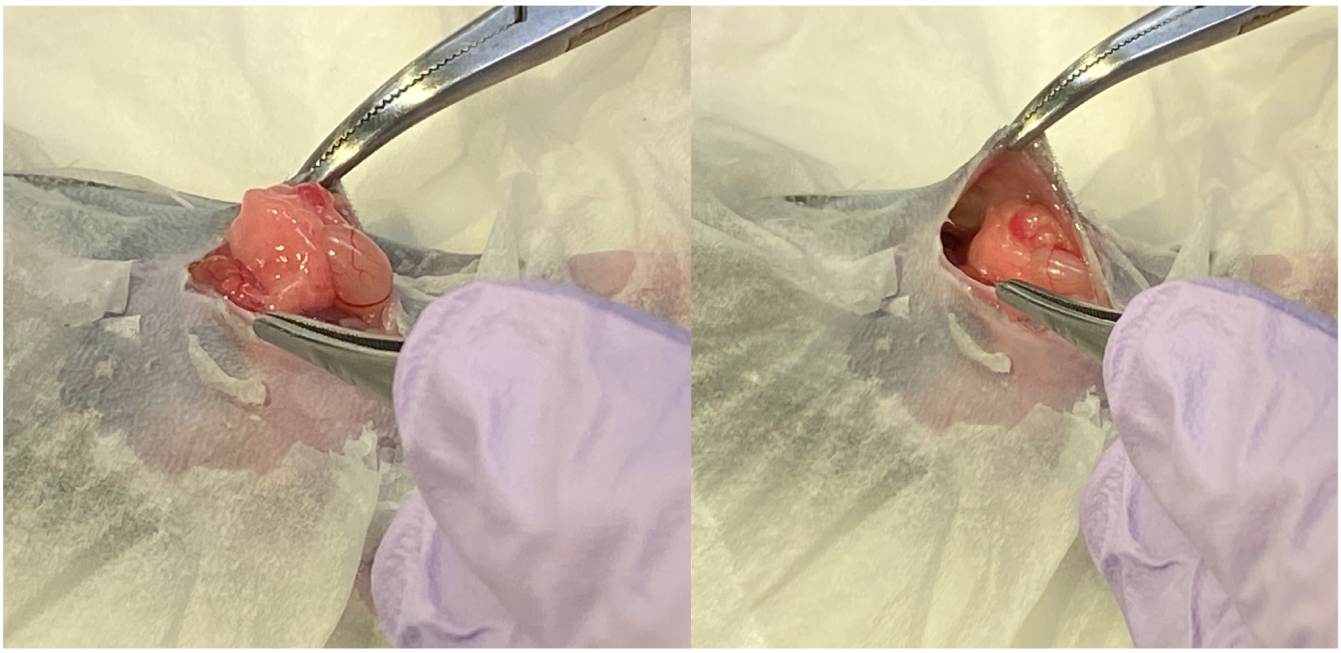

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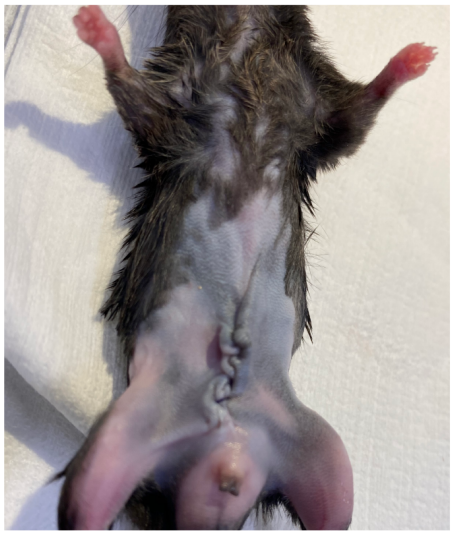
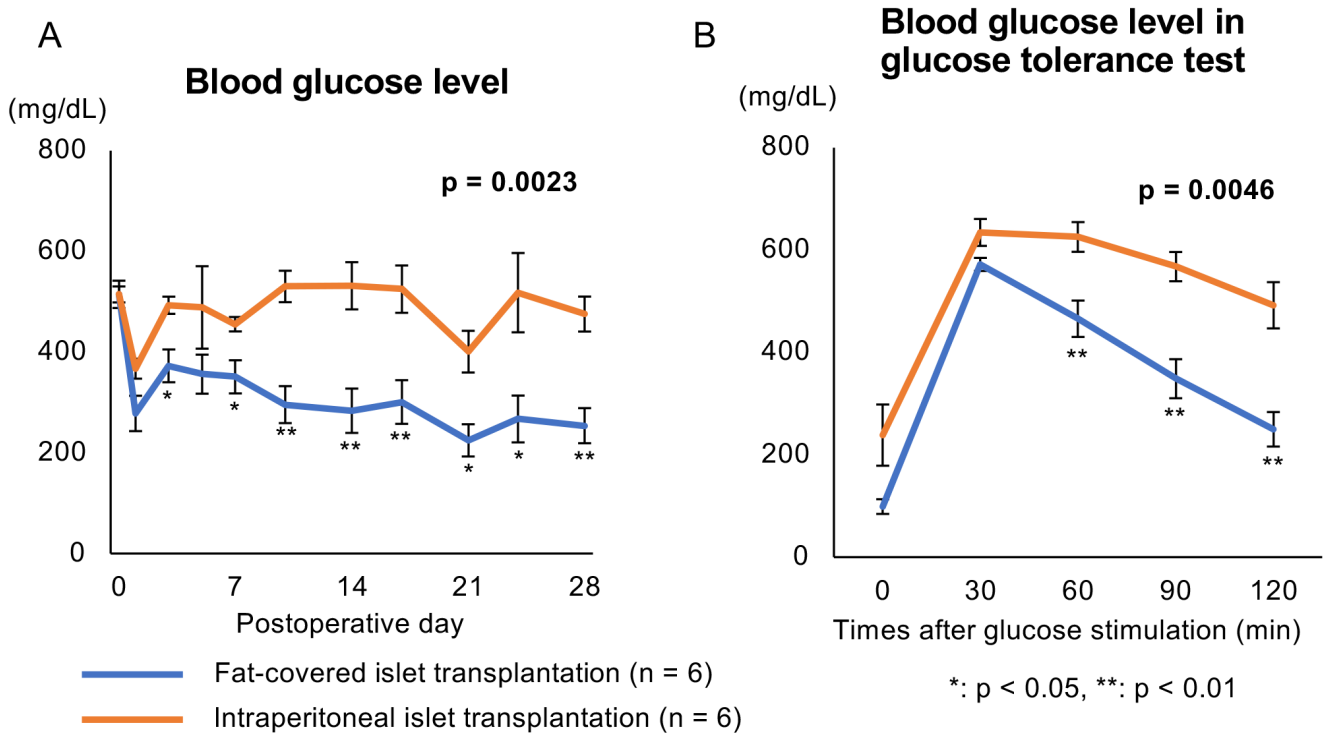
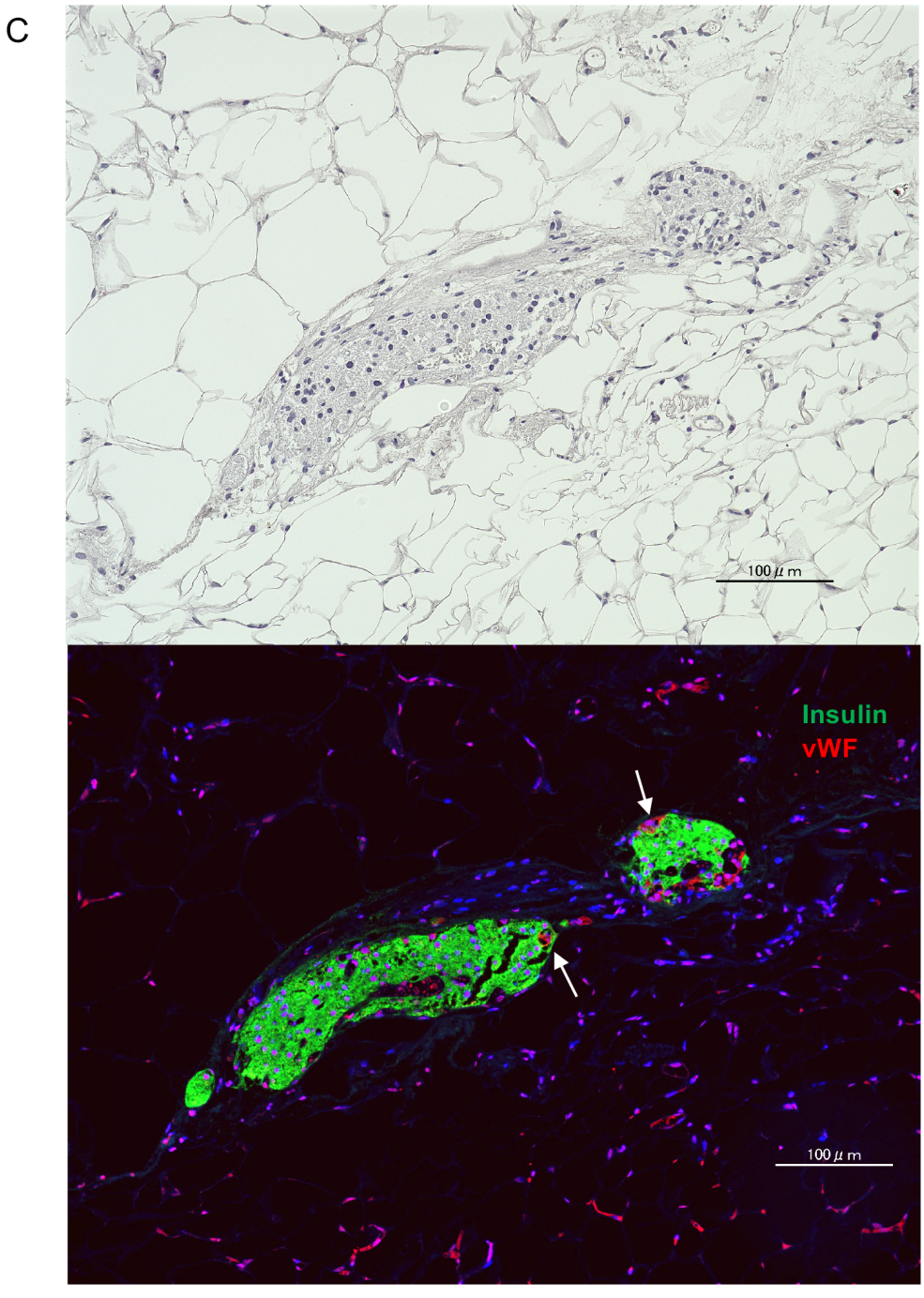


Figure

Click here to access/download;Figure;Fat-covered method resubmission Figure 3.pdf

Figure 3





Name of Material/Equipment	Company	Catalog Number	Comments/Description
4-0 Nylon	Alfresa	ER2004NA45-KF2	Closing abdomen
Alexa 488-conjugated donkey anti-guinea pig	Jackson Immunoresearch	706-546-148	Secondary antibody for insulin antibody
Alexa 647-conjugated donkey anti-rabbit	Jackson Immunoresearch	711-606-152	Secondary antibody for von Willebrand factor antibody
DMEM, low glucose, pyruvate	ThermoFisher Scientific	11885084	Culturing islets, transplanting islets
Eosin	Fujifilm Wako Chemicals	051-06515	Using for staining tissue by eosin
Eppendorf Safe-Lock Tubes, 1.5 mL	Eppendorf	30120086	Collecting islets
Falcon 15 mL Conical Centrifuge Tubes	Corning	352095	Collecting islets
Falcon 40 µm Cell Strainer	Falcon	352340	Using for separating islets from other pancreatic tissue
Falcon 50 mL Conical Centrifuge Tubes	Corning	352070	Discarding excessive medium/buffer
Guinea pig anti-insulin	Agilent Technologies Japan, Ltd. (Dako)	IR002	Primary antibody for murine insulin
Hematoxylin	Muto Pure Chemicals Co., Ltd.	30002	Using for staining tissue by hematoxylin
Isodine solution 10%	Shionogi&Co., Ltd.	no catalog number	Using for disinfection
Isoflurane	Fujifilm Wako Chemicals	095-06573	Using for anesthesia
Labcon 1000 µL ZapSilk Low Retention Pipette Tips	Labcon	1177-965-008	Using for separating islets from other pancreatic tissue
Labcon 200 µL ZapSilk Low Retention Pipette Tips	Labcon	1179-965-008	Using for seeding islets onto epididymal white adipose tissue

Mintsensor	Sanwa Kagaku Kenkyusho Co. Ltd.,	8AEB02E	Using for monitoring blood glucose
Pipetteman P-1000	Gilson	F123602	Using for separating islets from other pancreatic tissue
Pipetteman P-200	Gilson	F123601	Using for seeding islets onto epididymal white adipose tissue
Rabbit anti-vWF	Abcam	ab6994	Primary antibody for murine von Willebrand factor

Replies to editor and reviewers

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

- We received English proofreading service for this manuscript at the first submission from Enago group (www.enago.jp). Spells and grammars were checked and revised (ex. are to area: Page 5, Line 6). In this revision, further English proofreading was received because the protocol is thoroughly reconstructed with addition of more figures which may support the making video image. The definition of IEQ (newly included in the revised manuscript) was included (Page 4, Line 14-15).

2. Please provide an email address for each author.

- E-mail address for each author is described in title page (Page 1).

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sigma-Aldrich, St. Louis, MO, USA etc

- Commercial languages were removed in the revised manuscript (Page 3, Line 8).

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

- Many personal pronouns were avoided in the revised manuscript (ex. “We report a novel intraperitoneal islet transplantation…white adipose tissue.” to “An intraperitoneal islet transplantation…white adipose tissue is reported.”: Page 1 Line 32-33).

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

- We checked the original manuscript and also felt that explanation of the protocol was insufficient with few images and readers might hardly understand this protocol, as the editors and reviewers indicated. Therefore, we tried to include the detailed explanations and informative figures in the revised manuscript.

6. 1.2: Please provide details for islet isolation or cite a reference.

- The protocols about mouse islet isolation were widely published including in JOVE journal (Villarreal D, et al. J Vis Exp (150), e57048, doi:10.3791/57048 (2019)). Therefore, a reference about the method of islet isolation was included in revised version instead of detailed explanation about our islet isolation protocol (Gotoh M, et al. Transplantation. 40 (4), 437-438, (1985); Page 3, Line 15).

7. 2.5: How were the islets counted?

- Islets were counted by visual observation under microscope (Page 4, Line 15-16).

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

- The description of the centrifuge speed was changed to centrifugal force (Page 4, Line 20).

9. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

- A line space between the protocol steps was included. The pivotal steps of this

protocol were highlighted (Protocol section).

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

- The sizes of scale bar were included in Figure legends of new Figure 1B, C, 2D and 2F.

11. Lines 227-228: what do you mean by "it is important to minimize the contact of the top button of the micropipette…"? and line 230: "…refrain from pushing the button of the micropipette for implantation."?

- It was meant that "It is important to minimize pushing the plunger button (for aspiration and dispensation) of the micropipette for seeding the islets onto the adipose tissue without flushing them outside the tissue. The sentences were revised to try to clear the meaning (Page 8, Line 18, 21-22).

12. Please do not abbreviate journal names in the reference list.

- All journal names were changed to formal names. (Reference section)

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript mainly describes the specific steps of a new method of islet transplantation in mice. The remarkable feature of this method is that the islets are inoculated into a "pouch" formed by the folding of white adipose tissue of the epididymis, without the need for biological glue and suture. Compared with intraperitoneal islet transplantation, the efficiency of this transplantation site is significantly improved. The detailed steps in this manuscript, especially the emphasis on key procedures, are sufficient to enable colleagues in the field to understand and master the technology.

- This summary is what we expect to show in this protocol. Thank you for understanding.

Major Concerns:

1.The remarkable feature of the method described in the manuscript is that the islets are inoculated into a "pouch" formed by the white adipose tissue of the epididymis, without the need for biological glue and suture. As mentioned in the discussion section of the manuscript, this was an unexpected phenomenon and may due to the trapping ability of adipose tissue. In view of the fact that most of the previous islet transplants in the epididymal fat pad used sutures or biological glue to prevent islet loss, we are more or less concerned that the method in this manuscript may cause undetectable partial spills of islets. In those previous experiments, islet transplantation in the epididymal fat pad achieved excellent results. However, it is a pity that this article does not provide direct comparative evidence that its method is not inferior to the previously reported method. We expect the authors to provide more convincing evidence or explanations to encourage more colleagues to abandon the previous complicated transplantation method, accept and adopt this new method in the future study.

- Thank you. Actually, we have not compared the transplant efficacy of this fat-covered method with that of islet transplantation into epididymal adipose tissue. According to previous publications about islet transplantation into epididymal adipose tissue, syngeneic 500 islets are necessary for complete normoglycemia (Rios PD, et al. Biotechnol Bioeng 2016;113:2485–95. Doi.org/10.1002/bit.26005). Mao and colleague showed that there were no normoglycemic mice received syngeneic 300

islets transplantation (Mao D, et al. Acta Biomater 2017;59:210–20. https://doi.org/10.1016/j.actbio.2017.06.039.). Furthermore, Wang and colleague revealed that only 30% of mice acquired normoglycemia by syngeneic 250 islets transplantation (Wang K, et al. J Vis Exp 2017;(125). https://doi.org/10.3791/54995.; Wang X, et al. Biomaterials 2017;138:80–90. https://doi.org/10.1016/j.biomaterials.2017.05.033). Therefore, it is considered that the transplant efficacy of this fat-covered method is not inferior to islet transplantation into epididymal adipose tissue in previous publications (Page 8, Line 8-9)

2. We are confused by some of the results in Figures 2A and 2B. On the 28th day after transplantation, the blood glucose level of the Fat-covered group in Figure 2A was better than that of the Intraperitoneal group, but there was no difference in the serum insulin content in Figure 2B. This seemingly contradictory result may cause readers to worry about the long-term function of the islet grafts in the Fat-covered group. At the same time, unfortunately, this article does not show the changes in the blood glucose of the recipient mice after the graft is removed.

- Thank you. In general, some transplanted islets fail to be engrafted and are damaged in early stage of transplantation. Insulin in the damaged islets is leaked, and therefore, serum insulin level is temporally elevated and then decreased after islet transplantation. However, other islets succeed to engraft and provide insulin according to the blood glucose level. As a result, plasma insulin level after transplantation becomes higher than at pre-transplantation.
- Actually, as you detected, the difference of plasma insulin level became unclear at POD 28 in this series. I (Sakata) recognized this data was not good. And unfortunately, we did not certify the re-elevation of blood glucose level after graftectomy for this work.
- For the reasons mentioned above, we removed this plasma insulin data in the revised version. Instead, our previous work which revealed that the serum insulin of mice received fat-covered islet transplantation was higher than that of the mice with intraperitoneal islet transplantation and blood glucose level was re-elevated after graftectomy was included as a reference (Sakata N, et al. Transplantation 2020. doi: 10.1097/TP.0000000000003400) (Page 5, Line 38-40).

Minor Concerns:

1. The vwf fluorescent staining in Figure 2D seems to have obvious non-specific staining. It is recommended to improve the quality of the picture.

- Thank you. I tried to improve the quality of this figure. Furthermore, vWF positive capillaries were indicated by white arrow. (New Figure 3C)

2. A few sentences in the article may produce unnecessary ambiguity and may need to be modified appropriately. For example, a sentence in the discussion section: Our previous study proved that the islets covered by adipose tissue was successfully engrafted in the tissue without spilling and re-elevation of blood glucose after confirmed graftectomy.

- Thank you. This statement was not correct. We attempted to revise this statement to prevent ambiguity in the revised manuscript (ex. Page 8, Line 2-3).

Reviewer #2:

I have read with great interest the manuscript entitled, "Fat-covered islet transplantation using epididymal white adipose tissue" by Sakata and colleagues. The manuscript is useful addition to basic researchers conducting islet transplantation study. However, there are many confusing wording and seemingly confliction statements that need clarification.

- Thank you. We revised this manuscript with trying to remove the confusion and confliction of statements.

Epididymal fat site has been investigated for a long term by other researchers. For example, "Development of an ectopic site for islet transplantation, using biodegradable scaffolds" Tissue Engineering 2005; 11: 1323. "The epididymal fat pad as a transplant site for minimal islet mass" Transplantation 2007; 84: 122. The authors should consider including those previous studies into the reference list.

- Thank you. The references were included in Introduction section (Page 2, Line 32).

In the Introduction section, the authors blames intraportal site. However, any sites other than intraportal site are not clinically proven to be functioning and useful. Epididymal fat site cannot be a clinical relevant site, therefore the authors should not (or do not need to) blame intraportal site in this manuscript. Especially, the authors state that ischemia / hypoxia is a limitation of the liver as an optimal transplant site. This statement is true but not specific to the liver. All other sites suffer from ischemia / hypoxia as well.

- Thank you. We removed the limitations of liver as transplant site and changed the statements following, "However, it is important to discuss about the alternative transplant site if the liver hardly to be used (e.g. portal hypertension, hepatitis, and cirrhosis)." (Page 2, Line 11-13).

Protocol section is difficult to understand. For example, in sub-section 2. Preparation of islets for transplantation, the authors describe 15 mL plastic tube and 50 mL plastic tube and it is not clear how these tubes were used during the procedure. In the same section, the authors state that two hundred islets were divided equally into 1.5 mL sterilized plastic tubes. This statement is unclear. Does this mean 100 islets were

prepared for one transplant? Overall, the Protocol section should be re-written with more clarity.

- We also noticed that the descriptions of the protocol in original manuscript was insufficient. In the revised version, we revised the description with adding distinct explanations and informative figures. Hope this revision will contribute to clear understanding about this protocol.

The authors should clearly state that epididymal site does not provide portal circulation of insulin as opposed to omental pouch site.

- Thank you. We included these statement in Discussion, "As an experimental transplant site for islets, epididymal white adipose tissue has some merits in the size which enables to contain a certain volume of islets while rodent greater omentum is too small to contain them and in easiness of access. The only limitation is this site does not provide portal circulation of insulin." (Page 7, Line 35-38).

The authors did not conduct survival graftectomy, which can be easily done in case of epididymal site. The authors should recognize that this is a weakness of the study.

- Unfortunately, we did not perform graftectomy in this series. Instead, our previous study with the data about the re-elevation of blood glucose after graftectomy was referred in the revised version (Sakata N, et al. Transplantation 2020. doi: 10.1097/TP.0000000000003400) (Page 5, Line 38-40).

Minor point
No need for abbreviation DM.

- The abbreviation was removed. (Page 1, Line 43)

Reviewer #3:

Manuscript Summary:

Sakata et al., present in this manuscript a technique to realize an easy islet transplantation on the adipose tissue they named "fat-covered" islet transplantation.

Major Concerns:

Introduction part : Line 70-73 "To overcome the limitations of intraperitoneal islet transplantation, we developed a novel method named a "fat-covered method" using epididymal adipose tissue, which is a kind of peritoneum. Detection of transplanted islets can be achieved by covering the area with peritoneum." I never heard about considering EFP as part of the peritoneum (peritoneal cavity, yes, but not peritoneum), please document this claiming if true. The last sentence doesn't make sense and is not clear. What does it mean that islet can by detected by covering the area with the peritoneum?

- I consider serous membrane which encapsulated epididymal white adipose tissue is a kind of visceral peritoneum because this membrane and parietal peritoneum are in continuous. Actually, as far as I checked, there are no publications about the anatomy about the membrane, but it is considered that the researches about the anatomy has not been fully progressed. While it is hardly to detect where intraperitoneally transplanted islets are engrafted, transplanted islets by this method were engrafted in the epididymal adipose tissue, at least. In other words, detection of engrafted islets can be done if the epididymal adipose tissue, a kind of peritoneum, is examined.
- However, the description "by covering the area with peritoneum," certainly give the readers confusion, as you indicated. It is not clear whether visceral peritoneum or parietal peritoneum. Therefore, I revised this sentence by deleting "peritoneum" (Page 2, Line 29-30).

The bibliography is a bit old, a new CITR has been published and should be used as a reference. Same remark for intra-omental islet transplantation, work was done since 1983…

- The data about achieved normoglycemia and prevention of severe hypoglycemic events were referred from the most recent (the 10th) report of CITR (https://citregistry.org/system/files/10th_AR.pdf) (Page 2, Line 2-7). I think you indicated the oldness of the referred intraomental islet transplantation study. In revised

manuscript, recent works about intraomental islet transplantation were referred (for example, Kasoju N, et al. Biomater Sci 2020, 8 (2), 631-647) (Page 2, Line 14).

Epididymal fat pad is only found in male subjects, what about females? Also, the sex of the mice is not mentioned in the protocol section as well as mice strain, that's very important!

- The sex of the recipient mice was described in revised version (Page 3, Line 7). Regarding strain, I used C57BL/6J mice in this work, and previously performed this method to male BALB/c mice and certified that there were no problems in technical (no published data). I consider that this method can be done to animals having epididymal tissue like C57BL/6J, BALB/c, C3H, or Sprague-Dawley, Wistar…

Line 100-101: "Islets were cultured overnight using an incubator at 22 °C and 5% CO2." Can the author precise why using a low temperature? Also, what is the source of islet? Murine? Human?

- We agree with the opinion that culturing at 22°C is superior to at 37°C in preserving viability and preventing central necrosis of isolated islets (Brandhorst D, et al. Transplantation 67:1533–1541; 1999; Noguchi H, et al. Transplantation 89:47–54; 2010; Itoh T, et al. Cell Transplant. 21:1361–1370; 2012; Komatsu H, et al. doi: 10.1097/TP.0000000000002280) (Page 3, Line 18-19).
- We used mice as donor in this protocol (Page 3, Line 14). Of course, it is considered all animals are available, but some modification may be necessary according to the species, and the islet condition. For example, porcine islets are vulnerable, and tend to be damaged comparing with rodent and human islets. When the damaged porcine islets (due to over digestion) are gathered, it sounds like "paste". Though it is easier to deal with gathered islets than dispersed islets for this method, the therapeutic effect of transplantation is hardly expected because they are damaged islets. Contrarily, insufficient digestion bears "embedded" islets which are embedded undigested exocrine tissue. Remnant exocrine tissues may impair islets due to explosion of pancreatic enzyme in the tissue. Therefore, it is important to prepare islets in good condition and quality for the success of this method using porcine islets. Other difficulty is collecting islets from other pancreatic tissues. Porcine islets are hardly to distinguish from other tissue without staining dithizone. In other words, porcine islets cannot be selected by handpicking under microscope, different from rodent

islets which can be directly assessed the condition and size and selected in preferable condition. Therefore, porcine islets for transplantation are collected from total islets by sampling. That means sampled islets tend to be smaller, especially in case of damaged islets. So, it may need a longer time to drop islets at the tip of micropipette tip in porcine islets because they are smaller and lighter than rodent ones.

- Regarding human, human islets can be used for therapeutic transplantation for diabetic patients and are not be permitted for experimental use in Japan at present. If we can use them for research in future, I wish to promote studies including this work using them.

For the entire manuscript the precision of the protocol/method section must be improved. Some parts are very vague, this is not what we expect from a JOVE paper. (Some brief example: Medium or buffer? Which medium, what is the composition of the medium and the buffer? Additional and concomitant remark: the material table is poorly completed (anesthetics, mesh, plastic tubes, etc.) and must be correctly re-written.

- We rechecked the original version of this protocol and felt that it would need further explanations about the protocol, as editors and reviewers including you indicated. The description of the protocol was drastically revised including your indicated points (Page 2, Line 38 - Page 5, Line 28).

Line 154-155: "We implanted the same number of islets at left flank to act as the control." this need more details, what is the left flank, where it has been placed? Free in the peritoneum? In the peritoneum membrane? Not clear.

- "Left flank" means left paracololic sulcus (or space) in human. Different from human, descending colon of mouse is free from peritoneum, and therefore, have no idea the official name of this space in mouse. The islets were seeded onto, not into, the peritoneum at this space (Page 5, Line 32-33)

Line 168-169: "in contrast to intraperitoneal islet transplanted mice where no engrafted islets were seen in epididymal white adipose tissue or the abdominal wall." By placing the islet in the "left flank" (still don't know what is exactly), I don't see why islet would go to the adipose tissue…

- The transplanted islets in control group (intraperitoneal islet transplantation) were not fixed onto the peritoneum. Any biobonding agents were not used for fixation. In other words, transplanted islets might be moved according to the movement (walk and run) of the islet transplanted mice. It is considered that many of transplanted islets failed to engraft, but some islets might be engrafted on the peritoneum at "left flank" (or, should I mention as "the space named as left paracolic sulci/gutter in human"?) and others might be engrafted into other spaces in peritoneal cavity. Then, where was the possible site for engraftment of the islets? It is estimated that epididymal white adipose tissue is one of the candidates. I consider there are several possible reasons for that because epididymal white adipose tissue is one of the largest adipose tissue and it might be possible that some intraperitoneally transplanted islets were trapped by the tissue. Further, different from parietal peritoneum, colon or intestine, epididymal adipose has relative rough surface which can easily trap the transplanted islets. If the islets succeeded to be located on extra-adipose tissue sites like spleen, kidney or intestine, thick serous membrane might prevent the engraftment. These possibilities were the reason why I checked the engraftment of islets onto epididymal white adipose tissue. (Page 6, Line 4-5)

I'm not convinced that this transplant is an intra-peritoneal one. As islets are place onto the adipose tissue an covered by it, islets will not be in contact with the peritoneal membrane anymore. Furthermore, the goal of an islet tx is that newly formed blood vessel growth and carry out insulin through the blood flow and not that a passive insulin diffusion occurs from the adipose tissue to the IP cavity.

- Probably you can understand that intraperitoneal islet transplantation with islet engraftments onto parietal peritoneum and surfaces of stomach, small intestine, colon, greater omentum, liver, spleen and of course epididymal adipose tissue are defined as intraperitoneal islet transplant models. This fat-covered transplant model is characterized as islets seeded onto white adipose tissue just covering the adipose tissue. We did not use any biobonding agent and suturing, and therefore, the space in transplanted islets was not closed but opened. In other words, it is considered that the conditions as the transplant model between islets seeded onto epididymal adipose tissue and islets covered epididymal adipose tissue are same.
- I think that my understanding about the definition of islet engraftment is not different from yours. I understand that engraftment of transplanted islets is defined as the completement of vascular network between donor islets and recipient tissues.

Therefore, we showed the histological images of engrafted islets with staining von Willebrand factors-positive capillaries, which were proofs that insulin drainage was done via newly formed vessels (new Figure 3C).

The authors mentioned that the graft is easily removable to assess the real effect on the graft on tx recipients, but also said that, is it hard to remove later and even that removing/explant the graft doesn't modify glycaemia. Which statement to believe? Thus, I disagree on the "removable" characteristic of the graft. The authors also mentioned other grafts into the adipose tissue are not removable, that's false, they are removable but it requires the adipose tissue ablation. I propose the author also perform a full ablation of the grafted EFP, to prove the graft efficiency, this will be more potent as a proof of graft efficiency. See under in yellow the concerned paragraphs.

Our previous study proved that the islets covered by adipose tissue was successfully engrafted in the tissue without spilling and re-elevation of blood glucose after confirmed graftectomy. This was an unexpected phenomenon for us and may be due to the trapping ability of adipose tissue. Islets trapped by the adipose tissue may be difficult to be peeled off. The advantages of this method include a decreased technical difficulty as well as the capability to assess the therapeutic effect metabolically, historically, and in gene/protein assessment by graftectomy.

- Probably, this misunderstanding might be caused by our wrong description. The statement "…and re-elevation of blood glucose after confirmed graftectomy." was incorrect. Correctly, "…and re-elevation of blood glucose <u>was confirmed after</u> graftectomy" (Page 8, Line 2-3). Graftectomy (removal of epididymal adipose tissue with or without testis) could be safely done under general anesthesia. Re-elevation of blood glucose after graftectomy was certified in our previous study (Sakata N, et al. Transplantation 2020. doi: 10.1097/TP.0000000000003400). Sorry for misunderstanding.
- Thank you for suggestion about graft elimination. At least, ablation is not preferable for assessing the condition of engrafted islets using historical and genetic experiments.

The authors mentioned lipotoxicity, and claimed that doesn't happen during their procedure. I disagree, islets are in contact with adipose tissue, why will it be different? Please document.

- I consider the serous membrane (i.e. visceral peritoneum) encapsulated epididymal adipose tissue minimize the direct influence of lipotoxicity. Of course, there are no evidence and no way to certify the mechanism. Just my opinion. And therefore, I used the word “may” (Page 7, Line 42). At least, I recognize that Discussion is a part where the authors state their opinion/estimation based on their acquired results and other references. I understand you disagree with my opinion.

During mice follow-up, serum insulin decreased and reached the same level as the “IP left flank whatever” controls. Why? Especially if we considered the glycaemia, which stay stable. Which insulin is that (human/murine)?

- Some islets failed to be engrafted and are damaged in early stage of transplantation. Insulin leaks from the damaged islets. Therefore, plasma insulin level is temporally elevated and decrease. However, the level is higher than at pre-transplantation, if some islets succeed to be engrafted. Actually, I recognize this data is not good. For the reason, this figure is removed. Our previous work revealed that the plasma insulin of mice received fat-covered islet transplantation was higher than that of the mice with intraperitoneal islet transplantation (Sakata N, et al. Transplantation 2020. doi: 10.1097/TP.0000000000003400). By the way, what do you mean “IP left flank whatever”?

Minor Concerns:
Tenses and the narrative way used are not consistent, manuscript start with the “we” and goes to the passive voice.

- Tried to change to passive voices in many sentences (ex. Page 1, Line 32-33).

Line 231 : “It would be sufficient to attach the tip onto the adipose tissue and certify the islets seeded onto the tissue.” This sentence is not clear at all.

- Tried to show the meaning by adding new figures (new Figure 2F).

Centrifugation speed should be stated in rcf (g) and not rpm.

- Changed (Page 4, Line 20).

The islet number should be stated in IEQ, 200 small islets and 200 big islets will not be the same at all.

- Changed to IEQ (Page 4, Line 15-16). At least, I have chosen approximately 150 µm sized islets in this and previous rodent works.

A picture of the tip with islet next to the tissue pictures could be useful.

- Tried to show that in new Figure 2E and F.

Are you sure the figure 2D is H&E?

- Exactly (New Figure 3C).

Reviewer #4:

Manuscript Summary:

Sakata et al. aims at reporting the experimental procedure for transplantation of naked pancreatic islets in the epididymal fat pad.

Major Concerns:

The protocol is not novel an has been previously reported by different groups, including a recent JOVE publication (doi: 10.3791/54995).

- We consider that the aim of JOVE journal is to show the protocol/technique of experiments, not to show the novelty.

The comparison with the intraperitoneal transplant site for naked islets is not recommended, since it was previously reported that naked islets do no engraft in that site.

- As you indicated, it is true that intraperitoneal islet transplantation has limitation in detection of transplanted islets. However, we consider the intraperitoneal transplant site is the only appropriate control for this fat-covered method using epididymal adipose tissue on the point of view of equality in experimental condition. In detail, intraperitoneally transplanted islets are trapped by epididymal adipose tissue, which is considered as one of the largest abdominal white adipose tissue, or are dispensed on parietal peritoneum (i.e. intraperitoneal islet transplantation model).

Also, the rationale for this report is demonstrate the applicability of the procedure for bioengineered islets. However, islets encapsulated in traditional encapsulation systems would not fit in the fat pad due to their large volume.

- It is considered that this site enables to contain microencapsulated and small size (approximately 1 cm sized) microencapsulated islets, though some larger sized macroencapsulated islets may not be acceptable.

Minor Concerns:

The report requires copy-editing

- Thank you. We tried to fit this revised version to copy-editing format.