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

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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[2,3,4,5] 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[8,9,10]. 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[14,15,16]. 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. 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.

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.

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.

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.

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.

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.

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).

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

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. 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.

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

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.
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. 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.
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)

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.

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.
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.
    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.
    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. 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.
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.
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)

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

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.
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.

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

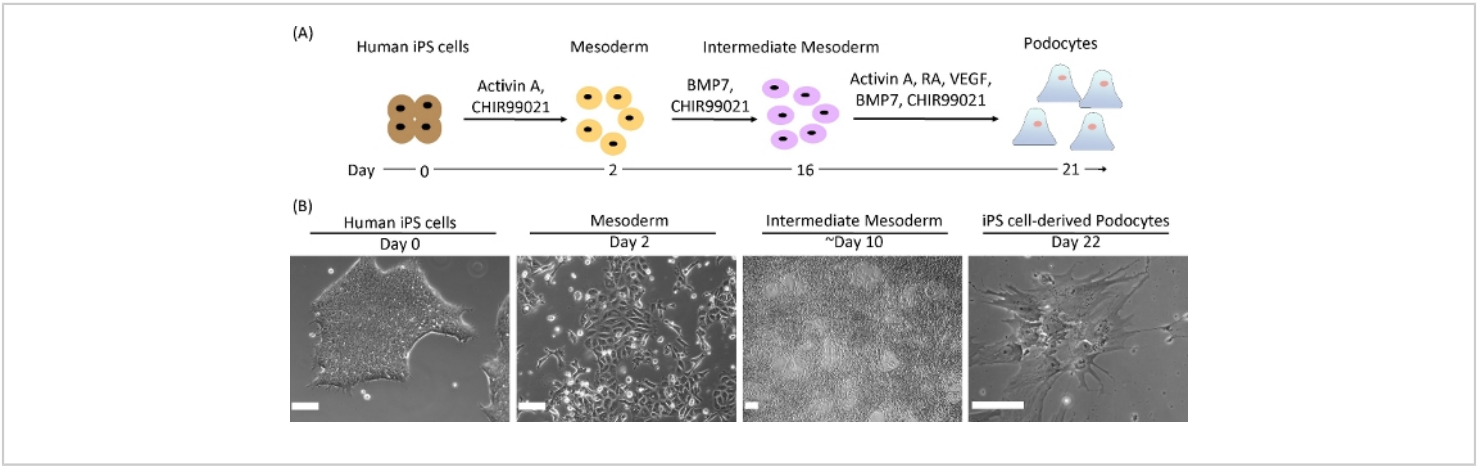

Figure 1: Differentiation of hiPS cells to podocytes.

(**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). Please click here to view a larger version of this figure.

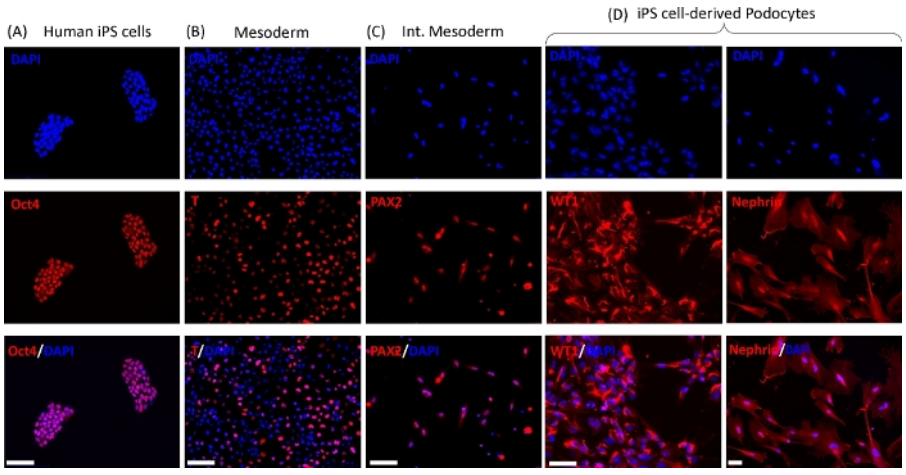

Figure 2: Immunofluorescent images of hiPS cells and their differentiated derivatives showing the expression of lineage-specific markers and nuclear counterstaining during the differentiation timeline.

(**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. Please click here to view a larger version of this figure.

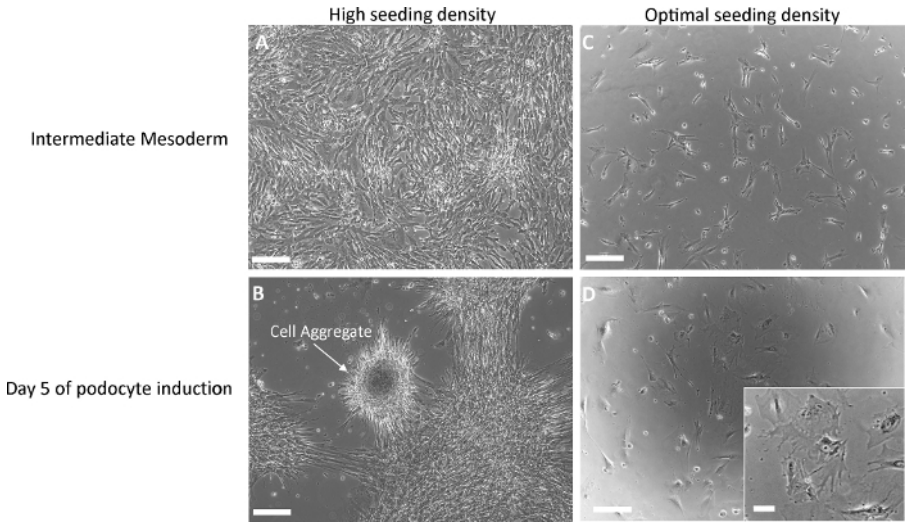

Figure 3: Effects of sub-optimal seeding density on the differentiation potential of intermediate mesoderm cells derived from the DU11 hiPS cell line.

(**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. Please click here to view a larger version of this figure.

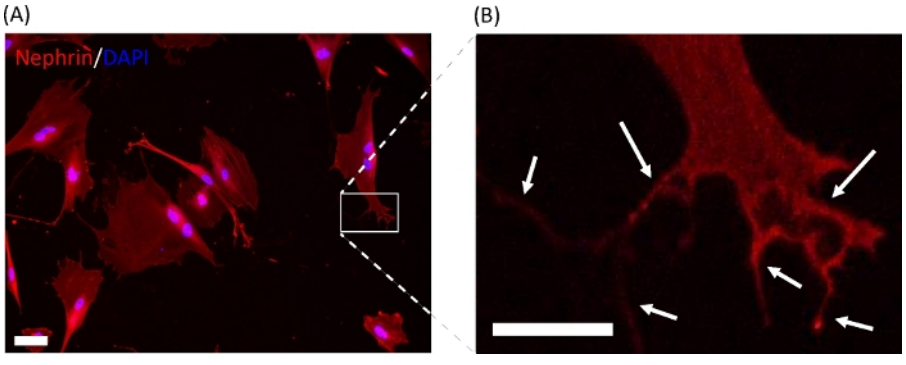

Figure 4: hiPS cell-derived podocytes exhibit morphologically mature phenotype *in vitro*.

(**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). Please click here to view a larger version of this figure.

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.

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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