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Isolating and culturing mouse podocyte cells to study diabetic nephropathy

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Running Head

Mouse podocyte isolation and culture

Key Words

Diabetes, diabetic nephropathy, glomerulus, kidney, podocyte

Abstract

Diabetic nephropathy is associated with injury and loss of podocytes, specialised epithelial cells that are critical for glomerular filtration. This chapter describes a method of isolating and culturing podocyte cells from mouse adult kidneys. In this way, podocytes with genetic modifications can be obtained from transgenic animals and they can be used to study the effects of the diabetic environment *in vitro*.

Introduction

Animal models are essential for the study of diabetic nephropathy (DN) and have been utilised extensively to understand the mechanisms that lead to DN and develop new therapeutic targets. **(1)** However, the study of individual cells *in vitro* is also instrumental in dissecting relevant mechanisms and pathways in a cell-specific manner. One of the earliest signs of DN is albuminuria, which arises due to defects of the glomerular filtration barrier consisting of podocyte cells, the glomerular basement membrane and endothelial cells. Podocyte injury is one of the early hallmarks of DN. In the context of DN, podocyte injury involves perturbation of the podocyte cytoskeleton leading to foot process effacement and podocyte loss **(2)** and changes to podocyte secretion of vascular growth factors (vascular endothelial growth factor A, angiopoietins) **(3)** and pro-inflammatory cytokines (monocyte chemoattractant protein-1) **(4, 5)**.

To enable the study of podocytes *in vitro*, immortalised cell lines of human **(6)** and mouse **(7)** podocytes have been established. This chapter describes the procedure of isolating and culturing primary podocytes from mouse adult kidneys. The procedure involves isolating glomeruli by perfusing mice with magnetic Dynabeads that are trapped in the glomerular capillaries, followed by glomerular purification by magnetic separation. Once in culture, the first cells to grow out of the glomeruli are podocyte cells, which can be separated from the glomeruli by filtration according to size. Podocytes obtained in this way display a differentiated phenotype and can be cultured at different conditions to mimic the diabetic environment *in vitro* and study podocyte morphology and function **(8, 9, 10)**.

Materials

Perfusion

1. Dynabeads (M-450 Epoxy magnetic beads, 4.5 μm bead size)
2. DynaMag-2 magnet
3. Phosphate Buffered saline (PBS)
4. 0.1% (w/v) Bovine Serum Albumin (BSA) in PBS
5. 0.1% BSA (w/v) in 0.2M Tris pH8.5
6. Sterile distilled water
7. Anaesthesia system for isoflurane administration
8. Dissection equipment
9. Venflon catheter
10. 20-ml syringes

Tissue digestion

1. 10 mg/ml Collagenase A in Hank's Balanced Salt Solution (HBSS); aliquots can be frozen at -20C.
2. DNase-I
3. Digestion solution: 1 mg/ml Collagenase A, 200 units/ml DNase I in PBS
4. Shaking waterbath
5. 100 μm cell strainer
6. 50 ml Falcon tubes
7. 5 ml syringes

Cell culture

1. Matrigel matrix
2. Culture media: DMEM/F12 with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, 1% L-Glutamine and 1% Insulin-Transferrin-Selenium
3. P100 cell culture dishes
4. Trypsin-EDTA (0.25%)
5. 30 μ m cell strainer
6. Normal glucose media: RPMI (powder reconstituted in 1L sterile dH₂O, no glucose) containing 0.2% (w/v) NaHCO₃, 25 mM Hepes, 3.3 mM NaOH, 10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine, 1% Insulin-Transferrin-Selenium, 5mM D-Glucose, 20 mM Mannitol
7. High glucose media: RPMI (powder reconstituted in 1L sterile dH₂O, no glucose) containing 0.2% (w/v) NaHCO₃, 25 mM Hepes, 3.3 mM NaOH, 10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine, 1% Insulin-Transferrin-Selenium, 25mM D-Glucose
8. Antibodies: anti-Nephrin, anti-nestin, anti-WT1
9. Phalloidin

Methods

Perfusion

The perfusion solution has to be prepared fresh on the day before the perfusion. Amounts are per mouse unless specified otherwise.

1. Resuspend Dynabeads by vortexing for 2 minutes.
2. Immediately pipette 250 μ l into an Eppendorf tube.
3. Place in a DynaMag-2 magnet, remove the supernatant and wash twice with 0.1% (w/v) BSA in PBS.
4. Resuspend in 250 μ l 0.1% BSA (w/v) in 0.2M Tris pH8.5 and incubate overnight with rotating at room temperature.
5. The next day, centrifuge for 10 minutes at 2000g, remove supernatant, place on the DynaMag-2 magnet and wash three times with PBS. Resuspend in 250 μ l distilled water and add to 40 ml PBS in a Falcon tube.
6. Take perfusion solution up in two 20 ml syringes with a venflon catheter attached and cut the catheter to a length of 5mm.
7. Anaesthetise the mouse and use toe pinch-response method to determine depth of anaesthesia. Animal must be unresponsive before continuing.
8. Secure in the supine position on a dissecting board.
9. Make an incision at the lower abdomen through the skin and abdominal wall with sharp scissors, extend to the left and right side of the ribcage and clamp open.
10. Cut through the connective tissue at the bottom of the diaphragm to allow access to the thoracic cavity and expose the heart, which should still be beating.
11. While holding the heart steady with forceps, incise the right atrium and make a small incision at the apex of the left ventricle.

12. Cannulate the left ventricle of the heart using the syringe with the perfusion solution prepared as above and perfuse with 40 ml of the Dynabeads solution. A successful perfusion is evident by a lightened colour of the liver (Note 1).
13. Remove the kidneys and keep in PBS on ice.

Glomerular isolation

Work under a class II biological safety cabinet and use sterile solutions.

1. Decapsulate the kidneys and mince into small (1 mm^3) pieces on a petri dish using a scalpel.
2. Digest each pair of kidneys in 1ml of digestion solution for 30 minutes at 37°C waterbath with gentle agitation.
3. Press digest through a $100 \mu\text{m}$ cell strainer on a 50 ml falcon tube using the plunger of a 5 ml syringe as a pestle.
4. Wash three times with PBS.
5. Centrifuge the cell suspension at 2000 g for 5 mins.
6. Discard the supernatant and resuspend the pellet in 1 ml PBS.
7. Transfer to an Eppendorf and place in the DynaMag-2 magnet. The glomeruli containing Dynabeads will be collected at the side of the Eppendorf facing the magnet.
8. Remove the supernatant and wash three times with PBS.
9. Remove from magnet and resuspend glomeruli in 1 ml culture media.

Culture and podocyte isolation

Work under a class II biological safety cabinet and use sterile solutions.

Prepare a Matrigel-coated P100 tissue culture dish in advance.

1. Thaw Matrigel on ice and keep on ice at all times (Note 2).
2. Dissolve Matrigel in cold media (without serum and supplements) at 1/100 and add 6 ml of this solution to the P100 dish to coat with Matrigel.
3. Incubate at 37°C for at least 1 hr.
4. Remove Matrigel solution and add 6 ml of cell culture media to the P100 dish.
5. Add the glomeruli and incubate at 37°C in a 5% CO₂ humidified atmosphere (Note 3).
6. Do not disturb the dishes to allow the glomeruli to attach to the bottom of the dish.
7. The glomeruli collected in this way are decapsulated (Figure 1a). Once they attach, the first cells to grow out of the glomeruli are podocytes. This starts approximately three days after plating (Figure 1b).
8. Seven days after plating, most of the podocytes will have grown out of the glomeruli and can be separated from them by filtration (Figure 1c).
9. To collect the podocyte cells, remove the culture media and rinse with PBS.
10. Add 2 ml 0.25% trypsin-EDTA to detach cells and glomeruli.
11. Add 8 ml culture media to deactivate the trypsin and filter through a 30 µm cell strainer on a 50ml Falcon tube. The glomeruli should stay on the top of the filter whereas the podocytes will pass through the filter and get collected in the filtrate.
12. Centrifuge the filtrate at 200g for 5 mins.
13. Discard the supernatant and resuspend the pellet (podocytes) in 10 ml culture media.
14. Plate into a new P100 dish coated with Matrigel (Figure 1d).
15. The podocytes obtained in this way express WT1, and mature podocyte markers nephrin and nestin and have prominent actin fibres (stained with phalloidin) (Figure

2). To assess the effects of the diabetic environment, the podocytes can be cultured with media containing normal (5 mM) or high (25 mM) glucose.

16. Isolated podocytes can be cryopreserved in liquid nitrogen in culture media containing 5% DMSO.

Notes

1. A successful perfusion is essential for efficient glomerular isolation. Ensure that the venflon catheter is short enough (5mm) to avoid puncturing the cardiac septum. Following a successful perfusion, the kidneys will have a pale colour.
2. When handling Matrigel, work quickly as it readily turns into a gel at room temperature.
3. The preparation of glomeruli can be assessed immediately after plating under the microscope to ensure that there is no contamination from other kidney structures.

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Figure Legends

Figure 1: Glomerular podocytes outgrowth

(a) A glomerulus in culture immediately post-isolation, magnetic beads trapped in the glomerulus are indicated by arrowheads. (b) Podocytes growing out of a glomerulus at day 3 post isolation are indicated by arrows. (c) Podocytes growing out of a glomerulus (indicated by *) on day 7 post-isolation. (d) Podocytes in culture following separation from glomeruli by filtration. Scale bar = 50 μm .

Figure 2: Podocyte characterisation

Representative pictures showing cultured podocytes stained for the podocyte markers WT1 (a), nephrin (b) and nestin (c). Actin filaments were visualised by staining for phalloidin (d). Scale bar = 20 μm .

Figures

Figure 1

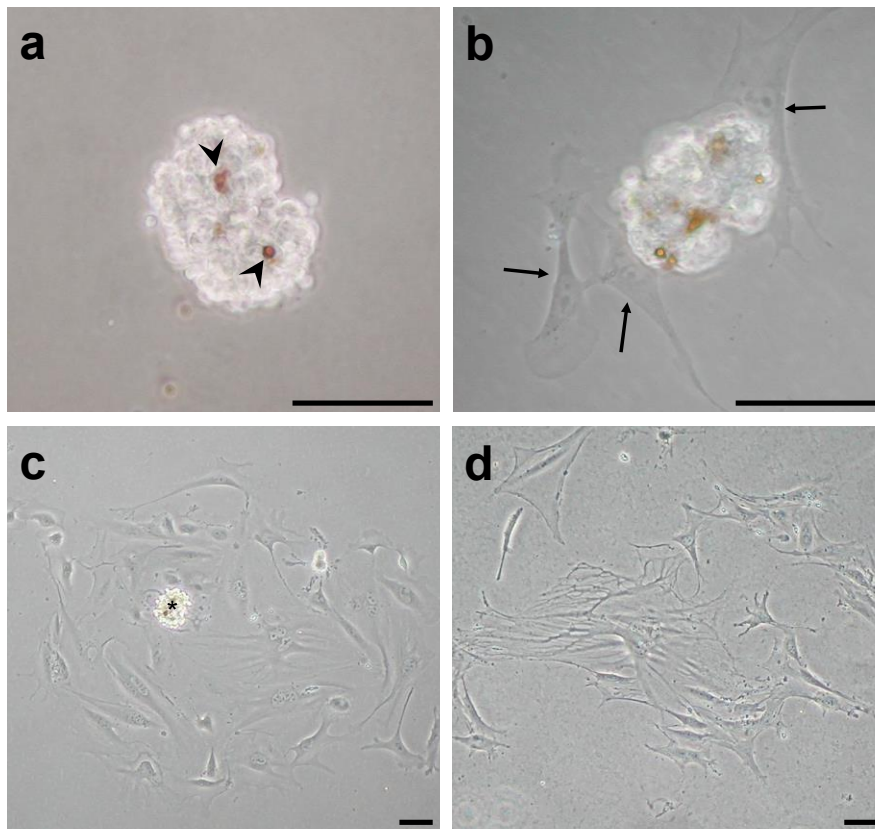


Figure 2

