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Guided differentiation of mature kidney podocytes from human induced pluripotent stem cells under chemically defined conditions

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May 13th, 2020

Dear Dr. Bajaj and Dr. Singh,

Enclosed please find the revised version of our manuscript (JoVE61299R1) entitled, "Guided differentiation of mature kidney podocytes from human induced pluripotent stem cells under chemically defined conditions", which we would like you to reconsider for publication as a Video Research Article in *JoVE*. Thank you for giving us the opportunity to respond to the concerns raised by the reviewers.

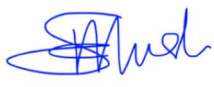
I believe that the prime revisions requested by your editorial team included the word count for our short abstract, the need for additional information for the DU11 human induced pluripotent stem cell line used in the study, the use of commercial language (such as Matrigel and Laminin) in the protocol, and a request to highlight 2.75 pages that identifies the essential steps of the protocol for filming. In our revised version of the manuscript, we have addressed the editorial comments as requested. These revisions include edits to reduce the word limit in the short abstract and ensuring that the long abstract is still within the specified word count; clarification that the DU11 stem cell line was previously derived at Duke University's Stem Cell Core Facility and citing relevant publication on their characterization and use to derive other organ-specific cell types including cardiomyocytes. We have also included a note clarify that our lab routinely tests the cells for mycoplasma (which was found to be negative) and our most recent karyotype analysis confirmed that the DU11 cells were normal. Additionally, we have removed commercial languages and provided relevant information in the Table of Materials per Dr. Bajaj's suggestions during our follow-up emails for clarification on this request. Furthermore, we have highlighted in the revised version of the manuscript the filmable content of the protocol as requested by the JoVE editorial team.

In regards to the more general intimation by Reviewer #3 that other reports that attempted to derive kidney cells from pluripotent stem cells using embryoid body and organoid approaches could have possibly generated podocytes under chemically defined conditions or with high purity (without subpopulation selection) has no basis given that these methods require the use of undefined animal-derived serum and by definition, organoids and embryoid bodies generate highly heterogenous populations that will also include non-kidney cell types (e.g.: skin cells, neurons, and other unidentified cells), thus producing a low yield of the desired cell types and necessitating subpopulation selection or sorting – which typically compromises the viability of the cells. The podocyte differentiation media we created in our protocol were formulated in our lab and are currently not available commercially. The term "chemically-defined" is used to indicate serum-free conditions and the fact that the composition, identity, and concentrations of all the chemicals used are known, such that readers of this manuscript who are interested in adapting the protocol will be able to create their own medium by following the step-wise directions provided in this protocol. The chemical components include soluble and insoluble (adhesion) proteins and small molecules, as well as the E8 fragment of Laminin 511 used as cell culture matrix. To the best of our knowledge, our method for the derivation of podocytes is the first in vitro stem cell differentiation method to produce podocytes with characteristics of the mature and functional phenotype with high yield (>90% yield) without subpopulation selection, genetic manipulation, or need for xenotransplantation to induce lineage specification.

In any case, in response to the reviewer concerns, we now clarify these key points that explain the differences and the limitations of the other differentiation approaches when trying to produce a more specific and specialized cell type such as kidney glomerular podocytes with specificity and efficiency. We have revised the Discussion section to place our work more clearly in an appropriate context.

Given the overall positive responses from most of the reviewers, the importance of these findings in illuminating the biology and mechanisms of human kidney tissue development, as well as providing new tools for nephrotoxicity testing, disease modeling, and drug screening, we hope that you'll be able to make a final decision regarding the publication of this article. To help expedite your review, we have tracked and underlined all changes to the manuscript in the revised version submitted.

Sincerely,



Samira Musah, Ph.D.

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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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KEYWORDS:

human iPS cells, stem cell differentiation, stem-cell derived podocytes, podocyte foot processes, kidney disease modeling, functional human kidney cells, nephrotoxicity, stem cells, extracellular matrix

SUMMARY:

Presented here is a chemically defined protocol for the derivation of human kidney podocytes from induced pluripotent stem cells with high efficiency (>90%) and independent of genetic manipulations or subpopulation selection. This protocol produces the desired cell type within 26 days and could be useful for nephrotoxicity testing and disease modeling.

ABSTRACT:

Kidney disease affects more than 10% of the global population and costs billions of dollars in federal expenditures. The most severe forms of kidney disease and eventual end-stage renal failure are often caused by the damage to the glomerular podocytes, which are the highly specialized epithelial cells that function together with endothelial cells and the glomerular basement membrane to form the kidney's filtration barrier. Advances in renal medicine have been hindered by the limited availability of primary tissues and the lack of robust methods for the derivation of functional human kidney cells, such as podocytes. The ability to derive podocytes from renewable sources, such as stem cells, could help advance current understanding of the mechanisms of human kidney development and disease, as well as provide new tools for therapeutic discovery. The goal of this protocol was to develop a method to derive mature, post-mitotic podocytes from human induced pluripotent stem (hiPS) cells with high efficiency and specificity, and under chemically defined conditions. The hiPS cell-derived podocytes produced by this method express lineage-specific markers (including nephrin, podocin, and Wilm's Tumor

1) and exhibit the specialized morphological characteristics (including primary and secondary foot processes) associated with mature and functional podocytes. Intriguingly, these specialized features are notably absent in the immortalized podocyte cell line widely used in the field, which suggests that the protocol described herein produces human kidney podocytes that have a developmentally more mature phenotype than the existing podocyte cell lines typically used to study human kidney biology.

INTRODUCTION:

Advances in human pluripotent stem cell culture are poised to revolutionize regenerative medicine, disease modeling, and drug screening by providing researchers with a renewable, scalable source of biological material that can be engineered to obtain almost any cell type within the human body[1]. This strategy is especially useful for deriving specialized and functional cell types that would otherwise be difficult to obtain. Human induced pluripotent stem (hiPS) cells[2-5] are particularly attractive due to their somatic cell origin and the potential they represent for personalized medicine. However, developing methods to derive other cell lineages from hiPS cells remain challenging due to the frequent use of poorly defined culture conditions which leads to low efficiency and non-specific generation of heterogenous cell populations[6,7].

Presented here is a method for the derivation of mature kidney podocytes from hiPS cells with specificity and high efficiency under chemically defined conditions. By considering the roles of multiple factors within the cellular microenvironment, a stem cell differentiation strategy was developed that involved the optimization of soluble factors presented in the cell culture medium as well as insoluble factors, such as extracellular matrix components or adhesive substrates. Given the importance of integrin signaling in podocyte development and function, the expression of integrin receptors on the cell surface was initially examined. β1 integrins were highly expressed not only in hiPS cells, but also in their derivatives including mesoderm and intermediate mesoderm cells[8-10]. Subsequent experiments confirmed that ligands that bind to β1 integrins (including laminin 511 or laminin 511-E8 fragment) support the adhesion and differentiation of hiPS cells into podocytes when used in conjunction with the soluble inductive media described below.

Induction of cell lineage commitment was initiated by first confirming that hiPS cells cultured on the laminin-coated surfaces for two days in the presence of a medium containing Activin A, CHIR99021, and Y27632 Rock inhibitor can differentiate into cells that express the early mesoderm markers HAND1, goosecoid, and brachyury[8,11]. Treatment of the mesoderm cells for 14 days with a medium supplemented with bone morphogenetic protein 7 (BMP-7) and CHIR99021 enabled the derivation of intermediate mesoderm cells that expressed the nephron-progenitor cell markers Wilm's Tumor 1 (WT1), odd-skipped related protein 1 (OSR1)[8,11], and paired box gene 2 protein (PAX2)[12]. To derive the mature kidney glomerular podocytes, the intermediate mesoderm cells were treated for 4–5 days with a novel medium consisting of BMP-7, Activin A, vascular endothelial growth factor (VEGF), all-*trans* retinoic acid, and CHIR99021. Flow cytometry and immunostaining were used to confirm that >90% of the resulting cells exhibited the molecular, morphological, and functional characteristics of the mature kidney podocyte[8,11,13]. These characteristics include the development of primary and secondary foot

processes; the expression of podocyte lineage-specific genes including SYNPO, PODXL, MAF, EFNB2[8] and the expression of proteins including podocin, nephrin, and WT1[14-16]. Additionally, it was found that the hiPS cell-derived podocytes can be maintained in culture for up to four weeks in vitro by using a commercially available medium[8,11] which provides an additional flexibility in the timing of downstream experiments. For more information regarding the flow cytometry panels used for determining the purity of the hiPS-podocytes, please refer to our previous publication[11].

PROTOCOL

1. Preparation of reagents

1.1. Dilute thawed 5x hiPS cell culture media (CCM) supplement in hiPS cell culture basal medium to obtain a 1x solution of hiPS CCM.

NOTE: Frozen 5x hiPS CCM supplement requires a slow thawing process, ideally in 4 ˚C for overnight. Aliquots of the 1x hiPS CCM can be stored for up to 6 months at -20 ˚C.

1.2. Preparation of basement membrane (BM) matrix 1-coated plates for hiPS cell culture: Thaw BM matrix 1 overnight on ice at 4 ˚C. Once thawed, prepare aliquots with appropriate dilution factors as suggested by the manufacturer.

NOTE: Typically, aliquots are prepared for subsequent dilution in 25 mL of cold DMEM/F12 in a 50 mL conical tube followed by thorough mixing to completely dissolve the BM matrix 1 and avoid the formation of residual crystals.

1.3. Transfer 1 mL of the BM matrix 1 solution to each well of a 6 well plate and incubate at 37 ˚C for 1-2 h or at 4 ˚C for a minimum of 24 h, wrapped in paraffin film. The BM matrix 1 -coated plates can be stored at 4 ˚C for up to 2 weeks.

1.4. Preparation of Basement membrane (BM) matrix 2 - coated plates: Dilute appropriate amounts of BM matrix 2 in 9 mL sterile distilled water to prepare a final concentration of 5 µg/mL. Add 700 µL of the BM matrix 2 solution to each well of a 12 well plate and incubate the plate at room temperature for 2 h or overnight at 4 ˚C.

1.5. Prepare 100 µg/mL stock solutions each of BMP7, Activin A, and VEGF as follows: reconstitute BMP7 in sterile distilled water containing 0.1% (wt/vol) BSA and reconstitute Activin A and VEGF separately in sterile PBS containing 0.1% (wt/vol) BSA. To avoid frequent freeze-thaw cycles, prepare 100 µL aliquots from each stock solution and store at -20 ˚C for up to 6 months.

1.6. Prepare a 10 mM stock solution of Y27632 by dissolving 10 mg of Y27632 in 3.079 mL of sterile distilled water. Aliquot 100 µL from the stock and store at -20 ˚C for up to 6 months.

1.7. Dissolve 2 mg of CHIR99021 in 143.4 µL of sterile DMSO to prepare a 30 mM stock solution. Prepare 5 µL aliquots and store at -20 ˚C for up to 1 month (or according to the manufacturer's recommendation).

1.8. Dissolve 10 mg of all-*trans* retinoic acid in 3.33 mL sterile DMSO. Prepare 500 µL aliquots and store at -20 ˚C for up to 6 months.

2. Preparation of culture media

2.1. Prepare the mesoderm differentiation medium by reconstituting corresponding stock solutions to a final concentration of 100 ng/mL Activin A, 3 µM CHIR99021, 10 µM Y27632, and 1x B27 serum-free supplement in an appropriate volume of DMEM/12 with glutamine supplement.

NOTE: The mesoderm differentiation medium should be freshly prepared before differentiation steps and at a volume appropriate for the scale of the experiment (typically, 50 mL of medium is adequate for two 12 well plates).

2.2. Prepare intermediate mesoderm differentiation medium by reconstituting corresponding stock solutions to a final concentration of 100 ng/mL BMP7, 3 µM CHIR99021, and 1x B27 serum-free supplement in DMEM/F12 with a glutamine supplement.

NOTE: If needed, the medium can be supplemented with 1% (vol/vol) Penicillin-Streptomycin. Adjust the volume of the medium by using DMEM/F12 with glutamine supplement. This medium can be prepared in large batches; however, it is recommended to store in smaller aliquots (e.g., 45 mL in 50 mL conical tubes) to avoid repeated freeze-thaw cycles. This media can be stored at -20 ˚C for up to 3 months and can be thawed at 4 ˚C overnight prior to use.

2.3. Prepare the podocyte induction medium by reconstituting to a final concentration 100 ng/mL BMP7, 100 ng/mL activin A, 50 ng/mL VEGF, 3 µM CHIR99021, 1x B27 serum-free supplement, and 0.1 µM all-trans retinoic acid in DMEM/F12 with glutamine supplement. Protect medium from light (e.g., by wrapping container with foil paper).

NOTE: This medium can be prepared in large batches and stored in the dark at -20 ˚C for up to 3 months. Frozen aliquots should be thawed overnight at 4 ˚C prior to use.

2.4. Prepare 25 mL of trypsin neutralizing solution by adding 10% (vol/vol) heat inactivated FBS in DMEM/F12 and filter under sterile conditions.

2.5. For post-differentiation maintenance of the stem cell-derived podocytes, prepare Complete Medium with podocyte maintenance media by adding the supplement to the basal medium as per the manufacturer's guidelines, and store at 4 ˚C for up to two weeks.

3. Feeder-free hiPS cell culture using hiPS cell culture medium

3.1. Aspirate the residual solution of BM matrix 1 from the pre-coated plates and wash the wells 3x with 1 to 2 mL of warmed DMEM/F12.

3.2. Aspirate spent hiPS CCM from the hiPS cells and rinse the cells 3x with warmed DMEM/F12. Add 1 mL of warm cell detachment solution and incubate for 1 min at 37 ˚C to help dissociate the cells. Perform visual inspection of cells under a tissue culture microscope and ensure that the edges of the cell colonies appear rounded, then quickly aspirate the cell detachment solution from the cells (ensuring that the cell colonies are still attached to the plate, albeit loosely). Gently rinse cells once with DMEM/F12 to remove the cell detachment solution.

3.3. Add 3 mL of hiPS CCM to the hiPS cells (in each well of a 6-well plate) that were treated with the cell detachment solution. Scrape colonies by using a cell lifter, and gently pipette cell suspension up and down to dislodge the loosely adhered cells. Wash the plate thoroughly to ensure all the cells are harvested.

NOTE: Use of a 5 mL pipette is recommended to avoid excess shear on the cells.

3.4. Transfer 0.5 mL of the cell suspension into each well of a new BM matrix 1-coated 6-well plate containing 2 mL of hiPS CCM per well. Move the plate in figure-eight fashion to distribute cell colonies within wells and incubate at 37 ˚C in a 5 % CO_2 incubator. Refresh medium daily until the cells are ready to be passaged or used for the differentiation experiment (approximately 70 % confluency).

NOTE: The ideal colony size for routine passaging of iPSCs is between 200-500 µm under feeder free conditions using hiPS CCM (without ROCK inhibitor). If cells are individualized during treatment with dissociation enzymes or buffers, the hiPS CCM can be supplemented with ROCK inhibitor (e.g., 10 µM Y27632) to improve cell viability.

4. Differentiation of hiPS cells into mesoderm cells (days 0-2)

4.1. While the hiPS cell cultures are in the exponential growth phase (approximately within 4 days of culture after passaging, and around 70% confluency), visually inspect for the presence of spontaneously differentiated cells within and around the edges of the colonies. If necessary, aseptically scrape-off areas of differentiation.

4.2. Aspirate hiPS CCM from hiPS cells and rinse the cells 3x with warm DMEM/F12. Incubate the cells with 1 mL of enzyme-free cell dissociation buffer for 10 min at 37 ˚C and check for the dissociation under a microscope. Due to inherent differences between different hiPS cell lines, the actual incubation time for the cell dissociation buffer must be determined for a given cell line.

4.3. Gently scrape the well with a cell lifter to dislodge loosely adhered cells and transfer the cell suspension to a 15 mL conical tube, followed by pipetting up and down several times to individualize the hiPS cells.

4.3.1. Bring cell suspension to the 15 mL volume with warm DMEM/F12 and centrifuge for 5 min at 290 *x g* at room temperature.

4.3.2. Gently aspirate the supernatant and resuspend the cells with warm DMEM/F12 for another round of centrifugation to remove residual BM matrix 1 and dissociation buffer components.

4.4. Aspirate the supernatant and resuspend cells in 1 mL of mesoderm induction medium as described in above. Count the total number of cells using a hemocytometer or coulter counter to determine the appropriate volume of mesoderm differentiation medium necessary to achieve a concentration of 1 x 10^5 cells/mL.

4.5. Aspirate ECM solution from the BM matrix 2-coated plates and rinse the plates twice with warm DMEM/F12. Mix the hiPS cell suspension gently by pipetting a few times. Transfer 1 mL of the cell suspension to each well of the BM matrix 2-coated 12-well plates and then gently shake the plates to distribute the cells more evenly.

4.6. Incubate the plate at 37 ˚C in a 5% CO_2 incubator. Refresh the mesoderm induction medium the next day.

NOTE: After 2 days, hiPS cell-derived mesoderm cells would be ready for intermediate mesoderm induction.

5. Differentiation of hiPS cell-derived mesoderm cells into intermediate mesoderm (days 2-16)

5.1. On the day 2 of the differentiation protocol, aspirate mesoderm induction medium and replenish with 1 mL per well intermediate mesoderm induction medium.

5.2. Refresh medium every day to maintain an accurate threshold of growth factors and small molecules for the metabolically active cells. If there is substantial cell growth and rapid depletion of media nutrients (indicated by the yellowing of the media), the volume of the intermediate mesoderm differentiation medium can be increased to 1.3 mL per well of the 12 well plates.

5.3. Culture cells for additional 14 days to obtain intermediate mesoderm cells. By day 16, these cells can be cryopreserved for later use.

6. Differentiation of hiPS cell-derived intermediate mesoderm cells into podocytes (days 16 to 21)

6.1. Rinse the intermediate mesoderm cells with warm DMEM/F12 followed by incubation of the cells with 0.5 mL per well of 0.05 % trypsin-EDTA for 3 min at 37 ˚C. Perform visual inspection to ensure that the cells are beginning to dissociate.

6.2. Scrape cells using a cell lifter and pipette the cell suspension several times using a 1,000 µL pipette tip to obtain individualized (or small clumps of) cells.

NOTE: At this stage, ensure that the cells are fully dissociated as aggregated cells may fail to acquire a terminally differentiated phenotype within the timeline of the protocol.

6.3. Add about 2 mL per well of trypsin neutralizing solution to stop the activity of trypsin.

6.4. Transfer cells to a 50 mL conical tube and bring the volume up to 50 mL using DMEM/F12, and then centrifuge the cell suspension for 5 min at 201 *x g* at room temperature.

6.5. Aspirate the supernatant and resuspend cells in the podocyte induction medium. For optimal results, ensure a final seeding density of approximately 100,000 cells/well of a 12 well plate. Add the cell suspension to the BM matrix 2-coated plates and gently shake the plate to help distribute the cells more evenly.

6.6. Incubate cells at 37 ˚C and 5% CO_2 and refresh medium daily for up to 5 days to obtain podocytes by day 21.

NOTE: The resulting hiPS cell-derived podocytes can be maintained in culture for 2-4 additional weeks by using complete medium with podocyte maintenance media. Once in podocyte maintenance media, the cells can be fed every other day and may be used for subsequent studies or downstream analyses.

REPRESENTATIVE RESULTS

The goal of this protocol was to demonstrate that mature human podocytes can be derived from hiPS cells under chemically defined conditions. The data presented in this manuscript were generated by using the DU11 hiPS cell line[17], which were first tested for, and found to be free of mycoplasma. Chromosomal analysis was also performed, and the cells were found to be karyotypically normal. Starting with the undifferentiated DU11 hiPS cells, the differentiation strategy (**Figure 1**) outlined in this report was employed to first differentiate the stem cells (**Figure 2A**) into mesoderm cell that express Brachyury (T) (**Figure 1B** and **Figure 2B**), followed by differentiation into PAX2-positive intermediate mesoderm cells (**Figure 2C**), and finally into mature kidney glomerular podocytes (**Figure 1B**, **Figure 2D**, **Figure 3D**, and **Figure 4**). Mesoderm cells were obtained by day 2 of the differentiation protocol, intermediate mesoderm cells were generated by day 16, and the mature podocytes were obtained by day 21 of the differentiation protocol. The resulting podocytes were about 150-200 µm in size when adhered to a flat tissue culture surface, such as the laminin-coated plates used in this experiment. The hiPS cell-derived podocytes exhibit numerous foot process-like extensions that were visualized using multiple complementary techniques including phase-contrast microscopy (**Figure 1**), immunofluorescent microscopy (**Figure 4**) and scanning electron microscopy[8,11]. The stem cell-derived podocytes also stained positive for WT1[15] (**Figure 2D**) as well as the lineage identification marker nephrin[14] (**Figure 4**). Intriguingly, the subcellular localization of nephrin was predominantly in the podocyte foot processes and cell cytoplasm, consistent with a mature podocyte phenotype (**Figure 2D** and

Figure 4A,**B**). During development and in podocyte physiology, the protein nephrin is shuttled between the cell nucleus and the cytoplasm, but nephrin becomes predominantly expressed in the cytoplasm and foot processes as podocytes mature and acquire a functional phenotype[18]. Thus, the observation that the podocytes produced by using the differentiation protocol described herein express nephrin largely in the cytoplasm and foot process-like structures underscores the utility of this protocol for generation of more mature or specialized podocytes. Scanning electron micrographs of hiPS cell-derived podocytes highlight the branching of the primary and secondary foot processes in the hiPS cell-derived podocytes as previously reported by our group[8,11]. Because WT1 is not by itself a definitive marker of kidney podocytes, it is highly recommended that researchers also characterize their cells for simultaneous expression of podocyte-specific markers such as podocin and nephrin. It was previously shown that terminally differentiated podocytes derived by using this method immunostain positive for podocin, nephrin, and WT1, with a corresponding decrease in expression of the progenitor cell marker PAX2[8,11]. Together, these results indicate that the podocytes derived using this protocol are developmentally mature or specialized, as required for a functional human kidney glomerular podocyte.

In optimizing conditions for the differentiation method, instances where the intermediate mesoderm cells were inadequately dissociated were observed. Cells seeded at densities that significantly exceeded the recommended density of 100,000 cells/well of a 12 well plate (before the final podocyte induction step) resulted in large clusters of cells that lacked the expected morphological phenotype of mature podocytes within the standard timeline of the protocol (**Figure 3A,B**). For these reasons, it is recommended that researchers use the seeding densities recommended in this protocol (**Figure 3C,D**) before experimenting with other conditions that may be desired for specific downstream applications. Together, these results represent the directed differentiation of hiPS cells into kidney glomerular podocytes within three weeks using the chemically defined media described above.

FIGURE LEGENDS:

Figure 1: Differentiation of hiPS cells to podocytes. (**A**) Schematic representation of the method for directed differentiation of hiPS cells into podocytes. BMP7, bone morphogenetic protein 7; RA, retinoic acid; VEGF, vascular endothelial growth factor. This figure has been modified from ref.[11]. (**B**) Representative images of hiPS cells at each stage of differentiation. From left to right, images show the characteristic human iPS cell colonies before dissociation for differentiation on day 0, followed by mesoderm cells after 2 days of differentiation, then intermediate mesoderm cells at around 10 days of differentiation, and finally the terminally differentiated podocytes at day 22. Scale bar, 100 µm for all images (in panel B).

Figure 2: Immunofluorescent images of hiPS cells and their differentiated derivatives showing the expression of lineage-specific markers and nuclear counterstaining during the differentiation timeline. (**A**) Human iPS cells expressing the pluripotency marker Oct4; (**B**) mesoderm cells expressing brachyury (T); (**C**) intermediate mesoderm cells expressing PAX2; and (**D**) the hiPS cell-derived podocytes expressing the lineage-identification marker , nephrin, and the associated marker, WT1 . Scale Bar, 100 µm for all images.

Figure 3: Effects of sub-optimal seeding density on the differentiation potential of intermediate mesoderm cells derived from the DU11 hiPS cell line. (**A**) When the initial cell seeding density is too high (approximately 500,000 cells/well of a 12 well plate), cells can form dense aggregates (**B**) during the podocyte induction stage. (**C**) Recommended seeding densities to prevent clumping (100,000 cells/well of a 12 well plate), and to microscopically observe the (**D**) extension of foot-process-like structures during the podocyte differentiation phase (inset). Scale bar, 200 µm for A-D; and 100 µm for the inset in D.

Figure 4: hiPS cell-derived podocytes exhibit morphologically mature phenotype *in vitro*. (**A**) Immunostaining of hiPS cell-derived podocytes for nephrin, and (**B**) higher magnification image of podocyte foot processes showing primary and secondary processes (white arrows) as well as the expression of nephrin in these specialized cell structures. Scale bar, 100 µm (A); 50 µm (B).

DISCUSSION

In this report, we describe a protocol for the generation of kidney glomerular podocytes from hiPS cells. The hiPS cell-derived podocytes exhibit morphological and molecular features associated with the mature kidney podocyte phenotype[13]. In previous publications, we showed that the hiPS cell-derived podocytes can mimic the structure and selective filtration function of the kidney glomerulus when co-cultured with glomerular microvascular endothelial cells in a perfusable microfluidic organ-on-a-chip device[8,11].

The following critical steps should be considered by researchers interested in adapting this protocol. First, the seeding of hiPS cells onto laminin-coated plates for the induction of mesoderm cells is a crucial step. While a seeding density of 100,000 cells/well is recommended, researchers can adjust the initial cell seeding density depending on the replication rate of the stem cell line used for the study. If necessary, this optimization will ensure adequate cell density during the mesoderm induction stage and prevent formation of cell aggregates or very large clumps that could potentially compromise the quality of the mesoderm, intermediate mesoderm, or podocyte phenotype (**Figure 3**). It is also important to note that differences in manufacturer specifications for some reagents such as CHIR99021 may affect the quality of differentiated cells. Thus, it is advisable to test reagents from different lot numbers or suppliers and vendors for their biological activity and consistency between independent experiments. Also, it is important to avoid excessive exposure of hiPS cells to dissociation enzymes as this could lead to undesired detachment from the adhesive matrix and possible loss of cells during medium aspiration. Another crucial step worthy of note is to ensure that the podocyte induction medium is protected from light to prevent photo-induced inactivation of retinoic acid.

In comparison to previous methods for the differentiation of human kidney podocytes, the method described here employs chemically defined factors (including small molecules, growth factors, and specific isoforms of basement membrane proteins, at desired concentrations) as needed to regulate specific cell signaling pathways involved in kidney development and podocyte function. Specifically, the generation of mesoderm cells from hiPS cells involved the activation of the canonical Wnt signaling pathway effected by using the small molecule CHIR99021[19]. The

canonical Wnt signaling pathway is known to regulate the transcriptional activity of genes involved in tissue development and patterning in vivo, as well as cell proliferation and migration[20,21]. The cell induction medium used in this protocol enables the derivation of podocytes from iPS cell-derived intermediate mesoderm cells. This podocyte induction medium consists of Activin A, BMP7, VEGF, retinoic acid, and CHIR99021. Notably, this podocyte induction medium does not require undefined serum components such as fetal bovine serum, which can obscure the contributions of specific factors and limit the applications of other cell differentiation methods for mechanistic studies of tissue development and disease, making it distinct from those employed in previous attempts to generate renal cell types[22]. Additionally, it was observed that FGF2 and FGF9 are not required for the differentiation of kidney podocytes from hiPS cells[6,7,22]. While previous methods dependented on the formation of multicellular aggregates such as embryoid bodies and organoids, the method described here produces cells that exhibit a mature phenotype, both morphologically and by the expression of lineage specific markers including nephrin and podocin, and down-regulation of progenitor cell markers such as PAX2. It is expected that the knowledge of the chemical components this protocol can facilitate mechanistic studies of human kidney tissue development, podocyte lineage specification, and disease pathogenesis in the future.

This method for hiPS cell differentiation also enables the generation of mature, postmitotic and functional podocytes with more than 90% efficiency, without subpopulation selection, genetic manipulations, or xenotransplantation. This is notably different from previous attempts where high levels of cellular heterogeneity[6,7,23] limited the use of the stem cell differentiation methods for translational medicine applications. This method also presents a significant advance for the field given the challenges associated with the extremely limited availability of human kidney podocytes from other sources such as primary tissues or organoids. Some of the challenges include low derivation efficiencies of less than 1% when using methods that rely on the formation of embryoid bodies or organoids[6,7].

One limitation to this protocol is the relatively high cost of the reagents required for the stem cell culture and differentiation steps, which may hinder its implementation in some laboratories or research groups. Also, because a lot of optimization for multiple factors in the cell's microenvironment (i.e., both soluble and insoluble or adhesive cues) were involved, we recommend that the protocol is followed as described. A common mistake noted from others who were interested in using this podocyte differentiation method included the use of inappropriate cell adhesion matrices such as BM matrix 1 or other poorly defined animal-derived proteins. Once the protocol is followed as described, further experiments can be performed to examine the efficacy of other modifications to the protocol. Furthermore, due to the inherent differences between different human stem cell lines, it is worth noting that some aspects of the differentiation method, e.g., the timing of exposure to differentiation medium or cell dissociation medium, and cell seeding densities, may need to be optimized. However, these conditions were not altered for the hiPS cell lines and embryonic stem cell lines tested to date. This podocyte differentiation method works across multiple hiPS cell lines including the PGP1, IMR-90–1, and IISH3i-CB6 as well as the H9 embryonic stem cell line[8,11]. In this report, we also demonstrate the successful differentiation of the DU11 hiPS cell line using the same protocol (**Figure 1** and **Figure**

2). Thus, given the consistency of this method across various cell lines including results from unpublished work from other independent research laboratories (pre-publication), we expect that this differentiation protocol will be useful for the derivation of kidney podocytes from a wide variety of hiPS cell lines, and therefore, not limited to the one used in this protocol.

Given the capacity of hiPS cells to self-renew indefinitely, this differentiation method could be used to provide researchers with a readily available source of human kidney podocytes. This could help advance the current understanding of human kidney biology and disease[24] as well as enable the development of new in vitro kidney systems for modeling human kidney function and responses to therapeutics. For example, the protocol described herein was recently employed and integrated with a microfluidic organ-on-a-chip system to develop a functional in vitro model of the human kidney glomerular capillary wall. This glomerular chip selectively filtered circulating molecules and recapitulated drug-induced nephrotoxicity when exposed to the chemotherapy drug, Adriamycin[8]. In the future, these results could potentially be integrated with 3D bioprinting technologies to engineer more complex in vitro models of the human kidney. Such advances could provide novel platforms for evaluating drug candidates, particularly those that target the forms of kidney diseases arising from the dysfunction of glomerular podocytes. This is especially important as the species-specific differences of animal models and lack of organ-level functionalities typically observed in standard tissue culture methods can impede the development of targeted therapeutics for various forms of human kidney diseases[25]. Thus, this protocol could, some-day, provide opportunities to unravel the signaling pathways involved in the development of the human kidney, as well as the pathogenesis of disease.

ACKNOWLEDGMENTS:

This work was supported by the Pratt school of Engineering at Duke University, the Division of Nephrology at Duke Medical School, A Chair's Research Award from the Department of Medicine at Duke University, and a Burroughs Wellcome Fund PDEP Career Transition *Ad Hoc* Award to S.M.. M.B was supported by the National Science Foundation's Graduate Research Fellowship Program. We thank the Bursac Lab for generously providing us with the DU11 stem cell line, and the Varghese Lab at Duke University for temporarily sharing their tissue culture facility with our group. This publication is dedicated to Prof. Laura L. Kiessling, Novartis Professor of Chemistry at the Massachusetts Institute of Technology, in celebration of her 60th birthday.

DISCLOSURES:

S.M. is an author on a patent pending for methods for the generation of kidney podocytes from pluripotent stem cells (US patent application 14/950859). The remaining authors declare that they have no competing interests.

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Figure 1

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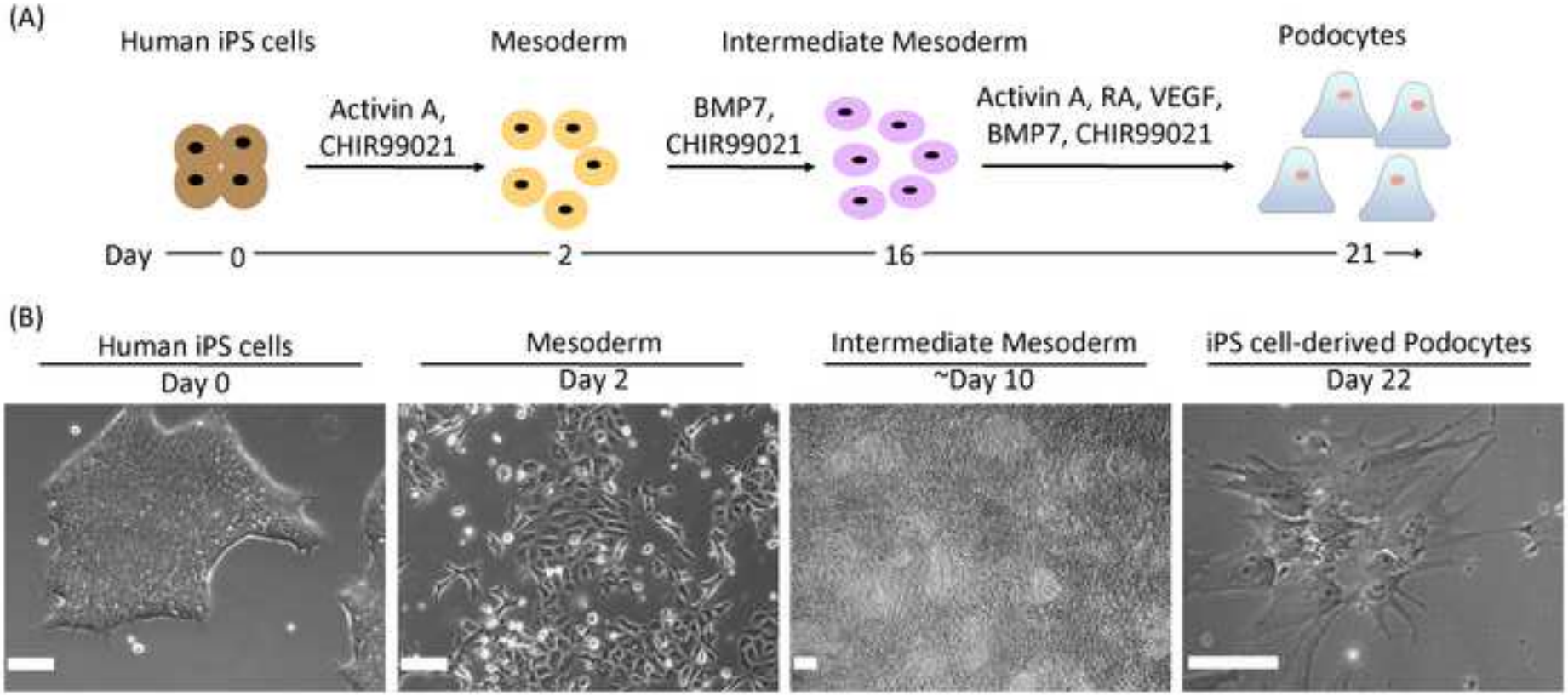

Figure 2

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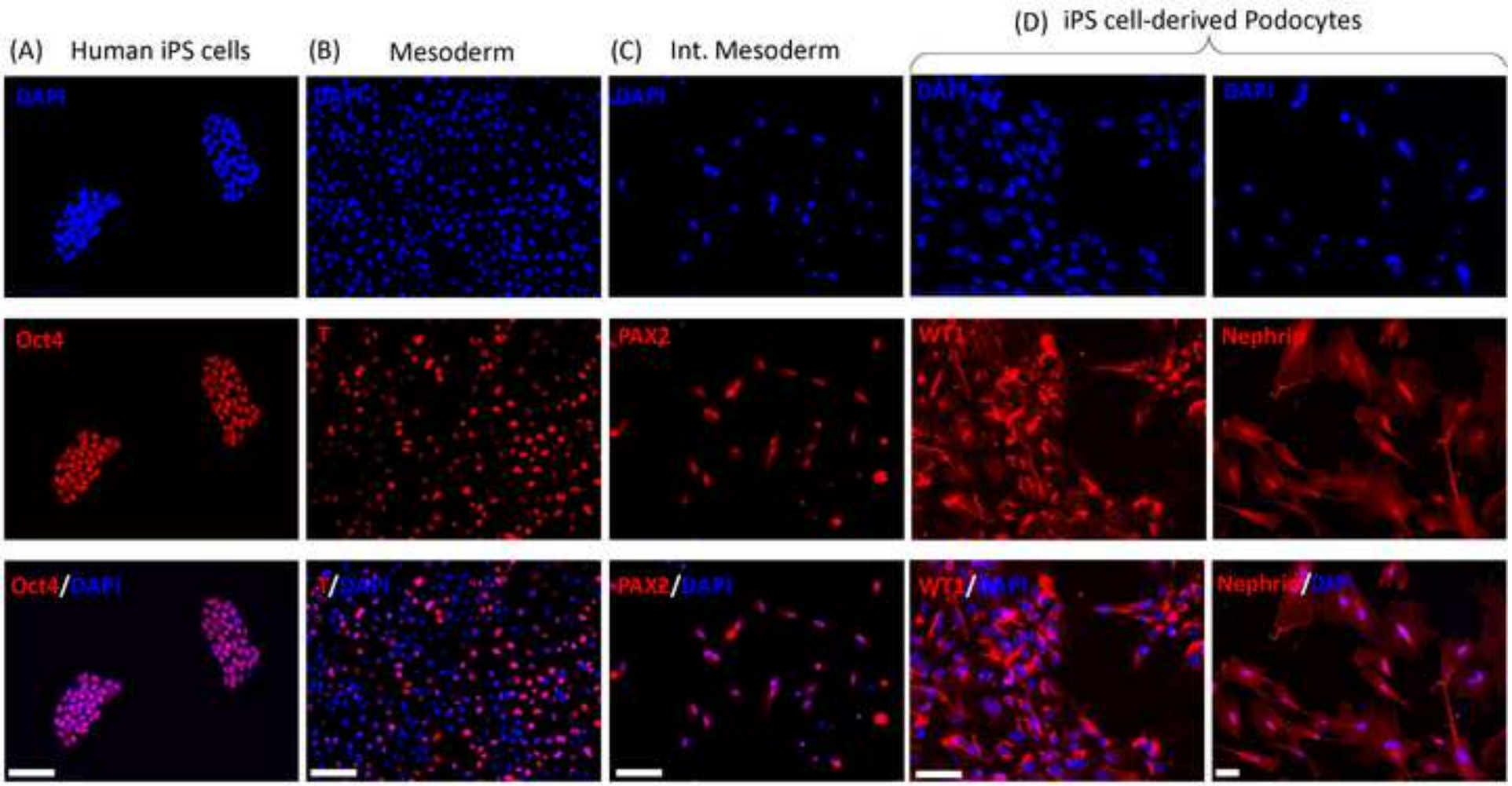

Figure 3

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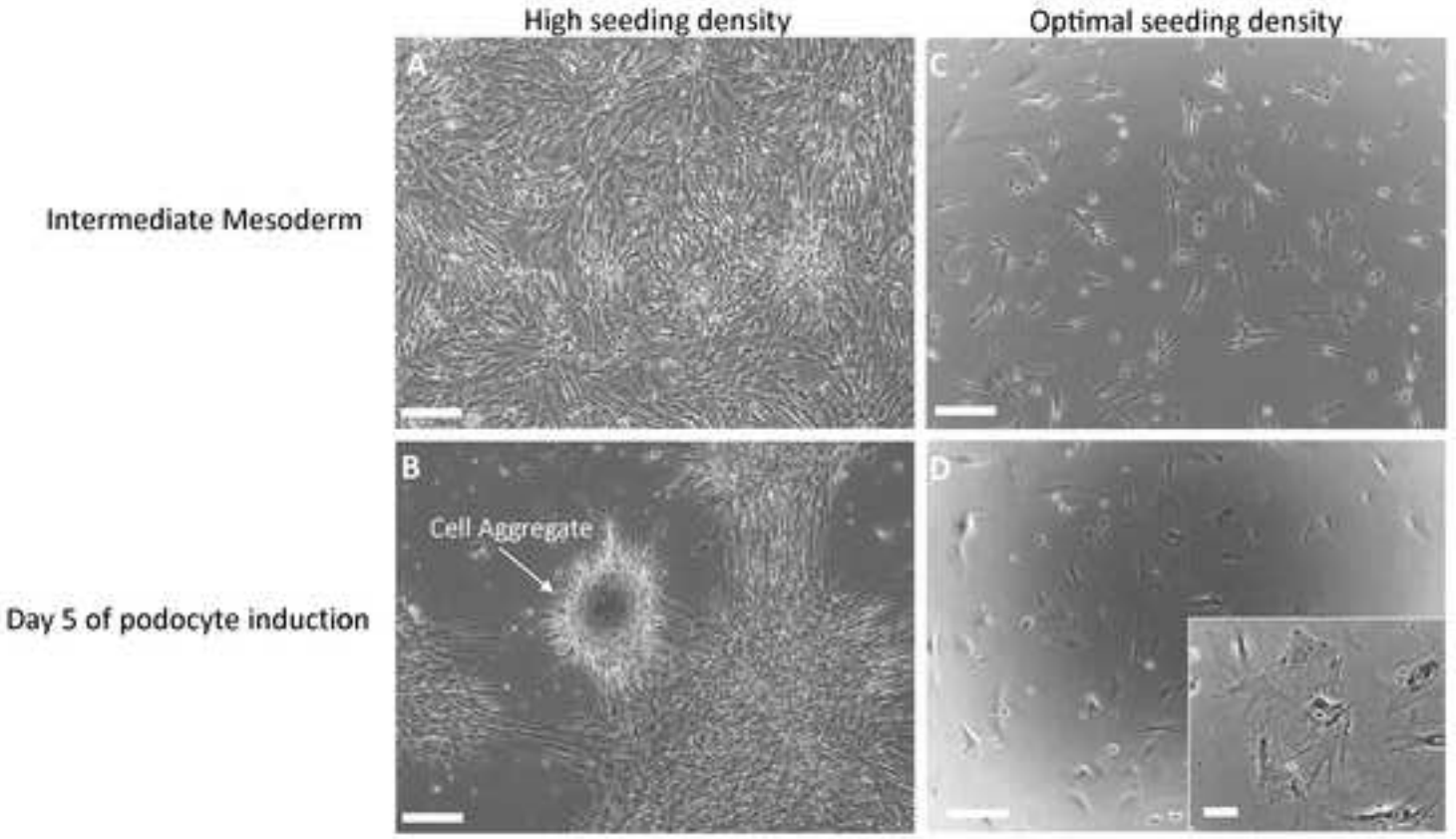

Figure 4

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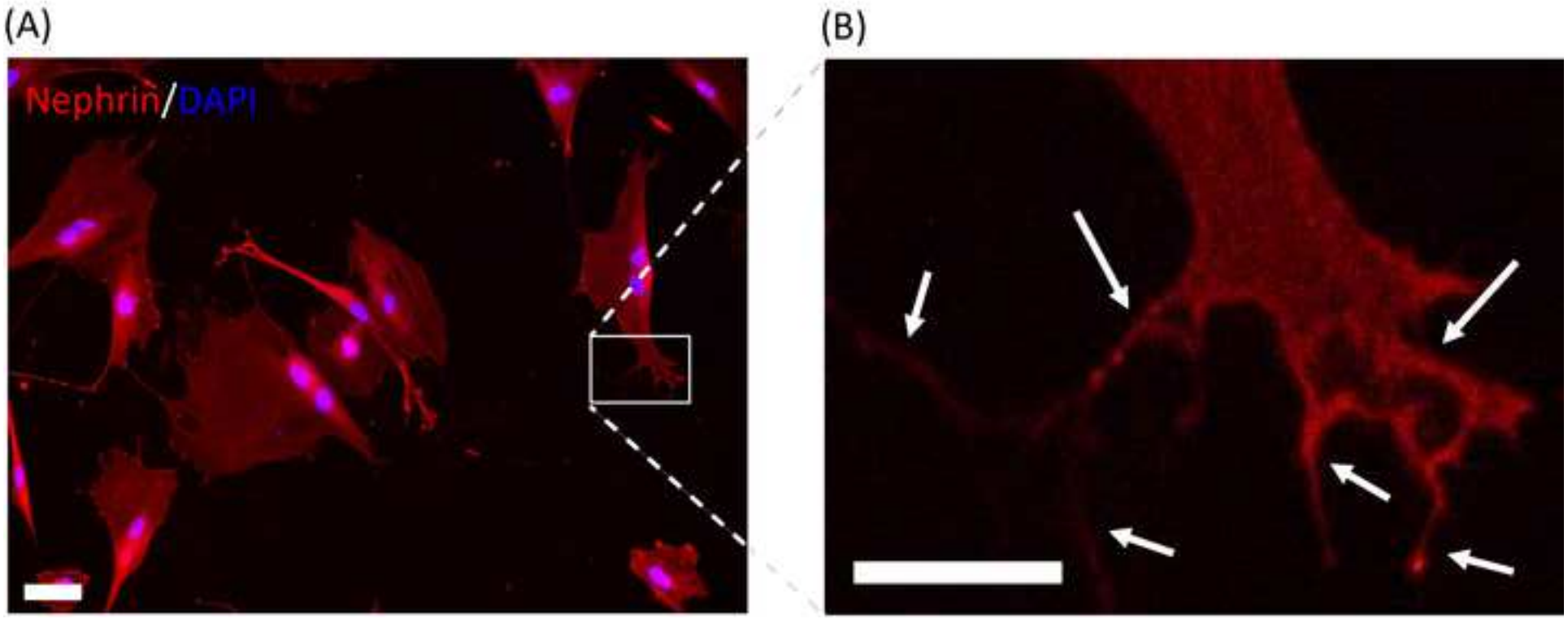

Click here to access/download;Table of Materials;20200514_Table of Materials_Revised.xlsx

Table of materials

Name of Material/Equipment	Catalog Number	Company	Comments/Description
Cells			
DU11 human iPS cells			The DU11(Duke University clone #11) iPS cell line was generated at the Duke University iPSC Core Facility and provided to us by the Bursac Lab at Duke University. This line has been tested for mycoplasma and was last karyotyped in July 2019 by our lab, and found to be karyotypically normal.
Growth Factors and Media Supplements			
All-trans retinoic acid (500 mg)	72262	Stem Cell Technologies	
B27 serum-free supplement	17504044	Thermo/Life Technologies	
CHIR99021	04-0004	Stemgent	May show lot-to-lot variation
Complete Medium Kit with CultureBoost-R	4Z0-500-R	Cell Systems	Podocyte maintenance media
DMEM/F12	12634028	Thermo/Life Technologies	
DMEM/F12 with GlutaMAX supplement	10565042	Thermo/Life Technologies	DMEM/F12 with glutamine supplement
Heat-inactivated FBS	10082147	Thermo/Life Technologies	
Human activin A	PHC9564	Thermo/Life Technologies	
Human BMP7	PHC9544	Thermo/Life Technologies	
Human VEGF	PHC9394	Thermo/Life Technologies	
mTeSR1 medium	05850	Stem Cell Technologies	hiPS cell culture media (CCM)
Penicillin–streptomycin, liquid (100×)	15140-163	Thermo/Life Technologies	
Y27632 ROCK inhibitor	1254	Tocris	
Antibodies			
Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary antibodies	A32744; A32754; A-11076; A32790	Thermo/Life Technologies	
Brachyury(T)	ab20680	Abcam	
Nephrin	GP-N2	Progen	
OCT4	AF1759	R&D Systems	
PAX2	71-6000	Invitrogen	
WT1	MAB4234	Millipore	
ECM Molecules			

iMatrix-511 Laminin-E8 (LM-E8) fragment	N-892012	Iwai North America	Basement membrane (BM) matrix 2
Matrigel hESC-qualified matrix, 5-mL vial	354277	BD Biosciences	Basement membrane (BM) matrix 1. May show lot-to-lot variation
Enzymes and Other Reagents			
Accutase	A1110501	Thermo/Life Technologies	Cell detachment solution
BSA	A9418	Sigma-Aldrich	
Dimethyl Sulfoxide (DMSO)	D2438	Sigma-Aldrich	DMSO is toxic. Should be handled in chemical safety hood
Enzyme-free cell dissociation buffer, Hank's balanced salt	13150016	Thermo/Life Technologies	
Ethanol solution, 70% (vol/vol), biotechnology grade	97065-058	VWR	Ethanol is flammable and toxic
FBS	431097	Corning	
Paraformaldehyde (PFA)	28906	Thermo/Life Technologies	PFA should be handled in a chemical fume hood with proper personal protection equipment, including gloves, lab coat, and safety eye glasses. Avoid inhalation and contact with skin
Phosphate-buffered saline (PBS)	14190-250	Thermo/Life Technologies	
Sterile Distilled Water	15230162	Thermo/Life Technologies	
Triton X-100	97062-208	VWR	
Trypsin-EDTA, 0.05%	25300-120	Thermo/Life Technologies	
Equipment			
Aspirating pipettes, individually wrapped	29442-462	Corning	
Avanti J-15R Centrifuge	B99516	Beckman Coulter	
Conical centrifuge tube, 15 mL	352097	Corning	
Conical centrifuge tube, 50 mL	352098	Corning	
Cryoboxes	3395465	Thermo/Life Technologies	For storing frozen aliquots
EVOS M7000	AMF7000	Thermo/Life Technologies	Flourescent microscope used to acquire images of fixed and stained iPS cells and their derivatives
Hemocytometer	100503-092	VWR	
Heracell VIOS 160i CO2 incu	51030403	Thermo/Life Technologies	For the routine culture and maintenace of iPS cells and their derivatives

Inverted Zeiss Axio Observe	491916-0001-000(microscope) ; 426558-0000-000(camera)	Carl Zeiss Microscopy	Used to acquire phase contrast images of live iPS cells and their derivatives at each stage of podocyte differentiation
Kimberly-Clark nitrile gloves	40101-346	VWR	
Kimwipes, large	21905-049	VWR	
Kimwipes, small	21905-026	VWR	
P10 precision barrier pipette tips	P1096-FR	Denville Scientific	
P100 barrier pipette tips	P1125	Denville Scientific	
P1000 barrier pipette tips	P1126	Denville Scientific	
P20 barrier pipette tips	P1121	Denville Scientific	
P200 barrier pipette tips	P1122	Denville Scientific	
Serological pipette, 10 mL, individually wrapped	356551	Corning	
Serological pipette, 25 mL, individually wrapped	356525	Corning	
Serological pipette, 5 mL, individually wrapped	356543	Corning	
Steriflip, 0.22 µm, PES	SCGP00525	EMD Millipore	
Sterile Microcentrifuge Tubes	1138W14	Thomas Scientific	For aliquoting growth factors
Tissue culture–treated 12-well plates	353043	Corning	
Tissue culture–treated six-well plates	353046	Corning	
VWR white techuni lab coat	10141-342	VWR	
Wide-beveled cell lifter	3008	Corning	

Rebuttal Letter
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Editorial Comments

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.

We have revised the manuscript as requested.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have revised the manuscript as requested.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

The short abstract has been revised to address this comment. The word count has been reduced to 50.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

We have made minor revisions to the long abstract to state the goal of the protocol more clearly. The long abstract is also under 300 words.

5. JoVE cannot publish manuscripts containing commercial language. 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 and Reagents.
For example: Matrigel, falcon tubes, Glutamax, Accutase, etc.

We have revised the main text of the manuscript such that all commercial languages were replaced with generic terminologies. We have referenced all commercial products in the Table of Materials and Reagents as requested.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We have revised sections 2.1, 2.3, 4.5, and 6.2 in the protocol to address this comment.

7. Please do not use personal pronouns in the protocol section.

Revised as requested (Section 2.2).

8. The Protocol should contain only action items that direct the reader to do something.

We have revised the manuscript text to ensure that the protocol section only contains action items.

9. Please ensure you answer the "how" question, i.e., how is the step performed?

We believe the information presented in the manuscript text, along with the revisions we have made adequately address the "how" question of the stem cell differentiation protocol we present in this manuscript.

10. What kind of hiPS cells are used in the study? Culture conditions?

The DU11 (Duke University clone #11) hiPS cells were used in this study. The cells were previously reprogrammed by another lab at Duke University by using BJ foreskin fibroblasts from a healthy newborn male (ATCC cell line, CRL-2522), and by using episomal vectors. The derivation and characterization of the DU11 hiPS cells was previously reported in Shadrin *et al., Nat. Comm*. **2017**, 8: 1825, and we obtained the cells from the same authors who originally derived these cells. We cited the relevant publication in the manuscript text (Representative Results, paragraph 1). We have also revised the acknowledgement section of our manuscript to thank the Bursac Lab for generously providing us with the cell line, which we accidently omitted in the previous draft. We also described the culture conditions we used for the DU11 hiPS cell line in section 3 on the manuscript text.

11. Please include citations for the markers used for confirming different stages.

We have revised the manuscript as suggested, and the citations below have been added to the Introduction section (paragraph 3) and Representative Results section (paragraph 1).

Citations:

(12) Torban *et al*., *J. Biological Chemistry*, **2006**, 18: 12705-12712 (PAX2)
(13) Kuusniemi *et al.,Pediatric Research.* **2004,** 55**:**774-781 (Nephrin)
(14) Guo *et al.,Human Molecular Genetics.***2002,** 11:651-659 (WT1)
(15) Roselli *et al.Am J Pathol.* **2002,** 160: 131-139 (Podocin)

12. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We have revised the protocol to ensure that individual steps in the protocol only contain 2 to 3 action statements.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Sections 3.1 to 6.6 have been highlighted (in blue) for the video component of this protocol.

14. Please ensure the results are described in the context of the presented technique i.e., you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title

Throughout the manuscript and especially in the "Representative Results" section, paragraph 1, we have made revisions to better contextualize our results with the technique as it is presented in the manuscript by also reiterating the goal of the protocol and adding more explicit references to the Figures.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All the data presented in this manuscript are new and not published previously. Thus, we do not require copyright permission for any of the figures included in this manuscript, which we are submitting exclusively to JoVE for review and publication.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

A paragraph highlighting critical steps within this protocol has been added to the Discussion section (paragraph 2). Also, we discussed the significance of this method with respect to previous efforts in the discussion section (paragraph 3, 4). The limitations, possible modifications as well as future applications have also been discussed (paragraph 5).

17. We do not have the author's contribution section in our manuscript. Please remove.

This section has been removed.

18. Please do not include the figure legends with the uploaded figures.

Figure legends have been removed from uploaded figures. The figure legends are now included only in the manuscript text.

19. Please sort the materials table in alphabetical order.

We have sorted the materials table in alphabetical order.

Reviewer #1

1. **Manuscript Summary: well written, easy to follow**

We thank the reviewer for his/her comment.

2. **Major Concerns: none**

We thank the reviewer for his/her feedback.

Minor Concerns:

3. Line 136 what is the storage time for the described stock solutions (BMP7, ActivinA and VEGF)? It is clearly stated for all other additives and media.

The manufacturer recommends storage at 20 ˚C for up to six months. We have revised the manuscript text (step 1.5) to include this recommendation for BMP7, Activin A, and VEGF stock solutions.

4. **Line 173 what is the ideal confluency of the cells in preparation for seeding?**

Generally, 70% confluency is considered ideal for iPSC seeding to initiate mesoderm induction. We have noted this in Section 3.4.

5. Line 185 what is the ideal size of clusters for seeding?

The ideal colony size for routine propagation of iPSCs should be around 200-500 µm in size when under feeder-free conditions using mTeSR1 medium (without ROCK inhibitor). We have now added a note to Section 3.4 in the protocol to more clearly state this. We have also included in the same note strategies for rescuing the cells if the colony size is too small or if the cells become individualized after treatment with dissociation enzymes or buffer.

Reviewer #2

1. Line 100: Abbreviations for days are sometimes "d" sometimes spelled out. Decide for one and keep consistent.

We thank the reviewer for noting this error. All occurrences of 'd' have been replaced with 'days' in the new version of the manuscript.

2. Line 126: Give exact concentration of Matrigel used.

The manufacturer provides the dilution factor, which varies between lots and can be found on the certificate of analysis of any given lot. Typically, aliquots (dilution factor) volume range from 270 to 350 µL and it is calculated for each lot based on protein concentration by the manufacturer. In any case, we dilute the aliquot into 25 mL of DMEM/F12 for use. In section 1.2 of the protocol, we noted that researchers should follow the manufacturer guidelines for appropriate dilutions.

3. Line 192: The term "Mitotic phase" does not make any sense here. Surely the cells reach mitotic phase several time within Day 4 (a typical cell cycle is usually 24 h and in iPSC cells much shorter). Also, in any given cell culture (unless the cells were synchronized) cells are always at mixed phases of the cell cycle with mitotic representing approximately 1% of the cells.

The doubling time for human iPS cells is around 20 h. However, when the culture density of these cells gets too high, they tend to grow slower or into aggregates, which is undesirable as it reduces the quality of the cell culture and increases the rate of spontaneous differentiation. To minimize these issues, it is established in the field that the use of human iPSCs while they are actively dividing (mitotic) and up to approximately 70% confluency (typically with the first 4 days of culture after passaging) is ideal for induction of the mesoderm lineage, which is also the first differentiation step in our protocol. Thus, our use of the term mitotic denotes actively dividing

cells. For clarity, we have changed the phrase 'mitotic phase' to 'exponential growth phase' (Section 4.1) in the revised version of the manuscript.

4. Line 225: Applying 1.3 ml of medium to a 12 well plate will cause hypoxic conditions. Also, what does "rapid depletion of the spent medium" mean here? How is this measured?

All our differentiation steps were performed using adherent cells in tissue-culture-treated plates that were functionalized with the Laminin 511-E8 fragment, and cell cultures were maintained in a 37°C and 5% CO_2 incubator. Under these conditions, we have not observed cellular effects that can be definitively attributed to hypoxia. As a note, we are aware that cells encapsulated in gels at a similar volume of medium will most likely experience hypoxia due to the thickness/volume of the gel and inadequate supply of oxygen within the gel. We wanted to emphasize that our cells were not encapsulated in a gel. Additionally, the volume of the differentiation medium we used is within the working range recommended by the well-plate manufacturer (i.e. 1 to 2mLs recommended, and we use a maximum of 1.3 mL). For all these reasons, we believe the cell culture conditions described in our protocol is normoxic.

Regarding the use of 1.3 mL volume of medium to help minimize nutrient depletion: since the cells are metabolically active even at the intermediate mesoderm differentiation stage, the nutrients in the medium can be depleted rapidly, and researchers may notice that the medium turns yellow (indicating an increase in acidity, as measured by the colorimetric pH indicator, phenol red, in the medium). If substantial color change (bright red to pale yellow) occurs frequently or between media changes, we recommend increasing media volume to around 1.3mL to help avoid prolonged nutrient depletion. We have revised this statement to encourage the reader to refer to the color of the media when deciding whether additional media supplementation is necessary (Section 5.2).

5. Line 241: 1:4 Splitting ratio depending on the density. This is really vague. More details should be given.

For added clarity and reproducibility, we have revised this statement to reflect the target cell number per well rather than target split ratio. For optimal results, we recommend seeding intermediate mesoderm cells at 100,000 cells/well of a 12 well plate (Section 6.5).

6. Line 265: The statement about this protocol works in every iPSC line we have tested today should be more specific. Please give a number, it worked in x ipsc cell lines. Are these listed below the only ones tested?

To date, this protocol has worked for all four human iPS cell lines we have tested (specifically, the PGP1, IMR-90-1, and IISH3i-CB6 described in our previous publications, and the DU11 line reported for the first time in this manuscript). The method has also worked for the derivation of podocytes from the H9 human embryonic stem cell line H9, also reported in our previous

publication. The relevant prior publications are (1) Musah *et.al, Nat. Protocols*, **2018**, 13:1662-1685 and (2) Musah *et.al Nat. Biomed. Engineering*, **2017**, 1:0069. Additionally, other independent research groups have also shared results of successful replication of the method by using completely different iPS cell lines (pre-publication).

7. Line 270: What does too dense mean? Too vague, please provide some numbers here.

The statement has been revised for clarity (see "Representative Results" section, paragraph 2). Please note that the issue of suboptimal seeding densities and its effect on the protocol is also described in Figure 3B in more detail.

8. Line 273 and 306: What is the author's definition of chemically defined medium here? Usually this refers to using only small molecules and not growth factors. However, in their protocol they also use the following growth factors: Activin A and BMP7. How is this then novel or different to other protocols that they cite which also use similar approaches: Ciampi et al. (Ref 14) uses also a combination of small molecules (CP21) with Growth factors (BMP4, BMP7, others). Similar for References 6 and 7. Also for completeness the authors should mention other studies including Song et al. and Rauch et al, that were previously reported.
I don't see the justification that the protocol reported here is described as "chemically defined" whereas the other cited ones are not.

The media used in the protocol were formulated in our lab and are currently not available commercially. The term "chemically-defined" is used to indicate serum-free conditions and the fact that the composition, identity, and concentrations of the chemicals used are known -- these include the soluble and insoluble (adhesion) proteins and small molecules, as well as the E8 fragment of Laminin 511 used as culture matrix. To the best of our knowledge, our method for the derivation of podocytes is the first in vitro stem cell differentiation method to produce podocytes with characteristics of the mature and functional phenotype with high yield (>90% yield) without subpopulation selection, genetic manipulation, or xeno-transplantation. We have described (in this manuscript and our previous publications in Nature Biomedical Engineering and Nature Protocols) some of the specialized differentiation features of the podocytes obtained using our method – this include post-mitotic (no incorporation of 5-ethynyl-2'-deoxyuridine, no cell division, and Pax2-negative) phenotype, development of elaborate primary and secondary foot processes, establishment of tissue-tissue interface resembling the glomerular capillary wall or filtration barrier when interfaced with human renal endothelial cells, and modeling selective molecular filtration function of the kidney as well as drug-induced proteinuria and foot process effacement when treated with the chemotherapy drug Adriamycin (Musah *et al*., 2017; and Musah *et al*., 2018). The chemically defined nature of our method and how it is distinct from previous attempts have been more explicitly highlighted in the Discussion section of the manuscript (Paragraphs 3 and 4). The three other studies cited in this reviewer's comment (Song *et al*., 2012, Ciampi *et al*., 2016, Rauch *et al*., 2018) all used undefined serum components such as fetal bovine serum and generated heterogenous populations of cells that exhibit phenotypes

associated with irrelevant cell types or with the immature/progenitor stage (Pax2-positive, little to no specialized primary and secondary foot processes). Additionally, none of these studies provided evidence to show whether the resulting cell types could form the filtration barrier necessary for kidney podocyte function in vivo. There is also no evidence that the cells can selectively filter molecules as functional kidney podocytes should. Thus, the physiological relevance of the cells generated in these other reports remain obscure. Finally, the studies reported by Morizane and colleagues and Takasato and colleagues produced organoids using undefined serum components as noted by the authors – thus, the resulting cell populations are inherently highly heterogenous (including non-kidney cell types such as skin and nerve cells), and it remains unknown what fraction of the cells in the organoids are actually kidney cells or more specifically podocytes or podocyte progenitors cells.

9. Line 318: The 90% efficiency how is this measured? Also do they mean efficiency or purity here? Can the authors add some data or refer to a previous study? Also, the protocols described by Song et al., Rauch et al. and Ciampi et al, describe protocols to retrieve podocyte without the need for selection or genetic manipulations. This should also be acknowledged.

We note that our method produces the desired cell type with 90% yield without the need for sub-population sorting. This value was quantified via flow cytometry and confirmed via immunofluorescent microscopy. For additional data concerning the flow cytometry panels used to determine cellular purity in this protocol, we direct the reader to our previous publication, Musah *et.al Nat. Biomed.Engineering*, **2017**, 1:0069. (Section 'Introduction', paragraph 3)

10. Figure 2: The authors need to add a counter stain for a nuclear marker to show specificity of the antibodies. It is not clear if WT1 and Pax2 expression is primarily in the nucleus. It also appears that the nephrin staining is unspecific at the main staining is observed in the nuclei, rather than in areas of foot proceeds.

Figure 2 has been revised to include the nuclear counterstain DAPI for all the lineage characterization markers shown in figure 2. It is worth noting that the lineage specific protein marker Nephrin is shuttled between the cell nucleus and the cytoplasm by the putative trafficker aPKCλ as we and others have previously reported (Satoh *et al*., *J. Biochem*. 2014; and Musah *et al*., *Nature Biomedical Engineering*. 2017). Thus, specialized podocytes could have nephrin both in the cytoplasm and foot processes (Figure 4) as well as in the nucleus, although we have noted that mature podocytes predominantly express Nephrin in the cytoplasm and foot processes. In the revised version of this manuscript, we have added an additional figure (Figure 4) and revised appropriate sections in the manuscript (Representative results, paragraph 1) to describe these results and their relevance more clearly.

11.Table: Give a reference to the DU11 iPSC cells that shows quality checks of this iPS line. Is this the first time this line was used then more information should be reported as well.

As addressed above (response to editorial comment #10), the DU11 iPSC line was generated from human neonatal fibroblasts and characterized by the Bursac Lab at Duke University (Shadrin *et al., Nat. Comm*. **2017**, 8: 1825) at the Duke iPSC core facility, and had been previously used to generate human cardiomyocytes. In this protocol, we show that the DU11 line can also give rise to mature human kidney podocytes. We have revised Representative Results (paragraph 1) and the Table of Materials and Reagents (Row 4) to also indicate that the DU11 line has been tested for mycoplasma (negative) and was last karyotyped in July 2019 by our lab, and found to be karyotypically normal.

12. Table: Please adjust the table size that it fits on one page so that catalogue number and product can be seen next to each other, it is impossible to keep scrolling up and down to match these.

The table has been revised as requested.

Reviewer #3

Manuscript Summary:
This protocol is based on the author's previous method which has been published on Nature Biomedical Engineering and Nature Protocol. As long as a method paper can be published in multiple journals, this method should be ready for publication

We thank the reviewer for his/her comments.

It is correct that we have previously reported this method in the journals Nature Biomedical Engineering and Nature Protocols. We have summarized and cited these reports throughout the text of this JoVE manuscript. The previous reports lacked a video component which will be produced and made available to researchers if the manuscript is accepted for publication in JoVE. We believe the clarity of this manuscript combined with the accompanying stepwise video demonstrating the protocol will provide useful resources for researchers who are interested in replicating the stem cell differentiation method and its applications to advance the field. We have also included statements throughout the manuscript encouraging readers to refer to the text of these previous publications for additional supporting data.

In our earlier publications, we provided results from the PGP1, IMR-90–1, IISH3i-CB6, and H9 human pluripotent stem cell lines -- In this JoVE manuscript, we present an entirely new set of additional data demonstrating for the first time the successful differentiation of the DU11 human induced pluripotent stem cell line by using the same podocyte differentiation protocol. These

new results provide additional evidence that the protocol works across multiple independent stem cell lines, which will greatly help in adaptation of the protocol by other research laboratories or institutions, either for experimental or instructional purposes.

Reviewer #4

1. In the current manuscript the authors have described the generation of mature podocytes from iPS cells and their characterization. The authors explain the methods carefully from preparation of the reagents to culture of the pluripotent stem cells and close with the differentiation to the podocytes.

We thank the reviewer for his/her comments.

Major Concerns:

2. Can the authors include a schematic timeline for the experiment? That would give a nice overview of the whole protocol.

We thank the reviewer for the suggestion. In the revised version of the protocol, we have included a schematic timeline for the directed differentiation of podocytes from human iPS cells (Figure 1A).

3. Have the authors considered including a description per day for the protocol - or to state it more specifically in the methods (on day 1…, day 2…. etc)? That would provide a clearer outline of the protocol.

We thank the reviewer for this suggestion, which we also believe improves clarity of the steps involved in the protocol. We have added the corresponding days for each of the major steps in the protocol in the method section.

4. It would be nice to extend Figure 2 and include more pictures of immunofluorescence results, for example characterization of podocin, NPHS1 and NPHS2. This can illustrate how should the cells look when using the markers specified in the protocol. Can some of them be combined?

We have added an additional figure (Figure 4) which highlights the expression of nephrin in differentiated podocytes. We believe that this figure, together with our previously reported results, illustrate the appearance of nephrin (NPHS1) in differentiated podocytes. We have recently reported data for podocin expression (at both protein and transcriptome levels) in two of our recent publication using the same podocyte differentiation protocol (Musah *et al*., 2017,

Nature Biotech. 1:0069, Musah *et al*., 2018, *Nature Protocol,* 13:1662-1685) which we have also referenced in the manuscript. Given the current situation with COVID-19 and a mandatory lab shutdown, we are unable to generate additional images for podocin (NPHS2), but we expect that the podocin expression data we provided in our previous reports adequately addresses the reviewer's comments. In the revised version of this manuscript, we have revised the text (Representative Results and Discussion sections) to more clearly direct readers to these additional sets of data.

5. In the protocol authors specify that podocytes are mature when they show the development of primary and secondary foot processes, the expression of podocyte lineage-specific genes including SYNPO, PODXL, MAF, EFNB2, and the expression of proteins including podocin, neprhin, and WT1. It would be valuable to include analysis and methods for detection of primary and secondary foot processes and analysis of the specified markers in here. How should the analysis after differentiation (e.g. immunofluorescence) be performed? That would help readers of the manuscript to determine whether their cells are mature podocytes or not.

Scanning electron microscopy (SEM) enables the detailed visualization of foot process branching, which we have described in our previous publications (Musah *et al, Nat. Protocols*, **2018**, 13:1662-1685 and Musah *et al Nat. Biomed. Engineering*, **2017**, 1:0069). We have also revised our manuscript to more clearly state this point as well as direct the readers to our previous publications (high resolution SEM images) showing these intricate features of the kidney podocytes derived from human iPS cells (Representative results section, Paragraph 1)

With regards to fluorescence microscopy analysis, we typically use this technique for additional confirmation of protein-level expression of podocyte markers such as nephrin and podocin. Additional characterizations to confirm the maturation stage of podocytes can include testing for BrdU incorporation (little to no incorporation), loss of progenitor cell markers (OSR1- and Pax2-negetive), ability to form tissue-tissue interface with endothelium and glomerular basement membrane components as well as perform selective filtration of toxins and waste from blood or vasculature as we have previously demonstrated using a microfluidic organ-on-a-chip device (Musah *et al, Nat. Protocols*, **2018**, 13:1662-1685 and Musah *et al Nat. Biomed. Engineering*, **2017**, 1:0069)

6. Can the authors include a section describing the maintenance of the cells after paragraph 6.6? How to maintain the cells after passaging in CultureBoost-R? Do they need to be split? How? How many cells to plate?

We have added a note to section 6.6 to address maintenance of the cells in CultureBoost-R (which we refer to as podocyte maintenance media, in the revised manuscript). Since the hiPS cell-derived podocytes are terminally differentiated by the end of the protocol and they do not self-replicate, it is not necessary to split the cells. We recommend that researchers change media every other day during the maintenance phase of the protocol

Minor Concerns:

7. The authors call the protocol completely defined, however the use of Matrigel in the maintenance of the pluripotent stem cells is a concern.

As noted by this reviewer, Matrigel was used only for the routine propagation of the human pluripotent stem cell lines, a standard protocol for the field. Importantly, there is no Matrigel or serum components used in the mesoderm, intermediate mesoderm, and podocyte differentiation steps described in this manuscript. Thus, our differentiation method is chemically defined because all the extracellular matrix and cell differentiation media have known compositions of specific types of proteins and small molecules at defined concentrations as indicated in the manuscript text. By using this protocol, one can more clearly examine the contribution of each molecule to tissue development in a way that cannot be fully understood if the differentiation method included serum or animal-derived matrices with unknown chemical composition. In any case, we have revised the text of the manuscript in the Discussions section (paragraph 3) to more clearly state this point.

8. Perhaps include in 1.2 that plates should be wrapped in parafilm

We thank the reviewer for noting this omission. We have revised this step (now step 1.3 in the revised copy of the manuscript) accordingly.

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