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Glomerular Outgrowth as an Ex vivo Assay to Analyze Pathways Involved in Parietal Epithelial Cell Activation

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Cover Letter

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May, 27th 2019

Editors and
members of the editorial board
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Dear Editors and members of the editorial board,

Please find our online submission of the manuscript entitled "Glomerular outgrowth - An *ex vivo* assay to analyze pathways involved in parietal epithelial cell activation" written by J. Eymael for the JoVE Biology section of the Editorial JoVE.
Parietal epithelial cell activation is one of the key factors involved in the development and progression of glomerulosclerosis. Inhibition of pathways involved in parietal epithelial cell activation could therefore be a tool to attenuate the progression of glomerular diseases. This paper describes a method to culture and analyze parietal epithelial cell outgrowth of encapsulated glomeruli isolated from mouse kidney and can therefore be used as a tool to study the effects of an altered gene expression in transgenic- or knockout - mice or the effects of culture conditions on parietal epithelial cell growth characteristics and signalling. Using this method, important pathways involved in the process of parietal epithelial cell activation and consequently in glomerulosclerosis can be studied.
We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to JoVE. The study was supported by a grant from the Dutch Kidney foundation (grant 14A3D104) and The Netherlands Organization for Scientific Research (NWO VIDI grant: 016.156.363). The authors have no conflicts of interest to declare.
Sincerely,

Dr. Bart Smeets, Ph.D.

TITLE:

Glomerular Outgrowth as an Ex vivo Assay to Analyze Pathways Involved in Parietal Epithelial Cell Activation

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KEYWORDS:

kidney, ex vivo assay, glomerular outgrowth, glomerulosclerosis, parietal epithelial cells, proliferation

SUMMARY:

This article describes a method for culturing and analyzing glomerular parietal epithelial cell outgrowths of encapsulated glomeruli isolated from mouse kidney. This method can be used to study pathways involved in parietal epithelial cell proliferation and migration.

ABSTRACT:

Parietal epithelial cell (PEC) activation is one of the key factors involved in the development and progression of glomerulosclerosis. Inhibition of pathways involved in parietal epithelial cell activation could therefore be a tool to attenuate the progression of glomerular diseases. This article describes a method to culture and analyze parietal epithelial cell outgrowth of encapsulated glomeruli isolated from mouse kidney. After dissecting isolated mouse kidneys, the tissue is minced, and glomeruli are isolated by sieving. Encapsulated glomeruli are collected, and

single glomeruli are cultured for 6 days to obtain glomerular outgrowth of parietal epithelial cells. During this period, parietal epithelial cell proliferation and migration can be analyzed by determining the cell number or the surface area of outgrowing cells. This assay can therefore be used as a tool to study the effects of an altered gene expression in transgenic- or knockout-mice or the effects of culture conditions on parietal epithelial cell growth characteristics and signaling. Using this method, important pathways involved in the process of parietal epithelial cell activation and consequently in glomerulosclerosis can be studied.

INTRODUCTION:

Glomerular diseases are an important group of kidney disorders and represent a major cause of end stage renal disease (ESRD). Unfortunately, specific treatment options are limited and progression to ESRD is inevitable. Glomerular diseases are defined by the presence of glomerular injury and can be grouped in inflammatory and non-inflammatory diseases. Although the initial insult is different, recent studies have shown that a common cellular mechanism leads to glomerular epithelial cell hyperplasia and ultimately to glomerulosclerosis in all glomerular diseases, irrespective of the underlying cause[1-4].

Specifically, it was shown that glomerulosclerotic lesions are mainly composed of activated parietal epithelial cells[5,6]. Under physiological conditions, parietal epithelial cells are flat quiescent epithelial cells that line the Bowman's capsule of the glomerulus. However, any glomerular injury either due to genetic mutations (e.g., podocyte specific or mitochondrial cytopathies), inflammation or hyperfiltration (e.g., caused by reduced renal mass, hypertension, obesity or diabetic mellitus) can trigger the activation of parietal epithelial cells. Activated parietal epithelial cells proliferate and deposit extracellular matrix which results in the formation of cellular crescents or sclerotic lesions[5,7,8]. Progression of these processes results in loss of renal function[9]. Therefore, parietal epithelial cell activation is a key factor in the development and progression of glomerulosclerosis in both inflammatory and non-inflammatory glomerular diseases[1-4,10].

The molecular processes mediating parietal epithelial cell activation are still unknown. Recent studies show that activated parietal epithelial cells de novo express CD44, a receptor that is important for the activation of different pathways involved in cellular proliferation and migration. Furthermore, inhibition of CD44 was shown to inhibit parietal epithelial cell activation and attenuate the progression of crescent formation and glomerulosclerosis in animal models of inflammatory as well as non-inflammatory glomerular diseases[11,12].

As parietal epithelial cell activation is a key player for the development of glomerulosclerosis and crescent formation, inhibition of these cells could slow down the progression of glomerular diseases. Elucidation of the molecular pathways driving parietal epithelial cell activation may lead to the development of specific therapeutic interventions that attenuate the formation of the hyperplastic and glomerulosclerotic lesions in glomerular disease.

In experimental animal models, it is frequently difficult to provide evidence for a direct effect of an altered gene expression (knock-out models or transgenic mouse models) or drug treatment

on the parietal epithelial cells. In a conventional knock-out mouse the observed in vivo changes might be explained by direct changes in parietal epithelial cells. However, since the gene expression is also altered in other cell types within the mouse, one cannot exclude indirect effects mediated by other cell types. The development of conditional *cre-lox* mice driven by promoters mainly active in parietal epithelial cells has provided a solution in some cases[13]. Nevertheless, conditional transgenic models are complex and although more conditional lines become available, for many of the conventional knock-out or transgenic mouse lines there is not yet a conditional substitute.

To study the direct effects on parietal epithelial cells, our group has developed an ex vivo assay using single encapsulated glomerular isolation from mouse kidneys to measure and analyze parietal epithelial cell proliferation and migration. This will enable us to determine parietal epithelial cell specific effects and to find responsible pathways for parietal epithelial cell activation and test treatment options to inhibit this activation.

PROTOCOL:

All animal experiments were performed according to the guidelines of the Animal Ethics Committee of the Radboud University Nijmegen.

NOTE: Untreated, healthy wild type (WT) mice (n = 4) and cd44–/– (n = 4) mice were sacrificed at the age of 12−16 weeks. Both male and female mice were used. All mice were on the C57Bl/6 background.

1. Mouse kidney dissection

1.1. Sacrifice healthy WT mice or genetically altered mice by cervical dislocation.

1.2. Dissect whole mouse kidneys directly after sacrificing the mice. For this, perform a median laparotomy using abdominal scissors, cutting the skin and then the abdominal muscles. Remove the intestine and place it next to the mouse.

1.3. Free the kidneys from connecting tissue and pull out the kidney using surgical forceps, cutting the renal artery, renal vein and ureter with scissors.

1.4. Remove the renal capsules from the kidneys by holding the kidney with surgical forceps and pull off the capsule using another pair of forceps.

1.5. Place the kidneys in a 6-well cell culture plate (2 kidneys/well) prepared with 2 mL of Hanks' balanced salt solution (HBSS) per well and place on ice.

2. Isolation of glomeruli from mouse kidney

2.1. Transfer the kidneys to a 100 mm Petri dish and mince the kidneys into small pieces of 1−2 mm using two scalpels. Keep the minced kidney pieces wet using 1−2 mL of HBSS.

2.2. Place the minced kidney pieces on top of a 300 µm metal sieve and press the kidney through the sieve using a plunger of a 20 mL syringe. Repeatedly rinse the sieve with HBSS in between and collect the flow-through in a clean Petri dish using a serological pipet. Collect also everything that remains/sticks to the bottom side of the sieve by scraping it off with the scalpel and transfer it to the collected flow-through (kidney homogenate).

2.3. Rinse the kidney homogenate through a 75 µm sieve with HBSS. Collect the flow-through and subsequently rinse this flow-through through a 53 µm sieve. Wash both sieves using HBSS to remove all smaller structures.

NOTE: In this step the flow-though is only rinsed but not pressed through the 75 µm and 53 µm sieve. Washing with HBSS is necessary to remove debris and smaller structures on the sieves. Therefore normally 200−300 mL of HBSS are used in total.

2.4. Collect the kidney structures/material that remain on the 75 µm and 53 µm sieve by washing the upper surface of the sieves with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) and transfer the material into a 6-well ultra-low attachment microplate.

NOTE: To wash the upper surface of the sieve, rinse the sieve in a tilted position (>45°) and collect the kidney material at the edge of the sieve. The collected material is enriched for encapsulated as well as decapsulated glomeruli, showing only a few tubular fragments. Encapsulated glomeruli are very sticky. To collect single glomeruli, it is therefore important to prevent glomeruli to adhere to the surface of a Petri dish or well plate. To avoid adherence, use medium with 20% FCS and use ultra-low attachment plates for this step.

3. Culturing of glomerular outgrowth

3.1. Bring the ultra-low attachment microplate to an inverted light microscope and collect single encapsulated and/or decapsulated glomeruli with a 20 µL pipette. Avoid pipetting other structures and debris. After catching a single glomerulus in the pipette tip, add fresh DMEM medium without collected kidney material into the same pipette tip to a volume of 20 µL.

3.2. Transfer the single glomerulus with the 20 µL DMEM medium to the center of a well of a 24-well cell culture plate and incubate for 3 h at 37 °C and 5% (v/v) CO_2 to allow attachment of the glomerulus to the center of the well. Carefully move the plate to avoid floating of the glomerulus to the boarders of the well.

3.3. After 3 h incubation, the glomerulus is attached to the center of the well. Carefully add 500 µL of endothelial basal medium (EBM) supplemented with a growth factor kit containing hydrocortisone, human endothelial growth factor, bovine brain extract and gentamicin sulfate-

amphotericin B (**Table of Materials**) and additional 5% (v/v) FBS and 1% (v/v) penicillin/streptomycin (pen/strep) to each well.

3.4. Culture the single glomeruli for 6 days at 37 °C, 5% (v/v) CO_2.

NOTE: Within 6 days the outgrowth consisting of parietal epithelial cells are formed. If one aims to test the effects of specific compounds or drugs on the parietal epithelial cells it should be added to the medium within this six-day period.

4. Analysis of parietal epithelial cell proliferation

4.1. Analyze the glomerular outgrowth after 6 days. Take microscopic images using a digital inverted light microscope.

4.2. Use an image analysis software (e.g., ImageJ/FIJI) to determine the surface area and the diameter of the glomerular outgrowth, and the number of outgrowing cells or outgrowing glomeruli.

4.2.1. To determine the surface area of glomerular outgrowth, open the tif. file of the glomerular outgrowth with scale bar in ImageJ. Draw a straight line on the scale bar and determine the distance in pixels by clicking on **analyze** and measure (e.g., 1 mm = 460 pixel).

4.2.2. Determine the scale by clicking on **analyze**, set scale and type the known distance in pixel (e.g., 460), also type the known distance (e.g., 1) and the unit of length (e.g., 1 mm). Click **ok**.

4.2.3. Determine which results will show up in the results table by clicking on **analyze** and then set measurements. To determine the surface area of glomerular outgrowth, activate the options area and display label. Click **ok**.

4.2.4. To determine the surface area of glomerular outgrowth, draw a freehand selection around the glomerular outgrowth. Click on **analyze** and then measure, a result table will pop up in ImageJ showing the surface area of outgrowth in the earlier determined scale (see step 4.4, e.g., mm^2).

5. Characterization of the glomerular cell outgrowth

NOTE: To assess the cellular composition of the outgrowth, immunofluorescence staining for cell-specific markers are performed on the glomerular outgrowths at t = 6 days.

5.1. Carefully remove the medium and gently wash the glomeruli twice using phosphate-buffered saline (PBS).

5.2. Fixate for 10 min at room temperature using 2% (v/v) paraformaldehyde (PFA) supplemented with 4% (w/v) sucrose in PBS and carefully wash 2x using PBS.

5.3. Incubate with the primary antibody with appropriate concentration (**Table of Materials**) diluted in PBS-ovine serum albumin (BSA) 1% (v/v) for 1 h at room temperature.

5.4. Remove the antibody solution and carefully wash 3x with PBS.

5.5. Incubate with secondary antibody (**Table of Materials**) diluted in PBS-BSA 1% (v/v) in the dark for 45 min at room temperature.

5.6. Carefully wash 3x with PBS and mount using 1−2 drops of mounting medium with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei and cover the well with a round cover slip.

5.7. Take microscopic images using a fluorescent microscope.

REPRESENTATIVE RESULTS:

A systematic diagram of the method to perform the glomerular outgrowth assay is shown in **Figure 1**. **Figure 2A-D** shows glomerular outgrowths of encapsulated glomeruli at different time points as observed using light microscopy. Outgrowths are shown at day 2, 4 and 6 (**Figure 2B-D**) in culture after glomerulus isolation from mouse kidney. In order to validate that the outgrowing cells are parietal epithelial cells, decapsulated glomeruli have also been isolated and cultured for 6 days as shown in **Figure 2E,F**. Decapsulated glomeruli showed no cell outgrowth during the incubation period within 6 days. In **Figure 3**, immunofluorescence staining was performed for different parietal epithelial cell markers, podocyte specific markers as well as endothelial cell markers. The results validate that the outgrowing cells indeed are parietal epithelial cells. **Figure 4** shows the outgrowth of isolated encapsulated glomeruli from CD44-/- vs WT mice after 6 days in culture. Glomeruli isolated from CD44-/- mice showed a decreased number of outgrowing cells as well as a decreased surface area of glomerular outgrowth compared to the glomeruli isolated from WT mice, suggesting an important role for CD44 in parietal epithelial cell activation as published previously[11]. In **Figure 5**, an example is given, in which the surface area of the outgrowing parietal epithelial cells is determined using ImageJ.

FIGURE LEGENDS:

Figure 1: Schematic overview of the method to perform a glomerular outgrowth assay to analyze parietal epithelial cell proliferation. (1) Kidneys are dissected from sacrificed mouse and minced into small pieces. (2) Kidney tissue is pressed through the 300 µm sieve and rinsed through the 75 µm and 53 µm sieves. (3) Glomeruli that remain on top of the sieves are collected using medium + 20% (v/v) FCS and are transferred to an ultra-low attachment plate. (4) Single glomeruli are captured using an inverted light microscope and are transferred to 24-well culture plates. (5) After incubation at 37 °C, 5% (v/v) CO_2 for 6 days, glomerular outgrowth can be analyzed using a digital inverted light microscope.

Figure 2: Glomerular outgrowth of isolated capsulated glomeruli from kidneys dissected from WT mice. The outgrowth of encapsulated glomeruli incubated at 37 °C is shown at different time points: (**A**) 0 days, (**B**) 2 days, (**C**) 4 days, and (**D**) 6 days. Decapsulated glomeruli were also isolated

and cultured at 37 °C and microscopic images were taken at (**E**) day 0 and (**F**) day 6 showing no outgrowing cells. Scale bars: (**A,E,F**) 200 µm, (**B**) 400 µm, (**C,D**) 1000 µm.

Figure 3: Outgrowing glomerular cells show expression of parietal epithelial cell marker. Immunofluorescence staining was performed at day 6 after isolation of single encapsulated glomeruli to characterize the outgrowing epithelial cells. Outgrowing cells stained positive for parietal epithelial cell marker (**A**) CD44, (**B**) SSeCkS, and (**C**) claudin-1, but did not show expression of (**D**) the podocyte specific marker synaptopodin or (**E**) the endothelial cell specific marker CD31, which were exclusively localized inside the glomerulus. Scale bars: (**A,B,D,E**) 100 µm, (**C**) 50 µm.

Figure 4: Glomerular outgrowth is impaired in glomeruli isolated from CD44 knockout mice. Encapsulated glomeruli were isolated from dissected kidneys of (**A**) WT mice and (**B**) CD44-/- mice. Microscopic pictures were taken after 6 days in culture using a digital inverted light microscope. The number of outgrowing parietal epithelial cells as well as the surface area of outgrowth was increased in the glomeruli from WT mice compared to CD44-/- mice suggesting an important role for CD44 in parietal epithelial cell activation. Scale bars: 1000 µm.

Figure 5: An example of the analysis of the surface area of the glomerular outgrowth as a marker for parietal epithelial cell proliferation using ImageJ (FIJI). (**A**) Glomerular outgrowth of an encapsulated glomerulus of a WT mouse after 6 days in culture at 37 °C. (**B**) First, the scale is determined to analyze the surface area in mm^2. Here: 1 mm = 460 pixel. (**C**) After setting the scale, a selection line is drawn around the area of glomerular outgrowth. (**D**) This selected area can then be measured (surface area in this example = 2.235 mm^2). Scale bars: 1000 µm.

DISCUSSION:

Using the protocol described in this article, one can use single encapsulated glomeruli to evaluate parietal epithelial cell proliferation which is a consequence of parietal epithelial cell activation. This ex vivo model will enable us to study in detail the molecular pathways, which are involved in parietal epithelial cell activation. The described method relies on the simple concept of kidney dissection and sieving to isolate and culture encapsulated glomeruli and to compare proliferation and/or migration of parietal epithelial cells under different experimental conditions. The outcomes that can be analyzed after 6 days in culture are, for instance, the surface area or diameter of the outgrowth or the number of outgrowing cells of a single capsulated glomerulus. Another application for this assay could be to study the effects of drugs that induce or inhibit molecular pathways that may be involved in parietal epithelial cell activation.

Immunofluorescence staining confirmed that the outgrowing cells after 6 days in culture are parietal epithelial cells as they stained positive for the parietal epithelial cell markers (CD44, claudin-1, SSeCKS) but did not express the podocyte-specific marker synaptopodin, nor the endothelial cell marker CD31. In line with the results of the staining, no outgrowing cells could be observed 6 days after isolation and culture of single decapsulated glomeruli, indicating that there is limited contamination of other glomerular cells in the cell outgrowth within this 6-day period. Another study also analyzed glomerular outgrowth and showed that the fast proliferating

cells derived from the glomerular outgrowth are indeed descendent from parietal epithelial cells[14].

The staining protocol that was used to analyze the marker expression of the outgrowing cells can also be adapted to test other molecules of interest. The immunostaining was performed inside the wells of the plates in which glomeruli were incubated for 6 days. These wells were not coated but glomeruli attached to the wells during the first 2−3 h incubation. Incubation on glass inserts or a chamber slide system which would result in better imaging was not possible as glomeruli did not attach completely to the surface and glomerular outgrowth was impaired. This specific protocol was set up recently to study the parietal epithelia cell outgrowth from glomeruli of healthy WT and cd44-/-mice[11]. Using this method, it was shown that *cd44*-deficient parietal epithelial cells show a decreased proliferation rate, which is also demonstrated in **Figure 4**. This method can also be used for mice of other strains and also for other genetically altered mice. In a previous study for instance, a comparable approach was used to analyze the effects of glucocorticoid receptor signaling[15].

The use of this technique to isolate glomeruli from mice and analyze the cellular outgrowths has many advantages towards the use of immortalized parietal epithelial cell lines for the analysis of pathways involved in parietal epithelial cell activation or drugs that could influence the process of epithelial cell proliferation. First, in this method, primary cells are used which directly grow out of the glomerulus and are only 6 days in culture. Therefore, the parietal epithelial cells from glomerular outgrowths underwent fewer changes in phenotype compared to immortalized cell lines, which need additional growth passages to create the cell line[16]. Furthermore, the method described here can be used to compare the effect of specific gene knockout on parietal cell proliferation also for pathways that are difficult to knock out in cell lines because of impaired cell growth or efficiency of gene knockout using silencing methods.

To adapt the protocol to other animal models or to human kidney tissue, the size of the sieves should be optimized to obtain the best result. This is because the glomerular size differs between species and therefore the size of the sieves on which the glomeruli can be collected varies. Also, it is important to isolate intact encapsulated glomeruli for the purpose of this method. Therefore, the glomeruli should not be pressed but gently rinsed through the smaller sieves.

Another critical step in the protocol is the collection of the encapsulated glomeruli after sieving. Here, it is important to use medium with 20% FCS to avoid attachment of glomeruli to each other. In addition, the solution enriched with glomeruli should be directly transferred to ultra-low adhesion plates because, otherwise, glomeruli will directly attach to the surface of regular cell culture plates and even to the surface of plastic tubes which makes it difficult to capture and isolate single glomeruli.

After collection of single encapsulated glomeruli, these should be cultured 3 h in a small volume of culturing medium at the center of the well to allow adherence. Floating of the glomeruli towards the boarder of the wells should be avoided to optimize the read-out during image analysis.

To obtain the best results using the protocol described here, we would recommend to culture single glomeruli and perform the read-out at day 6. At this time point, a homogenous cellular outgrowth consisting of parietal epithelial cells can be observed. At later time points, glomerular outgrowth becomes phenotypically heterogenous indicating outgrowth of other cell types. Therefore, the protocol does not seem to be suitable for very long incubation times. One should keep in mind that incubation times for parietal epithelial cell outgrowth could vary between species or between different mouse strains. Therefore, culture times should be tested and optimized for each mouse strain or species. In addition, the origin of glomerular outgrowth should always be validated by staining for parietal epithelial cell specific markers.

ACKNOWLEDGMENTS:

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DISCLOSURES:

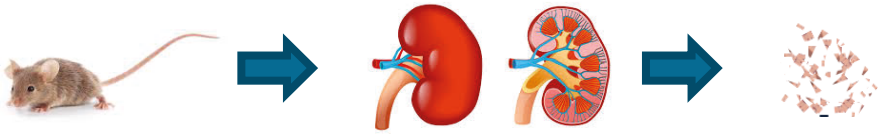
The authors have nothing to disclose.

REFERENCES:

1. Fatima, H. et al. Parietal epithelial cell activation marker in early recurrence of FSGS in the transplant. *Clinical journal of the American Society of Nephrology: CJASN.* **7** (11), 1852-1858 (2012).
2. Dijkman, H. B. et al. Proliferating cells in HIV and pamidronate-associated collapsing focal segmental glomerulosclerosis are parietal epithelial cells. *Kidney International.* **70** (2), 338-344 (2006).
3. Kuppe, C. et al. Common histological patterns in glomerular epithelial cells in secondary focal segmental glomerulosclerosis. *Kidney International.* **88** (5), 990-998 (2015).
4. Dijkman, H., Smeets, B., van der Laak, J., Steenbergen, E., Wetzels, J. The parietal epithelial cell is crucially involved in human idiopathic focal segmental glomerulosclerosis. *Kidney International.* **68** (4), 1562-1572 (2005).
5. Smeets, B. et al. Parietal epithelial cells participate in the formation of sclerotic lesions in focal segmental glomerulosclerosis. *Journal of the American Society of Nephrology: JASN.* **22** (7), 1262-1274 (2011).
6. Smeets, B. et al. Tracing the origin of glomerular extracapillary lesions from parietal epithelial cells. *Journal of the American Society of Nephrology: JASN.* **20** (12), 2604-2615 (2009).
7. Smeets, B., Moeller, M. J. Parietal epithelial cells and podocytes in glomerular diseases. *Seminars in Nephrology.* **32** (4), 357-367 (2012).
8. Ryu, M. et al. Plasma leakage through glomerular basement membrane ruptures triggers the proliferation of parietal epithelial cells and crescent formation in non-inflammatory glomerular injury. *The Journal of Pathology.* **228** (4), 482-494 (2012).
9. Eymael, J., Smeets, B. Origin and fate of the regenerating cells of the kidney. *European Journal of Pharmacology.* **790**, 62-73 (2016).
10. Smeets, B. et al. Renal progenitor cells contribute to hyperplastic lesions of podocytopathies and crescentic glomerulonephritis. *Journal of the American Society of Nephrology: JASN.* **20** (12),

2593-2603 (2009).
11. Eymael, J. et al. CD44 is required for the pathogenesis of experimental crescentic glomerulonephritis and collapsing focal segmental glomerulosclerosis. *Kidney International.* **93** (3), 626-642 (2018).
12. Roeder, S. S. et al. Activated ERK1/2 increases CD44 in glomerular parietal epithelial cells leading to matrix expansion. *Kidney International.* **91** (4), 896-913 (2017).
13. Appel, D. et al. Recruitment of podocytes from glomerular parietal epithelial cells. *Journal of the American Society of Nephrology: JASN.* **20** (2), 333-343 (2009).
14. Yaoita, E. et al. Visceral epithelial cells in rat glomerular cell culture. *European Journal of Cell Biology.* **67** (2), 136-144 (1995).
15. Kuppe, C. et al. Investigations of Glucocorticoid Action in GN. *Journal of the American Society of Nephrology: JASN.* **28** (5), 1408-1420 (2017).
16. Ohse, T. et al. Establishment of conditionally immortalized mouse glomerular parietal epithelial cells in culture. *Journal of the American Society of Nephrology: JASN.* **19** (10), 1879-1890 (2008).

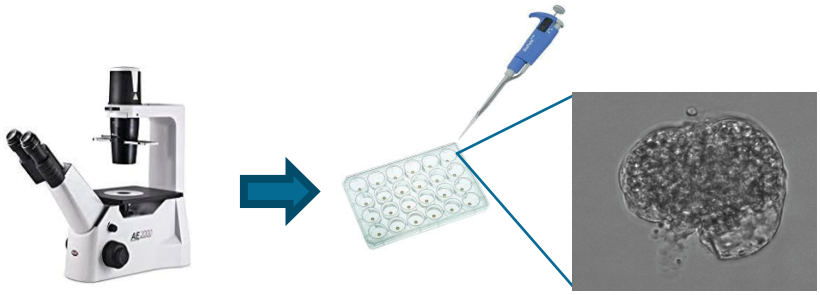
Figure 1



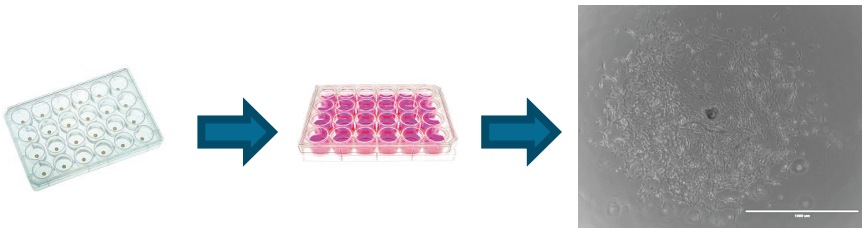
1. Dissect kidneys and mince with a scalpel. Directly put on ice.

2. Press through 300 µm sieve. Rinse through 75 and 53 µm sieves.

3. Collect glomeruli and transfer to ultra-low attachment plate.



4. Capture single capsulated glomeruli, transfer to 24-well plate.



5. Incubate 3 h in 20 µL, add medium. Incubate 6 days at 37 °C.

Figure

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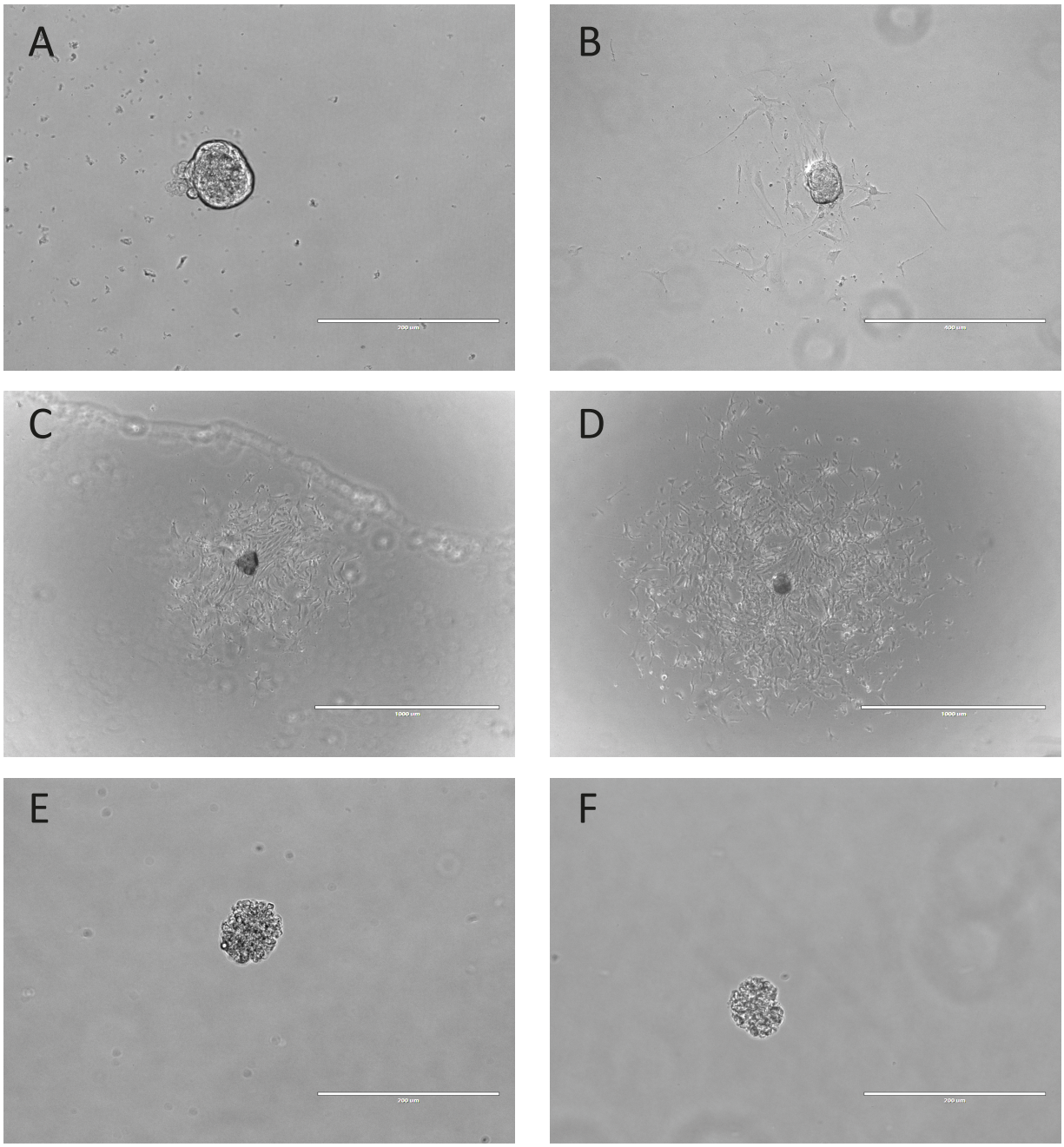

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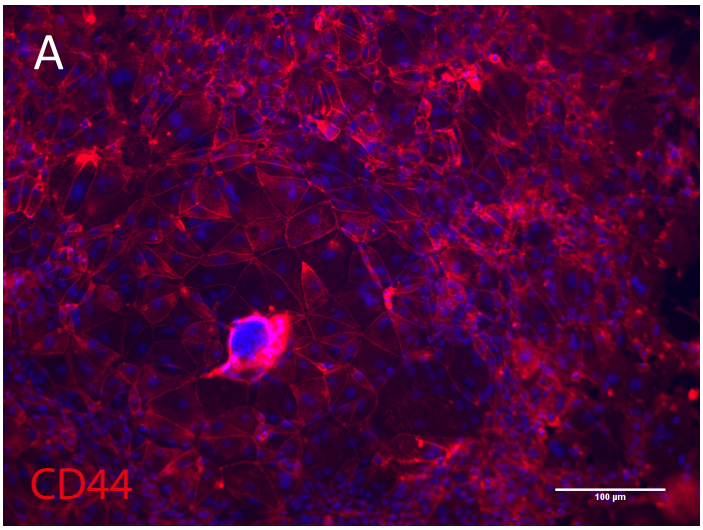

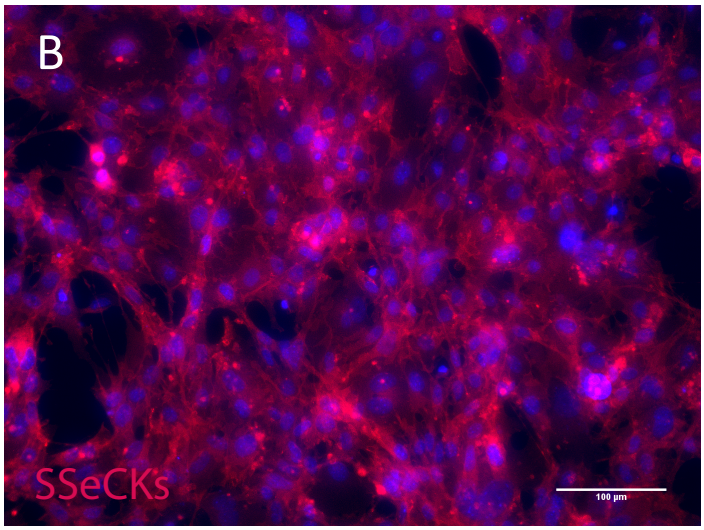

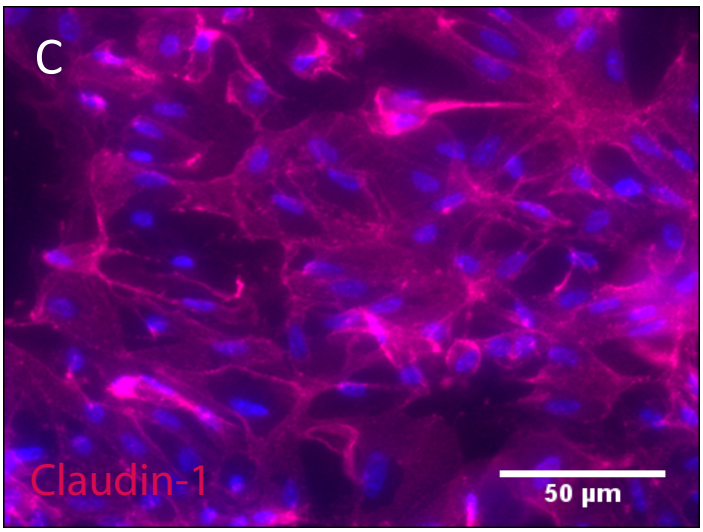

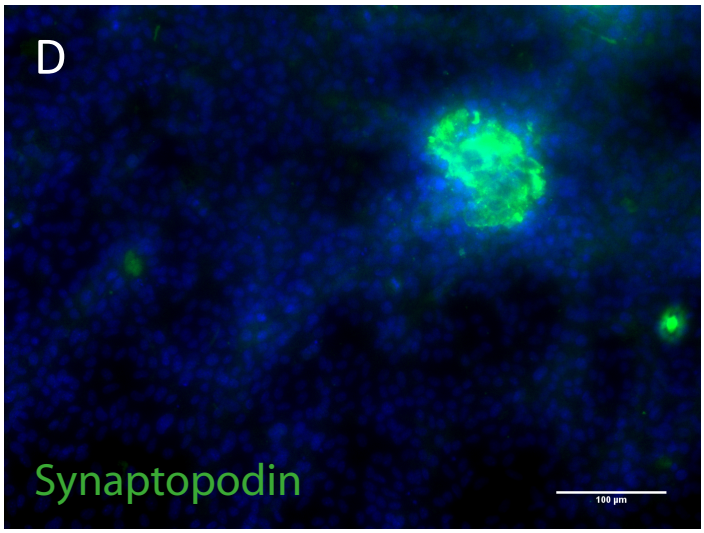

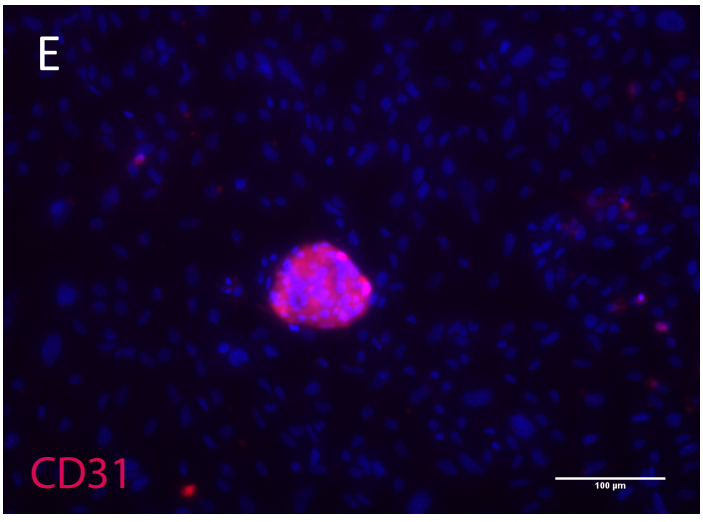

Figure

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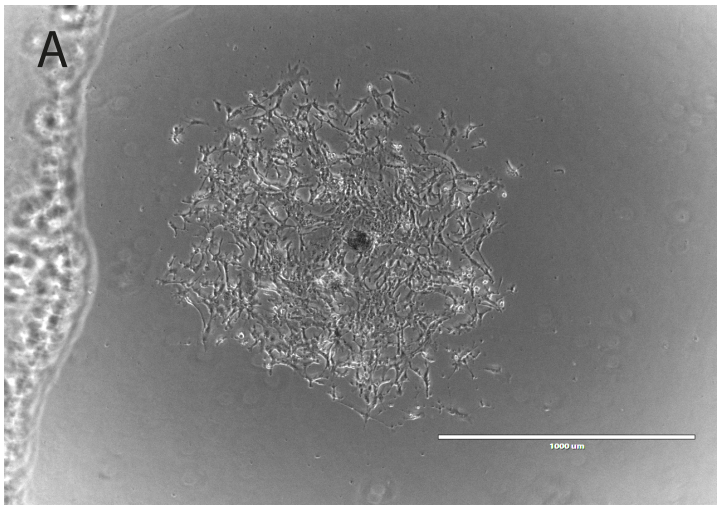
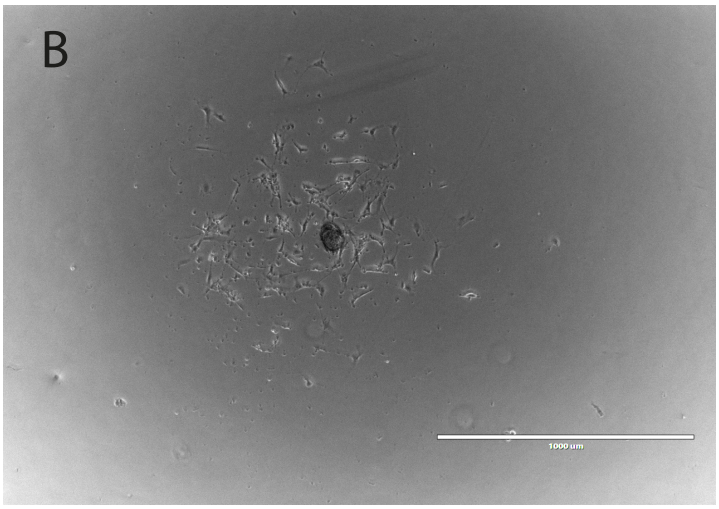
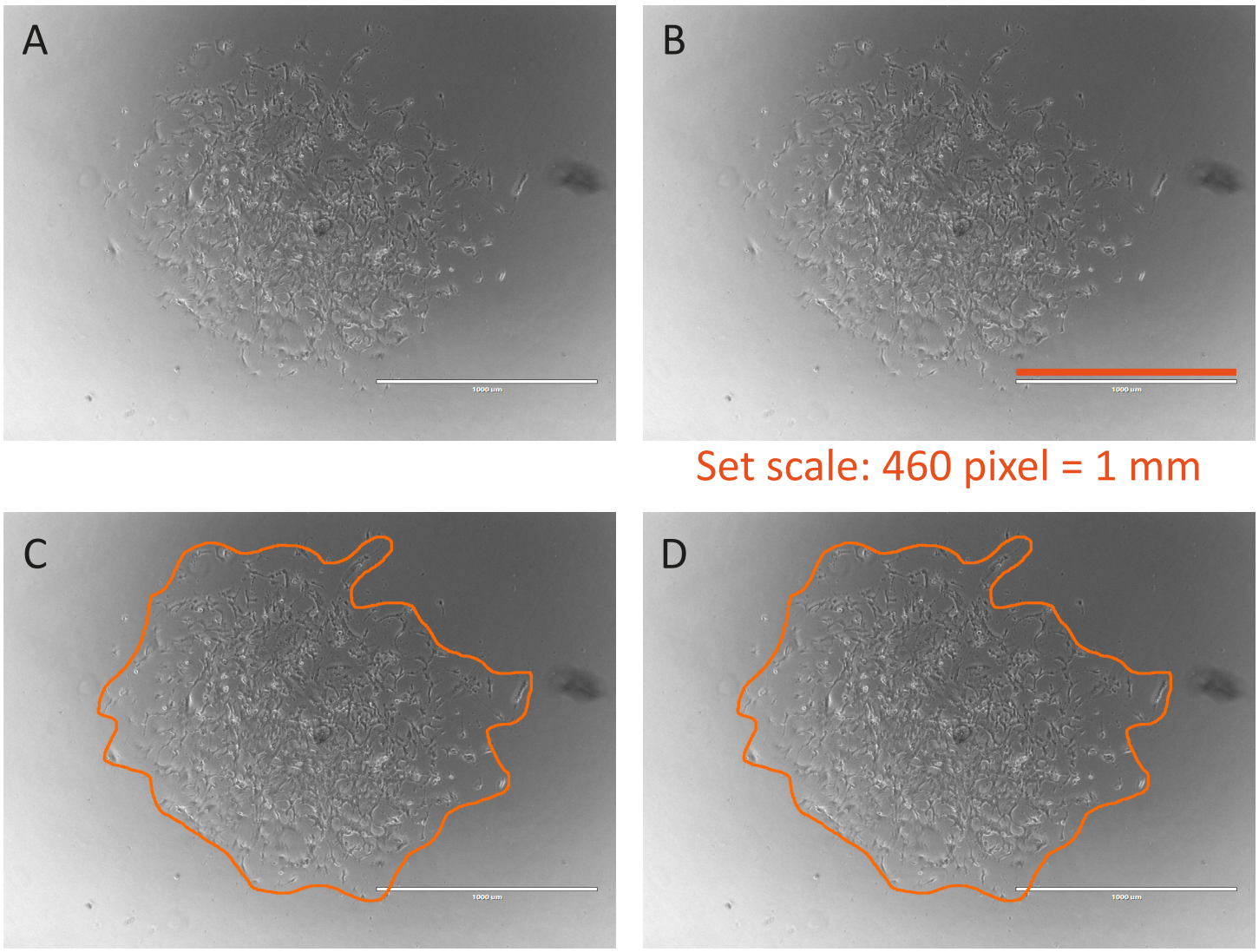




Figure

Click here to access/download;Figure;JOVE figure 5.ai



Name of Material/Equipment	Company
24-well cell culture plate	Corning Costar
anti-CD31	BD Pharmingen
chicken-anti-rat Alexa 647	Thermo Fisher
DAPI-Fluoromount G	Southern Biotech
Digital inverted light microscope	Westburg, EVOS fl microscope
donkey-anti-goat Alexa 568	Thermo Fisher
donkey-anti-rabbit Alexa 568	Thermo Fisher
Dulbecco's Modified Eagle's medium	Lonza
EBM Medium	Lonza
EBM-MV Single Quots kit	Lonza
Fetal Bovine Serum	Lonza
Fetal Calf Serum	Lonza
Fluorescent microscope	Leica Microsystems GmbH
goat-anti-synaptopodin	Santa Cruz
Hanks'Balanced Salt Solution	Gibco
ImageJ software	FIJI 1.51n
petri dish	Sarstedt
rabbit-anti-claudin1	Abcam
rabbit-anti-SSeCKs	Roswell Park Comprehensive Cancer Center,Buffalo, NY, USA
rat-anti-CD44	BD Pharmingen
scalpel	Dahlhausen
Sieves	Endecotts Ltd
syringe	BD Plastipak
Ultra-Low Attachment Microplates	Corning Costar

Comments/Description
Endothelial cell marker (used concentration 1:200)
(used concentration 1:200)
Mounting medium containing DAPI
(used concentration 1:200)
(used concentration 1:200)
containing hydrocortisone, hEGF, GA-1000, FBS and BBE
Podocyte marker (used concentration 1:200)
size 100
Parietal epithelial cell marker (used concentration 1:100)
kindly provided by Dr. E. Gelman, Parietal epithelial cell marker
Parietal epithelial cell marker (used concentration 1:200)
size 10
size 300 µm, 75 µm, 53 µm, steel
size: 20 ml
6-well plates

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Title of Article: Glomerular outgrowth - An ex vivo assay to analyze pathways involved in parietal epithelial cell activation , Author(s): Jennifer Eymael, Laura Miesen, Fieke Mooren, Jitske Jansen, Jack Wetzels, Johan van der Vlag, Bart Smeets

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CORRESPONDING AUTHOR

Name:	BART SMEETS
Department:	PATHOLOGY
Institution:	RADBOUD UMC
Title:	DR.
Signature:	B Smeets
Date:	27/5 2019

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Dear Dr. Smeets,

Your manuscript, JoVE60324 "Glomerular outgrowth - An ex vivo assay to analyze pathways involved in parietal epithelial cell activation," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Jul 15, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

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Editorial comments:

RE. We thank the editors for their comments and positive evaluation of our manuscript. We have addressed all editorial comments and concerns of the reviewers as described in point-to-point rebuttal.

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.

RE: We have proofread the whole manuscript to ensure that there are no spelling or grammar issues.

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

RE: We have added an ethical statement in the protocol (section 1.0). The following text was added: “1.0) All animal experiments were performed according to the guidelines of the Animal Ethics Committee of the Radboud University Nijmegen.”

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: EGM-MV Single Quots, -Fluoromount G, etc.

RE: We have removed any commercial language from the manuscript. We changed EGM MV Single Quots to the following sentence: “500 µl Endothelial Basal Medium (EBM) medium supplemented with a growth factor kit containing hydrocortisone, human endothelial growth factor, bovine brain extract and Gentamicin sulfate-Amphotericin B (see table of materials)”.

Also we have changed ‘Fluoromount G’ into ‘mounting medium with DAPI’.

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

RE: We have checked every step in the protocol whether the “how” question was answered and added additional protocol information whenever necessary (see also answers to 5.-10.).

5. 1.1: Please specify the age and gender of mice used. How many mice are used?

RE: The following text was added to section 1.0: “Untreated, healthy WT mice (n = 4) and cd44–/– (n = 4) mice were sacrificed at the age of 12-16 weeks. Both male and female mice were used. All mice were on the C57Bl/6 background.”

6. Please specify all surgical tools used throughout the protocol.

RE: The following information was added to the surgical procedure of kidney dissection: “1.2) Dissect whole mouse kidneys directly after sacrificing the mice. For this, perform a median laparotomy using abdominal scissors, cutting the skin and then the abdominal muscles. Remove the intestine and place it next to the mouse. Free the kidneys from connecting tissue and pull out the kidney using a surgical forceps, cutting the renal artery, renal vein and ureter with a scissor. 1.3) Remove the renal capsules from the kidneys by holding the kidney with a surgical forceps and pull off the capsule using another forceps.” Also additional information about the surgical instruments used is provided in the table of materials (see also answer to 14.)

7. 1.3: How many kidneys are placed in each well of the plate?

RE: The following text was added to the manuscript (section 1.4): 1.43) Place the kidneys in a 6-well cell culture plate (2 kidneys/well) prepared with 2 ml Hanks’ Balanced Salt Solution (HBSS) per well and place on ice.

8. 2.1: How many kidneys are transferred to one petri dish? How large is the petri dish? How small are the minced tissues?

RE: Additional information was added to the following steps of the protocol: “1.4) Place the kidneys in a 6-well cell culture plate (2 kidneys/well) prepared with 2 ml Hanks’ Balanced Salt Solution (HBSS) per well and place on ice. 2.1) Transfer the kidneys to a 100 mm petri dish and mince the kidneys into small pieces of 1-2 mm using two scalpels. Keep the minced kidney pieces wet using 1-2 ml HBSS.”

9. 4.1: Please use the active/imperative voice.

RE: We have proofread the whole method section and changed 4.1 and 5.1 into the following sentences:” 4.1) Analyze the glomerular outgrowth after 6 days. Make microscopic images using a digital inverted light microscope.” And “5.1) Perform immunofluorescence staining for cell-specific markers on the glomerular outgrowths at t=6 days to assess the cellular composition of the outgrowth.”

10. 4.2: Please describe the analysis step in more detail. For actions involving software usage, please provide all specific details (e.g., button clicks, software commands, any user inputs, etc.) needed to execute the actions.

RE: We agree that additional information about the analysis method should be provided in the protocol. Therefore, we added the necessary steps for analysis of glomerular outgrowth using ImageJ (FIJI) in section 4. Analysis of parietal epithelial cell proliferation. Now, the following steps are described for analysis:" 4.1) Analyze the glomerular outgrowth after 6 days. Make microscopic images using a digital inverted light microscope. 4.2) Use an image analysis software, like ImageJ (FIJI), to determine the surface area and the diameter of the glomerular outgrowth, and the number of outgrowing cells or outgrowing glomeruli. 4.3) To determine the surface area of glomerular outgrowth, open the tif. file of the glomerular outgrowth with scale bar in ImageJ. Draw a straight line on the scale bar and determine the distance in pixels by clicking on analyze and measure (e.g. 1 mm = 460 pixel). 4.4) Determine the scale by clicking on analyze, set scale and type the known distance in pixel (e.g. 460), also type the known distance (e.g. 1) and the unit of length (e.g. 1 mm). Click ok. 4.5) Determine which results will show up in the results table by clicking on analyze and then set measurements. To determine the surface area of glomerular outgrowth, activate the options area and display label. Click ok. 4.6) Determine the surface area of glomerular outgrowth. For this, draw a freehand selection around the glomerular outgrowth. Click on analyze and then measure, a result table will pop up in ImageJ showing the surface area of outgrowth in the earlier determined scale (see 4.4, e.g. mm^2)."

11. Please remove the titles from the uploaded figures.

RE: We have done this in all 5 figures.

12. Figure 1: Please include a space bweteen numbers and their corresponding units (300 µm, 75 µm, 37 °C). However please delete the space between the number and the degree symbol (i.e., change 45 ° to 45°). Please abbreviate liters to L (µL) to avoid confusion.

RE: This was changed in figure 1.

13. Figure 3: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.

RE: Scale bars were added to figure 3 at the lower right corner for all microscopic images. The scale was defined in the appropriate figure legend. Therefore the following was added to figure legend 3: " Scale bars: A-B, D-E) 100 µm, C) 50 µm."

14. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

RE: This was checked and the materials were sorted alphabetically by name. Additional information is provided about ImageJ software, syringes, sieves, petri dish and scalpel in the table of materials.

15. References: Please do not abbreviate journal titles; use full journal name.

RE: This was done using JoVE Style in EndNote. Now, all journal titles in the reference list are written-out.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors presented the methods of primary culture of glomerular parietal cells from the mice.

Major Concerns:

1. The methods of parietal cell culture in the rodent has already been described by Yaoita et al. (Eur J Cell Biol. 1995 Jun;67(2):136-44) and others. The process is similar to it. Author should cite such papers and state advantage of the current method. In addition, reference may be better focusing on the culture technique, not the importance of parietal cell in various animal models.

RE: We agree and have cited this article in the discussion section: Another earlier study also analyzed glomerular outgrowth and showed that the fast proliferating cells derived from the glomerular outgrowth are indeed descendent from the parietal epithelial cells.

2. How many percent of glomeruli forms outgrowth? In our experience, the ratio is not very high. The success may be based on some critical points in this method, including isolation of encapsulated glomeruli under 20% FCS. Did authors try to investigate % of outgrowth under different conditions and confident the condition described here is the best? In this context, authors should provide the background data to recommend the particular condition.

RE: The method works very well with the used condition. Earlier results showed that isolation of glomeruli from wildtype mice resulted in ca. 80% outgrowing glomeruli which was published in our research paper “CD44 is required for the pathogenesis of experimental crescentic glomerulonephritis and collapsing focal segmental glomerulosclerosis (Kidney International (2018) 93, 626–642)” and is a good percentage of outgrowth to work with. Furthermore, in the same study it was shown that glomeruli isolated from CD44-/- mice only showed outgrowth of 30% of the isolated glomeruli. Therefore, the percentage of outgrowing glomeruli highly depends on the genotype and is very likely to also differ between different species and different experimental conditions. Optimal culture conditions should be optimized to answer the specific research questions individually. The protocol described in this method paper tends to give a optimal step by step overview of a practical good working standard protocol which can be used and was validated for the used animal model.

3. The image of immunostaining with CD44, Claudin1 and SSeCKs are unclear, because they are not sheet-like features; may have some artefact during staining process. Better to revise. At least the authors should describe the % of positive cells in each staining and time course expression of these markers. Better to include PAX8 that express in the parietal cell nuclei in vivo. In addition, large magnification of one or two cells that show intracellular localization clearly.

RE: An additional set of mice would be necessary for the collection of glomeruli . Currently, we have no approval of the ethical commission to perform additional glomerular isolation for new experiments. In order to deliver a new set of outgrowths for additional staining would require months, mainly due to the time consuming process of the ethical approval application. Therefore, we were not able to repeat all the necessary animal experiments to perform a PAX8 staining. Indeed parietal epithelial cells show high expression of markers such as PAX8 or PAX2 which would also be interesting to investigate. For this study we have tested 3 other parietal epithelial cell markers which were earlier also successfully used by our group and also described by others. Staining for all 3 markers was performed at day 6 after glomerular isolation and the vast majority of the cells show expression of all 3 parietal epithelial cell markers.

Furthermore, a CD44 immuno-fluorescence staining of isolated glomeruli from wildtype versus CD44 -/- mice was published earlier in our research paper “CD44 is required for the pathogenesis of experimental crescentic glomerulonephritis and collapsing focal segmental glomerulosclerosis (Kidney International (2018) 93, 626–642)”. Here, clearly the glomerular outgrowth of CD44 -/- showed no CD44 expression whereas the vast majority of outgrowing cells of glomeruli isolated from wild type mice showed CD44 expression in locations typical for a receptor staining which can also be observed in the recent staining (figure 3A). This also indicates that no artefact is shown. Also expression of SSecKs (cytoplasmatic) and Claudin-1 can be observed at the typical locations.

Minor Concerns:

1. Please provide efficient use of this technique other than immunolabeling and the merit comparing with immortalized cell line established by Ohse et al. J Am Soc Nephrol. 2008 Oct;19(10):1879-90.

RE: To make the advantages of our method clear, we added some explanation about the advantages of our technique to the discussion section. The use of this technique to isolate glomeruli from mice and analyze the cellular outgrowths has a lot of advantages towards the use of immortalized parietal epithelial cell lines for the analysis of pathways involved in parietal epithelial cell activation or drugs which could influence the process of epithelial cell proliferation. First, in this method, primary cells are used which directly grow out of the glomerulus and are only 6 days in culture. Therefore, the parietal epithelial cells from glomerular outgrowths underwent less changes in phenotype compared to immortalized cell lines, which need additional growth passages to create the cell line.{Ohse, 2008} Furthermore, the method described here, can be used to compare the effect of specific gene knock out on parietal cell proliferation also for pathways which are difficult to knock out in cell lines because of impaired cell growth or efficiency of gene knock out using silencing methods.

Reviewer #2:

Manuscript Summary:
The manuscript „Glomerular outgrowth - An ex vivo assay to analyze pathways involved in parietal epithelial cell activation" by Eymael et al. provides a detailed protocol for the culturing and visualization of parietal epithelial outgrowths from capsulated glomeruli to study PEC activation ex vivo. The steps are described clearly and comprehensively. The inability for glomeruli to adhere to microscopy-compatible plates/slides is a pity. This would have improved the versatility of the method.

Major Concerns:
- Is CD44 present in all outgrowth cells? Has a mesangial cell marker been used to control for outgrowth of mesangial cells?

RE: CD44 is present in the vast majority of outgrowing cells at the specific time point tested in our experimental conditions. Also we have not only tested CD44 as parietal epithelial cell marker but a combination of 3 markers which are expressed by parietal epithelial cells including SSecKs and Claudin-1. As we know so far, all 3 markers are *in vivo* not expressed by mesangial cells. For sure, this may change during in vitro culturing conditions but it seems very unlikely that mesangial cells will show expression of all 3 markers during the time frame of 6 days in culture. Therefore we can be sure that the vast majority of outgrowing cells are indeed parietal epithelial cells at the recommended experimental time points (6 days after glomerular isolation).

Contamination with a small number of other cells in the glomerular outgrowth cannot be completely excluded. Importantly, at later time points, not only parietal epithelial cells can be observed in the glomerular outgrowth but also outgrowth of other cell types as also stated in the discussion of the paper.

- Scale bars sare missing in Figure 3.

RE: Scale bars were added to figure 3 at the lower right corner for all microscopic images. The scale was defined in the appropriate figure legend. Therefore the following was added to figure legend 3: ” Scale bars: A-B, D-E) 100 µm, C) 50 µm.”

- Is this method sensitive to the type of medium used (in regards to numbers of PEC outgrowths)? Can the authors elaborate why PECs are activated by their method? What might the stimulus for PEC migration and activation in in vitro culture?

RE: The method works very well with the used medium. Therefore, it was not necessary to test other media, so we don’t know if better results would be obtained in another medium. Furthermore, isolation of glomeruli from wildtype mice resulted in ca. 80% outgrowing glomeruli which was published earlier in our research paper “CD44 is required for the pathogenesis of experimental crescentic glomerulonephritis and collapsing focal segmental glomerulosclerosis (Kidney International (2018) 93, 626–642)” and is a good percentage of outgrowth to work with.“

It is indeed striking that PECs in culture seem to be activated based on their CD44 expression. Normally, PEC proliferation and migration is limited due to contact inhibition signaling. However, In the early outgrowth these cells can proliferate and migrate. CD44 is important for cell migration and can stimulate cell proliferation. Therefore, CD44 may be necessary for PECs to migrate from the glomerulus into the culture flask. In fact CD44 deficient PECs show a limited potential to form glomerular outgrowths. Nevertheless, It would be interesting to find the culture conditions in which PECs show less CD44 expression as PECs in vivo.

Minor Concerns:
- Add sieves to materials list

RE: ”Sieves were added to the materials list.”

- Which age of the mice has been used/tested for PEC outgrowth? (in mice age 7 days and less podocytes are also able to do outgrowths)

RE: The following text was added to step 1.0: “Untreated, healthy WT mice (n = 4) and cd44–/– (n = 4) mice were sacrificed at the age of 12-16 weeks. Both male and female mice were used. All mice were on the C57Bl/6 background. “

- Is kidney perfusion with PBS necessary or would it be helpful?

RE: We have not tested kidney perfusion with PBS for this method. It is in our experience not necessary. We don't think that perfusion with PBS would influence the glomerular outgrowth or the method of glomerulus isolation because kidneys are minced into small pieces and washed with HBSS directly after kidney dissection from sacrificed mice.

- What is the approximate volume of HBSS rinsing solution used (total or per step)

RE: Additional information was added to step 2.3 of the protocol: " Rinse the kidney homogenate through a 75 µm sieve with HBSS. Collect the flow-through and subsequently rinse this flow-through through a 53 µm sieve. Wash both sieves using HBSS to remove all smaller structures. Note: In this step the flow-though is only rinsed and NOT pressed through the 75 µm and 53 µm sieve. Washing with HBSS is necessary to remove debris and smaller structures on the sieves. Therefore normally 200-300 ml HBSS are used in total. "

- Is the flowthrough poured directly from the Petri dish or with a serological pipette?

RE: This information was added to step 2.2. of the method section.