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Assessment of β-cell replication in isolated rat islets of Langerhans

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Assessment of β-cell replication in isolated rat islets

Abstract

Pancreatic β-cells in the Islets of Langerhans secrete insulin in response to the rise in glucose levels following food intake. The hypoglycemic action of insulin applies a strong evolutionary brake on β-cell division. However, under some conditions β-cells can be stimulated to enter cell cycle progression and divide, for example following exposure to increased glucose levels or during pregnancy. Here, a protocol is described for the isolation of rat adult islets of Langerhans, followed by culture of intact islets in Matrigel and measurement of β-cell replication by the incorporation of ethynyldeoxyuridine (EdU). EdU positive cells are revealed by a click-reaction, nuclei are visualized using a DNA-binding fluorophore (Hoechst 33342) and β-cells are identified using immunofluorescence detection.

Running title

Assessment of β-cell replication in isolated islets

Key words

Islet of Langerhans, β-cell, Proliferation, Mitosis, Ethynyldeoxyuridine (EdU), Glucose, Insulin, Hoechst 33342

1. Introduction

In the adult, proliferation of the pancreatic β-cells occurs at a slow rate under steady-state conditions. However, the β-cell mass has been shown to increase in the face of insulin resistance during obesity and pregnancy [1-7]. The production of adult β-cells predominantly occurs through replication, rather than differentiation although this is still debated [8,9]. The total β-cell mass of the pancreas is regulated by at least 5 different mechanisms: Proliferation, neogenesis, hypertrophy, apoptosis and atrophy [7].

During progression into type 2 diabetes (T2D) an initial increase in β-cell mass occurs, likely driven by increases in postprandial glycaemia [10], but after a while the apoptosis rate increases and the β-cell mass decreases. Diabetes may arise as a consequence of failure to properly increase the β-cell mass; analyses show that the pancreatic mass donated from obese patients diagnosed with T2D is lower compared to obese individuals without diabetes [11]. Therefore, understanding the mechanisms of beta-cell replication would allow for novel therapeutic interventions in preventing or treating diabetes.

Here, I present a detailed protocol for the isolation of islets from adult rats, followed by culture of intact islets on Matrigel coated glass cover slips with incorporation of Ethynyldeoxyuridine (EdU) followed by its detection using click chemistry and identification of β-cells by insulin immunofluorescent staining.

The specific protocol outlined here identifies replicating β-cells. However, the protocol may easily be modified to identify other cell populations of interest: Other mature endocrine cells of the islet (α-cells, δ-cells, PP-cells, respectively Glucagon, Somatostatin or Pancreatic Polypeptide positive) or progenitor-cells (Neurogenin 3 and/or Nestin positive) using suitable antibodies.

2. Materials

2.1 Isolation of adult rat islets of Langerhans

1. All media and buffers should be sterile, but the actual procedures for islet isolation may be carried out at the bench.
2. Collagenase IV (Note 1).
3. Instruments for dissection: A small peang, 2 small serrafine Dieffenbach clamps, 1 pair of fine serrated forceps, 1 pair of hooked forceps, 1 pair of large scissors for the opening of the abdomen, 1 pair of fine scissors for dissecting out the distended pancreas. Sterilize instruments. (Note 2).
4. Magnifier LED Lamp with Magnifying Lens (10x) (well stocked office supplies)
5. Two adult rats, weight 201-225 g, WistarHannover GALAS
6. Hanks buffered saline solution (HBSS)
7. Deoxyribonuclease I (DNase) (5mg/mL dissolved in HBSS, store at -20°C) in 500 µL aliquots
8. Fetal bovine serum (FBS). Heat inactivate at 56°C for 10min and aliquot aseptically into 15mL and 37.5mL aliquots and store at -20°C
9. Penicillin-Streptomycin solution (10.000U/mL). Aliqout aseptically into 5mL aliquots and store at -20°C
10. Collagenase mix: For 500mL collagenase mix add 0.7g Collagenase IV (540U/mg) and 500µL DNase. Sterile filter using a cellulose acetate filter and store at -20°C in 30-50mL aliqouts.
11. Quenching medium: 500 mL HBSS, 15 mL FBS, 5mL penicillin-streptomycin. Keep on wet ice. The small amount of FBS in the Quenching medium inhibits trypsin activity from the exocrine pancreas and impurities in the collagenase.

12. Histopaque 1.077. Allow to equilibrate to room temperature before use
13. Sterile 50mL conical glass bottle, preferably with screw lids
14. Cover for a 14G Venflon needle and 10mL plastic syringes
15. Metal sieve CD-1 with Mesh 40 (Sigma S1145 and S0770)
16. RPMI 1640 with Glutamax 1M HEPES pH 7.4 Complete islet medium: 500mL RPMI1640 with Glutamax, 37.5mL FBS (7.5%), 10mL 1M HEPES, 5mL penicillin-streptomycin

2.2 Culture of isolated islets, free floating

1. Stereo microscope such as Olympus SZ51 or similar preferably with illumination from below
2. Sterile polystyrene bacterial petri dishes (10 cm diameter)
3. Complete islet medium (see above)

2.3 Matrigel culture of islets and EdU incorporation

1. Complete human serum (HS) islet medium: 500mL RPMI 1640 with Glutamax, 10 mL (2%) pooled HS, 10mL 1M HEPES, 5mL penicillin-streptomycin
2. RPMI1640
3. Matrigel Matrix Growth Factor Reduced
4. Pipette tips, 200µL, stored at -20°C for use with Matrigel, which is liquid at temperatures below 0°C.
5. 12 well cell culture plate
6. Glass cover slips (16mm diameter) Click-iT EdU Alexa Fluor 555 Imaging Kit
7. Phosphate Buffered Saline (PBS)
8. Bovine Serum Albumin (BSA)

9. DMSO
10. Paraformaldehyde (PFA)
11. PBT buffer: PBS with 0.2% BSA, 0.5% Triton X-100. Store at -20°C.
12. Animal Free Blocker (Vector Labs)
13. Microscope slides
14. Guinea pig anti-insulin (Abcam ab7842)
15. Goat Anti-Guinea pig Dylight 488 (Abcam ab102374)
16. Nail polish for sealing cover slips
17. Prolong Gold Antifade Mountant
18. Confocal imaging facility

3. Methods

3.1 Perfusion of the pancreas via the common bile duct

1. On the day of islet isolation: Thaw collagenase mix and keep on ice. Calculate 12.5mL collagenase-mix solution for each rat pancreas.
2. Histopaque 1.077 is removed from the refrigerator and must not be used before it has reached room temperature.
3. Add 4mL collagenase mix to each 50mL conical glass bottle, one per pancreas, and place on wet ice.
4. Items to bring for the animal facility/perfusion bench: Instruments, syringe (10mL) and needles (27G), wet ice, 50mL conical flasks to place the perfused pancreata in, ethanol for disinfection, gloves, bench-kote and wipes, and a bag for disposal of waste.

5. Fill up the 10mL syringe with diluted collagenase mix, bend the needle 90° and attach it and leave on the wet ice (Figure 1).
6. The euthanization of the rats should follow local institutional animal care and utilization guidelines (Note 3)
7. Immediately following euthanization, rinse the abdomen with 70% ethanol and cut open the abdomen using the large scissors. First cut the skin and then the peritoneum. Open the abdomen from side to side and push the intestines and the top liver lobes to the side in order to have access to the common bile duct (ductus choledochus communis) and the duodenum. Place the rat with its tail towards you and the head pointing away (Figure 1)
8. Clamp the entrance of the common bile duct into the duodenum (Ampulla of Vater) using a small Dieffenbach clamp.
9. Clamp the upper bile duct before the entrance to the liver using a second Dieffenbach clamp (Figure 1)
10. Perfusion of the pancreas: Use the fine forceps held in the left hand to lift up the common bile duct. Under the lamp with magnifying lens use the right hand to insert the needle into the duct pointing the needle towards you. Avoid rolling of the duct by lifting the duct slightly with the left hand forceps, while simultaneously piercing the duct with the needle. Gently inject collagenase mix into the duct. The needle may, if necessary, be kept in place using a clamp. The signs of a successful perfusion are: 1) A slight resistance in the syringe, 2) No fluid leaking, and 3) The pancreas swells slowly and entirely including the distal part (the tail of the pancreas).
11. One pancreas will use 5-10mL collagenase-mix. If the perfusion is not complete, then collagenase may be injected directly at different positions in the pancreas, especially in

the distal part, which is most rich in islets. However, this will result in a much lower islet yield compared to a successful perfusion.

12. The pancreas is carefully dissected from the intestines using the fine scissors. Both the ventral and the dorsal part of the pancreas should be distended and dissected out. Place the pancreas in a petri dish on ice and remove fat and obvious connective tissue.
13. Repeat perfusion with the next rat (Note 4).

3.2 Digestion of pancreas and isolation of islets

1. Each perfused pancreas is transferred to a sterile 50mL conical glass bottle on ice. Digest the pancreas at 37°C in a shaking water bath (200 rotations/min) for 4 min.
2. Add 75 mL cold Quenching medium, mix carefully and let the conical glass bottle stand tilted at a 45° angle in ice for 3 min.
3. Pour off the supernatant one glass bottle at a time, until 25mL is left.
4. Add an extra 2.5mL collagenase mix solution per pancreas.
5. Further incubate in the shaking water bath at 37˚C and 200 rotation/ min for 2 min.
6. Transfer to ice bath for 1 min.
7. Vigorously shake the tissue for 1 min to enhance dissociation.
8. Pour the entire content of each glass bottle into a 50mL 'blue cap' (plastic)conical tube, rinse the glass bottle with ice-cold Quenching medium and add to the tube. Fill up the tube to the 50mL mark. Keep the islets cold from now on.
9. Incubate 10 min on ice.
10. Centrifuge the 50 mL tube at 4˚C, 1 min at 900 x g using a swinging bucket rotor.
11. Pour off the supernatant (take care to remove floating fat) and resuspend the pellet in 20mL Quenching medium by pipetting up and down using a serological pipette.

12. Filter the tissue blend through a metal sieve with mesh to remove undigested connective tissue or fat into a new 50 mL centrifuge tube. Wash the previous tube with 2 x 15mL Quenching medium.
13. Centrifuge the 50 mL tube at 4˚C, 1 min at 900 x g using a swinging bucket rotor.
14. Pour off the supernatant until 10 mL remains in the conical tube.
15. Homogenize tissue by pipetting 6-10 times using the plastic cover for a 14G Venflon needle and a 10mL plastic syringe. Avoid foaming.
16. From now, work at room temperature: Carefully add 10 mL Histopaque 1.077 below the homogenized tissue.
17. Centrifuge at 900 x g, 20˚C, without brakes and slow acceleration and deceleration, 22 mins.
18. Hold tube to light source, locating the main part of islets in the intersection between the top and bottom phases.
19. Transfer islets to a new 50 mL conical tube and fill up with Quenching medium.
20. Then transfer the remaining top and middle phase to a new 50 mL conical tube and fill to the 50 mL mark with Quenching medium and mix well through inversion.
21. Centrifuge both 50 mL conical tubes at 200 x g, 20˚C, with brake, 7 mins.
22. Remove supernatant through decanting, flick and add 5mL Quenching medium.
23. Pour the contents of the 50mL conical tubes into sterile 10 cm bacterial petri dishes, wash the conical tube 2 times with new Quenching medium to make certain all islets are transferred into the petri dish.

3.3 Culture of islets free floating

1. Hand pick the islets under a stereo microscope into complete islet medium. Expected yield of islets: 200-300 per pancreas.
2. Pick islets into new petri dish with fresh complete islet medium until all exocrine tissue has been removed.
3. When clean, move islets to a clean petri dish with complete islet medium and culture at 37°C in CO_2 incubator. Circling movements with the petri dish will collect the islets in the center, from where they can be easily transferred. Do not culture more than 150 islets in the same 10 cm petri dish as they tend to stick together – be sure to gently move plate sideways when placing it in the incubator in order to move islets away from the center during incubation.
4. Pick islets clean every day and transfer to fresh islet medium. After 2-3 days, all exocrine tissue is degraded and islets may be seeded on to Matrigel coated glass cover slips or used for other experiments.

Matrigel culture of islets and EdU incorporation

1. Prepare for matrigel coating the following day: A) Thaw matrigel overnight at 4°C on ice, B) Store pipette tips (200µL) at -20°C overnight.
2. Coat cover slips (16mm diameter) with Matrigel (Thin Gel Method): A) Put slides into 12-well plate on ice. B) Thaw Matrigel Matrix Growth Factor Reduced as recommended on ice. C) Using cooled pipette tips, mix the Matrigel to homogeneity on ice and while keeping culture plates on ice, add 50 µL per square centimeter of growth surface (Ø16mm ~ 2 cm2 ~ 100µL). D) Incubate plates at 37°C for 30 minutes (Note 5) E) Remove excess Matrigel by washing in serum-free RPMI 1640.
3. Seed islets onto Matrigel: Pick islets clean onto new petri dish, fill up with fresh RPMI. Pick 10-20 islets of similar sizes/cover slip. Do not scrape the bottom of the cover slip.

Make sure islets do not touch each other on the slide. Let islets attach in small droplets on the cover slip for 30 min in the incubator, while ensuring that the droplets do not dry out. Carefully add 1000 µL complete HS islet medium (37°C) with 2% Human Serum (hinders fibroblast formation).

4. Incubate at 37°C CO_2 48-96 hr, during which time the islets will be treated with stimulators or inhibitors as per experimental conditions and pulsed with EdU for 24- 48 hrs.
5. EdU labeling: Prepare the stock solutions for the Click-iT EdU imaging kit according to manufacturers' instructions.
6. Carefully remove 500 µL RPMI from each well on the 12-well plate and add to respective sterile 1.5mL centrifuge tube (this is to ensure that the treatment initiated earlier continues).
7. Add 2.0 µL EdU (10mM stock solution, component A of MP10338) to each 1.5mL centrifuge tube and pipette carefully, avoid foaming.
8. Restore respective 500 µL EdU-mix into each well of the 12-well plate (final conc. 10 µM EdU/well).
9. Incubate 37°C CO_2 24-48 hours. Note 6.

Detection of EdU and staining for insulin

10. Islets fixation: Remove the media and wash once in 500 µL PBS. Add 200 µL 4% Paraformaldehyde in PBS (PFA) per well and incubate at room temperature for 2hrs.
11. Wash in PBS 200 µL 3 x 10 min.
12. Continue with step 13 or keep wrapped in multiwall plate at 4°C in 500 µL PBS until later use.

13. Islet permeabilization: Add 200 µL 17% DMSO vol/vol. E.g. for total volume 3 mL; 510 µL DMSO + 2490 µL PBS. Incubate in hood 2hrs.
14. Wash in 200 µL PBT 3*15 min.
15. Add 200 µl PBT and incubate 1h RT.
16. Wash in 3% BSA/PBS 2*10 min.
17. EdU detection: Prepare the click-iT reaction cocktail, 200µl per well as per Table 1.
18. Reaction buffer additive -- Solution F: Dilute the stock (10X) to 1:10 in milliQ water (or deionized water).
19. Add 200 µL Click-iT reaction cocktail per cover slip and incubate RT dark 1hr
20. Wash in 200 µL 3% BSA/PBS 1 x 5 min.
21. Wash in 200 µL PBT 3*15 min.
22. Block with 200 µL Animal Free Blocker (1:5) for 30 min (e.g. for 3000 µL animal-free blocker use 600 µL blocker and 2400 µL deionized H_2O).
23. Wash in 200 µL PBT 2*5 min.
24. Add 250 µL 1° antibody: Guinea pig anti-insulin diluted 1:500 in PBT, incubate over night at 4°C.
25. Wash in 200 µl PBT, 3 x 20 min.
26. Add 250 µL 2° antibody: Goat α-guinea pig, Alexa Fluor 488 labeled (1:200) and Hoechst (5µg/mL) in PBT, incubate 3 hrs at room temperature in the dark. Note 7.
27. Wash 200 µL PBS, 3 x 20 min.
28. View under the fluorescent microscope and if the staining is satisfactory, proceed with mounting of the cover slip.
29. Mount with Prolong Gold antifade mounting medium: A) Place one drop of Prolong Gold on a microscope slide, B) Pick up the cover slip from the well using the blunt side

of a broad scalpel, C) Turn it down (islets facing downwards) onto the droplet of mounting medium on the microscope slide. Dry away surplus mounting medium.

30. Let the mounting medium solidify overnight at room temperature on a level surface.
31. Next day, seal the edges of the cover slip using nail polish.
32. Image the islets on the cover slip by confocal microscopy.
33. The fluorescence excitation/emission wavelength maxima for the fluorophors are (in nm): EdU stain: 555/565, Dylight 488: 495/519, Hoechst 33342: 350/461.
34. Whole islets are scanned using the 20x objective on the z-axis with acquisition for every 10µm. For exemplary images see Figure 2.

4. Notes

1: Collagenase mix for rodent islet isolation: Collagenase IV isolated from Clostridium histolyticum isavailable from Sigma Aldrich (C6079), ThermoFisher (17104019) and other suppliers: Various preparations of collagenases of different purity grades are available for islet isolation. Liberase TL has replaced Liberase RI (Roche) as a more refined and pure enzyme product, which is reported to result in 28% increased islet yield [12]. Optimization of the digestion time should be anticipated.

2: Fine surgical instruments may be purchased at Roboz.

3: Euthanization using CO_2 followed by cervical dislocation is recommended. The rat must be perfused immediately following euthanization. If this is not possible, the recommendation is to anesthetize the animal using hypnorm-dormicum (Fentanyl/fluanisone/midazolam) according to local guidelines.

4: Up to 6 rats may be perfused at the same time, but all perfusions must be done within 1hr as the pancreata will start to digest although they are kept on ice.

5. Matrigel contains chloroform, known to cause cancer. This procedure should be done in a ventilated hood.

6. 48hrs of pulse with EdU should yield 2-3% incorporation, though not all of the labeled cells will be β-cells. We never observe labeled clusters and therefore consider this pulse interval suitable. Extended pulse periods is discouraged as EdU incorporates into the DNA and causes DNA damage, which will limit the viability of incorporated cells.

7. Suitable controls for the antibody staining could be to leave out either the primary or the secondary antibody to ensure that the expected specificity of the signal is obtained.

References

1. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC (2008) Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 57 (6):1584-1594
2. Butler PC, Meier JJ, Butler AE, Bhushan A (2007) The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 3 (11):758-768
3. Van Assche FA, Aerts L, de PF (1978) A morphological study of the endocrine pancreas in human pregnancy. Br J Obstet Gynaecol 85 (11):818-820
4. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, Butler PC (2010) Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 53 (10):2167-2176. doi:10.1007/s00125-010-1809-6 [doi]
5. Saisho Y, Butler AE, Meier JJ, Monchamp T, Allen-Auerbach M, Rizza RA, Butler PC (2007) Pancreas volumes in humans from birth to age one hundred taking into account sex, obesity, and presence of type-2 diabetes. Clin Anat 20 (8):933-942. doi:10.1002/ca.20543 [doi]
6. Rhodes CJ (2005) Type 2 diabetes-a matter of beta-cell life and death? Science 307 (5708):380-384
7. Ackermann AM, Gannon M (2007) Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. J Mol Endocrinol 38 (1-2):193-206
8. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429 (6987):41-46
9. Georgia S, Bhushan A (2004) Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J Clin Invest 114 (7):963-968. doi:10.1172/JCI22098 [doi]
10. Schmidt SF, Madsen JG, Frafjord KO, Poulsen L, Salo S, Boergesen M, Loft A, Larsen BD, Madsen MS, Holst JJ, Maechler P, Dalgaard LT, Mandrup S (2016) Integrative Genomics Outlines a Biphasic Glucose Response and a ChREBP-RORgamma Axis Regulating Proliferation in beta Cells. Cell Rep 16 (9):2359-2372. doi:10.1016/j.celrep.2016.07.063
11. Donath MY, Halban PA (2004) Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia 47 (3):581-589

12. Yesil P, Michel M, Chwalek K, Pedack S, Jany C, Ludwig B, Bornstein SR, Lammert E (2009) A new collagenase blend increases the number of islets isolated from mouse pancreas. Islets 1 (3):185-190. doi:10.4161/isl.1.3.9556

Figure 1

Perfusion of the rat pancreas through the common bile duct. The stomach is cut across the abdomen and the skin pulled away. The intestines and the liver lobes are pushed aside. Two clamps are placed; one across the Ampulla of Vater, where the common bile duct enters the duodenum, and the other just below the liver. These clamps will ensure that the pancreatic duct is the only route available for the collagenase perfusion. The needle is bend 90 degrees and inserted into the duct, which is held up by serrated fine forceps.

Figure 2

Images of an adult rat islet of Langerhans cultured on Matrigel, stained for Hoechst (blue), insulin (green), EdU incorporation (red) and a composite image of all 3 stains (overlay).

Table 1: Overview of the EdU reactions for 1, 2, 5 and 10 cover slips for the Click-iT EdU Imaging kit from Thermo Fisher Scientific.

Reaction components	Number of cover slips			
	1	2	5	10
1 x Click-iT reaction buffer (component D, MP10338, step 1.4)	430 µL	860 µL	2.2 mL	4.3 mL
$CuSO_4$ (component H)	20 µL	40 µL	100 µL	200 µL

Alexa Fluor azide (MP10338, step 1.3)	1.2 µL	2.5 µL	6 µL	12.5 µL
Reaction buffer additive (component F, MP10338, step 4.1)	50 µL	100 µL	250 µL	1.25 mL
Total volume	500 µL	1 mL µL	2.5 mL	5 mL
Note: Add components in the order listed				