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Generation of Functional Kidney Organoids In Vivo Starting from a Single-Cell Suspension

Valentina Benedetti*, Valerio Brizi*, and Christodoulos Xinaris

Abstract

Novel methods in developmental biology and stem cell research have made it possible to generate complex kidney tissues in vitro that resemble whole organs and are termed organoids. In this chapter we describe a technique using suspensions of fully dissociated mouse kidney cells to yield organoids that can become vascularized in vivo and mature and display physiological functions. This system can be used to produce fine-grained human–mouse chimeric organoids in which the renal differentiation potential of human cells can be assessed. It can also be an excellent method for growing chimeric organoids in vivo using human stem cells, which can differentiate into specialized kidney cells and exert nephron-specific functions. We provide detailed methods, a brief discussion of critical points, and describe some successfully implemented examples of the system.

Keywords: Kidney organoids, Stem cells, Implantation, Kidney engineering, Glomerulogenesis, Dissociation-reaggregation assay, VEGF, Cell suspensions, Kidney development

1 Introduction

Kidney tissue generated from single cells is a powerful tool for investigating human kidney development, modeling disease, developing new drugs as well as for evaluating novel regenerative medicine strategies.

One classic, pioneering study, reported that after dissociation into a single cell suspension and then reaggregation, mouse metanephric mesenchyme (MM) can be induced by spinal cord cells to generate three-dimensional (3D) renal tissue in vitro that contains rudimental nephron-like structures [1]. Based on this study, a new method has been established wherein whole embryonic kidneys are dissociated and then reaggregated and cultured in vitro in the presence of a Rho-associated protein kinase (ROCK) inhibitor to prevent apoptosis. This protocol leads to the formation of immature nephrons and multiple collecting ducts without using any exogenous tissue [2]. However, in vitro culture systems neither

* Author contributed equally with all other contributors.

support the long-term survival of renal tissues nor, more importantly, the development of vascularized glomeruli, both indispensable for achieving filtering function.

To overcome this limitation, we devised a new system that starts with mouse embryonic kidney cell suspensions to generate, in vitro, mouse renal tissues—called renal organoids—that can grow and mature in vivo after implantation under the kidney capsule of rat hosts [3]. The resulting tissue displays a high degree of maturation, including vascularized glomeruli containing fully differentiated podocytes [3, 4]. Furthermore, the organoid exerts kidney-specific functions, such as permselective blood filtration, tubular reabsorption of filtered macromolecules, and the production of erythropoietin. The following two crucial steps are key to our technology's success: the construction of large cell aggregate (LCA)-derived organoids that enable organoid survival and growth in vivo; and treating the organoids and rat hosts with vascular endothelial growth factor (VEGF), a molecule essential for both glomerulogenesis and nephrogenesis [5]. These technical maneuvers allow implanted organoids to integrate into the host tissue, and grow and develop functional nephrons with filtering glomeruli [3, 4].

The ability of mouse embryonic kidney cells to self-organize and generate kidney tissue after dissociation and reaggregation has been exploited to incorporate and evaluate the nephrogenic potential of different human cell types in vitro, such as amniotic fluid stem cells (AFSCs) [6], adult proximal tubule cells reprogrammed into nephron progenitors [7], bone marrow stromal cells reprogrammed into proximal tubule-like cells [8] and human pluripotent stem cell-derived renal progenitor cells [9–12]. Very recently, by mixing mouse embryonic kidney cells with AFSCs modified to temporarily express glial cell-derived neurotrophic factor (GDNF)—a molecule secreted by MM—we generated functional human–mouse chimeric organoids in vivo. The human cells preferentially localized into vascularized glomeruli in which they differentiated into highly specialized and functional podocytes [4].

Our technology can be used as a platform for testing the nephrogenic potential of human stem or renal progenitor cells, and for growing chimeric organoids in vivo where human stem cells can differentiate into specialized and functional kidney cells. Here, we provide detailed instructions of our methods for (1) the construction and culture of mouse and human–mouse chimeric organoids in vitro, (2) immunofluorescence analysis of organoids in vitro, (3) organoid implantation, and (4) histological analysis of organoids in vivo.

2 Materials

2.1 Construction and Culture of Mouse and Chimeric Organoids In Vitro

1. Glass Pasteur pipettes.
2. P1000 and P200 micropipettes.
3. 3.5 cm and 6 cm sterile petri dishes.

4. 1.5 ml Eppendorf tubes and 3–5 ml plastic tubes.
5. Embryonic kidney isolating medium: Eagle's Minimum Essential Medium (MEM; Sigma-Aldrich, St. Louis, MO, USA).
6. Culture medium: Advanced DMEM (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 2 % Embryonic Stem cells Fetal Bovine Serum (ES-FBS, Gibco), 1 % L-glutamine (Invitrogen Corporation, Carlsbad, CA), and 1 % penicillin/streptomycin (Invitrogen).
7. Trypsin–EDTA solution 0.1 % in phosphate-buffered saline (PBS) 1× without Ca^{2+} (Biochrom AG, Berlin, Germany).
8. Rho-associated protein kinase (ROCK) inhibitor. We use glycyl-H1152 dihydrochloride (Tocris) at a final concentration of 1.25 μM in culture medium.
9. Tracker to detect human cells. We use green fluorescent chloromethyl derivative of fluorescein diacetate probe CellTracker (Molecular Probes Inc., Eugene, OR, USA).
10. 40 μm cell strainer (BD Falcon, Oxford, UK).
11. 5 μm pore polycarbonate filter (Merck Millipore Ltd., Ireland).
12. Stainless steel culture grids. These are used as a support of the culture filter at the air–medium interface. A description of how to produce grids was reported previously [13]. Briefly, pieces of stainless steel mesh are cut in the shape of small squares with sides 1.5–2 cm long, and the corners are bent down to function as grid "legs" of approximately 2–3 mm.
13. Trypan blue (Sigma-Aldrich).
14. Automatic cell counter or Burker counting chamber.
15. Microcentrifuge.
16. We use the ZOOM 2000 Model Z45 E and M205 FA (Leica) stereomicroscopes.

2.2 Immunofluorescence Analysis of Organoids In Vitro

1. Paraformaldehyde 8 % aqueous solution (Electron Microscopy Sciences, Hatfield, PA, USA) made up to 4 % in PBS 2×.
2. Methanol stored at −20 °C.
3. Phosphate-buffered saline (PBS).
4. Primary antibodies: chicken anti-laminin (Sigma-Aldrich), mouse anti-calbindin D28k (Abcam, Cambridge, MA, USA), rabbit anti-paired box 2 (Pax-2) (Zymed Laboratories, San Francisco, CA, USA), goat anti-megalin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 5B8 anti-NCAM (1:2; developed by Jessel T.M., Dodd J. and Brenner-Morton S. from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-synaptopodin (Progen Biotechnik GmbH, Heidelberg, Germany), mouse anti-E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), FITC-conjugated human nuclear antigen (HNA) (Merck Millipore Ltd.).

5. Secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).
6. FITC-conjugated *Bandeiraea simplicifolia* Isolectin B4 (BSLB4) (Vector Laboratories, Burlingame, CA).
7. Dako Fluorescence Mounting Medium (DAKO Corporation, Denmark).
8. Optional: green fluorescent chloromethyl derivative of fluorescein diacetate (CMFDA) probe CellTracker (Molecular Probes Inc., Eugene, OR).
9. We use the inverted confocal laser scanning microscope LS 510 Meta Zeiss (Carl Zeiss, Jena, Germany) to reconstruct three-dimensional (3D) images of organoid tissues. We also use the Apotome fluorescence microscope Axio Vision Imager 2Z Zeiss.

2.3 Implantation of Mouse and Chimeric Organoids

Animal studies must be approved by the Institutional Animal Care and Use Committees and conducted according to the guidelines, in compliance with national and international law and policies. Given the complexity and invasiveness of the surgery required for these experiments (uninephrectomy and implantation under the kidney capsule) they should be performed on anesthetized animals, only by experienced personnel.

Below we provide a list of the materials necessary for treating and handling the organoids generated for in vivo implantation.

1. P200 micropipettes and glass Pasteur pipettes.
2. 96-well plate.
3. Recombinant rat vascular endothelial growth factor (VEGF) protein (Gibco, Invitrogen) reconstituted according to manufacturer's instructions to 0.1 mg/ml.
4. Culture medium.
5. Catheter. This homemade device is composed of a sterile polypropylene 1-ml syringe with a 5–6 cm long rubber cannula instead of the needle and a 3–4 cm long piece of the tapered end of a glass Pasteur pipette.

2.4 Renal Histology of Organoids In Vivo

1. Periodate-lysine paraformaldehyde (PLP).
2. Optimal Cutting Temperature compound (OCT).
3. Cryostat.
4. Hematoxylin and eosin (Bio-Optica, Milan, Italy).
5. Dako Faramount Aqueous Mounting Medium (DAKO Corporation).
6. Light microscope. We use Olympus BH2-RFCA (Olympus America Inc., Melville, NY, USA).

2.5 Animals

1. CD1 mice (Charles River Italia SpA, Calco, Italy).
2. Male 6–8-week-old athymic nude rats (Harlan Laboratories Inc., Indianapolis, IN, USA).

3 Methods

3.1 Construction and Culture of Mouse Organoids In Vitro

The dissection and isolation of embryonic day (E) 11.5 or 12.5 CD1 mouse kidneys have been described in detail previously [13]. The technology we describe here involves meticulous micromanipulation of tissues in open-air and in media buffered against 5 % CO_2. Therefore it is crucial to observe best-practice sterile techniques while preparing and handling instruments, solutions, and equipment to avoid culture contamination.

1. Isolate fresh E11.5 CD1 mouse kidneys in isolating medium.
2. Prepare petri dishes, culture filters, and grids for later use. Cut small squares of 5 μm polycarbonate filter about 8 mm per side using sterile scissors and tweezers. Place the metal grid in a 6 cm petri dish and add 7–8 ml (or until the medium level reaches grid height and the filter on the grid is wet) of culture medium containing 1.25 μM Glycyl-H1152 dihydrochloride. Place the filter on top of the grid and the whole dish in the 37 °C, 5 % CO_2 incubator.
3. Using the glass Pasteur pipette, transfer the embryonic kidneys to a 3.5 cm petri dish containing 2.5 ml trypsin–EDTA 0.1 % and incubate E11.5 kidneys for 3 min and E12.5 kidneys for 4 min at 37 °C, 5 % CO_2.
4. Using a P1000 micropipette, transfer trypsin-treated embryonic kidneys to a 3.5 cm petri dish containing 3 ml of isolating medium supplemented with 10 % ES-FBS to quench the trypsin–EDTA action.
5. Using a P1000 micropipette, transfer the embryonic kidneys into a 1.5 ml Eppendorf tube containing 300 μl of isolating medium supplemented with 10 % ES-FBS. Dissociate the kidneys into single-cell suspensions by pipetting them up and down through a P200 micropipette tip.
6. Filter the single-cell suspension through the 40 μm cell strainer. Wash the Eppendorf tube with 150 μl of isolating medium and filter the washing medium. Then, wash the filter with 150 μl of isolating medium (again) and finally transfer the cell suspension into a new 1.5 ml Eppendorf tube.
7. To determine cell viability, mix 20 μl cell suspension with 20 μl Trypan Blue and visually examine cells using a microscope to determine whether they internalize or exclude the dye. If dissociation and filtering steps are performed rapidly and carefully, more than 92 % of cells should be viable.

8. Count the cells using an automatic cell counter or a Burker counting chamber. Place aliquots of 1.2×10^5 or 4×10^5 freshly dissociated renal cells for in vitro and in vivo studies, respectively, in new 1.5 ml Eppendorf tubes. Add fresh medium to obtain a final volume of 500–600 μl per tube and mix the suspension gently using a P1000 micropipette.
9. Centrifuge the cell suspensions at $900 \times g$ for 4 min to form a pellet.
10. During centrifugation, place the previously prepared petri dishes (*see* **step 2**) on the stereoscope. Check there are no air bubbles under the filter, because they would disturb tissue development by precluding nutritional supply to the cells. Repeat this step for each petri dish.
11. To detach the pellet from the tube, aspirate a little medium using a P200 micropipette within the centrifuged Eppendorf tube and then expel it very carefully over the top portion of the pellet (*see* **Note 1**).
12. Using a glass Pasteur pipette, immediately collect the floating pellet and gently place it on top of the filter. Repeat this step for each pellet.
13. Place the petri dish containing the pellet in the incubator in a humidified atmosphere with 5 % CO_2 at 37 °C. After only a few minutes, ureteric bud (UB) cells already start to reaggregate into multiple UB structures.
14. After 24 h change the medium by replacing it with fresh warm culture medium without ROCK inhibitor. At this stage, the pellet grows into tissue—defined mouse renal organoid—containing UB tubules and metanephric mesenchyme (MM) derivatives.
15. Change medium every 2 days. We cultured mouse organoids for up to 21 days (Fig. 1).

3.2 Construction and Culture of Human–Mouse Chimeric Organoids In Vitro

For chimeric organoid construction, single-cell suspensions of E11.5 or E12.5 mouse kidney cells are mixed with human cells, aggregated and cultured as above. The human cell types that were tested are amniotic fluid stem cells (AFSCs) [4]; HK2 renal proximal tubular epithelial cells, and bone marrow-mesenchymal stem cell-derived renal proximal tubular-like epithelial cells [8]; embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (*see* **Note 2**).

1. Trypsinize human cells into homogenous single-cell suspensions and harvest them. Make sure there are no residual cell clusters. Cell viability can be evaluated by Trypan Blue exclusion test. Count human cells using an automatic cell counter or a Burker counting chamber.

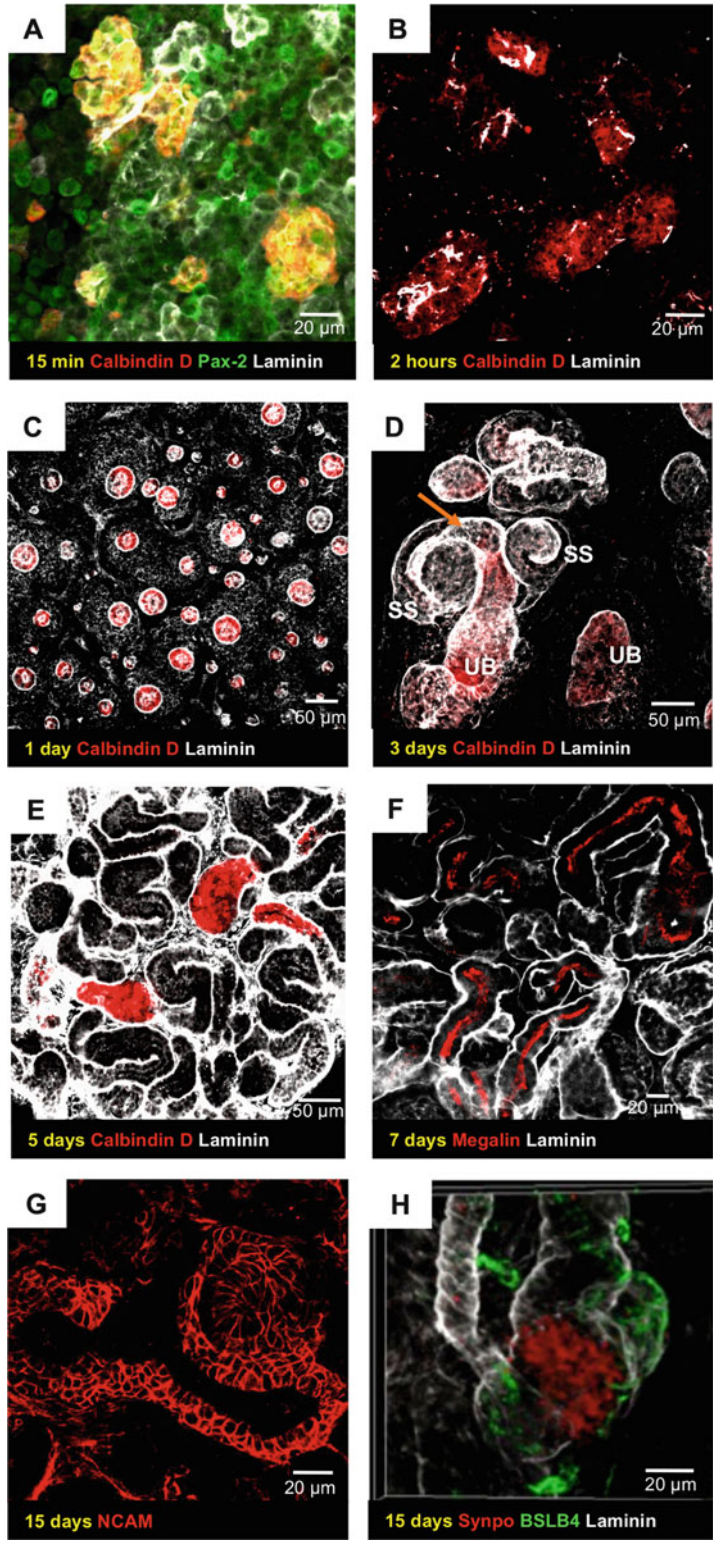

Fig. 1 In vitro development of mouse renal organoids. (**a**) At 15 min, ureteric bud (UB) epithelia expressing calbindin D28k (*red*) were reformed. A number of cells were positive for the UB- and nephron marker Pax-

2. Dissociate embryonic kidneys as described in Section 3.1 in **steps 3–8**.
3. Mix 1.2×10^4 or 4×10^4 human cells with 1.2×10^5 or 4×10^5 freshly dissociated mouse renal cells (1:10, human–-mouse), respectively. Mix the chimeric cell suspension by pipetting.
4. Centrifuge the cell suspensions at $900 \times g$ for 5 min to form a pellet.
5. Human stem cells can also be labeled with 4 μM green-fluorescent chloromethyl derivative of fluorescein diacetate probe CellTracker, following the manufacturer's instructions, before mixing with mouse cells.
6. Proceed with stages described in Section 3.1 in **steps 10–14**.
7. Change medium every 2 days. Chimeric organoids can be cultured in vitro for several days. We cultured them for up to 5 days (Fig. 2a–f).

3.3 Immunofluorescence Analysis of Organoids In Vitro

1. Transfer the filter with the mouse or chimeric organoid to a closable plastic tube with PBS and wash for 10 min.
2. Fix the organoids in 4 % paraformaldehyde for 10 min at room temperature. Once fixed, the organoids can be preserved in PBS at 4 °C.
3. Permeabilize the organoids with 100 % cold methanol for 10 min at room temperature.
4. Wash in PBS and replace the PBS with a solution of primary antibodies diluted in PBS and incubate overnight at 4 °C.
5. Wash in PBS and incubate with the specific secondary antibodies (and the lectin if necessary) diluted in PBS overnight at 4 °C.
6. Wash again in PBS, then mount with Dako Fluorescence Mounting Medium and observe with an inverted confocal laser scanning microscope or an Apotome fluorescence microscope.

Fig. 1 (Continued) 2 (*green*). Cells positive for the general basement membrane marker laminin (*white*) were randomly distributed within the reforming tissue. (**b**) At 2 h, reformed UB cells expressed calbindin D28k (*red*) and some of these were laminin-positive (*white*). (**c**) At 1 day, laminin positive membranes (*white*) surrounded UBs. (**d**) At 3 days, S-shaped bodies (SS) were connected to calbindin D28k-positive (*red*) UBs. *Arrow*: connection between UB and SS. (**e**) At 5 days, developing tubuli expressed calbindin D28k (*red*) in UB and distal domains. (**f**) At 7 days, megalin (*red*) was found in the proximal portions. (**g**) At 15 days, an elongated nephron with well-defined tubular portions and glomerular pole was visualized, by neural cell adhesion molecule (NCAM) immunostaining. (**h**) At the same time, the 3D image showed more mature tubuli connected to glomeruli containing podocytes positive for synaptopodin (*red*). BSLB4-positive endothelial progenitors (*green*) were also visible within the organoid. *Synpo* synaptopodin, *BSLB4 Bandeiraea simplicifolia* Isolectin B4

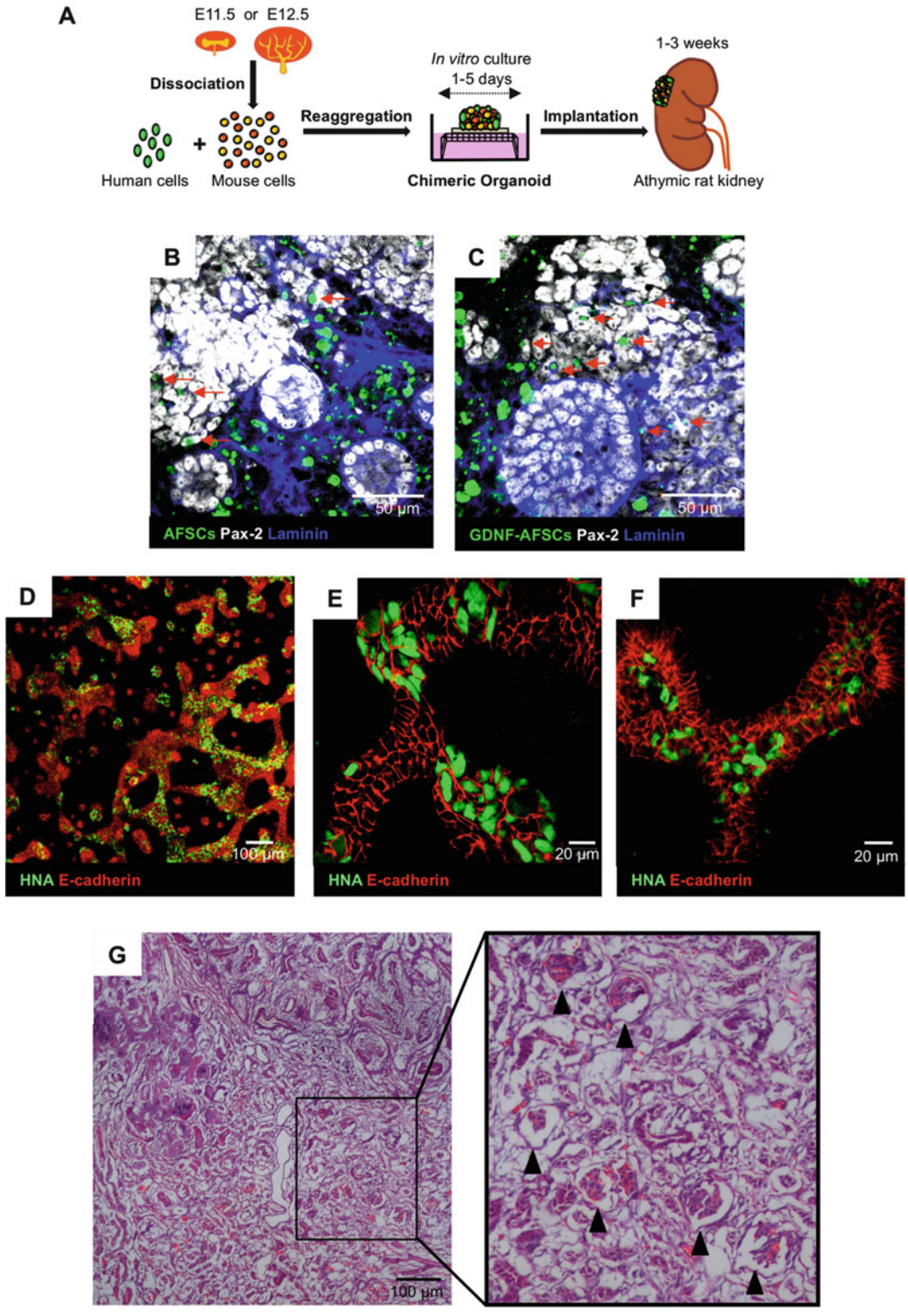

Fig. 2 (**a**) In vitro construction and implantation of mouse and human–mouse chimeric organoids. (**b**–**f**) Integration of human cells into the chimeric organoids after 2 days in vitro. (**b**) AFSCs (*green*, *arrows*) were

3.4 Implantation of Mouse and Chimeric Organoids

For in vivo studies, chimeric organoids are cultured in vitro for 1 day, and mouse organoids for 5 days, and then implanted under the kidney capsule of athymic rats. These animals are immunocompromised, enabling xenotransplantation experiments without the need for immunosuppressive treatment. Rats are subjected to right nephrectomy just before implantation to accelerate the development of implanted organoids, and are euthanized 1–3 weeks later [3, 4] (Fig. 2g).

1. To detach the organoid from the filter, aspirate medium using a P200 micropipette and then carefully expel it close to the organoid. Repeat this procedure until the organoid has been detached completely.
2. Using a glass Pasteur pipette, transfer the floating organoid to a well of a 96-well plate (*see* **Note 3**).
3. Remove as much medium as possible from the well.
4. Add 2 μg recombinant rat VEGF prepared according to the manufacturer's instructions, to the well to soak and precondition the tissue, and incubate for 4 h at 37 °C, 5 % CO_2 in a humidified atmosphere.
5. Put the 96-well plate on ice. Anesthetize athymic rat and perform unilateral nephrectomy.
6. Aspirate the organoid in the catheter.
7. Carefully insert the end of the catheter containing the organoid into the hole that has been generated previously in the kidney capsule, and gently expel the solution drop by drop until organoid comes out. The organoid looks whitish and roundish (*see* **Note 4**).
8. Extract the catheter from the hole, taking care not to damage the kidney capsule, and cauterize. The organoid can now be considered as having been implanted beneath the kidney capsule.
9. Inject recipient rat locally with VEGF (1 μg) into the area of implantation before performing the suture.
10. Inject recipient rat intravenously with VEGF (1 μg, 3 times per week) into the tail vein until euthanasia.

Fig. 2 (Continued) mainly concentrated in areas among Pax-2-positive renal structures. (**c**) GDNF-expressing AFSCs integrated into Pax-2-positive developing structures more efficiently compared with control cells. *Red arrows*: human cells integrated into Pax-2-positive structures. (**d**) Induced pluripotent stem cells (iPSCs), positive for the human marker human nuclear antigen (HNA, *green*) were almost entirely localized in E-cadherin-positive UB. (**e**) Human–mouse chimeric UB at higher magnification. (**f**) Similarly, HNA-positive human embryonic stem cells (ESCs) were found in developing UB. (**g**) Histology of mouse organoids at 2 weeks in vivo showed vascularized glomeruli (*inset*, *arrowheads*) containing red blood cells (*bright pink*)

3.5 Renal Histology of Organoids Grown In Vivo

1. Euthanize the host rat by CO_2 inhalation 1–3 weeks after implantation and remove the rat kidney.
2. Fix the implanted organoids in PLP and embed in OCT compound.
3. Stain 3-μm cryosections with hematoxylin for 15 min at room temperature
4. After washing in running water, stain the slices with eosin for 5 min at room temperature [3, 4].
5. Wash in running water, mount with Dako Faramount Aqueous Mounting Medium and observe by light microscopy.

4 Notes

1. Detaching the pellet from the Eppendorf is a very delicate step because of the extreme fragility of the pellet. To avoid pellet fragmentation be careful to expel the aspirated medium gently but firmly. Note that repeating centrifugations may reduce cell viability.
2. Previous studies reported that when mouse embryonic kidneys cells were reaggregated with undifferentiated human pluripotent stem cells (PSCs) (i.e., hESCs or hiPSCs) and cultured for 3–4 days, human cells did not integrate into renal structures and severely disrupted 3D renal tissue development [10, 11]. In contrast to these data, in our experimental conditions we observed a massive integration of both undifferentiated hESCs and hiPSCs almost exclusively into the developing UB epithelia (Fig. 2d–f). This is an important point to take into consideration in studies aimed at validating the integration potential of in vitro hPSC-derived renal progenitors by using undifferentiated hPSCs as negative controls in reaggregation assays, especially if the in vitro differentiation protocol is designed to generate UB progenitor cells.
3. To aspirate the LCA for transplantation without damaging it, we suggest using a glass Pasteur pipette broken manually in order to obtain a larger hole at the end.
4. During LCA implantation under the kidney capsule, we recommend pushing the syringe plunger very slowly and making sure that the implant is positioned away from the intervention point. This will minimize the risk of it sliding out from the site of implantation.

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