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.