Video Article

Assessing Replication and Beta Cell Function in Adenovirally-transduced Isolated Rodent Islets

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Abstract

Glucose homeostasis is primarily controlled by the endocrine hormones insulin and glucagon, secreted from the pancreatic beta and alpha cells, respectively. Functional beta cell mass is determined by the anatomical beta cell mass as well as the ability of the beta cells to respond to a nutrient load. A loss of functional beta cell mass is central to both major forms of diabetes [1-3]. Whereas the declining functional beta cell mass results from an autoimmune attack in type 1 diabetes, in type 2 diabetes, this decrement develops from both an inability of beta cells to secrete insulin appropriately and the destruction of beta cells from a cadre of mechanisms. Thus, efforts to restore functional beta cell mass are paramount to the better treatment of and potential cures for diabetes.

Efforts are underway to identify molecular pathways that can be exploited to stimulate the replication and enhance the function of beta cells. Ideally, therapeutic targets would improve both beta cell growth and function. Perhaps more important though is to identify whether a strategy that stimulates beta cell growth comes at the cost of impairing beta cell function (such as with some oncogenes) and vice versa.

By systematically suppressing or overexpressing the expression of target genes in isolated rat islets, one can identify potential therapeutic targets for increasing functional beta cell mass [4-6]. Adenoviral vectors can be employed to efficiently overexpress or knockdown proteins in isolated rat islets [4,7-15]. Here, we present a method to manipulate gene expression utilizing adenoviral transduction and assess islet replication and beta cell function in isolated rat islets (**Figure 1**). This method has been used previously to identify novel targets that modulate beta cell replication or function [5,6,8,9,16,17].

Video Link

The video component of this article can be found at https://www.jove.com/video/4080/

Protocol

1. Adenoviral Transduction and Culturing of Rat Islets

1. Prepare a 6-well non-tissue culture coated plate by adding 2 ml of media (RPMI 1640 media containing 8 mM glucose, 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin) to the required number of wells. For example, a typical experiment may require three wells – one each for a no-virus control, a virus control (e.g., GFP-expressing adenovirus), and the experimental group.
2. Warm the plate to 37 °C by placing it into a tissue culture incubator for at least 30 min.
3. Immediately following rat islet isolation [18,19], place 100-200 islets into individual wells of the 6-well non-tissue culture coated plate. Sixty islets are required for the insulin secretion and thymidine incorporation assays. The remaining islets can be used for RNA isolation for gene expression studies or protein isolation for immunoblotting.

[Note: From this point forward, please follow institutional protocols for the handling, use, and disposal of biohazardous materials.]

4. Gently swirl the plate to bring the islets to the center of the well.
5. Pipette the adenovirus directly onto the islets in the center of the dish. Use 100-500 multiplicities of infection (MOI, the ratio of target cells to viral plaque-forming units).
6. Let the islets rest for 5 min.
7. Place the plate in the tissue culture incubator (37 °C, 5% CO_2).
8. After 24 h, gently swirl the plate to bring the islets to the centers of the wells and transfer the islets using a P200 micropipette to a new well containing fresh media. If the islets become attached to the plate, they can be gently dislodged with the pipette tip.

[Note: To verify adequate transduction efficiency, the use of a control virus expressing GFP is beneficial, as islets can then be imaged via confocal microscopy to verify penetration of the adenovirus into the islet core.]

9. Culture the islets for an additional 24-72 h, depending on the desired timing of the experiment from optimization pilot studies. For example, induction of a proliferative response may require times ranging from 24-72 h or knockdown of the gene of interest may require 48 or 72 hours. Transfer the islets to fresh media each day.
10. For the final 24 h of the experiment, culture the islets in media containing 1 µCi [methyl-^{3}H]-thymidine/ml media (generally 1 µl thymidine/ml media).

[Note: From this point forward, please follow institutional protocols for the handling, use, and disposal of radioactive materials.]

2. Insulin Secretion Assay

1. Prepare the secretion assay buffer (SAB) 10X stock solution (1.14 M NaCl, 47 mM KCl, 12 mM KH_2PO_4, 11.6 mM $MgSO_4$) and $CaCl_2$ 100X stock solution (0.25 M $CaCl_2$). These stock solutions may be prepared ahead of time and stored at room temperature.
2. Freshly prepare 50 ml of the working SAB (5 mL of 10X SAB, 1 ml of 1 M HEPES, 0.5 ml of 100X $CaCl_2$, 0.28 ml of 35% BSA, 0.11 g $NaHCO_3$, and sterile water to 50 ml) in a 50-ml conical tube and warm to 37 °C by placing in a 37 °C waterbath.
3. Pipette 10 ml of the working SAB into a 15-ml conical tube and add 66.8 µl of 2.5 M D-glucose to prepare the high glucose (16.7 mM) SAB.
4. Add 44.8 µl of 2.5 M D-glucose to the remaining 40 ml of the working SAB to prepare the low glucose (2.8 mM) SAB.
5. Label three 1.7-ml microcentrifuge tubes for each well of the 6-well plate and add 1 ml of phosphate-buffered saline (PBS).

[Note: As the islets are radioactive, please follow institutional protocols for the handling, use, and disposal of radioactive materials.]

6. Place 20 islets into each microcentrifuge tube. Make every attempt to add comparably sized islets to each microcentrifuge tube. For example, each tube may contain 5 small-, 10 medium-, and 5 large-sized islets (see **Figure 1**).

[Note: Islets can be visualized using either a dissecting stereoscope or a standard microscope.]

7. After the islets have settled on the bottom of the tube by gravity (~2 min), aspirate the PBS with a micropipette and discard.

[Note: As an alternative to settling by gravity, the tubes may be centrifuged at 300 x g for 1 min.]

8. For pre-incubation, add 400 µl of the low glucose SAB, place the tubes (with their caps open) into the tissue culture incubator (37 °C, 5% CO_2), and pre-incubate for 60 min. Aspirate the pre-incubation low glucose SAB and discard.
9. For basal insulin secretion, add 400 µl of the low glucose SAB, place the tubes (with their caps open) into the tissue culture incubator (37 °C, 5% CO_2), and incubate for 60 min. Collect the low glucose SAB and save for the insulin radioimmunoassay.
10. For stimulated insulin secretion, add 400 µl of the high glucose SAB, place the tubes (with their caps open) into the tissue culture incubator (37 °C, 5% CO_2), and incubate for 60 min. Collect the high glucose SAB and save for the insulin radioimmunoassay.

3. Thymidine Incorporation Assay

1. Add 1 ml PBS; after the islets have settled on the bottom of the tube by gravity, aspirate the PBS with a micropipette, discard, and repeat this step once.
2. Add 500 µl ice cold trichloroacetic acid (TCA, 10% w/v) and incubate on ice for 30 min.
3. Centrifuge the tubes at 16 000 x g for 3 min at 4 °C.
4. Aspirate the TCA, add 80 µl of 0.3 N NaOH, and incubate for 30 min at room temperature. During this time, vigorously vortex the samples for 5-10 s every 10 min.
5. Add 4 ml of Econo-safe counting cocktail to 7 ml liquid scintillation counting tubes.
6. Add 50 µl of the sample to the scintillation counting tube, cap the tube, shake briefly, and count in a liquid scintillation counter.
7. Measure the protein concentration using the bicinchoninic acid (BCA) assay and 10 µl of sample according to the manufacturer's protocol.

4. Data Analysis

1. Perform the insulin radioimmunoassay following the manufacturer's protocol.
2. Normalize the insulin secretion and thymidine incorporation data with the protein concentration.

5. Representative Results

An example of the experiment to assess islet replication and beta cell function in rat islets is shown in **Figure 2**. This example shows that adenoviral overexpression of hypothetical "Gene #6" robustly stimulates islet replication without altering beta cell function. In the top panel, the results from the thymidine incorporation assay demonstrate that increasing the expression of "Gene #6" increases DNA synthesis, as measured by the incorporation of thymidine. Because most of the cells in the rat islet are beta cells, it is likely that this increase in thymidine incorporation indicates an increase in beta cell replication. However, confirmatory experiments must be performed to firmly establish this. In the bottom panel, the results from the insulin secretion assay demonstrate that overexpression of "Gene #6" did not alter one of the primary beta cell functions, i.e., insulin secretion at low and high glucose. The quality of the islet isolation and health of the islets following treatment with adenoviruses is indicated by the fold increase in insulin secretion at low and high glucose concentrations. If increasing the expression of "Gene #6" impaired beta cell function, this would likely be reflected as a decrease in the insulin secreted at high, stimulatory glucose concentrations (16.7 mM). A dose-response curve for varying glucose concentrations could also be performed.

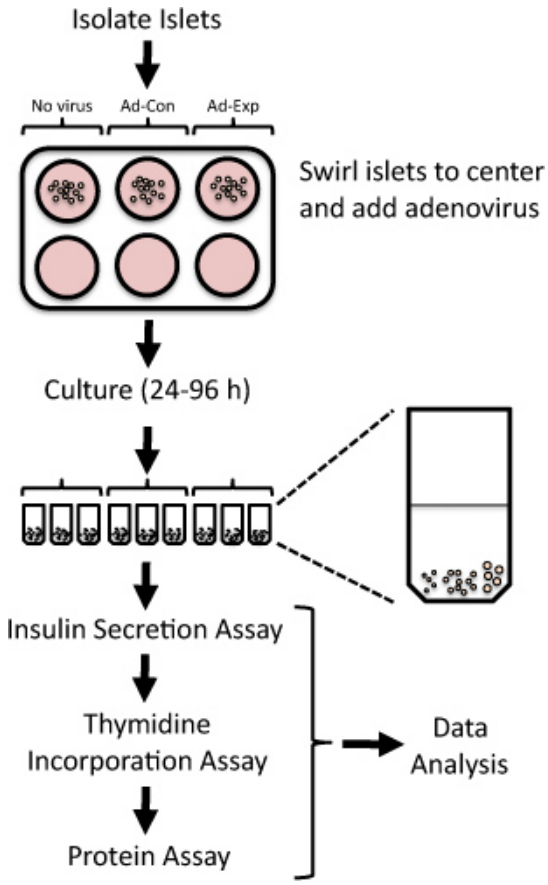

Figure 1. Overview of protocol to assess islet replication and beta cell function in isolated rat islets following adenoviral-mediated alterations in gene expression. Freshly isolated rat islets are exposed to adenoviruses for 24 h and then cultured up to 96 h. Thymidine incorporation is assessed in the final 24 h, followed by the measurement of insulin secretion at low and high glucose.

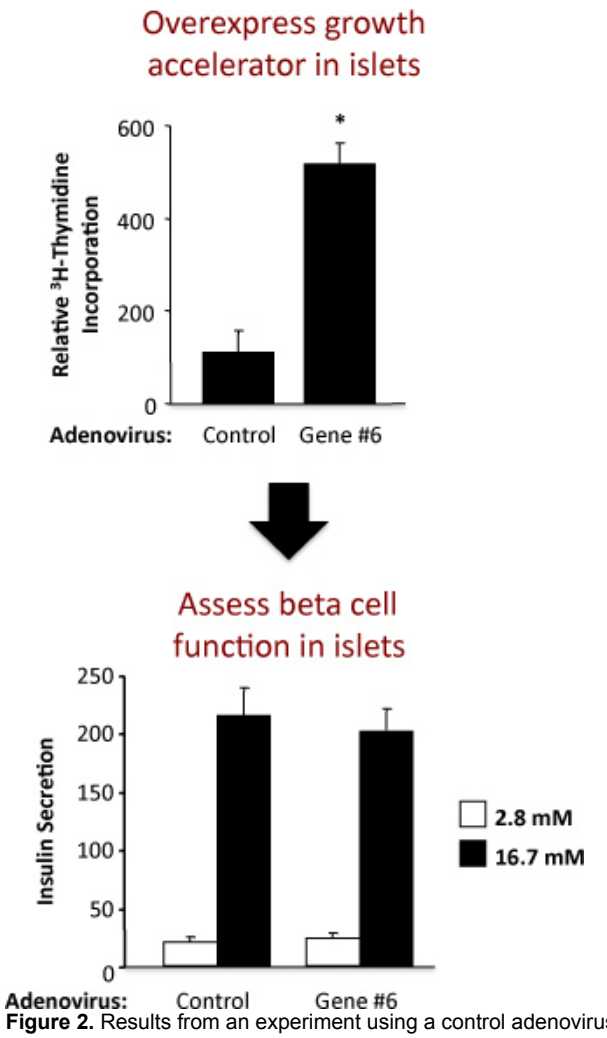

Figure 2. Results from an experiment using a control adenovirus and an adenovirus overexpressing a hypothetical gene labeled as "Gene #6". The top panel shows the thymidine incorporation and the bottom panel the insulin secretion.

Discussion

Establishing pathways that can be modulated to stimulate the replication and enhance the function of beta cells are relevant to both major forms of diabetes. Because functional beta cell mass is dependent on the existence and function of insulin-secreting cells, assessing these determinants simultaneously has its advantages. This protocol describes a streamlined protocol for identifying whether overexpression or suppression of a protein leads to changes in functional beta cell mass *in vitro*, which can then be tested for efficacy *in vivo*.

One limitation of this protocol is that the islet is a micro-organ consisting of many cell types, including but not limited to alpha, beta, delta, epsilon, and PP cells. A change in islet replication may not completely translate to a change in beta cell replication because 80-90% of the cells in the rat islet are beta cells. The possibility that an observed change in islet replication is due to non-beta cell replication exists. Thus, a logical and confirmatory next step to this protocol might be examining beta cell replication with the use of thymidine analogs coupled to immunofluorescence or FACS analysis [5,6]. This confirmatory analysis can also alleviate the potential concern of non-specific thymidine incorporation into islets independent of proliferation.

Another potential drawback lies in the transduction efficiency of adenoviruses. A transduction efficiency of 60-70% is reasonable to achieve, but a key determinant of the effectiveness is the timing of adenoviral transduction. It is essential to culture the isolated islets in the adenoviruses as soon as possible to maximize transduction efficiency. Within a few hours after the islet isolation the islet begins to contract, thereby limiting the ability of the adenovirus to penetrate deep into the core of the islet. The use of a reporter construct, such as an adenovirus expressing GFP, coupled with confocal microscopy may be beneficial for evaluating transduction efficiency.

The primary advantages of this protocol are: 1) the efficiency of testing multiple determinants of functional beta cell mass on the same pool of islets and 2) the small numbers of islets required to perform the protocol (a typical rat islet isolation yields 400 islets). These advantages allow this protocol to be used as a screening tool for multiple genes at a moderate pace.

Disclosures

No conflicts of interest declared.

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