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Podocyte Shape Regulation by Semaphorin 3A and MICAL-1

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Abstract

Podocytes are complex epithelial cells with foot processes that are essential for the integrity and function of the kidney glomerular filters. Podocyte foot processes linked by slit diaphragms constitute signaling platforms that tightly regulate the cell shape and the function of the filtration barrier. Semaphorin (Sema) 3A is a class 3 semaphorin secreted by podocytes that has autocrine and paracrine functions in the kidney. We have shown that Sema3A regulates podocyte shape and that excess Sema3A signaling induces glomerular disease and aggravates diabetic nephropathy. MICAL-1 is an actin-binding protein that mediates Sema3A signals in podocytes. This chapter describes the methods used to examine how Sema3A signaling regulates podocyte shape.

Keywords

Immortalized podocytes; Sema3A; F-actin; Xanthofulvin; MICAL-1; shRNA; siRNA

1 Introduction

Podocytes are epithelial cells that wrap around kidney glomerular capillaries and together with endothelial cells form the filtration barrier. Mature podocytes have primary processes and interdigitating F-actin-based secondary processes called foot processes, linked by modified adherens junctions called slit diaphragms. The structural integrity of foot processes and slit diaphragms is critical for normal podocyte function [1]. Podocyte foot processes and slit diaphragms are signaling platform functionally and the backbone of the cell shape structurally. The signaling pathways that regulate podocyte shape involve F-actin directly or indirectly and are tightly controlled.

Semaphorin 3A (Sema3A) is a secreted class 3 semaphorin produced by several cell types, including podocytes [2, 3]. We have shown that Sema3A is required for podocyte development and functions as a negative regulator of angiogenesis and branching morphogenesis [4, 5]. In the adult kidney, excess Sema3A signaling induces glomerular disease and aggravates diabetic nephropathy [6, 7]. Sema3A influences podocyte shape and function in vivo and in culture [3 – 7]. Exposure to Sema3A induces podocyte contraction and F-actin collapse in a time and dose–response manner [6]. MICAL-1 is a flavoprotein monooxygenase known to link Sema3A signals to the actin cytoskeleton [8 – 10], which we recently identified in podocytes [7]. Podocyte MICAL-1 knockdown abrogates Sema3A-induced podocyte shape changes [7]. This chapter describes the methods used to examine how Sema3A signaling regulates podocyte shape.

2 Materials

2.1 Podocyte Culture

1. Cell culture incubators with air/5 %CO_2 at 33°C and 37 °C.
2. Immortalized podocytes [6, 7] (*see* Note 1).
3. Complete podocyte culture medium: RPMI 1640 medium + 10 % fetal bovine serum (FBS) heat inactivated.
4. Mouse interferon γ (Sigma): Make 1000 U/ml stock solution in 10 mM Na_2HPO_4, pH 8.
5. Slide chambers (Nunc Lab-Tek II, 4 well).
6. Cell culture dishes (60–100 mm) or T25–T75 cell culture flasks (sterile).
7. Dulbecco's phosphate buffer saline (DPBS): To prepare 1 L place about 100 ml deionized water in 1 L graduated cylinder; add 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 0.2 g KCl; add water to 1 L, mix; adjust pH to 7.4 with HCl or NaOH; and autoclave to sterilize.
8. Rat Collagen I: Dilute Rat Collagen I (Sigma-Aldrich) to 0.1 mg/ml in DPBS using sterile technique and keep at 4 °C.
9. Collagen I-coated flasks: In laminar flow hood add 1 ml 0.1 mg/ml rat collagen I to T25 cell culture flasks and spread on bottom surface, and incubate for 1 h. Remove the non-attached collagen I, wash with sterile DPBS, aspirate, and discard the wash. Turn the flask downward and let dry uncapped overnight in the hood. Avoid UV light exposure (it cross-links collagen). The following day flasks are ready to use and may be kept at 4 °C until use.
10. 100 mm Collagen I-coated culture dishes, collagen I-coated slide chambers (Nunc Lab-Tek II, 4 well), and collagen I-coated 6-well plate: coat in similar manner as described above to coat the T25 flasks (except decreasing the volumes).

2.2 Microperfusion System

1. Injectman NI2 micromanipulator/Femtojet microinjector semi-automated microperfusion system (Eppendorf).
2. Glass microcapillary needles (Femtotips II, Eppendorf).
3. Recombinant Sema3A [5] or (R&D). Store at −80 °C.

2.3 F-actin Labeling

1. Recombinant Sema3A [5] or (R&D). Store at −80 °C.
2. Xanthofulvin [11, 12]: Make 0.1 mM stock solution in DPBS, aliquot to avoid freezing/thawing, and store at −20 °C.

3. 4 % Paraformaldehyde in DPBS: Dilute 16 % paraformaldehyde aqueous solution (Electron Microscopy Sciences) 1:4 in DPBS immediately prior to use, and keep at room temperature.
4. 0.5 % Triton X-100 in DPBS: Add 0.5 % Triton X-100 (vol:vol) to DPBS, mix, and keep at room temperature.
5. F-actin fluorescent labeling solution: Dilute rhodamine phalloidin (Molecular Probes) 1:100 (vol:vol) in DPBS.
6. DAPI containing mounting medium.
7. Cover slips.
8. Fluorescence or confocal microscope.
9. Image analysis software (ImageJ-NIH or Zeiss Axiovision).

2.4 MICAL-1 Knockdown

1. Oligofectamine (Invitrogen).
2. Opti-MEM medium (Gibco).
3. MICAL-1 shRNA expression plasmid DNA : Dilute mouse MICAL-1 shRNA (Origene, catalog #TF506165) in dH 2 O to 100 ng/ml, vortex, and store at −20 °C until used.
4. Scrambled shRNA DNA stock solution: Dilute scrambled DNA in dH2O to 100 ng/μl, vortex, and store at −20 °C until used (*see* Note 2).
5. Diluted DNA: Mix 1 μg shRNA DNA (either MICAL-1 shRNA expression plasmid DNA or scrambled shRNA DNA stock solution) with 150 μl Opti-MEM or serum-free medium, and incubate for 5 min at RT.
6. Diluted transfection reagent: Mix 10 ml Lipofectamine 2000 (Invitrogen) in 100 ml Opti-MEM or serum-free medium (1:10 vol:vol), and keep for 5 min at RT.
7. 0.5 %Trypsin medium (Gibco).

3 Methods

3.1 Podocyte Cell Culture and Differentiation

1. Mouse podocyte cell lines [6,7] are maintained in complete podocyte culture medium in humidified incubators with air-5 % CO_2, and are propagated on collagen I-coated flasks at 33 °C in the presence of recombinant mouse interferon. Removal of interferon and temperature switch to 37 °C inactivate the SV40 T antigen and induce podocytes to differentiate.
2. Plate 2×10^5 undifferentiated immortalized podocytes in collagen I-coated flasks and incubate in 10 ml complete podocyte culture medium with 10 U/ml interferon γ at 33 °C, allowing cell proliferation until 70 % confluent (this

usually takes 2–3 days). Note that the culture volume will vary depending on the size of the flask or chamber, as stated in the corresponding packages.

3. Gently remove the medium from **step 2** by tilting the flask and removing the media with a pipet. Replace with complete podocyte culture medium without interferon γ and transfer to 37 °C for 7–10 days to induce differentiation (*see* Note 3). Replace culture medium every 2 days.

3.2 Time-Lapse Microscopy of Sema3A-Induced Podocyte Contraction

1. Plate 2×10^5 differentiated podocytes in 100 mm collagen I-coated culture dishes, and incubate overnight at 37 °C in 10 ml complete podocyte culture medium.
2. Place dish in a microinjection workstation mounted on the microscope.
3. Program the micromanipulator injection level (*Z*-axis) and angle (30–60°).
4. Program Femtojet parameters: injection time 3–30 s, injection pressure 50–200 hPa, and injection volume (100 pL–1 μl).
5. Fill microcapillary with RPMI 1640 (serum-free) medium with 500 ng/ml Sema3A in micromanipulator holder and place at 30–60° angle in proximity of 1–2 podocytes. Inject 100 pL–1 μl amount of the Sema3A/RPMI 1640 solution in the vicinity of the podocytes.
6. Program camera to image the podocytes every 15 s for 30 min following injection/release of the Sema3A.

3.3 Evaluation of Sema3A-Induced Podocyte Contraction and F-actin Collapse

1. Plate 6×10^4 differentiated podocytes in collagen I-coated slide chambers and incubate in complete podocyte culture medium overnight at 37 °C.
2. Remove culture medium, wash with DPBS, replace with RPMI 1640 without serum, or RPMI 1640 with 100 ng/ml Sema3A, and incubate at 37 °C for 8 h (*see* Note 4).
3. Sema3A-binding inhibitor xanthofulvin may be added to 0.1 μM final concentration in culture medium 1 h prior to exposure to Sema3A (*see* Note 5).
4. Remove culture medium, and wash cells for 5 min with DPBS (1×).
5. Fix cells with 4 % paraformaldehyde in DPBS for 10 min at room temperature.
6. Wash cells with DPBS for 5 min (3×).
7. Permeabilize cells with 0.5 % Triton X-100 in DPBS for 10 min.
8. Incubate cells in F-actin fluorescent labeling solution for 2 h at room temperature (or overnight at 4 °C).
9. Wash cells with DPBS for 5 min (3×).
10. Remove DPBS, add DAPI containing mounting medium, and cover slip.

11. Keep slides at −20 °C until performing imaging to minimize fading of fluorescent signal.

12. Measure individual podocyte cell area by tracing around the perimeter of the cell, count the number of cellular processes and F-actin fibers, and determine the extent/degree of F-actin collapse by using image analysis software to compute the integrated fluorescence (OD/area) (*see* Note 6).

3.4 Evaluation of Sema3A-Induced Podocyte Contraction and F-Actin Collapse Following MICAL-1 Knockdown

1. Plate 2×10^5 podocytes/well on collagen I-coated 6-well plate, and incubate at 37 °C until ~70% confluent.

2. Wash podocytes with DPBS, add 500 μl Opti-MEM medium, and incubate for 30 min.

3. During the 30-min incubation described in **step 2**, add diluted DNA containing (1) MICAL-1 shRNA expression plasmid DNA and (2) scrambled shRNA DNA stock solution separately to diluted transfection reagent and mix gently. Incubate for 20 min at room temperature (*see* Note 7).

4. Add the mixtures from **step 3** separately to podocytes, rock briefly to mix, and incubate for 4 h at 37 °C.

5. Add 1 ml complete podocyte culture medium, and incubate overnight at 37 °C.

6. Remove medium, add 2 ml complete podocyte culture medium, and incubate for additional 48–72 h.

7. Trypsinize podocytes with 0.5 % trypsin medium for 5 min or longer at 37 °C. Re-plate 6×10^4 podocytes from each condition (non-transfected, scrambled shRNA, MICAL-1 shRNA) in duplicate on collagen I-coated slide chambers and incubate in complete podocyte culture medium overnight at 37 °C (*see* Note 8).

8. Incubate podocytes in complete medium with or without 100 ng/ml Sema3A for 8 h at 37 °C as described in Subheading 3.3, **step 2**.

9. Fix, stain with F-actin fluorescent labeling solution, and analyze as described in Subheading 3.3, **steps 4**–**12**.

4 Notes

1. Primary mouse podocytes and human immortalized podocytes [13] can also be cultured in these conditions.

2. Scrambled shRNA has the same nucleotide composition as the MICAL shRNA sequence but does not match with any mouse mRNA, and has no known miRNA recognition sequence.

3. The temperature switch and removal of interferon γ inactivate the SV40T antigen, thereby stopping cell proliferation and allowing cell differentiation.

Immortalized podocytes continue to proliferate for 2–3 days after, and thus need to be switched to 37 °C when they are 60–70 % confluent. Cell differentiation occurs within 10 days and becomes apparent by a change in shape. Changes in gene expression associated with differentiation have been well documented [1, 14, 15].

4. If time–response experiments longer than 12 h are performed, 1 % FBS should be added to prevent cell shape change due to serum starvation [7].

5. Sema3A inhibitor xanthofulvin is used as a control for Sema3A effect specificity and to ascertain that Sema3A is not toxic [7].

6. Image analysis can be performed using available image analysis software, including Zeiss Axiovision, ImageJ-NIH, and Metamorph®.

7. Additional recommended controls for shRNA transfections (in addition to transfection with scrambled shRNA) are (1) non-transfected podocytes, (2) transfection with empty plasmid, and (3) Lipofectamine only. Alternatively MICAL-1 knockdown can also be accomplished using siRNA oligonucleotides [7] and controls for siRNA transfections are (1) scrambled siRNA and (2) Oligofectamine alone.

8. Use the remainder of the podocytes to confirm MICAL-1 knockdown by western analysis using standard techniques (1:500 MICAL-1 antibody [Proteintech, catalog #14818–1AP]). Podocyte MICAL-1 knockdown of ~70 % can be achieved with both shRNA and siRNA; the efficiency varies slightly with specific podocyte lines.

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