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Abstract

During development, the mammalian kidney arises from the intermediate mesoderm, an early embryonic germ layer, which further gives rise to the metanephric mesenchyme and the ureteric bud, the two progenitor populations of the kidney. The advancements in the field of human pluripotent stem cells (hPSCs), characterized by their capacity to differentiate into every cell type of our body, have made possible the development of procedures to recapitulate early events of kidney development in vitro. This is achieved through direct differentiation of hPSCs into primitive streak, followed by intermediate mesoderm and nephron progenitor cells, which are further aggregated and cultured in three-dimensional spheroids to generate kidney organoids. Here we describe in detail the stepwise protocol that we have recently developed to generate kidney organoids in a process that lasts a total timeline of 20 days.

Keywords (separated by '-')

Kidney organoid - Human pluripotent stem cells - Differentiation - Primitive streak - Intermediate mesoderm - Nephron progenitor cells - 2D Monolayer - 3D Organotypic culture - Nephrons - Flow cytometry - Immunocytochemistry

Chapter 12

Directed Differentiation of Human Pluripotent Stem Cells for the Generation of High-Order Kidney Organoids

Idoia Lucía Selfa, Maria Gallo, Núria Montserrat, and Elena Garreta

Abstract

During development, the mammalian kidney arises from the intermediate mesoderm, an early embryonic germ layer, which further gives rise to the metanephric mesenchyme and the ureteric bud, the two progenitor populations of the kidney. The advancements in the field of human pluripotent stem cells (hPSCs), characterized by their capacity to differentiate into every cell type of our body, have made possible the development of procedures to recapitulate early events of kidney development in vitro. This is achieved through direct differentiation of hPSCs into primitive streak, followed by intermediate mesoderm and nephron progenitor cells, which are further aggregated and cultured in three-dimensional spheroids to generate kidney organoids. Here we describe in detail the stepwise protocol that we have recently developed to generate kidney organoids in a process that lasts a total timeline of 20 days.

Key words Kidney organoid, Human pluripotent stem cells, Differentiation, Primitive streak, Intermediate mesoderm, Nephron progenitor cells, 2D Monolayer, 3D Organotypic culture, Nephrons, Flow cytometry, Immunocytochemistry

1 Introduction

Human pluripotent stem cells (hPSCs) can be exposed to a series of developmental cues in form of cytokines, growth factors, chemical compounds, and biophysical cues [1] to direct their differentiation toward specific cell lineages occurring during kidney development [2]. The self-aggregation and self-organization of the differentiating cells result in three-dimensional (3D) spheroids that resemble in structure and function of the mammalian developing kidney, known as kidney organoids (reviewed in [3–5]). Several protocols have described different approaches to generate organoids containing nephron-like structures [1, 6–9]. Most of the procedures developed up to date account with a first stage of differentiation in where undifferentiated hPSCs are guided to the posterior primitive streak (PPS) fate by the endogenous activation of WNT using the GSK-3ß

Mo R. Ebrahimkhani and Joshua Hislop (eds.), *Programmed Morphogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 2258, https://doi.org/10.1007/978-1-0716-1174-6_12,

inhibitor CHIR99201 (CHIR). To that end, different lengths and doses of CHIR treatment have been described [1, 6–11]. In the same manner, different laboratories have shown that the commitment of PPS toward intermediate mesoderm (IM) and nephron progenitor cells (NPCs) can vary among protocols [1, 6–11]. In this chapter, we explain in detail the kidney organoid protocol recently developed in our laboratory, during which we have put in place a procedure to differentiate hPSCs for a short period of 4 days in a two-dimensional (2D) culture fashion toward IM-like cells that are then aggregated into 3D spheroids and cultured under organotypic conditions for 16 additional days. Under these permissive conditions, IM-committed spheroids are kept in the presence of growth factors for 5 days to induce NPC commitment. Three additional days are then required for the formation of renal vesicle (RV) structures—the precursor structures of the nephrons—that appear within the organoids 24 h after complete growth factor removal due to a process of mesenchymal to epithelial transition. During the following 8 days, RVs acquire proximal–distal polarity and develop into nephron-like structures by recapitulating in vitro the process known as nephron patterning (Fig. 1). Kidney organoids reveal the presence of segmented nephron-like structures containing glomeruli with podocyte-like cells connected to proximal and distal tubular structures [1]. In contrast to recent works that showed the possibility to differentiate hPSCs into kidney organoids transcriptomically matching first trimester gestational kidney in a process of 25 days, the method described here takes advantage of the aggregation of IM-like cells very early during the differentiation process to boost cell-to-cell contact and cell-to-extracellular matrix contact to efficiently generate kidney organoids with a similar transcriptomic profile as that found in second-trimester human gestational kidney [1].

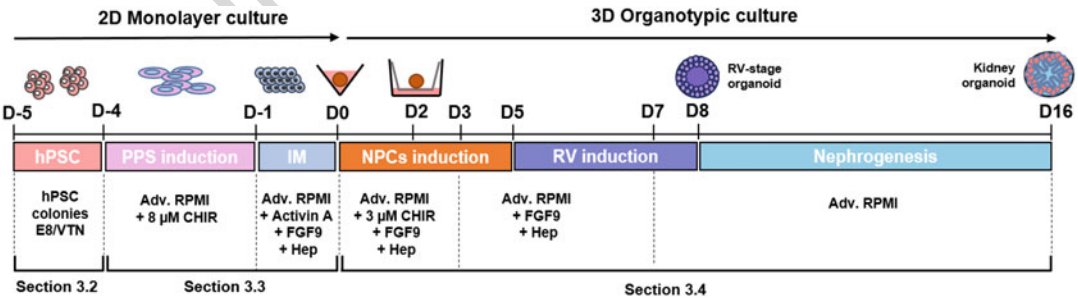

Fig. 1 Kidney organoid differentiation protocol. The days of the protocol are indicated as "D". hPSCs: human pluripotent stem cells. *PPS* posterior primitive streak, *IM* intermediate mesoderm, *NPCs* nephron progenitor cells, *RV* renal vesicles, *E8* essential 8 medium, *VTN* vitronectin, *Adv RPMI* advanced RPMI 1640 basal medium, *Hep* heparin

During the last decade, several laboratories have demonstrated the generation of organoids from hPSC resembling not only kidney but also a variety of organs including eye cup, brain, intestine, and lung among others [5]. The field is rapidly evolving, and several studies have recently highlighted organoids' potentiality for modeling organ development and disease in the human context, opening the door to future drug discovery and regenerative medicine applications. However, the organoid field is still facing major challenges, in part associated to the lack of control in the self-organizing events occurring during organoid formation in current methodologies, leading to high organoid variability as well as lack of essential cellular components (i.e., vascularization) and incomplete maturation. In this regard, emergent bioengineering technologies including biomimetic materials, microtechnologies, and 3D bioprinting [12, 13] can be used to harness and control organoid morphogenesis by precisely tuning the organoid niche (i.e., cell–cell and cell–extracellular matrix interactions) (reviewed in [5, 14]). In this regard, we have shown that kidney organoids can vascularize upon implantation onto the chick chorioallantoic membrane (CAM), thus acquiring relevant features of podocyte maturity [1]. Of note, when the soft CAM microenvironment was mimicked in vitro using polyacrylamide hydrogels, these accelerated the formation of kidney organoids that indeed contained more nephron-like structures in comparison to a rigid microenvironment [1]. Overall, the exploitation of bioengineering tools as well as other emergent disciplines (i.e., gene editing, single-cell analysis, force mapping by in toto imaging, computational modeling, among others) in combination with the morphogenetic potential of organoids is revealing as a promising scenario toward the next-generation organoid models.

2 Materials

2.1 Culture and Passage of hPSCs

1. Air-vented 10 cm Petri dishes, Nunclon Delta™ (150350, Thermo Scientific).
2. Phosphate-buffered saline (PBS) pH 7.4 (1×) (1001–015, Life Technologies).
3. Vitronectin (VTN-N) (A14700, Fisher Scientific). The vitronectin coating is prepared by diluting the vitronectin stock solution (0.5 mg/mL) at a 1:100 ration in 1× PBS. Coating of an air-vented 10 cm Petri dish is performed by diluting 50 μL of the vitronectin stock solution in 5 mL of PBS to have a final concentration of 0.5 μg of vitronectin/mL. For 24-well plates (*see* Subheading 2.2), 400 μL of diluted vitronectin (5 μg/mL) per well is used. Plates containing the vitronectin solution are left at room temperature for 1 h or kept at 4 °C overnight until further use.

4. Essential 8 medium (A1517001, Life Technologies). It is provided as a two-component kit (500 mL basal medium bottle and 10 mL supplement). Besides adding the 10 mL supplement, add 5 mL of Penicillin/Streptomycin (10,000 U/mL, 15140122, Life Technologies).
5. 0.5 EDTA (15575-038, Life Technologies): dilute an EDTA stock solution (0.5 M) at a 1:1000 ratio in 1× PBS.

2.2 Plating of hPSCs for Differentiation

1. 24-Well plate, Nunclon Delta™ (142475, Thermo Scientific).
2. Round coverslips, diameter 12 mm, #1.5 (CBA-D00120RAC20MNZ#0, Fischer Scientific). Autoclave the coverslips before use for cell culture.
3. Microfuge tubes, 1.5 mL (200400P, Deltalab).
4. Accumax (07921, Stem Cell Technologies).
5. Dulbecco's modified eagle medium (DMEM) (11966025, Life Technologies).
6. Fetal Bovine Serum (FBS) (10270-106, Gibco).
7. Neubauer chamber (0640010, Superior Marienfeld).

2.3 Differentiation of hPSCs Toward IM-Committed Cells and Generation of Kidney Organoids

1. Advanced RPMI 1640 basal medium (12633-012, Life Technologies): A bottle of 500 mL of advanced RPMI 1640 basal medium is supplemented with 5 mL of L-GlutaMAX (200 mM, 35050–038, Life Technologies) and 5 mL of penicillin/ptreptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, 15140122, Life Technologies).
2. 12 µM CHIR99021 (SML1046-5MG, Sigma): dilute 5 mg of CHIR99021 in 896 µL of dimethyl sulfoxide (D2650, Sigma), aliquot, and store at −20 °C.
3. 50 µg/mL Recombinant human FGF9 (100-23B, Peprotech): reconstitute in cell-culture-grade distilled water (15230-089, Life Technologies), aliquot, and store at −20 °C.
4. 50 mg/mL Heparin (H3149-10KU, Sigma): reconstitute in cell-culture-grade distilled water (15230-089, Life Technologies), aliquot, and store at 4 °C.
5. 50 µg/mL Activin A (338-AC-050, R&D Systems): reconstitute in sterile 4 mM hydrochloric acid (HCl), aliquot, and store at −20 °C.
6. 96-well plate, V-bottom, Nunc™ (249935, Thermo Scientific).
7. Corning® Transwell® polyester membrane cell culture inserts (CLS3460, Sigma).
8. Thin glass Pasteur pipette, 150 mm (5426015, Normax).
9. 200 µL-wide orifice pipette tips (E1011-8400, Starlab). Tips need to be autoclaved for cell culture use.

2.4 Flow Cytometry Analysis of Differentiation Markers

1. eBioscience™ Foxp3/Transcription factor staining buffer set (00-5523-00, Invitrogen). Components are fixation/permeabilization concentrate (00-5123), fixation/permeabilization diluent (00-5223) and 10× permeabilization buffer (00-8333). Prepare fresh Foxp3 fixation/permeabilization working solution by mixing one part of Foxp3 fixation/permeabilization concentrate with three parts of Foxp3 fixation/permeabilization diluent. Prepare a 1× working solution of permeabilization buffer by mixing one part of 10× permeabilization buffer with nine parts of distilled water.
2. Accumax (07921, Stem Cell Technologies).
3. Falcon® 5 mL Round-Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap (352235, Corning).
4. LIVE/DEAD™ Fixable violet dead cell stain kit (L34963, Invitrogen).
5. Fetal bovine serum (FBS) (10270-106, Gibco).

Antibodies and Isotype Controls

6. Oct3/4, mouse IgG1 κ, Alexa Fluor (AF) 488, Clone 40/Oct3 (560253, BD Pharmigen).
7. Mouse IgG1 κ, AF488-conjugated isotype control, clone MOPC-21 (557721, BD Pharmigen).
8. Brachyury, goat IgG, APC (IC2085A, R&D Systems).
9. Goat IgG, APC-conjugated isotype control (IC108A, R&D Systems).
10. PAX2, goat IgG (AF3364, R&D Systems). Before use, conjugate PAX2 antibody to AF488 using the Lightning-Link® Rapid conjugation kit (322-0010, Innova Biosciences) following manufacturer instructions.
11. Goat IgG, AF488-conjugated isotype control (IC108G, R&D Systems).

2.5 Immunocytochemistry Analysis of Differentiation Markers

1. Fixation solution of 4% paraformaldehyde. In the fume hood, prepare this solution by mixing 2.5 mL of 16% paraformaldehyde (153799, Aname) with 7.5 mL of 1× PBS. Prepare fresh and use it upon preparation.
2. Tris–buffered saline (TBS), 10× (pH 7.4–7.5). To prepare this buffer, dissolve 132.2 g of Trizma–HCl (T6666, Sigma), 19.4 g of Trizma base (T6791, Sigma), and 90.0 g of NaCl (S7653, Sigma) in 855 mL of distilled water. Keep the solution at 4 °C and use it within 1 month.
3. TBS, 1× (pH 7.4–7.5). To prepare this buffer, dilute 10× TBS at 1:10 ratio by mixing 900 mL of distilled water and 100 mL of 10× TBS. Keep the solution at 4 °C and use it within 2 weeks.

4. TBS, 1× (pH 7.4–7.5) supplemented with 1% Triton X-100 (50 mL). To prepare this buffer, dilute 500 μL of Triton X-100 (T8787, Sigma) in 50 mL of TBS, 1× (pH 7.4–7.5). Keep the solution at 4 °C and use it within 2 weeks.
5. TBS, 1× (pH 7.4–7.5) supplemented with 0.5% Triton X-100 (50 mL). To prepare this buffer, dilute 250 μL of Triton X-100 (T8787, Sigma) in 50 mL of TBS, 1× (pH 7.4–7.5). Keep the solution at 4 °C and use it within 2 weeks.
6. Blocking solution (10 mL) containing 1% Triton X-100 and 6% donkey serum. To prepare this solution, dilute 600 μL of donkey serum (S30-KC, Sigma) in 9.4 mL of TBS, 1× (pH 7.4–7.5) containing 1% Triton X-100. Prepare fresh blocking solution and use it upon preparation.
7. Streptavidin/Biotin blocking kit (SP-2002, Vector Labs) is used as an additional blocking step when biotinylated Lotus Tetragonolobus Lectin (LTL) is used to stain proximal tubule-like structures. Briefly, use 4–5 drops of streptavidin solution and incubate 20 min. Wash once with 1× TBS for 15 min at RT. Then use 4–5 drops of biotin solution and incubate 20 min. Wash once with 1× TBS for 15 min at RT.
8. For diluting the antibodies and for the washing steps, prepare a solution (25 mL) of 1× TBS containing 0.5% Triton X-100 and 6% donkey serum by mixing 1500 μL of donkey serum (S30-KC, Sigma) in 23.5 mL of 1× TBS containing 1% Triton X-100. Prepare fresh and use it upon preparation.
9. In case LTL is used to stain proximal tubule-like structures, for diluting the antibodies and LTL, and for the washing steps, prepare a solution (25 mL) of 1× TBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA) by dissolving 0.25 g of BSA (A4503, Sigma) in 25 mL of 1× TBS containing 0.5% Triton X-100. Prepare fresh and use it upon preparation.

2.6 Primary Antibodies

1. Brachyury, goat IgG (AF2085, R&D Systems).
2. Oct4, mouse IgG, clone C10 (Sc-5279, Santa Cruz Biotechnology).
3. PAX2, goat IgG (AF3364, R&D Systems).
4. SALL1, mouse IgG2a, clone K9814 (PP-K9814-00, R&D Systems).
5. SIX2, rabbit IgG (11562-1-AP, Proteintech).
6. WT1, rabbit IgG, clone CAN-R9(IHC)-56-2 (ab89901, Abcam).
7. E-Cadherin, mouse IgG2a,κ, clone 36 (610181, BD Bioscience).
8. Podocalyxin, goat IgG (BAF1658, R&D Systems).

2.7 Secondary Antibodies and Other Reagents

1. Donkey anti-goat IgG AF488 (705-545-147, Jackson ImmunoResearch).
2. Donkey anti-mouse IgG Cy3 (715-165-151, Jackson ImmunoResearch).
3. Donkey anti-mouse IgG AF488 (A21202, Fischer Scientific).
4. Donkey anti-rabbit IgG Cy3 (711-165-152, Jackson ImmunoResearch).
5. Donkey anti-goat IgG AF555 (A21432, Fischer Scientific).
6. Donkey anti-mouse IgG AF647 (715-605-151, Jackson ImmunoResearch).
7. Biotinylated Lotus Tetragonolobus Lectin (LTL) (B-1325, Vector Laboratories).
8. DyLight 488 Streptavidin (SA-5488, Vector Labs).
9. 4′,6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) (D1306, Invitrogen). To make a 5 mg/mL (14.3 mM) DAPI stock solution, dissolve the contents of one vial (10 mg) in 2 mL of distilled water. For long-term storage, aliquot the stock solution and store at −20 °C.

2.8 Other Materials

1. Histology mold, plastic (20447200820, Laboquimia). To embed the organoids, prepare a solution of 0.8% low gelling temperature type VII agarose (A4018, Sigma) by dissolving 0.8 g of agarose in 100 mL of distilled water. Use the plastic mold to place the organoids together with pre-warmed 0.8% agarose. Leave at 4 °C to obtain an agarose block containing the organoids.
2. Dako Pen (S200230, Agilent). Use it to surround tissue sections on the slide to create a smaller area for antibody incubation.
3. Fluoromount-G (0100-01, Southern Biotech).
4. Round coverslips, diameter 12 mm, #1.5 (CBA-D00120RAC20MNZ#0, Thermo Scientific).
5. Rectangular coverslip, 24 × 50 mm, #1.5 (BBAD02400500#SC13MNZ#0##, Thermo Scientific).
6. Microscope slide, 25 × 75 × 1.0 mm (J1800AMNZ, Thermo Scientific).
7. Nail polish to fix the coverslips when mounting the samples.

3 Methods

The methodology to generate kidney organoids from hPSCs (Fig. 1) has been divided into four methodological stages involving the culture and passage of hPSCs (Subheading 3.1), the plating of hPSCs for differentiation (Subheading 3.2), the differentiation of

hPSCs toward posterior primitive streak (PPS) and intermediate mesoderm (IM) (Subheading 3.3), and the formation and culture of kidney organoids (Subheading 3.4). Next, by means of flow cytometry (Subheading 3.5) and immunocytochemistry (Subheading 3.6) analyses, a methodology to analyze the differentiation outcomes at different time points during the generation of kidney organoids is also described.

3.1 Culture and Passage of hPSCs

1. hPSCs are grown on vitronectin-coated air-vented 10 cm Petri dishes in Essential 8 Medium and incubated with 5% CO_2 at 37 °C. In order to avoid their spontaneous differentiation, hPSCs need to be passaged before they reach 100% confluency.
2. For enzymatic passaging, aspirate the Essential 8 medium from hPSCs monolayers at approximately 80% confluency.
3. Perform a quick and gentle wash by rinsing twice with 8 mL PBS.
4. Add 5 mL of 0.5 mM EDTA. Place the cells in an incubator at 37 °C for 3–4 min. Aspirate the EDTA and gently disaggregate the cells by flushing 1 mL of Essential 8 media to the hPSCs colonies in order to detach the cells into small clusters (*see* **Note 1**). Collect the clumps of cells into a tube. To prevent excessive dissociation of cells, use a fresh 1 mL of Essential 8 Media each time and repeat the process until complete detachment of all cells in the plate. Normally, 10 mL of Essential 8 Media is used to collect the cell clumps in a 15-mL tube.
5. Take 250 μL of the cell suspension and complete to 10 mL with Essential 8 media in a new 15-mL tube to passage the cells at a 1:40 ratio (*see* **Note 2**).
6. Aspirate the vitronectin of a new air-vented 10 cm Petri dish (for vitronectin coating preparation, *see* Subheading 2.1, **item 3**) and directly plate the diluted cell suspension. Gently move the plate in all directions to evenly distribute the cell clusters on the plate.
7. Culture the cells in an incubator at 37 °C with 5% CO_2 for approximately 1 week, changing the Essential 8 media every second day.

3.2 Plating of hPSCs for Differentiation

1. Prepare fresh 24-well plates coated with vitronectin. Prepare also an additional 24-well plate containing 12 mm round glass coverslips coated with vitronectin, which will be further used to analyze the extent of differentiation into PPS and IM by immunocytochemistry (*see* Subheading 3.6).
2. Start the procedure as if it is a normal hPSCs passage and collect the undifferentiated cells in 10 mL of Essential 8 media in a 15-mL tube (*see* Subheading 3.1, **steps 1–4**).

3. For cell counting, take 1 mL of the cell suspension, place it in a 1.5 mL microfuge tube, and centrifuge for 5 min at 100 *g* to obtain a cell pellet (*see* **Note 3**).
4. Remove the Essential 8 media, add 1 mL of 1× PBS to wash the cell pellet, and centrifuge again for 5 min at 100 *g*.
5. Remove the 1× PBS and add 300 μL of Accumax and incubate at 37 °C for 5 min to allow single-cell dissociation for cell counting.
6. After the incubation time, cancel Accumax activity by adding 700 μL of DMEM media supplemented with 10% FBS.
7. Take 10 μL of the single-cell suspension and place it in a Neubauer chamber for cell counting (*see* **Note 4**).
8. Based on cell number, resuspend the cells in the appropriate volume of Essential 8 media to have 2 × 10^5 cells/mL. Plate 500 μL of the resultant cell suspension per well of a 24-well plate to have 10^5 cells in each well (*see* **Note 5**).
9. Plating density and cell colony distribution is essential for an efficient differentiation. In order to prevent cell clusters to come together in the center of the wells of a 24-well plate, shake vigorously the plate in all directions and carefully place the plate in the incubator. Avoid opening and closing the incubator during the next 2 h to ensure an even distribution of the adhered cells in the plate.
10. Culture the cells at 37 °C with 5% CO_2 for 24 h before starting the differentiation (Fig. 2a, b; *see* **Note 6**) (Fig. 1, day −5).

3.3 Differentiation of hPSCs Toward Posterior Primitive Streak (PPS) and Intermediate Mesoderm (IM)

1. Once cell colony density and distribution are adequate (Fig. 3a), aspirate the Essential 8 media from the 24-well plate and rinse once with 1× PBS to remove remnants of it (Fig. 1, day −4).
2. Add 500 μL/well of advanced RPMI 1640 basal media supplemented with 8 μM CHIR (Fig. 1, day −4).
3. Every 24 h, replace the media with fresh advanced RPMI 1640 basal media supplemented with 8 μM CHIR for two consecutive days (Fig. 1, day −3 and day −2). Note that hPSCs start to change their morphology (Fig. 3b).
4. After the 3 days of 8 μM CHIR treatment (Fig. 1, day −1), cultured cells show an appearance of dense clusters (Fig. 3c). In order to confirm that cells have started to acquire a PPS-related fate, the differentiation extent is measured at the molecular level by the collection of cells and analysis for the loss of pluripotency-related markers such as OCT4, and the acquisition of the primitive streak marker Brachyury through both flow cytometry (*see* Subheading 3.5) and immunocytochemistry analysis (Fig. 4a; *see* Subheading 3.6).

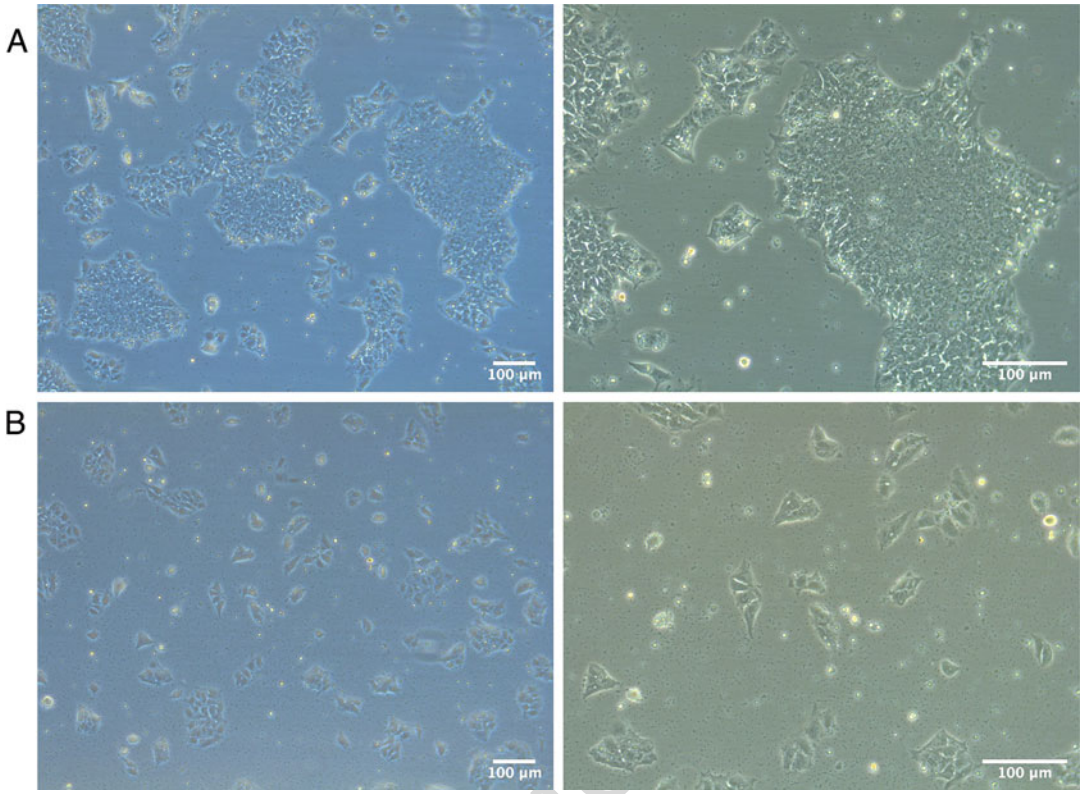

Fig. 2 Plating density and colony distribution of undifferentiated hPSCs cultured in Essential 8 media for differentiation. Representative bright-field images of ES [4] hPSCs 24 h after plating (day −4). (**a**) Differentiation is started from hPSCs colonies presenting a good compaction. (**b**) When hPSCs colonies are too small or are not well compacted, wait 12–24 h more before starting the differentiation. If waiting 24 h does not improve hPSCs colony compaction of the right confluency is not achieved, then repeat the plating of hPSCs. Higher magnification images (10×) on the right correspond to the images shown on the left (5×). Scale bars: 100 µm

5. On day −1, remove the advanced RPMI 1640 basal media supplemented with 8 µM CHIR and gently rinse once with 1× PBS (*see* **Note 7**).
6. Add 500 µL per well of advanced RPMI 1640 basal media supplemented with 200 ng/mL FGF9, 1 µg/mL heparin, and 10 ng/mL activin A (Fig. 1, day −1).
7. After 24 h (Fig. 1, day 0), cells should appear under the microscope as a tight monolayer (Fig. 3d).
8. Cells at this point can be analyzed for the expression of the IM marker PAX2, using both flow cytometry (*see* Subheading 3.5) and immunocytochemistry analysis (Fig. 4b; *see* Subheading 3.6).

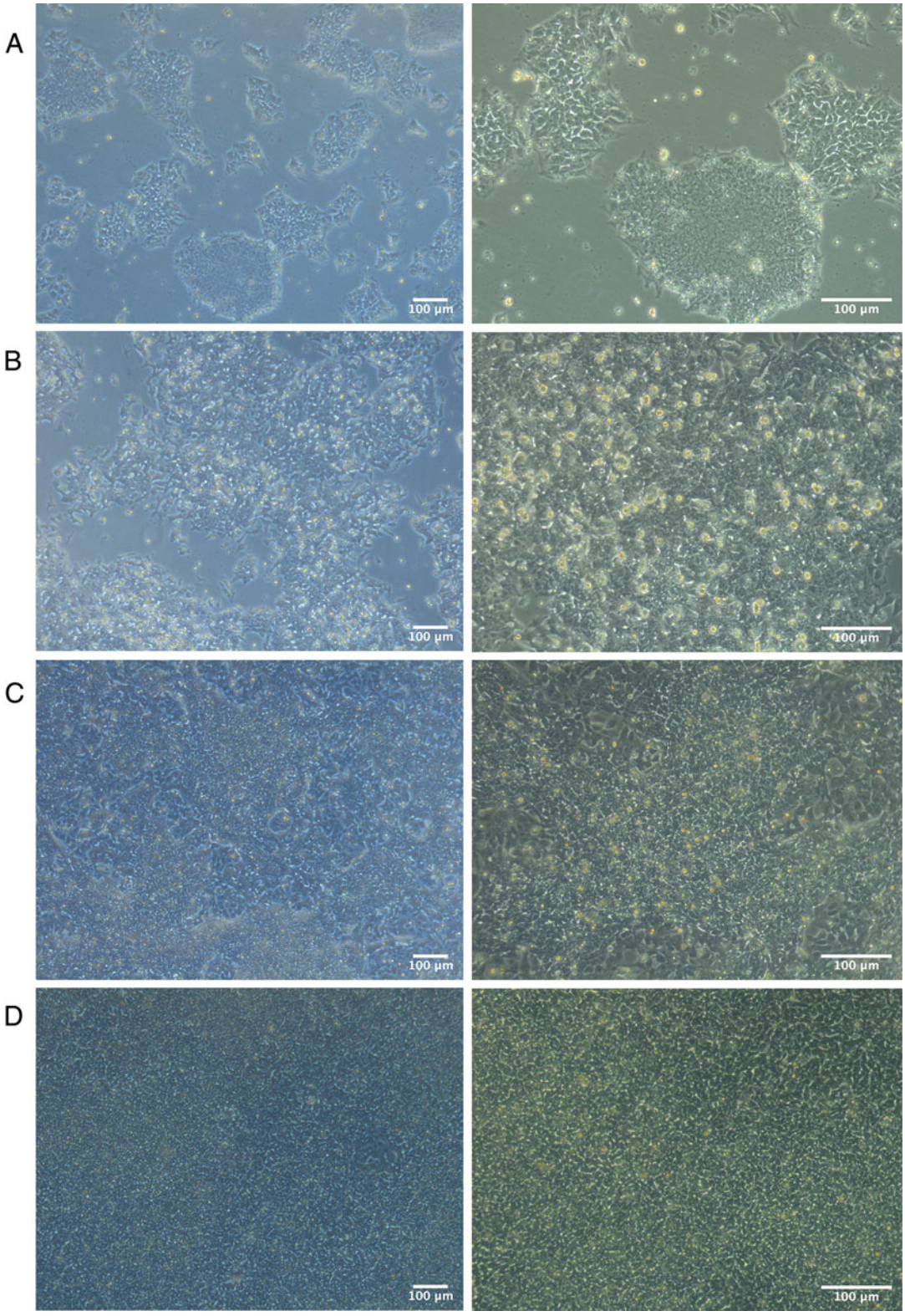

Fig. 3 Morphological changes in hPSCs during the first days of differentiation. Representative bright-field images. (**a**) Day −4: ES [4] hPSCs colonies 24 h after plating showing a typical confluency (40–50%) ready to

3.4 Formation and Culture of Kidney Organoids

1. On day 0, remove the media and rinse twice with 1× PBS. Place 500 μL of Accumax per well of the 24-well plate and incubate for 1 min at 37 °C. Carefully, aspirate the Accumax without disrupting the cell monolayer. Then, use 500 μL of fresh advanced RPMI 1640 basal media to dissociate the cell monolayer in each well and collect the single-cell suspension in a 15-mL tube (*see* **Note 8**). Normally, 12 mL of advanced RPMI 1640 basal media is used to collect the cells from a 24-well plate.

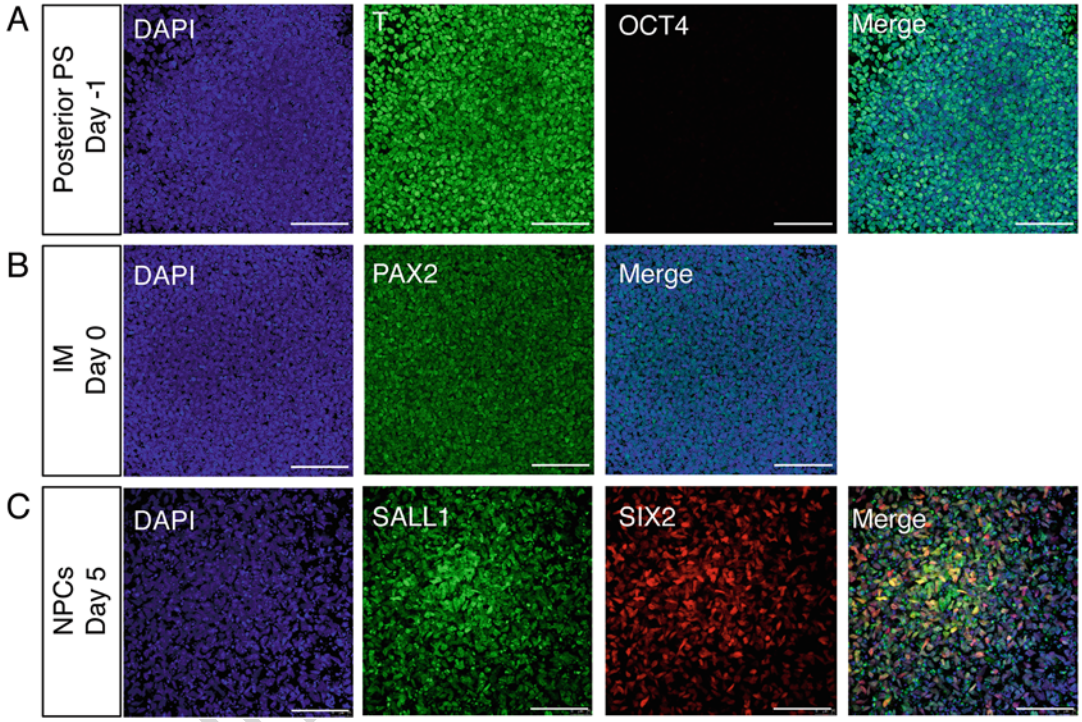

Fig. 4 Immunohistochemistry analysis for the assessment of the differentiation extent of monolayer cell cultures at different time points during the differentiation protocol. (**a**) Immunohistochemistry is performed to detect the expression of Brachyury (T), one of the major markers related to PS identity. Notice that T-positive cells do not express OCT4, one of the core pluripotency-related markers. (**b**) The commitment of PS toward the IM is assessed by the detection of PAX2 at day 0. (**c**) The NPC signature in day 5 cells is assessed by the detection of SALL1 and SIX2 markers. Scale bars: 100 μm

Fig. 3 (continued) start 8 μM CHIR treatment. (**b**) Day −2: morphology of hPSCs after 48 h of 8 μM CHIR treatment. (**c**) Day −1: appearance of cells when the monolayer is starting to form after 72 h of 8 μM CHIR treatment. Still loose clusters of cells are found at this stage. (**d**) Day 0: appearance of cells after treatment with FGF9, activin A, and heparin for 24 h. The cell monolayer appears uniform and compacted. At this stage, monolayers are ready to aggregate into 3D organoids. Higher magnification images (10×) on the right correspond to the images shown on the left (5×). Scale bars: 100 μm

2. Usually, since large amounts of cells are recovered after cell dissociation, an aliquot of the cell suspension is diluted at 1:4 ratio in advanced RPMI 1640 basal media to have a proper cell density to correctly perform the cell counting. Then, 10 µL of the single-cell suspension are placed in a Neubauer chamber and cells are counted (*see* **Note 3**).
3. Based on cell number, resuspend the cells in the appropriate volume of advanced RPMI 1640 basal media supplemented with 3 µM CHIR, 200 ng/mL FGF9, and 1 µg/mL heparin to have 5×10^6 cells/mL. Pipette 150 µL of the cell suspension in each well of a 96-well plate (V-bottom) to have 5×10^5 cells per well (*see* **Note 9**).
4. Centrifuge the 96-well plate (V-bottom) for 3 min at 300 *g* (Fig. 1, day 0).
5. Maintain the 96-well plate in an incubator at 37 °C with 5% CO_2 for 48 h without medium change to allow cells to self-aggregate and induce the differentiation toward nephron progenitor cells (NPCs).
6. After 48 h (day 2), the self-aggregated spheroids are transferred to 12-well plate transwells. Carefully transfer one spheroid per transwell by placing the spheroid on top of the transwell membrane (*see* **Note 10**).
7. Immediately, add 450 µL of advanced RMPI 1640 basal media with 3 µM CHIR, 200 ng/mL FGF9, and 1 µg/mL heparin to the base of each transwell to generate an air–liquid interface organotypic culture condition (Fig. 1, day 2).
8. On day 3, aspirate the media from the transwells with thin glass Pasteur pipettes and replace it with 450 µL of advanced RMPI 1640 basal media containing only 200 ng/mL FGF9 and 1 µg/mL heparin, and culture for 48 h without media changes.
9. On day 5, aspirate the media from the transwells with thin glass Pasteur pipettes and replace it with 450 µL of fresh advanced RMPI 1640 basal media containing 200 ng/mL FGF9 and 1 µg/mL heparin, and culture for 48 h without media changes. At this point, NPCs induction can be assessed by analyzing the expression of NPC-associated markers by immunocytochemistry (Fig. 4c, *see* Subheading 3.6).
10. On day 7, growth factors are removed by replacing the media with advanced RMPI 1640 basal media. After 24 h, multiple renal vesicles (RVs)—the precursor structures of the nephrons—clearly appear within the spheroid (Fig. 1, day 8 RV-stage organoid). RVs can be visualized in bright field, and further analyzed by performing hematoxylin and eosin staining of kidney organoid sections and immunocytochemistry for the expression of RV-associated markers (Fig. 5a; *see* Subheading 3.6).

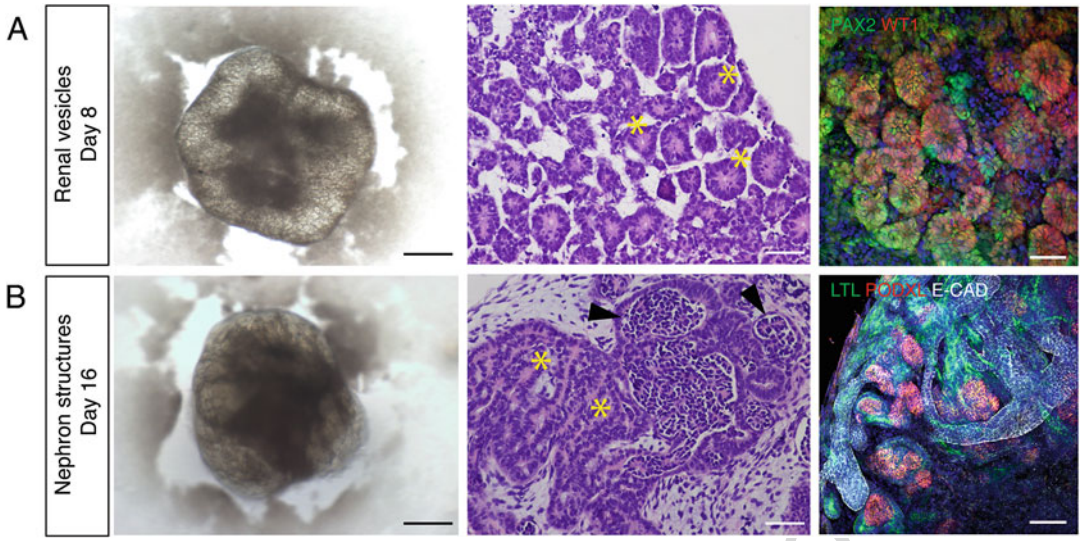

Fig. 5 Characterization of renal vesicles (RVs) and segmented nephron-like structures in hPSC-derived kidney organoids. (**a**) RV-stage kidney organoids contain RV-like structures that can be detected by different techniques. From left to right: bright-field image of a RV-stage kidney organoid, hematoxylin-eosin staining on a paraffin section of a RV-staged kidney organoid, and immunocytochemistry for the detection of PAX2 and WT1 in a RV-staged kidney organoid at day 8 of differentiation. Asterisks point to renal vesicle structures. (**b**) Segmented nephron-like structures are analyzed at day 16 during differentiation by different techniques. From left to right: bright-field image of a day 16 kidney organoid, hematoxylin-eosin staining on a paraffin section of a day 16 kidney organoid, and immunocytochemistry for the detection of Podocalyxin (PODXL) in podocyte-like cells, and Lotus Tetragonolobus Lectin (LTL) and E-cadherin (ECAD) in proximal and distal segments of the tubular-like structures, respectively. Arrowheads point to glomerular-like structures, and asterisks point to tubular-like structures. Scale bars in bright-filed images correspond to 500 μm. Scale bars in hematoxylin-eosin staining correspond to 50 μm. Scale bars in immunocytochemistry staining correspond to 75 μm

11. Change the media every second day until day 16, when kidney organoids are fully developed and contain nephron-like structures (Fig. 1, day 16 kidney organoid). The nephron structures can be visualized in bright field and further analyzed by performing hematoxylin and eosin staining of kidney organoid sections and immunocytochemistry for the expression of nephron markers (Fig. 5b; *see* Subheading 3.6).

3.5 Flow Cytometry Analysis of Differentiation Markers

1. For analysis of PPS differentiation efficiency, harvest cells at day −1. PPS induction is characterized by a marked decrease in the expression of the pluripotency-associated marker OCT4 and upregulation of the primitive streak marker Brachyury. For analysis of IM differentiation efficiency, harvest cells at day 0. IM commitment is assessed by analyzing the expression of PAX2.
2. For intracellular staining of all the above markers, use the Foxp3/transcription factor staining buffer set (*see* Subheading 2.4 for buffer preparation).

3. A minimum number of 10^5 cells/tube should be used for the analysis hereafter. Therefore, harvest approximately 3–4 wells of the 24-well plate at day −2 and 2–3 wells of the 24-well plate at day −1, to perform the analysis.
4. Remove media and gently rinse twice with 1× PBS.
5. Add 300 µL/well of Accumax and incubate for 2–5 min at 37 °C.
6. Aspirate the Accumax, dissociate cells by flushing with 1× PBS, and collect them in 1× PBS. Collect the necessary wells and resuspend the cells in 1 mL of 1× PBS.
7. Pass the cell suspension to a flow cytometry tube with 35-µm-filter caps to ensure a single-cell suspension (*see* Subheading 2.4, **item 3**).
8. Add 1 µL of reconstituted LIVE/DEAD fixable violet stain (*see* **Note 11**; reconstituted reactive should be used in 2 weeks) and incubate for 30 min at room temperature (RT) in the dark. AU1
9. Wash two times with 3 mL of 1× PBS and centrifuge for 3 min at 300 *g*. Discard the supernatant and pulse vortex the sample to completely dissociate the pellet.
10. Add 500 µL of Foxp3 fixation/permeabilization working solution to each tube and resuspend the cells in the solution.
11. Incubate for 30 min at RT in the dark.
12. Centrifuge the sample for 3 min at 300 *g* and discard the supernatant.
13. For permeabilization, wash two times with 3 mL of permeabilization buffer. Centrifuge the sample for 3 min at 300 *g* and discard the supernatant.
14. Resuspend the pellet with permeabilization buffer. At this point, divide the sample into the necessary tubes to perform the isotype control staining and the correspondent antibody stainings. For PPS analysis (day −1), resuspend in 2.5 mL of permeabilization buffer and pipette 500 µL of cell suspension per tube (five tubes). For IM analysis (day 0), resuspend in 1.5 mL of permeabilization buffer and pipette 500 µL of cell suspension per tube (three tubes).
15. Block the sample tubes with 2% FBS by adding 10 µL directly to each tube containing 500 µL of the cell suspension. Incubate for 15 min at RT.
16. Centrifuge the tubes for 3 min at 300 *g* and discard the supernatant by gently pouring the solution without disrupting the pellet.
17. Add 100 µL of permeabilization buffer to each tube and pulse vortex to dissociate the pellet.

18. Add the recommended amount of conjugated isotype controls and antibodies for detection of intracellular antigens to each tube, pulse vortex and incubate for 30 min at RT in the dark.
19. For PPS analysis (day −1), add the indicated isotype controls and antibodies:
    (a) Tube 1: do not add any reagent.
    (b) Tube 2: Goat IgG APC-conjugated isotype control (10 μL) + Mouse IgG1 AF488-conjugated isotype control (5 μL).
    (c) Tube 3: Brachyury-APC (10 μL).
    (d) Tube 4: Oct4-AF488 (20 μL).
    (e) Tube 5: Brachyury -APC (10 μL) + Oct4-AF488 (20 μL).
20. For IM analysis (day 0), add the indicated isotype controls and antibodies:
    (a) Tube 1: do not add any reagent.
    (b) Tube 2: Goat IgG AF488-conjugated isotype control (5 μL).
    (c) Tube 3: PAX2-AF488 (0.5 μL).
21. After incubation, add 1 mL of permeabilization buffer to each tube and centrifuge the tubes for 5 min at 300 *g*. Discard the supernatant.
22. Wash two times with 2 mL of permeabilization buffer to each tube, centrifuging the tubes for 5 min at 300 *g* and discard the supernatant.
23. Resuspend each cell pellet in 500 μL of 1× PBS supplemented with 2% FBS.

Acquire the sample tubes in a flow cytometer and analyze the data using a flow cytometry software such as FlowJo. The expected percentage of Brachyury$^+$ OCT4$^-$ cells in the PPS analysis (day −1) is around 80%. The expected percentage of PAX2$^+$ cells in the IM analysis (day 0) is around 85%.

3.6 Immunocytochemistry Analysis of Differentiation Markers

For the analysis of the extent of differentiation into PPS and IM, cells differentiated onto glass coverslips coated with vitronectin (*see* Subheadings 3.2 and 3.3) are collected at day −1 and day 0, respectively. For the analysis of the extent of differentiation into NPCs, RVs, and nephron structures (*see* Subheading 3.4), organoids are collected at days 5, 8, and 16, respectively. Immunocytochemistry on organoids is performed in toto. Alternatively, paraffin sectioning of organoids can be also performed (*see* **Note 12** for details on kidney organoid sample processing for paraffin sectioning and immunohistochemistry).

1. Remove the media from the correspondent wells containing the glass coverslips with cells. For organoids, remove the media from the transwells. Wash samples once with 1× PBS.
2. In the fume hood, fix the samples by adding 1 mL of 4% paraformaldehyde to each well. For organoids on transwells, add 0.5 mL of 4% paraformaldehyde inside the transwell and 1 mL of 4% paraformaldehyde in the base of the transwell. Incubate for 20–30 min at RT in the case of cells and 1 h at RT in the case of organoids.
3. In the fume hood remove the fixative and wash the samples three times with 1× PBS at RT for 5 min each in the case of cells and three times for 15 min each in the case of organoids.
4. Block the samples with 500 μL of 1× TBS containing 1% Triton X-100 and 6% donkey serum. For organoids on transwells, add 0.5 mL of the blocking solution inside the transwell and 1 mL of the blocking solution in the base of the transwell. Incubate for 1 h at RT in the case of cells and 4 h at RT in the case of organoids. In this step and during the next steps (**steps 5–12**), place the samples over a shaker when possible.
5. For nephron structure analysis in which the biotinylated Lotus Tetragonolobus Lectin (LTL) is used to stain proximal tubule-like structures, an additional blocking step with the Streptavidin/Biotin blocking kit is required to block the organoid endogenous biotin. Briefly, use 4–5 drops of streptavidin solution and incubate for 20 min. Wash once with 1× TBS for 15 min at RT in the case of cells and 15 min at RT in the case of organoids. Then use 4–5 drops of biotin solution and incubate 20 min. Wash once with 1× TBS for 15 min at RT in the case of cells and 15 min at RT in the case of organoids. Notice that after using the Streptavidin/Biotin blocking kit, the solutions to dilute primary and secondary antibodies contain 1% BSA instead of donkey serum (*see* next **steps 6–10**).
6. After blocking, prepare the correspondent primary antibody combinations. At this point, the transwell membranes containing organoids are cut using a scalpel and placed into separate wells of a 24-well plate. Primary antibody combinations and antibody dilutions are detailed bellow. Antibodies in combinations a., b., c., and d. are diluted in 1× TBS containing 0.5% Triton X-100 and 6% donkey serum. Antibodies and LTL in combination e. are diluted in 1× TBS containing 0.5% Triton X-100 and 1% BSA.
   (a) For PPS analysis (day −1): Brachyury (1:20) + Oct4 (1:25).
   (b) For IM analysis (day 0): PAX2 (1:20).
   (c) For NPCs analysis (day 5): SALL1 (1:100) + SIX2 (1:500).

(d) For renal vesicles analysis (day 8): PAX2 (1:20) + WT1 (1:100).

(e) For nephron structures analysis (day 16): LTL (1:200) + Podocalyxin (PODXL) (1:100) + E-cadherin (ECAD) (1:50).

7. Remove the blocking buffer and incubate the samples with primary antibodies overnight at 4 °C. Use 250-µL volume per well of a 24-well plate.
8. The following day, wash the samples with 1× TBS containing 0.5% Triton X-100 and 6% donkey serum (instead of donkey serum, use 1% BSA for samples assayed for LTL) three times for 5 min each at RT in the case of cells and three times for 15 min each at RT in the case of organoids.
9. Prepare the correspondent secondary antibody combinations and dilutions as detailed below. Antibodies in combinations a., b., c., and d. are diluted in 1× TBS containing 0.5% Triton X-100 and 6% donkey serum. Antibodies and LTL in combination e. are diluted in 1× TBS containing 0.5% Triton X-100 and 1% BSA:
   (a) For PPS analysis (day −1): anti-goat 488 (1:100), anti-mouse Cy3 (1:100).
   (b) For IM analysis (day 0): anti-goat 488 (1:100).
   (c) For NPCs analysis (day 5): anti-mouse 488 (1:200), anti-rabbit Cy3 (1:100).
   (d) For RVs analysis (day 8): anti-goat 488 (1:100), anti-rabbit Cy3 (1:100).
   (e) For nephron structures (day 16): DyLight 488 Streptavidin (1:40), anti-goat 555 (1:200), anti-mouse 647 (1:100).
10. Incubate the samples with secondary antibodies diluted in 1× TBS containing 0.5% Triton X-100 and 6% donkey serum (instead of donkey serum, use 1% BSA for samples assayed for LTL) for 2 h in the case of cells and 4 h in the case of organoids at RT in the dark. Use 250 µL volume per well of a 24-well plate.
11. Wash the samples three times with 1× TBS for 5 min each at RT in the case of cells and three times for 15 min each at RT in the case of organoids.
12. For nuclei detection, incubate the samples with DAPI at a 1:5000 ratio in 1× TBS for 30 min at RT in the case of cells and 1 h at RT in the case of organoids.
13. Remove the DAPI solution and mount the samples with coverslips using Fluoromount-G as mounting medium. For cells, deposit one or two drops of Fluoromount-G on top of a

microscope slide, and then place the coverslip on top of it, with the growing cells facing the microscope slide. For transwell organoids, place first the transwell membrane with the organoid on top of the slide, add one or two drops of Fluoromount-G on top of the organoid, and then carefully place a round coverslip to cover the sample. Dry the excess of mounting medium and cover the borders with nail polish to fix the coverslips to the slides.

14. Keep the slides at 4 °C in darkness until microscopic observation. Sample fluorescence is well preserved for about 2 weeks. After this time, the intensity of the fluorescence staining could substantially decrease.
15. Acquire images on a confocal microscope.

4 Notes

1. It is essential for hPSCs not to be dissociated into single cells to prevent reduced attachment and poor survival. Therefore, it is best to first check if undifferentiated hPSCs are ready to detach by flushing a small volume of EDTA after 3 min of incubation at 37 °C. If cells do not come off, wait an extra minute before checking again and removing the EDTA. It is also important to visually check under the microscope the borders of the colonies since when hPSCs colonies are ready to be detached, the borders are shinier and refracting.
2. The plating dilution can vary among the hPSC cell line. When starting to culture hPSCs, try different passaging dilutions until finding the one that allows growth of the cells for approximately 5–6 days until reaching 80% confluency for next passage.
3. Always keep the cell suspension in the 15-mL tube in an incubator at 37 °C during the preparation of the cells for cell counting (Subheading 3.2, **steps 3**–**7**, and Subheading 3.4, **steps 1** and **2**).
4. The number of hPSCs collected out of a 10-cm Petri dish range from seven to ten million. Such differences relay in the starting cell density and cell confluency before starting the experiment.
5. To have a 24-well plate for differentiation, a total number of 2.4 million cells are needed. Generally, cell suspensions are performed in 12 mL of Essential 8 media at a concentration of 2×10^5 cells/mL. In this manner, each 500 μL contain the needed number of cells (10^5 cells) per well of a 24-well plate. The starting plating density of 10^5 cells was optimized for ES [4], H1 and H9 hPSCs lines. Importantly, the starting cell density should be tested in case other hPSCs lines are used.

6. As mentioned, plating density and cell colony distribution are essential for an efficient differentiation (Fig. 2a). If colonies are too small after 24 h of plating, wait additional 12–24 h before starting the differentiation (Fig. 2b).
7. After starting the differentiation, media changes and 1× PBS washes should be performed very gently. As cells start to differentiate, they tend to become looser and can easily detach from the plate, leaving spaces without cells that can greatly detriment differentiation efficiency and formation of monolayer on day 0.
8. On day 0, cells are very loosely attached to the plate surface, so for a mild enzymatic treatment is sufficient to detach them and obtain a single-cell suspension.
9. Using the ES [4] human embryonic stem cell line, the monolayer differentiation of one 24-well plate (from day −4 to day 0) should be sufficient enough to generate one 96-well plate of kidney organoids (from day 0 to day 16). Expected cell numbers on day 0 should be approximately 2.5–3 × 10^6 cells per well of a 24-well.
10. To transfer the spheroid onto transwells, use a 200-μL-wide orifice pipette tip to prevent damaging or fragmenting the spheroid. Carefully place the spheroid in the middle of the transwell membrane and remove with the 200 μL-micropipette, the remaining medium from the membrane to leave only the spheroid. Afterwards, add 450 μL of media through the side of the transwell into the base of the well to maintain the organoid under the air–liquid interface organotypic culture condition.
11. Once LIVE/DEAD fixable violet stain solution has been reconstituted, it should be used in the following 2 weeks.
12. For performing organoid sections, after organoid fixation (*see* Subheading 3.6, **steps 1–3**), place the organoids in a 7 × 7 × 5 mm histology mold and embed the organoids in 0.8% low gelling temperature agarose. Once solidified, embed the block of agarose containing the organoids into paraffin following the classic histology procedure. Then, perform 5 μm thick sections using a microtome. Next, dewax and rehydrate organoid sections following the classic histology procedure. Then, proceed with an antigen retrieval consisting of citrate buffer (pH 6) at 95 °C for 30 min. Afterwards, continue with the blocking step and antibody incubations steps using 3% instead of 6% donkey serum in blocking, washing, and antibody solutions (*see* Subheading 3.6, **steps 4–9**). Use 1 mL of blocking buffer to incubate each slide. After blocking, use Dako pen to surround the tissue section on the slide to create a smaller area for antibody incubation. Create an area with one tissue section for negative control (only

secondary antibody incubation). The Dako pen ink is water repelling and prevents diffusion of the antibody dilutions during incubations. Use 300 μL for primary and secondary antibody dilutions and incubation per slide, following the combinations described in the protocol (Subheading 3.6, **steps 6–12**). After DAPI incubation, add three or four drops of Fluoromount-G on top of the slide and carefully cover it with a coverslip. Dry the excess of mounting medium and seal the borders with nail polish.

Acknowledgments

I.L.S. has received financial support through the "la Caixa" INPhINIT Fellowship Grant for Doctoral studies at Spanish Research Centres of Excellence, "la Caixa" Banking Foundation, Barcelona, Spain. The fellowship code is LCF/BQ/IN17/11620003. This project has received funding from the European Union´s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 713673.

This work has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (StG-2014-640525_ REGMAMKID to E.G. and N.M.), the Spanish Ministry of Economy and Competitiveness/FEDER (SAF2015-72617-EXP to N.M., SAF2017-89782-R to M.G. and N.M., and RYC-2014-16242 to N.M.), the Generalitat de Catalunya and CERCA program (2017 SGR 1306 to N.M.), la Asociación Española contra el Cáncer (LABAE16006 to N.M.). N.M. is also supported by CardioCel (TerCel, Ins5tuto de Salud Carlos III). IBEC is the recipient of a Severo Ochoa Award of Excellence from MINECO.

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