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Corresponding Author:	Yumi Imai UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	yumi-imai@uiowa.edu
Order of Authors:	Siming Liu
	Mikako Harata
	Joseph A. Promes
	Anthony J. Burand
	James A. Ankrum
	Yumi Imai
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TITLE:

Lentiviral Mediated Gene Silencing in Human Pseudoislet Prepared in Low Attachment Plates

AUTHORS AND AFFILIATIONS:

Siming Liu[1,2], Mikako Harata[1,2], Joseph A. Promes[1,2], Anthony J. Burand[2,3], James A. Ankrum[2,3], Yumi Imai[1,2]

[1]Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa
[2]Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, Iowa
[3]Roy J. Carver Department of Biomedical Engineering, University of Iowa, Iowa City, Iowa

Corresponding author:
Yumi Imai (yumi-imai@uiowa.edu)

Email addresses of co-authors:
Siming Liu (siming-liu@uiowa.edu)
Mikako Harata (mikako-harata@uiowa.edu)
Joseph A. Promes (promes@healthcare.uiowa.edu)
Anthony J. Burand (tony.burand@gmail.com)
James A. Ankrum (james-ankrum@uiowa.edu)

KEYWORDS:

shRNA, lentivirus, insulin secretion, culture, single cell, reaggregation

SUMMARY:

A protocol to create gene modified human pseudoislets from dispersed human islet cells that are transduced by lentivirus carrying short hairpin RNA (shRNA) is presented. This protocol utilizes readily available enzyme and culture vessels, can be performed easily, and produces genetically modified human pseudoislets suitable for functional and morphological studies.

ABSTRACT:

Various genetic tools are available to modulate genes in pancreatic islets of rodents to dissect function of islet genes for diabetes research. However, the data obtained from rodent islets are often not fully reproduced in or applicable to human islets due to well-known differences in islet structure and function between the species. Currently, techniques that are available to manipulate gene expression of human islets are very limited. Introduction of transgene into intact islets by adenovirus, plasmid, and oligonucleotides often suffers from low efficiency and high toxicity. Low efficiency is especially problematic in gene downregulation studies in intact islets, which require high efficiency. It has been known that enzymatically-dispersed islet cells reaggregate in culture forming spheroids termed pseudoislets. Size-controlled reaggregation of human islet cells creates pseudoislets that maintain dynamic first phase insulin secretion after prolonged culture and provide a window to efficiently introduce lentiviral short hairpin RNA (shRNA) with low toxicity. Here, a detailed protocol for the creation of human pseudoislets after lentiviral transduction using two commercially available multiwell plates is described. The

protocol can be easily performed and allows for efficient downregulation of genes and assessment of dynamism of insulin secretion using human islet cells. Thus, human pseudoislets with lentiviral mediated gene modulation provide a powerful and versatile model to assess gene function within human islet cells.

INTRODUCTION:

The loss of functional beta cell mass is the central pathology for both type 1 and type 2 diabetes[1]. While beta cells are the producers of insulin in pancreatic islets, communication between beta cells and non-beta cells plays a critical role in the regulation of insulin secretion[2]. In addition, dysregulation of glucagon secretion contributes to hyperglycemia in diabetes[3]. Thus, there is strong interest to modulate gene expression of cells within pancreatic islets to address the mechanism behind the development of islet dysfunction in diabetes. A variety of approaches including transgenic mice are available to modulate gene expression of mouse islets. However, human and mouse islets show distinct innervation, cell distribution, ratio of beta to alpha cells, and response to secretagogues[4]. Therefore, direct assessment of gene function in human islets is extremely important for understanding the pathophysiology of human pancreatic islets.

Adenoviral vector is the most widely used viral vector to transduce pancreatic islets in vitro due to the high efficiency of transduction in non-dividing cells. However, adenovirus does not penetrate to the core of islets efficiently, especially in human islets[5], and is cytotoxic at high doses[6]. Comparatively, lentiviral vector is less cytotoxic and delivers exogenous genes permanently into the chromosome of post-mitotic cells, making it a widely tested vehicle for gene therapy[7]. However, the ability of the lentivirus to penetrate the core of intact human islets is also limited, thus requiring partial dispersion by enzymatic digestion to increase the transduction efficiency[8]. The caveat with the dispersion of intact human islets is the interruption of cell-cell and cell-matrix communication, which compromises the dynamic regulation of insulin secretion critical for the maintenance of glucose homeostasis in humans[9]. Thus, it has been challenging to assess the impact of gene modulation on the dynamic regulation of islet function in a model of human islets.

It has been known that dispersed islet cells from human and rodent islets autonomously reaggregate into islet-like structures called “pseudoislets”. Pseudoislets show beta and non-beta cell distribution similar to native islets[10,11]. Additionally, after long-term culture, native islets progressively lose robust first phase insulin secretion[5,10–12]. Yet, pseudoislets demonstrated better preservation of first phase insulin secretion in response to glucose compared with native islets after the same culture period[5]. In addition to having better preservation of insulin secretion, size-controlled reaggregation of human islet cells in low attachment plates[11] provides a window of opportunity to introduce lentivirus vectors prior to their reaggregation into pseudoislets. Several studies have demonstrated the utility of pseudoislets combined with lentiviral mediated transduction. Caton et al.[13] reported that the introduction of the green fluorescent protein (GFP) expressing lentivirus had little effect on insulin secretion while achieving homogenous expression of GFP in rat pseudoislets compared with non-infected control. They also demonstrated the specific effect of different connexins on insulin secretion by overexpressing connexins 32, 36, and 43 via lentivirus[13]. Human pseudoislets prepared with a commercially available 96-well ultra-low attachment plate demonstrated that lentiviral-mediated overexpression of transcription factor SIX3 improves insulin secretion assessed by static incubation[14]. Recently, human pseudoislets prepared with a 96-well ultra-low attachment plate were used to downregulate glucokinase via lentiviral short hairpin RNA (shRNA) as a proof of principle to show that glucose-stimulated insulin secretion is reduced, while KCl-stimulated insulin secretion was preserved[5]. The study also demonstrated that human pseudoislets are similar to native islets in gene expression and secretory profiles, further supporting the utility of human pseudoislets to dissect the regulation of islet function[5]. Although perifusion was not performed, a bioengineered micro well culture plate that recently became commercially available, was also reported to be compatible for lentiviral transduction and produced human pseudoislets that exhibited excellent insulin secretion in vitro and in vivo after transplantation[11]. Collectively, human pseudoislet formation combined with lentiviral transduction is a simple and efficient approach to investigate human islet pathophysiology, providing a valuable tool to perform mechanistic studies in human islets.

In the current report, a protocol to form human pseudoislets transduced with lentivirus using two commercially available platforms, a 96-well ultra-low attachment plate and a micro well culture plate is presented. Both achieve efficient modulation of gene expression and create human pseudoislets that are compatible for downstream assessments including static incubation and perifusion.

PROTOCOL:

Prior to commencement of studies, a human subjects research determination was made by the University of Iowa Institutional Review Board, who determined that the study did not meet the criteria for human subjects research. Consult the local review board before the initiation of the study to determine if the source of islets and planned study requires prior approval.

NOTE: Typically, 1200−1400 islet equivalent (IEQ) of human islets are required for the formation of 192 pseudoislets at the size of 3,000 cells/pseudoislets in a 96-well ultra-low attachment plate or 1200 pseudoislets at the size of 500 cells/pseudoislets in a micro well culture plate. IEQ of islets required varies between different preparations of human islets as donor factors (age, health, weight), isolation efficiency, and culture conditions affect the yield of the single cell suspension. In this protocol, lentivirus containing shRNA targeting a gene of interest is used. The cytomegalovirus (CMV) and human phosphoglycerate kinase (hPGK) promoter based lentiviral vectors are reported to down-regulate gene efficiently in human pseudoislets[5,15]. The use of lentivirus requires precaution as biohazard[16]. Contact the local biosafety committee prior to the initiation of the use of lentivirus.

1. Overnight culture of human islets for recovery after shipment

1.1. Prepare Connaught Medical Research Laboratories 1066 (CMRL-1066) medium supplemented with 1% human serum albumin (HSA) by combining 50 mL of CMRL-1066, 0.5 g of HSA, 0.5 mL of penicillin-streptomycin, and 0.5 mL of 100 mg/mL glutamine (1% HSA CMRL) in biological safety cabinet (BSC) and passing through a 0.2 µm filter for sterilization.

1.2. Gently swirl the shipping bottle to keep islets in suspension. Transfer the shipping medium containing islets to a 50 mL conical centrifuge tube. Let the tube sit in BSC for 15 min so that islets settle to the bottom of the tube.

1.3. Remove shipping medium gently without disturbing the islet pellet using a 10 mL pipette. Re-suspend the islet pellet in 1% HSA CMRL to a concentration of 400 IEQ/mL.

1.4. Transfer islets into a non-tissue culture treated dish. If islets are split into multiple dishes, keep islets evenly suspended in medium by gently swirling before splitting. Culture islets at 37 °C in a 5% CO_2 incubator overnight.

NOTE: The use of a non-tissue culture treated dish is required to prevent the attachment of islets to the plate.

2. Preparation of single cell suspension from human islets

2.1. Prepare the following: CMRL-1066 medium with 10% heat-inactivated fetal bovine serum (HiFBS), penicillin-streptomycin, and glutamine (10% HiFBS CRML) at room temperature (RT), a 40 µm strainer, a 35 mm Petri dish, a 1 mL tuberculin syringe, and a hemocytometer.

2.2. Transfer human islets after overnight culture into a 15 mL conical centrifuge tube. Centrifuge at 190 x *g* for 5 min in a swinging-bucket rotor. Aspirate medium with a 5 mL pipette without disturbing the islet pellet.

2.3. Wash the pellet by adding 10 mL of phosphate-buffered saline (PBS) into the tube, mix gently, and centrifuge at 190 x *g* for 5 min. Aspirate PBS without disturbing the islet pellet.

2.4. Re-suspend the islet pellet in 0.5 mL of a pre-warmed proteolytic and collagenolytic enzyme mixture and pipette 5x using a P1000 pipette to mix islets. Incubate at 37 °C for 5 min. Mix by pipetting up and down gently 1−5 times.

NOTE: Aggressive pipetting will increase cell loss.

2.5. Check for cloudiness (single cells) and the number of flakes (undigested islets). Add 2−3 min to 37 °C incubation depending on the extent of digestion judged by cloudiness and the number of flakes. Stop digestion when flakes are reduced to ~10% of predigestion and solution is cloudy.

NOTE: Time required for digestion differs depending on the islet size distribution of each human islet preparation.

2.6. Place a 40 µm strainer in a 35 mm Petri dish and wet the strainer by adding 1 mL of 10% HiFBS CMRL and pressing with 1 mL syringe plunger. Transfer all the cell suspension on top of the strainer and collect the pass-through in a fresh 15 mL tube.

2.7. Wash the tube used for islet digestion with 0.5 mL of fresh CMRL medium to collect leftover cells and pass the wash through the strainer. Combine the pass-through in a 15 mL tube. Repeat once.

2.8. Next, dissociate undigested islets remaining on the strainer by pressing the strainer placed in a 35 mm dish with 1 mL syringe plunger. Collect pass-through again and wash the strainer with fresh CMRL-1066 to remove all remaining digested islets from the strainer and the dish. A total ~3 mL of single cell suspension will now be in the 15 mL tube.

2.9. Record the total volume of the cell suspension and take 10 µL aliquot of cells to count the cell number on a hemocytometer.

2.10. Centrifuge the cell suspension for 5 min at 200 x *g*. Remove medium without disturbing the pellet. Proceed to step 3.1.1 if using a 96-well ultra-low attachment plate or step 3.2.1 if using a 24 well micro well culture plate to reaggregate the cells.

3. Pseudoislet formation and transduction by lentivirus

3.1. Protocol using a 96-well ultra-low attachment plate

3.1.1. Determine the desired number of cells per pseudoislet and the number of pseudoislets to create. Typically, 1000–3000 cells are used for each pseudoislet for a 96-well ultra-low attachment plate. For 3000 cells per pseudoislet, adjust the cell suspension to 1 x 10^5 cells/mL by resuspending the islet pellet from step 2.10 in 10% HiFBS CMRL so that 30 µL of cell suspension has 3000 cells. Calculate the total volume of 1 x 10^5 cells/mL of single cell suspension (mL) required using the following equation:

The total volume of 1 x 10^5 cells/mL of single cell suspension (mL) = (number of cells per pseudoislet) x (number of pseudoislets being made) / 1 x 10^5.

NOTE: Adjust the concentration of the cell suspension based on the desired number of cells per pseudoislet so that 30 µL of cell suspension makes one pseudoislet.

3.1.2. Transfer the required volume (30 µL x number of pseudoislets being made) of single cell suspension to a fresh 15 mL tube. Add 250 transduction units (TU)/cell of lentivirus containing shRNA targeting a gene of interest or control.

CAUTION: Lentivirus is classified as biosafety level 2 and can be integrated into DNA of infected cells.

NOTE: Use concentrated lentivirus so that the volume of lentivirus added is minimal. Titer required per cell for efficient gene silencing may differ depending on the lentiviral construct.

3.1.3. Mix cell suspension with virus by pipetting gently 5x with a P1000 pipette. Transfer mixed cells into a 50 mL sterile reagent reservoir if using an 8-channel pipette.

3.1.4. Dispense 30 µL per well of single cell suspension mixed with lentivirus into each well using an 8-channel pipette or a P200 pipette depending on the number of the wells.

3.1.5. Centrifuge the 96-well plate in a swinging-bucket plate centrifuge at 270 x *g* at RT for 7 min. Check whether cells are gathered in the center of each well. If not, centrifuge again as gathering of all cells in the center of the well is critical for pseudoislet formation. Culture at 37 °C in a humidified 5% CO_2 incubator overnight.

3.1.6. Add 100 µL of pre-warmed 10% HiFBS CMRL per well next morning to avoid drying of cells during subsequent culture. Centrifuge at 270 x *g*, RT for 7 min. Culture at 37 °C in a 5% CO_2 incubator. Pseudoislets will complete formation in 5−7 days.

3.1.7. When harvesting pseudoislets, pre-warm the desired volume of 10% HiFBS CMRL (100 µL per islet), prepare one 50 mL sterile reservoir, one sterile 10 cm Petri dish, and one 8-channel pipette in BSC.

3.1.8. Remove the 96-well plate from the incubator and place in BSC. Pipette 100 µL per islet of 10% HiFBS CMRL into a reservoir.

3.1.9. Pipette 100 µL per well of 10% HiFBS CMRL from a reservoir to pseudoislets and pipette up and down 2−3 times gently in the well to lift islets up. Then, aspirate medium in the well containing a pseudoislet and eject into a 10 cm Petri dish. Use of an 8-channel pipette allows transfer of 8 pseudoislets at one time.

3.1.10. Check the plate under a light microscope to ensure the complete removal of all the pseudoislets. Pseudoislets form firm aggregates and remain aggregated after lifting. The pseudoislets are now ready for downstream experiments.

3.2. Protocol using a 24-well micro well culture plate

3.2.1. Warm up the anti-adherence rinse solution (**Table of Materials**) to RT for efficient spheroid formation. Also, pre-warm plain CMRL-1066 and 10% HiFBS CMRL.

3.2.2. Add 500 µL per well of the anti-adherence rinsing solution to each well of the 24-well micro well culture plate to be used for pseudoislets. Centrifuge at 1300 x *g* for 5 min in a swinging-bucket plate centrifuge.

3.2.3. Observe the plate under a microscope to ensure that air bubbles are removed from micro wells. If air bubbles are trapped in micro wells, centrifuge at 1300 x *g* for 5 min again.

3.2.4. Aspirate the anti-adherence rinsing solution from the wells in a BSC. Rinse each well with 2 mL of warm plain CMRL-1066 once. Aspirate CMRL-1066. Add 0.5 mL/well of warm 10% HiFBS

CMRL to each well planned for use. Wells are now ready for loading dispersed human islet cells prepared in step 2.10.

3.2.5. Determine the total number of cells needed for each well. One well of 24-well micro well culture plate contains 1200 micro wells and forms 1200 pseudoislets. 500 cells per pseudoislet x 1200 micro wells = 6 x 10^5 cells. Resuspend single cells from step 2.10 to 6 x 10^5 cells in 0.8 mL of 10% HiFBS CMRL in a sterile 1.5 mL tube.

NOTE: The maximum volume per well is 2 mL. Volume of cell suspension should not exceed 1.5 mL. The protocol describes steps for creating one well of pseudoislet transduced by lentivirus. Scale up depending on the number of wells to be made for each lentivirus.

3.2.6. For viral transduction, add 125 TU/cell to the single cell suspension. Keep the volume of virus below 0.2 mL. Incubate the cell and virus mixture at 37 °C with occasional gentle mixing for 1 h to allow contact of cells with virus before condensation of cells in step 3.2.8.

NOTE: Use concentrated lentivirus so that the volume of lentivirus added is minimum. Lower number of TU per cell is used for Protocol 2 compared with Protocol 1 as the total volume of medium per cell number is less. However, titer required per cell for efficient gene silencing may differ depending on the lentiviral construct and needs optimization.

CAUTION: Lentivirus is classified as biosafety level 2 and can be integrated into DNA of infected cells.

3.2.7. After 1 h, adjust the total volume of the islet cell and virus mixture to 1 mL by adding 10% HiFBS CMRL. If cells form clump after 1 h incubation, disperse into single cell suspension by gentle and quick pipetting 2−3 times. The pipetting is very important for even distribution of cells across micro wells. Transfer cell suspension to one well of the 24-well micro well culture plate.

3.2.8. Immediately following pipetting, centrifuge at 100 x *g* for 3 min at RT to capture cells into all micro wells. Observe under a microscope to verify that cells are evenly distributed in all micro wells.

3.2.9. Culture the micro well culture plate at 37 °C in a 5% CO_2 incubator. Pseudoislets will form in 24−48 h. Pseudoislets can be cultured without medium change for up to 7 days.

3.2.10. When changing the medium for culture beyond 7 days, replace 50%−75% of medium for each medium change as follows. Slowly remove 0.5−1 mL of medium using a P1000 pipette from each well. Add 0.5−1 mL of fresh 10% HiFBS CMRL slowly by placing a tip to the wall of the well to avoid dislodging pseudoislets from the micro well culture plate.

3.2.11. To prepare for harvesting pseudoislets, warm up the 10% HiFBS CMRL medium. Pseudoislets tend to float up in serum free CMRL-1066 making it hard to pick them.

3.2.12. Aspirate 0.5 mL of medium from well using a 1 mL pipette and dispense media forcefully back to plate surface to lift up pseudoislets from the micro well culture plate.

3.2.13. Gently aspirate dislodged pseudoislets using the 1 mL pipette and transfer islets into a non-tissue culture treated 6-well plate. Pass through a small 37 µm reversible strainer placed on a 15 mL conical tube. Pseudoislets will remain on the filter; any unincorporated single cells will flow through.

NOTE: To avoid loss of smaller size pseudoislets through strainer, the strainer may be omitted.

3.2.14. Dispense 1 mL of 10% HiFBS CMRL across the entire surface of the well to dislodge any remaining pseudoislets, aspirate dislodged pseudoislets, and pass through the strainer. Repeat 3x to ensure complete collection of all pseudoislets from wells.

3.2.15. Observe the micro well culture plate under an invert microscope to ensure that all pseudoislets are collected. Repeat the wash as in step 3.2.14 if pseudoislets remain.

4. RNA extraction for evaluation of gene silencing efficiency

4.1. Pick pseudoislets into the RNase free PBS in a 1.5 mL microcentrifuge tube and centrifuge at 300 x *g* for 3 min at 4 °C. Remove PBS without disturbing the islet pellet and wash once with PBS followed by centrifugation at 300 x *g* for 3 min at 4 °C.

4.2. Aspirate most of PBS using a P1000 pipette. Then, change to a P10 pipette to remove the rest of PBS without disturbing the islet pellet.

4.3. Add 0.5 mL of guanidinium thiocyanate RNA extraction reagent (**Table of Materials**) per tube. Homogenize the pseudoislets using a motor-driven pestle for 2−3 times. The homogenate in the guanidinium thiocyanate RNA extraction reagent can now be stored at -80 °C or processed for RNA purification.

NOTE: 24 of the 3000 cell-pseudoislets formed in a 96 well plate or 48 of the 500 cell-pseudoislets formed in a micro well culture plate are sufficient to obtain 0.5−1 µg of RNA.

REPRESENTATIVE RESULTS:

Figure 1 illustrates key steps in the production of pseudoislets using a 96-well ultra-low attachment plate and a micro well culture plate. **Figure 2a** shows sequential changes in morphology during the formation of pseudoislets from 3 x 10^3 human islet cells in a 96-well ultra-low attachment plate. Monolayer or loose clumps of cells observed in day 1 changed into solid aggregates with a smooth, round border by day 5 to 7 (**Figure 2a**). In a micro well culture plate, the formation of solid pseudoislets is usually visible within 4 days (**Figure 2b**). When 600 cells/micro well were plated in the micro well culture plate, human islet cells were condensed into spheroids of uniform size. It is observed that a micro well culture plate allows the successful formation of pseudoislets from a small number of cells compared with a 96-well ultra-low attachment plate. Typically, over 1500 cells/pseudoislet are needed for a 96-well ultra-low attachment plate, while 500 cells/pseudoislet are sufficient for a 24-well micro well culture plate. Successfully formed pseudoislets remain as spheroids after recovery from a 96 ultra-low attachment plate or a micro well culture plate and are compatible for downstream applications including static incubation (**Figure 3a**) and perifusion (**Figure 3b**). The uniform size of pseudoislets reduces the variation within a test group and allows static incubation using as little as 5 pseudoislets per measurement (**Figure 3a**). Also, human pseudoislets maintained robust first phase insulin secretion in response to glucose after 7 days of culture when the original human islets cultured for a similar period of time showed blunted first phase glucose-stimulated insulin secretion (**Figure 3b**)[5]. The introduction of lentivirus into a single cell suspension ensures the efficient and homogenous transduction of islet cells and achieves highly efficient down-regulation of genes as shown in **Figure 3c**. All results shown were obtained using human islets from non-diabetic donors.

FIGURE AND TABLE LEGENDS:

Figure 1: Process of human pseudoislet preparation. (**a**) The suspension containing 4000 IEQ of human islets becomes cloudy after digestion by a proteolytic and collagenolytic enzyme mixture and mild pipetting. (**b**) Human islets after dispersion are passed through a strainer. Undigested islets remaining on top of the strainer are dispersed using a 1 mL syringe plunger. (**c,d**) Microscope images of the single cell suspension containing 3000 cells/well in a 96-well ultra-low attachment plate before (**c**) and after (**d**) centrifugation. (**e,f**) Microscope images of the single cell suspension containing 500 cells/micro well in a 24-well micro well culture plate before (**e**) and after (**f**) centrifugation. Scale bar = 250 µm.

Figure 2: Morphology of human pseudoislets. Sequential changes in morphology of human pseudoislets created (**a**) in a 96-well ultra-low attachment plate from 3000 cells and (**b**) in a 24-well micro well culture plate from 500 cells. Scale bar = 100 µm.

Figure 3: Examples of functional assays using human pseudoislets. (**a**) Representative static incubation performed using human pseudoislets created in a micro well culture plate from a single donor at the size of 500 human islet cells per pseudoislet. Four sets of 5 pseudoislets were incubated for 1 h in Krebs-ringer bicarbonate buffer supplemented with either 2 mM or 16.8 mM glucose. Each symbol represents insulin secretion from one set of 5 pseudoislets. Mean ± standard error of the mean (SEM) is shown. *, $p < 0.05$ by student's t test. Representative results from three donors. (**b**) Representative perifusion testing insulin secretion from human pseudoislets created in a micro well culture plate in response to 16.7 mM glucose and 30 mM KCl. Method for perifusion was previously published[5]. Mean ± SEM of insulin secretion from two sets of 40 pseudoislets created in a micro well culture plate from a single donor at the size of 500 human islet cells per pseudoislet plate is shown. Representative data from six donors. (**c**) Pseudoislets were created with lentivirus carrying shRNA targeting human *ATGL* (targeting CCTGCCACTCTATGAGCTTAA, left) or *PLIN5* (targeting GACAAGCTGGAAGAGAAGCTT, right). Control pseudoislets were transduced with lentivirus expressing scrambled sequence (Scr) previously published[5]. The mRNA expression of each gene was determined by real time

polymerase chain reaction (PCR) as previously published[5]. Data were expressed using $2^{-\Delta\Delta CT}$ taking peptidylprolyl Isomerase B (PPIB) as an internal control[17]. Each dot represents data from each donor for an indicated primer and a data set from the same donor is connected by a line. N = 3 donors. *; $p < 0.05$ by student's t test.

DISCUSSION:

Here, a detailed protocol to generate human pseudoislets that are transduced by lentivirus using a 96-well ultra-low attachment plate or a micro well culture plate is presented. Pseudoislets have been reported to demonstrate morphology and secretory functions similar to native human islets and can be cultured for prolonged time in vitro[5,11,18]. Unlike native human islets that show a wide variation in size, pseudoislets are relatively uniform in size, reducing variation between donors and experimental replicates[5,11]. Downregulation of genes requiring high efficiency of transduction can be performed easily prior to the formation of pseudoislets in single cell suspension. This method avoids the difficulty of viral penetration through layers of cells in intact islets. Thus, this simple, highly efficient, and reproducible protocol for the creation of human pseudoislets has wide applications.

While several different platforms have been reported for the formation of pseudoislets[12,14,19,20], both a 96-well ultra-low attachment plate and a micro well culture plate are commercially available, allowing this technique to be adopted by any laboratory. Although the hanging drop method[12] also allows the formation of human pseudoislets using common labware, potential limitations include the difficulty in controlling size and reaggregation duration of pseudoislets. These limitations were due to the limited volume per drop of pseudoislet and ongoing evaporation during the 5−7 day culture required for pseudoislet formation. Additionally, it is easier to contain lentivirus with the use of a 96 well ultra-low attachment plate or a micro well culture plate compared with the hanging-drop method.

Several steps within the protocol require close attention. Optimizing the digestion of intact islets with the proteolytic and collagenolytic enzyme mixture is critical since both under- and over-digestion will reduce yield of single cell suspension and subsequently affect aggregation of pseudoislets. During digestion, it is important to closely monitor islets for the disappearance of clumps and the increase in cloudiness as islets dissociate into single cells. It is important to note that the optimal time for digestion varies between islets from different donors. The optimal time is dependent on several factors including medical history and age of each donor, the length of ischemia time, the islet isolation procedure used, islet size, islet purity, islet viability, and shipping conditions. Typically, human islets with viability and purity higher than 80% and within 5 days of isolation are used. Careful and gentle pipetting during dispersion is also important to maintain cell viability and recovery that will ultimately affect cell aggregation and the final size of pseudoislets being formed. When dispensing islet cell suspension to wells (steps 3.1.4 and 3.2.7), gentle and thorough mixing of cells is important to achieve an even distribution of single cells into micro wells. If cells form clumps after 1 h incubation with lentivirus, it requires gentle pipetting to break the clumps into single cell suspension prior to the final centrifugation.

We have had similar success in creating human pseudoislets after lentiviral transduction using both a 96-well plate and micro well culture plate. The choice between the two platforms depends on the size and number of pseudoislets desired. A micro well culture plate has small, pyramid shaped bottoms allowing condensation of a smaller number of cells compared with a 96 well round bottom plate. Thus, the number of cells per pseudoislet can be reduced for a micro well culture plate. Also, a single centrifugation step creates all pseudoislets simultaneously in a micro well culture plate while multiple pipetting is required for creating pseudoislets in a 96-well plate. Thus, scaling up the creation of pseudoislets is easier in a micro well culture plate. However, the currently available micro well culture plate does not offer flexibility in the number of pseudoislets being created. Currently, the minimum number of pseudoislets created using a micro well culture plate is 1200 and can be increased only by the factor of 1200. Thus, we typically use a 96-well plate for small scale pilot experiments and for an experiment in which small quantity of samples is sufficient such as an insulin secretion assay and RNA extraction for gene expression. We have used pseudoislets from a micro well culture plate for assays that require large numbers of cells such as Western blot, oxygen consumption rate determination by a metabolic analyzer, and triglyceride extraction.

The major limiting factor for the generation of human pseudoislets is the loss of cells during the preparation of single cell suspension. While 1 IEQ of human islet is considered to contain around 2000 cells, the recovery of single cell suspension is typically 30% or lower due to multiple washing and passing through a strainer. Heterogeneity of islet size also makes it difficult to dissociate all islets simultaneously. While gentle pipetting and the use of the proteolytic and collagenolytic enzyme mixture in the protocol are efforts to combine mechanical and enzymatic forces for maximum recovery of single cells, there still is an inevitable loss of cells. Thus, the application of pseudoislets requires clear justification over studying intact human islets. It also needs to be reminded that insulin secretion from pseudoislets is more robust than islets cultured for the same period of the time but tends to be lower compared with freshly isolated islets[5,11].

Although limitations exist, stable and highly efficient gene silencing combined with better preservation of glucose-stimulated insulin secretion for prolonged time in culture enable the assessment of gene function in human islet cells. Additionally, the complex intercellular communication between beta-beta and beta-non beta cells is proposed to have a regulatory role in islet function. However, there is currently limited information regarding intercellular communication within human islets. With increased availability of cell specific markers[21], it is feasible to create human pseudoislets with defined cell composition, as recently reported in mouse islet cells[22], which will facilitate improved understanding of the cell-cell communication between human islet cells. The recent advancement of imaging of three-dimensional tissues also potentially increases the utility of human pseudoislets as a model to unmask how cellular polarity and intercellular communication are regulated in human islets. Thus, human pseudoislets provide a useful model to dissect the functions of genes of interest and other questions in the field of islet biology.

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DISCLOSURES:

The authors have nothing to disclose.

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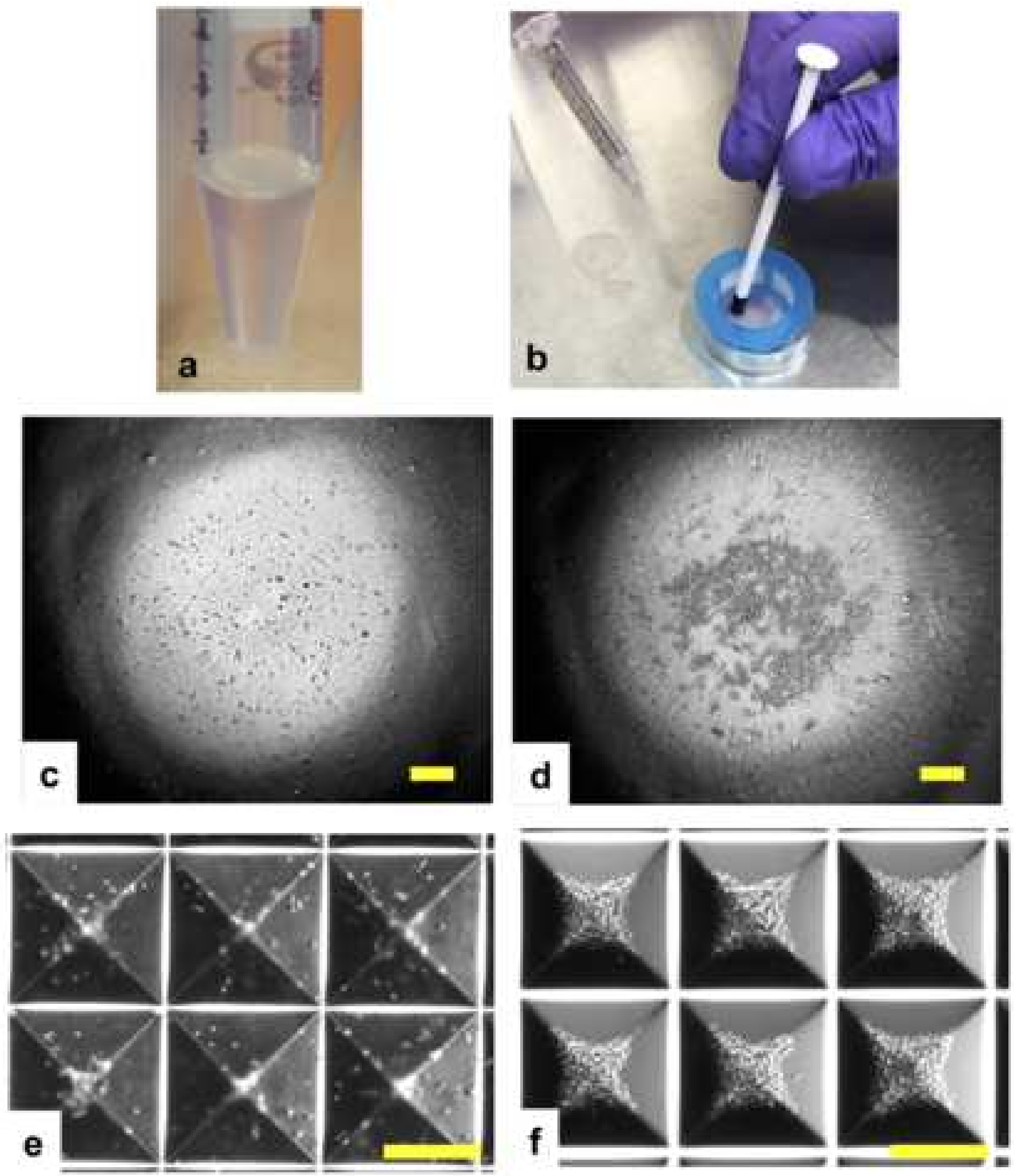
Figure. 1



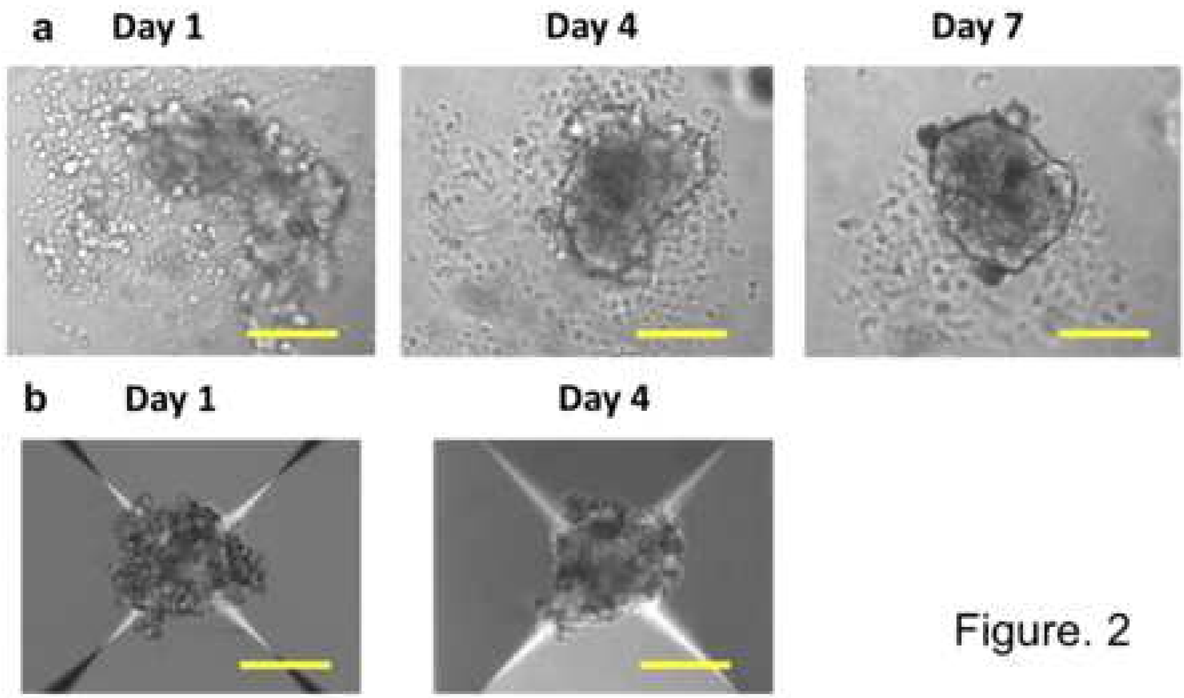
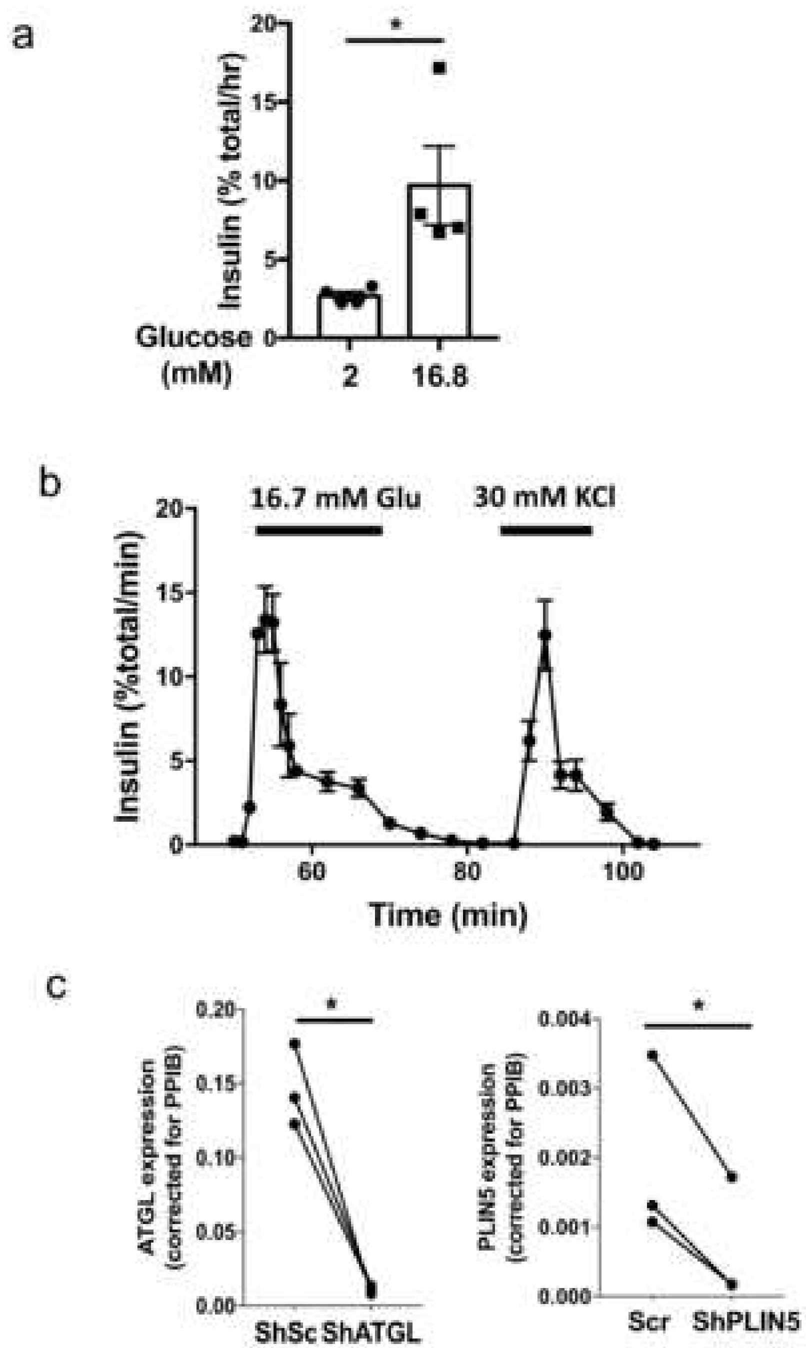


Figure. 2

Figure. 3


a
Insulin (% total/hr)
Glucose (mM)
2
16.8
*
b
16.7 mM Glu
30 mM KCl
Insulin (%total/min)
Time (min)
c
ATGL expression (corrected for PPIB)
ShSc
ShATGL
PLIN5 expression (corrected for PPIB)
Scr
ShPLIN5

Name of Material/ Equipment	Company
Anti-adherence rinsing solution	technologies
Biological safety cabinet	Thermo Scientific
cell strainer, 40 micrometer	Corning
CMRL-1066	ThermoFisher
CO_2 incubator	Thermo Scientific
conical centrifuge tube, 15 mL	VWR
conical centrifuge tube, 50 mL	VWR
fetal bovine serum	ThermoFisher
guanidinium thiocyanate RNA extraction reagent	ThermoFisher
glutamine	ThermoFisher
Hemocytometer	Marien Feld
Human serum albumin	Sigma
inverted microscope	Fisher brand
microcentrifuge	Beckman Coulter
microcentrifuge tube, 1.5 mL	USA Scientific
microwell culture plate	Stemcell technologies
motor-driven pestle	GAMUT
non-tissue culture treated dish, 10 cm	Fisher Scientific
PBS	ThermoFisher
Penicillin-streptomycin	ThermoFisher
Petri dish, 35 mm	Celltreat
pipette, 5 mL	DOT Scientific,
pipette, 8-channel	VWR
pipette, 10 mL	VWR
pipette, P10	Denville
pipette, P200	Denville
pipette, P1000	Denville
proteolytic and collagenolytic enzyme mixture	Sigma
reagent reservoir, 50 mL	VWR
reversible strainer, 37 micrometer	Stemcell technologies
swing bucket plate centrifuge	Beckman Coulter

swing bucket rotor	Beckman Coulter
tuberculin syringe, 1 mL	BD
ultra low attachment microplate, 96 well	Corning

Catalog Number	Comments/Description
7919	
1300 Series Type A2	
431750	
11530037	
Heracell VIOS 160i	
89039-666	
89039-658	
26140079	
15596026	Trizol
25030164	
Neubauer-Improved Bright line	
A1653	
11-350-119	
Microfuge 20	
1615-5500	
34411	Aggrewell 400, 24 well
#399X644	
FB0875713	
14190250	
10378016	
229638	
667205B	
#613-5253	
667210B	
UEZ-P-10	
UEZ-P-200	
UEZ-P-1000	
A6965	Accutase
89094-680	
27251	
Allegra X-14R	

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309659	
4515	

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Name:	Yumi Imai
Department:	Internal medicine
Institution:	University of Iowa, Carver college of Medicine
Title:	Associate Professor
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Dear Dr. Dsouza

Thank you very much for an opportunity to submit our revised manuscript. We carefully reviewed valuable comments and addressed as below.

We greatly appreciate your review.

Editorial comments:

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

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2. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.

Done

3. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Done

4. Please be consistent with the scale bars. Some are black and some are yellow. Some are placed in the lower right corner and some are in the lower left.

Changes made

5. Please shorten the title by removing the words “A protocol for”.

Changes made

6. Please provide complete author affiliations including city and state.

Changes made

7. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. You can use substeps instead: 2.4.1, 2.4.2, etc.

Changes made

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

Changes made

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For example: Accutase, Trizol, etc.

Changes made

10. Please use SI abbreviations for time: h instead of hr, etc.

Changes made

11. Please do not abbreviate journal titles.

Changes made

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript from Siming Liu et al. describes a protocol for the creation and culture of human islets from human donor pancreatic islets. The advantages of such constructs include uniform size of islet preparations, enhanced function during longer-term culturing, and increased efficiency of viral gene manipulation with reduced toxicity. These distinct advantages make the technique potentially very valuable to the field, so having a detailed video protocol of this technique would also be valuable. There were a few concerns that should be addressed in this paper and protocol that are enumerated below.

We greatly appreciate encouraging comments and valuable inputs. We have carefully considered the comments/suggestions; and in response to those comments, we have revised the manuscript accordingly.

Major Concerns:

1. *The authors make claims that pseudoislets perform better than cultured intact islets related to Figure 3. This really should be directly shown in the manuscript. For the N=4 sets of pseudoislets in Figure 3A, there must be data on the donor islets for similar static incubation studies since it is part of standard protocol to assess human islet viability and function. These data should be included to compare intact islets from the same donors as pseudoislets.*
We appreciate reviewer's point. Figure 3A is shown as an example of static incubation to demonstrate that small number of pseudoislets are sufficient to measure insulin in static incubation. We and others have published detail comparison of insulin secretion from intact and pseudoislets. This is included in introduction (L81-86).

2. *The authors also reference a previous paper (reference 5) that shows the comparatively better insulin release from pseudoislets over intact cultured islets. However, the authors did not note that the perifusion data for insulin secretion from freshly acquired intact islets were actually superior to pseudoislets. This should be identified as a limitation of the technique.*
We agree. Accordingly, we added the limitation in the discussion (L500).

3. *The presentation of the data in Figure 3C-D is a bit confusing. Since it is a line graph, is this a "before" vs. "after" display. None of the following terms are defined anywhere: PPIB, ShSc, Scr (presumably scrambled). Presumably, this is looking at gene expression levels between a sham knockdown (left side of each graph) and lentiviral shRNA (right side), but this is not very clear. Finally, the y-axes are 0.20 and 0.004, respectively. This is not conventional fold-change over control values, so again, some clarification would be helpful.*
We apologize for the lack of clarity. We revised Figure legend 3 extensively.

4. *It would be really interesting to include pseudoislet co-staining for glucagon and insulin to get an estimate of the relative numbers of alpha and beta-cells for each pseudoislet. Since alpha and beta-cells differ in size, are they equally distributed throughout each pseudoislet or are there differences between first-deposited vs. last-deposited pseudoislets?*
We do appreciate Reviewer's valid point. Indeed, alpha and beta cell distribution and interaction are important for islet function. We have not performed the staining because there are published studies that addressed cell distribution and composition in pseudoislets (L79-80). Unfortunately, we cannot add a new experiment within a limited timeframe allowed for the revision.

We agree that it will be important to confirm even distribution of alpha and beta cells in the future study. We think a number of cells used for a 96 well (3,000 cells/pseudoislet) makes it less likely to have significant unevenness in cell distribution. For a multiwell culture plate, all pseudoislets are made simultaneously during a single centrifuge. However, we stressed the importance of mixing single cell suspension for even distribution in discussion (L468).

5. *Regarding the protocol itself, it seems fairly clear and straightforward. The authors did an excellent jump identifying and describing limitations and pitfalls. One thing that might be helpful for the video would be a zoom-in to the Aggrewells after depositing pseudoislets. It wasn't visually intuitive from the description exactly how one effectively deposits the correct number of islet cells into each tiny micro-well.*
Thank you very much for highlighting an important step. We will include a detailed presentation of this step in the video. Also, we revised 3.2.7. and 3.2.8 to stress the importance of pipetting prior to adding cell suspension followed by prompt centrifugation for the even deposition of the exact number of cells. Reference 11 reports narrow size distribution of pseudoislets created by this methods and our experience agrees with the report.

Minor Concerns:
1. Line 40: reword to clearly indicate increased or greater toxicity by better separating the modifier 'low'. Try "...suffer from toxicity and low efficiency" or "suffer from low efficiency and high toxicity".
We appreciate reviewer's suggestion and modified the text (L42).

2. *Line 66: especially human islets requires a citation of a direct human vs rodent comparison of virus efficiency. Human islets may actually be easier to transfect to the core due to architectural differences unless there is substantial evidence to say otherwise.*
In our earlier published data, we demonstrated that viruses have low penetration to intact human islets (ref 5, Fig. 5a).

3. *There were a few other grammatical errors that should be checked more carefully.*
We apologize for errors. We reviewed carefully and corrected grammatical errors throughout the text.

Reviewer #2:
Manuscript summary:
This protocol by Liu and colleagues describes the procedure of preparing genetically modified human pseudoislet from dissociated human islet. They argue that to study the role of different gene in human islet, this method ensure a proper gene silencing in islet by using a lentiviral vector. However, the protocol as currently written requires revisions to address the following concerns.

First, we would like to express our deep gratitude for a number of valuable comments that have tremendously improved our manuscript. We have carefully considered the comments/suggestions; and in response to those comments, we have revised the manuscript accordingly.

Global comments:
1. *The focus of the manuscript need to be revised, as here the useful part of the protocol is the efficient gene silencing in whole islets. Some parts of the manuscript distract from the original message and the power of the technique, as for example the cell-matrix interactions that are finally, not explored. Some consistency problems are recurrent, please homogenize (P1000, 1mL pipettor, P20, CMRL1066…).*

While we greatly appreciate Reviewer's point, we believe it is worthwhile to include potential future applications of pseudoislets in discussion. As L112 to L368 of the manuscript is dedicated to describe the protocol and the efficiency of gene silencing in whole islets, we think it has sufficient focus on the subject.
We revised text to keep the consistency throughout the manuscript.

2. *The protocol is realizable by following the presented steps but does not contain critical steps that require a full video report. However, if we consider the highlighted part we miss pseudoislet harvesting that represent the most technical part of the protocol.*
We have highlighted the harvesting steps for the video presentation (Steps 3.1.8 to 3.1.10).

3. *For a fully validation of the protocol, a functional comparison between islets formed from 96 well and Aggrewell looks mandatory (as here only the Aggrewell are used). As well as, a comparative evaluation of the functionality of islet transfected with shRNA and control islets/pseudoislets.*
We agree with Reviewer's point. At the same time, the interpretation of data will be confounded by difference in the size of islets between two platforms. Our objective is to make readers aware of two commercially available platforms rather than comparing performance between the two. We reported insulin secretion evaluated by perifusion for non-transduced and lentivirus transduced pseudoislets in reference 5.

4. *Further than functionality, evaluation of lentivirus transfection effect on islet using a simple viability assessment (FDA/PI) will bring more consistency to purpose in term of toxicity.*
We agree with Reviewer that viability test will provide an important index of toxicity. Unfortunately, we are unable to perform the test with limited time allowed for revision.

Other concerns:

5. *As pseudoislet formation is common, the originality of this technique is the efficient use of the lentiviral vector, manuscript and title has to be axed on the gene silencing rather than pseudo islet formation. Please, revise the title in this way.*
We modified the title.

6. *Line 81. "Controlled re-aggregation of human islet": how is the re-aggregation controlled?*
Clarified as "size-controlled" and reference is added (L84).

7. *Line 96. "was not preformed", is there a spelling mistake?*
Corrected to "performed".

8. *Line 121. "overnight culture after shipment". If islets are isolated in the same center, how long does they be cultured before to be dissociated into single cells?*
We do not isolate human islets on site, like the majority of potential readers/audience of the manuscript, and cannot comment. In Ln464, time in culture before shipment is discussed, which will provide a guide for those isolate islets on site.

9. *Line 130. Spelling mistake "HAS CMRL".*
Corrected

10. *Line 149. (2.3) Does the accutase be pre-warmed? Please precise.*
Added "pre-warmed".

11. *Line 155. Could you be more precise about the few flakes. Indeed, this part appear critical to ensure proper and not overdigestion of cells. For a certain IEQ for example, what does it represent (1 or 10?).*
We added more details in this step (L174).

12. *Line 164. Does CMRL be cold to stop enzymatic digestion?*
"Room temperature" is added.

13. *Line 168. Count cell number, how?*
By a hemocytometer. The information is added in L191.

14. *Line 187. Lentiviral vector appears suddenly without information about its content, or preparation. TU must be defined.*
Lentivirus is now introduced in L121-124.
Transduction unit (TU) is defined.

15. *Line 197. 30µL per well will not dry during overnight culture?*
No, 30 µL will not dry in a humidified 37 °C cell culture incubator. "Humidified" is added.

16. *Line 205. Does the CMRL be warmed before ? No medium change for 5 to 7 days will not result in a cell mortality increase ?*
"Pre-warmed" is added to L236.
Since one well contains only one pseudoislets and most of cells are not dividing, ~130 µl of media provides sufficient nutrients and maintains pH.

17. *Line 215. "lift medium in the well containing a pseudoislet…" , please can you comment on the fragility of pseudoislets ?*
Formed pseudoislets tolerate the lifting process and remain aggregated (L253).

18. *Line 224. "CMRL1066" replace HiFBS CMRL, be consisten*
HiFBS CMRL is defined in L154.

19. *Line 240. Can you precise which size of pseudoislet we get with the concentration range of cell you define.*
500 cell per well generates pseudoislets of ~100 µm as shown in Fig. 2B, .

20. *Line 246. Why are the concentration of the lentiviral vector different from the previous part with the 96 plate? Please justify.*
We have found that lower titer is sufficient for obtaining efficient down regulation in a microwell culture plate. This could be due to higher number of cells/volume (lower total volume) in a microwell culture plate compared with a 96 well. However, as stated, we recommend that viral titer to be optimized for each application. L292 discusses this point.

21. *Line 247. Here, a mixing period of one hour appears toward just a "five time pipetting" in the first part using 96-wells plate. Is it mandatory? Why don't do the same incubation period in the first part of the protocol? Please justify.*
Thank you very much for noting the difference between the two protocols. For a 96 well plate, we have achieved efficient down-regulation without one hour mixing, which is a standard protocol widely employed for transduction of cells by lentivirus. For a multiwell culture plate, cells and viral mixture is centrifuged down after being added to a well, which will quickly condense cells (L289). Thus, we recommend preincubation.

22. *Global comment on the protocol 2. Volumes needed and steps are unclear. Why presenting a concentration of cell/0.8mL, a concentration in cell/mL will be easier to understand and to adapt to user needs. Here I supposed that the protocol is presented for one well of the AggreWell, what about if we want to do more, please adapt this part of the protocol to be more clear. Also, should we aliquot cell suspension and then apply lentiviral vector or first the lentiviral vector then aliquot in wells. Globally this part lack of consistency.*
While we appreciate Review's point, we would like to stress that it is very important to prepare each cell suspension in total of 0.8 ml. However, we revised the section 3.2.5 and 3.2.6 to increase the clarity.

23. *Line 271. If the optional use of CMRL without serum is hard, why use it? If we think about transplantation, an additional wash can be easily performed after handpicking.*
We apologize for confusing wording. 3.2.11 was rephrased to communicate that the presence of serum aids the collection of pseudoislets.

24. *Line 277. Part 3.2.14. Why this step is not perfomed with the 96 wells formed pseudoislets?*
The answer to this question truly takes advantage of a video format of this journal by demonstrating difference between a 96 well and a multiwell culture plate. This step is unique to a multiwell culture plate that produces high number of (1200) pseudoislets in a small volume.

25. *Line 288. If the strainer is optional and will provoke loss of pseudoislets, it has to be omitted in all cases. As number of pseudo islet obtain is already decreased by the procedure from the original IEQ number, loss of cell material appear unfunded.*
We thinks that it is beneficial for readers/audience to be aware of both options. Depending on downstream applications, users can choose between two options as the strainer will be helpful to remove cell debris when all pseudoislets are used for a single down-stream application.

26. *Line 291. The last part (number 4) is useless. You must detailed how the silencing is validated. A total description of PCR will not be useful, however, the use of shRNA or the reference gene should appear here as we don't have any information on the lentiviral vector and it's content and how it is validated.*
While an experienced researcher may find number 4 "useless", we think it will aid those new to islet research considering that some may find it difficult to obtain sufficient RNA from a small quantity of samples such as pseudoislets after gene modification. Considering that this is a protocol paper, we think it is appropriate to include this.
Functional validation were previously published (L91-97) providing a basis of this manuscript. It also is a beyond the scope of this manuscript to cover functional validation that could differ depending on genes being targeted.

27. *Figure 1. If you present the shape of cells before and after centrifuge for the Aggrewell, you should also present the pictures for the 96 plate.*
Figure 1 is revised to add the pictures. Please note that a size of one 96 well and one microwell is significant different. Please refer to a scale bar.

28. *Figure 2. Pictures doesn't have the same size.*
We keep the size of a scale bar the same between a 96 well and a multiwell culture plate. As a number of cells per aggrewell is less than a 96 well, the picture is smaller for a multiwell culture plate.

29. *Figure 3. Number of n for the static incubation is missing. It looks like a single n as pseudo islet are coming from a single islet donor. Performed a statistic analysis on that seems incorrect (part A). Line 347, sentence cannot start by 40.*

We apologize that n was missing. Each data point in the figure represents insulin secretion from 5 pseudoislets. The message of this figure is that n=4, each from 5 pseudoislets, is sufficient to statistically separate insulin secretion between 2 mM and 16.8 mM glucose. This is a technical protocol and the focus is not to demonstrate variations among donors. To clarify the point, figure legend 3 is revised.

The figure legend (part C) is uncomplete and not consistent (ShSc and Scr), and the shRNA, PPIB and genes haven't be defined before in the protocol, the readability of the figure is not optimal and hard to understand without the missing informations.
Missing information is added to figure legend 3.

30. *Line 364. Author claim that the reproducibility is high, however, with just n=2 the reproducibility cannot be a reliable point.*
We respectfully would like to point out that line 364 states “highly efficient.” This manuscript is a protocol reporting stemming from publications that reported validation data (reference 5, 11, 15) as discussed in introduction. However, to avoid confusion, we updated figure legends.

31. *Line 367. Authors talk about the hanging drop but any results in term of functionality or lentiviral efficiency transfection are presented or compared with existing ones. Can you comment on that?*
We did not perform extensive experiments using hanging drop after noticing shortcomings. While direct comaprision between the hanging drop and two plate platforms is not the focus of the manuscript, we discussed the hanging drop so that readers will be aware of a potential alternative.

32. *Line 390. "The choice between the two platforms depend on the size and number of pseudo islet desired". Any information is given toward the size in the protocol.*
The size of pseudoislets formed in each method is presented in Figure 2a and 2b.

33. *Line 393. "Number of cell can be reduce", no, number of cell will be reduce in any case compare to 96 wells plate.*
We reduced number of cells in a multiwell culture plate in order to save islets required for each experiment. However, if desired, number of cells per one aggrewell may be increased to 2,000 or more. Thus, “can be reudced.”

34. *Line 399-403. Author claim to use 96 well-formed pseudoislet for perifusion and AggreWell for oxygen consumption rate for example, but size of islet is a key point on the oxygen consumption and will affect for sure functionality of pseudoislet. The techniques are both valuable, but cannot be used as the same batch on different experiment as pseudoislet size are different.*
We agree that the statement can be misunderstood. We revised L485 to add “when small quantity of samples are sufficient.”

35. *Line 414. Author insist on the beta-non-beta communication however, here nothing is proven in term of correct structural re-aggregation or communication without impairment. Here the discussion and globally the whole article need to be concentrated on the "efficient gene silencing".*
We added a recent publication that utilized pseudoislets to address beta and non-beta cell communication in mouse islet cells in L513 so that readers will be aware of a potential future application.

36. *Concerning material list, some references are missing (10cm petri dishes), lentiviral vector, RNA sequences.*
We supplemented the missing information.