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Title: Guided Differentiation of Mature Kidney Podocytes from Human Induced Pluripotent Stem Cells Under Chemically Defined Conditions

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y, EVOS XL CORE with Mechanical Stage**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **46**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Samira Musah**: Protocols for the differentiation of stem cells into functional kidney cells remain elusive. This method produces mature podocytes with specificity and efficiency, providing new tools for studying kidney disease mechanisms **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Rohan Bhattacharya**: This method employs cell culture medium and extracellular matrix proteins with a chemically defined composition to produce human iPS cell-derived podocytes with a high purity without using subpopulation selection or genetic manipulation **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Samira Musah:** There are limited therapeutic options for patients with kidney disease. This method has implications for modeling and understanding disease mechanisms, which could facilitate the development of novel biomarkers and therapeutics **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

NOTE: The authors have uploaded the protocol footage to the FTP server.

2. Feeder-Free Human Induced Pluripotent Stem Cell (hiPSC) Culture

2.1. To set up a feeder-free human iPSC culture, aspirate the residual solution of basement membrane matrix 1 from pre-coated plates **[1]** and wash the wells three times with 1-2 milliliters per well of warmed basal medium **[2-TXT]**.

2.1.1. WIDE: Talent aspirating matrix
2.1.2. Talent washing well(s), with medium container visible in frame **TEXT: See text for all medium preparation details**

2.2. Aspirate the spent cell culture medium from a human iPSC culture **[1]** and rinse the cells three times with warmed medium **[2]**.

2.2.1. Medium being aspirated
2.2.2. Well(s) being rinsed, with medium container visible in frame

2.3. Add 1 milliliter of warm cell detachment solution to each well for a 1-minute incubation at 37 degrees Celsius **[1]**.

2.3.1. Talent adding solution to well(s), with solution container visible in frame

2.4. After confirming rounded cell colony edges under a tissue culture microscope **[1]**, quickly aspirate the cell detachment solution **[2]** and gently rinse the cells with cell culture medium **[3]**.

2.4.1. SCOPE: Shot of rounded cell colony edges
2.4.2. Solution being aspirated
2.4.3. Well(s) being rinsed, with medium container visible in frame

2.5. Add 3 millimeters of human iPSC culture medium to the human induced pluripotent stem cells **[1]** and use a cell lifter to scrape the colonies **[2]**.

2.5.1. Talent adding medium to well(s), with medium container visible in frame
2.5.2. Colonies being scraped

2.6. Gently pipette the cell suspension to dislodge any loosely adhered cells **[1]** and wash the plate thoroughly to ensure harvesting of all of the cells **[2]**.

2.6.1. Well being pipetted
2.6.2. Talent washing well

2.7. Transfer 500 microliters of cells into each well of a new basement membrane matrix 1-coated 6-well plate containing 2 milliliters of human induced pluripotent stem cells per well **[1]** and move the plate in figure eight fashion to evenly distribute the cell colonies within the wells **[2]**.

2.7.1. Talent adding cells to well(s)
2.7.2. Plate being moved in figure 8

2.8. Place the plate in the cell culture incubator **[1]**, refreshing the medium daily until the cells reach approximately 70% confluency **[2]**.

2.8.1. Talent plate into incubator
2.8.2. Talent adding medium to well(s), with medium container visible in frame

3. hiPSC Differentiation into Mesoderm Cells (Days 0-2)

3.1. When the human induced pluripotent cell cultures reach the exponential growth phase, visually inspect the approximately 70% confluent culture **[1]** for the presence of spontaneously differentiated cells within and around the edges of the colonies **[2]**. **NOTE: Not filmed**

3.1.1. WIDE: Talent at microscope, inspecting culture
3.1.2. SCOPE: Shot of differentiated cells around edges culture *Videographer: Important step*

3.2. If necessary, aseptically scrape-off areas of differentiation **[1]** before aspirating the supernatant from the wells **[2]**. **NOTE: Not filmed**

3.2.1. Talent scraping well *Videographer: Important step*
3.2.2. Supernatant being aspirated *Videographer: Important step*

3.3. After washing the wells three times, dissociate the cells as demonstrated **[1]** and pool the resulting cell suspensions in a 15-milliliter conical tube **[2]**.

3.3.1. Talent adding dissociation solution to well(s), with solution container visible in frame
3.3.2. Talent adding cells to tube

3.4. After mixing by pipetting, bring the final volume in the tube to 15 milliliters with warm medium **[1]** and collect the cells by centrifugation **[2-TXT]**.

3.4.1. Talent adding medium to tube, with medium container visible in frame
3.4.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 290 x g, RT**

3.5. Resuspend the pellet in fresh medium for a second centrifugation **[1]** and resuspend the cells in 1 milliliter of mesoderm induction medium for counting **[2]**.

3.5.1. Shot of pellet if visible, then medium being added to cells, with medium container visible in frame
3.5.2. Talent adding medium to tube, with medium container visible in frame

3.6. After counting, resuspend the cells to a 1 x 10^5 cells/milliliter of mesoderm induction medium concentration **[1]** and aspirate the extracellular matrix solution from the basement membrane matrix 2-coated plates **[2]**.

3.6.1. Talent counting cells
3.6.2. Talent aspirating medium

3.7. Rinse the plates two times with warm medium **[1]** and mix the human induced pluripotent stem cell suspension with gentle pipetting **[2]**.

3.7.1. Talent rinsing well(s), with medium container visible in frame
3.7.2. Talent pipetting cells

3.8. Add 1 milliliter of cells to each well of the basement membrane matrix 2-coated 12-well plates **[1]** and gently shake the plates to distribute the cells more evenly **[2]**.

3.8.1. Talent adding cells to well(s)
3.8.2. Plate being gently shaken

3.9. Then place the plate into the cell culture incubator **[1-TXT]**.

3.9.1. Talent placing plate into incubator **TEXT: Refresh medium after 24 h**

4. hiPSC-Derived Mesoderm Cell Differentiation (Days 2-15 and 16-21)

4.1. On days 2 to 15 the differentiation, replace the mesoderm induction medium with 1 milliliter of intermediate mesoderm induction medium per well **[1]**.

4.1.1. WIDE: Talent adding medium to well(s), with medium container visible in frame

4.2. If substantial cell growth and a rapid depletion of nutrients is observed, as indicated by yellowing of the medium **[1]**, the volume of the intermediate mesoderm differentiation medium can be increased to 1.3 milliliters per well **[2-TXT]**.

4.2.1. Shot of yellow medium *Videographer: Difficult step*
4.2.2. Talent adding medium to well(s), with medium container visible in frame *Videographer: Difficult step* **TEXT: Alternative: Passage or split cells into new plates**

4.3. On day 16 of culture, rinse the intermediate mesoderm cells with warm medium **[1]** and incubate the cells with 500 microliters of 0.05 % trypsin-EDTA per well for 3 minutes at 37 degrees Celsius **[2]**.

4.3.1. Talent rinsing well(s), with medium container visible in frame *Videographer: Difficult step*
4.3.2. Talent adding trypsin-EDTA to well(s), with trypsin-EDTA container visible in frame *Videographer: Difficult step*

4.4. When the cells begin to dissociate, scrape the cells with a cell lifter **[1]** and gently mix the cells by pipetting **[2]**.

4.4.1. Cells being scraped *Videographer: Important step*
4.4.2. Cells being mixed *Videographer: Important step*

4.5. Stop the reaction with about 2 milliliters of trypsin neutralizing solution per well **[1]** and transfer the cells to a 50-milliliter conical tube **[2]**.

4.5.1. Talent adding solution to tube, with solution container visible in frame
4.5.2. Talent adding cells to tube

4.6. Bring the volume up to 50 milliliters with medium **[1]** and collect the cells by centrifugation **[2-TXT]**.

4.6.1. Talent adding medium to tube, with medium container visible in frame
4.6.2. Talent adding tube(s) to centrifuge **TEXT: 5 min, 201 x g, RT**

4.7. Resuspend the pellet in podocyte induction medium at a 1 x 10^5 cells/milliliter of medium concentration **[1]** and add the cells to basement membrane matrix 2-coated plates **[2]**.

4.7.1. Talent adding medium to tube, with medium container visible in frame
4.7.2. Talent adding cells to well(s)

4.8. Then gently shake the plate to help distribute the cells more evenly **[1]** and place the cells into the incubator for up to 5 days **[2-TXT]**.

4.8.1. Plate being gently shaken *Videographer: Important step*

4.8.2. Talent placing plate into cell culture incubator *Videographer: Important step*
TEXT: Refresh medium daily

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
3.1., 3.2., 4.4., 4.8.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.2., 4.3. The most challenging part of this procedure is maintaining the health of the IM cells, and preventing their overgrowth. This can be accomplished by closely monitoring the media color and splitting the cells if they become too confluent.

Results

5. Results: Representative Mature Kidney Podocyte Differentiation Characterization

5.1. Using the culture strategy as demonstrated **[1]**, the stem cells can be first differentiated into mesoderm cell that express Brachyury **[2]**, followed by differentiation into PAX2 (packs-two)-positive intermediate mesoderm cells **[3]**, and eventually mature kidney glomerular podocytes **[4]**.

5.1.1. LAB MEDIA: Figure 1B *Video Editor: please sequentially add/emphasize images from Day 0 to Day 22*
5.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2B images*
5.1.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2C images*
5.1.4. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2D images*

5.2. Stem cell-derived podocytes stain positive for WT1 (W-T-one) **[1-TXT]** as well as for the lineage identification marker nephrin **[2]**.

5.2.1. LAB MEDIA: Figure 2D *Video Editor: please emphasize red signal in WT1 image*
TEXT: WT1: Wilm's tumor 1
5.2.2. LAB MEDIA: Figure 2D *Video Editor: please emphasize red signal in Nephrin image*

5.3. Intriguingly, the subcellular localization of nephrin is predominantly within the podocyte foot processes and cell cytoplasm **[1]**, consistent with a mature podocyte phenotype **[2]**.

5.3.1. LAB MEDIA: Figure 4 *Video Editor: please add/emphasize arrows in Figure 4B*
5.3.2. LAB MEDIA: Figure 4

5.4. Cells seeded at densities that significantly exceed the recommended density of 1 x 10^5 cells/well of a 12 well plate **[1]** result in large clusters of cells that lack the expected morphological phenotype of mature podocytes within the standard timeline of the protocol **[2]**.

5.4.1. LAB MEDIA: Figure 3A
5.4.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize Cell Aggregate and/or add text and arrow*

5.5. Seeding at the densities recommended in this protocol **[1]**, however, result in cultures of cells with the desired morphology **[2]**.

5.5.1. LAB MEDIA: Figure 3C
5.5.2. LAB MEDIA: Figure 3D

Conclusion

6. Conclusion Interview Statements

6.1. **Rohan Bhattacharya**: We recommend using the appropriate basement membrane protein for cell attachment and maintaining the correct cell seeding density for mesoderm and podocyte induction **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.6., 3.8., 5.5.)

6.2. **Samira Musah**: This podocyte differentiation method can be integrated with organ-on-chip microfluidic systems or 3D bioprinting technologies to develop functional models of the human kidney for drug screening and nephrotoxicity testing **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.3. **Morgan Burt**: The lack of appropriate cell culture models has impeded progress in understanding kidney disease mechanisms. This protocol provides researchers with an inexhaustible source of patient-derived podocytes for disease modeling **[1]**.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera