Chapter 2

In Vitro Culture of Embryonic Kidney Rudiments and Isolated Ureteric Buds

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Abstract

In vitro culture of embryonic kidney rudiments has been utilized to study a variety of cellular processes and developmental mechanisms. Here, we describe two-dimensional (2D) culture of embryonic kidney rudiments on Transwell filters and three-dimensional (3D) cultures in collagen gels in detail, and 3D culture of isolated ureteric bud (UB) in Matrigel with BSN-conditioned media.

Key words: Microdissection, Embryonic kidney rudiments, Ureteric bud, Three-Dimensional culture

1. Introduction

The development of the metanephric kidney begins with the reciprocal inductive interactions between the ureteric bud (UB) and the metanephric mesenchyme (MM). The UB invades the surrounding MM where it undergoes branching morphogenesis giving rise to the tree-like kidney collecting system. In a reciprocal fashion, the UB signals the MM to condense near the newly formed UB tips, undergo the process of mesenchymal-to-epithelial transition (MET) followed by a series of morphological stages to form the nephrons (1). Many genes (2, 3) and molecules, including growth factors, extracellular matrix proteins, integrins, etc., have been reported to regulate these two distinct processes (4–7). A variety of such cellular processes and developmental mechanisms are capable of being studied during organogenesis. Therefore, in vitro cultures of whole embryonic kidney rudiments or progenitor tissues isolated from the embryonic kidney, including the isolated MM and UBs, have been utilized to study these mechanisms (8–12).

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A number of in vitro three-dimensional (3D) culture systems have been devised to obtain greater spatial growth of isolated embryonic kidneys and UBs (13–16). In this protocol, two-dimensional (2D) culture of embryonic kidney rudiments on Transwell filters and three-dimensional (3D) cultures in extracellular matrix gels are described in detail. The difference between 2D and 3D cultures are compared.

2. Materials

2.1. Reagents

1. Phosphate-Buffered Saline (PBS) without calcium or magnesium.
2. Phosphate-Buffered Saline (PBS) with calcium or magnesium.
3. 70% Ethanol.
4. Liebovitz's L-15 medium with L-glutamine.
5. Trypsin (0.1% solution in L-15 medium): dissolve powdered Trypsin (porcine pancreas; Sigma) in L-15 medium to a concentration of 1 mg/mL.
6. DNase I.
7. DMEM–F12 (50:50) mixture growth medium with L-glutamine and 15 mM HEPES.
8. 10× Dulbecco's modified Eagle's medium (DMEM).
9. Fetal Bovine Serum (FBS).
10. Type I collagen (BD Biosciences).
11. Type IV collagen (BD Biosciences).
12. Growth factor-reduced Matrigel (BD Biosciences).
13. Antibiotic-antimycotic solution.
14. Growth factors: Rat recombinant glial cell line-derived neurotrophic factor (rrGDNF) (R&D Systems); Fibroblast growth factors (FGF)—recombinant human FGF1 (Calbiochem).

2.2. Equipment

1. Stereozoom dissecting microscope.
2. Fiber-optic external light source (eliminates a potential source of heat during dissections).
3. Blunt operating scissors.
4. Potts-Smith forceps with teeth either straight or curved.
5. Dumont #55 forceps.
6. Minutien pins held in pinholder.
7. 100 × 15 mm Petri dishes.
8. Tissue culture dishes (60 × 15 mm; 35 × 10 mm).

9. Corning Transwell permeable supports 0.4 μM pore size for 12- or 24-multiwell plate.
10. Tissue culture plates: 12- or 24-multiwell plate.
11. Insert pin held in pinholder with the final ½ in. of the pin bent at ~45° angle.
12. Drummond Wiretrol I calibrated micropipettes, 50 μL and 100 μL.
13. 500 mL filter system.
14. Amicon Ultra-15 centrifugal tubes.
15. Allegra™ 25R Centrifuge.

3. Methods

3.1. Isolation of Embryonic Kidneys from Time-Pregnant Mice/Rats

1. The uterine horns from the pregnant rodents (mice—gestational day 10.5–11, or rats—gestational day 12.5–13; day 0 of gestation coincides with appearance of the vaginal plug) are dissected free from surrounding tissues and transferred to a separate 10-cm Petri dish filled with L-15 medium kept on ice.
2. The embryos are isolated and transferred to a new 10-cm Petri dish with L-15 medium kept on ice.
3. The paired embryonic urinary tracts [i.e., mesonephros, Wolffian ducts, kidneys, ureters, and urogenital sinus (under the cloacal ridge)]—which lie against the back body wall arranged in an anterior to posterior fashion running parallel to the dorsal aorta—are dissected free from surrounding tissues and the entire structure is transferred to a separate 60-mm tissue culture dish containing L-15 medium.
4. The kidneys will lie at the posterior end of the isolated urinary tract, just under the bifurcating dorsal aorta. Remove and isolate the kidneys by dissecting away the surrounding tissue. If the whole embryonic kidney is to be cultured, it should be comprised of just the ureteric bud and its surrounding metanephric mesenchyme. Transfer kidneys with a micropipette to a 35-mm tissue culture dish containing DMEM/F12 medium.

3.2. Preparation of Extracellular Matrix Gel

1. Type I collagen gel is prepared such that the final solution consists of 80% sterile type I collagen solution, 10% 10× DMEM, and 10% sterile 1 M Hepes solution. Adjust pH to 7.4 with 1 M NaOH solution and keep the solution on ice to prevent gelation before use for tissue culture (see Note 4).
2. Type IV collagen gel is prepared by mixing 75% type IV collagen, 10% 10× DMEM, 10% 1 M Hepes, 5% 20× $NaHCO_3$. Adjust pH to 7.4 with 1 M NaOH solution and keep the solution on ice to prevent gelation before use for tissue culture (see Note 4).

3. Growth factor-reduced Matrigel is obtained from BD Biosciences. A 50% Matrigel solution is prepared by diluting the original Matrigel 1:1 with 1× DMEM/F12 medium (see Note 5).

3.3. Culture of Embryonic Kidney Rudiment

1. Under the dissection microscope carefully clean away any tissues surrounding the embryonic kidney. The embryonic kidney to be cultured should be comprised of just the ureteric bud and its surrounding metanephric mesenchyme.
2. Prepare Transwell tissue culture inserts for the whole embryonic kidneys and transfer one to two whole embryonic kidneys directly onto the filter (2D culture) (see Note 2). Remove excess liquid from top of each filter and position the kidneys on the filters (see Note 3).
3. For 3D culture, pipette 600 μL type I or IV collagen solution into a 12-multiwell Transwell filter (see Note 8). Using a 50-μL Wiretrol micropipette, transfer one to two embryonic kidneys directly into the collagen gels. Using an insert pin with the angled tip position and suspend each kidney within the collagen gel; this must be done until the collagen matrix solidifies, as the kidneys will sink to the bottom of the insert (see Notes 6 and 7).
4. In the biological safety cabinet, transfer the Transwell tissue culture inserts from either the 2D or 3D culture system containing the whole embryonic kidneys cultures into the individual wells of a separate tissue culture plate containing DMEM/F12 supplemented with 10% FBS, 1× antibiotic-antimycotic solution [12-multiwell (600 μL) or 24-multiwell plate (400 μL)].
5. Culture for 7–14 days (without media changes) at 37°C with ~95% humidity. Examine and photograph the growth of the whole embryonic kidneys (Figs. 1 and 2).

3.4. Culture of Isolated UB

1. Pipette 2,000 μL of 0.1% trypsin/L-15 solution, as well as 10 μL of DNase I in a 35-mm tissue culture dish, and mix well (see Note 1).
2. Using the 50 μL Wiretrol micropipette, transfer the whole embryonic kidneys to the trypsin/DNase I solution. Place the lid on the tissue culture dish and incubate the kidneys in the trypsin/DNase I solution for 20 min at 37°C.
3. Stop the enzymatic activity by adding 200 μL of FBS to the trypsin/DNase I solution and swirl to mix (see Note 1). Remove the kidneys to a separate 35-mm tissue culture dish containing 2 mL of L-15 supplemented with 10% FBS and 10 μL of DNase I.
4. Under the dissection microscope, gently grasp the trypsinized kidney with the Dumont #55 forceps and, using the minutien

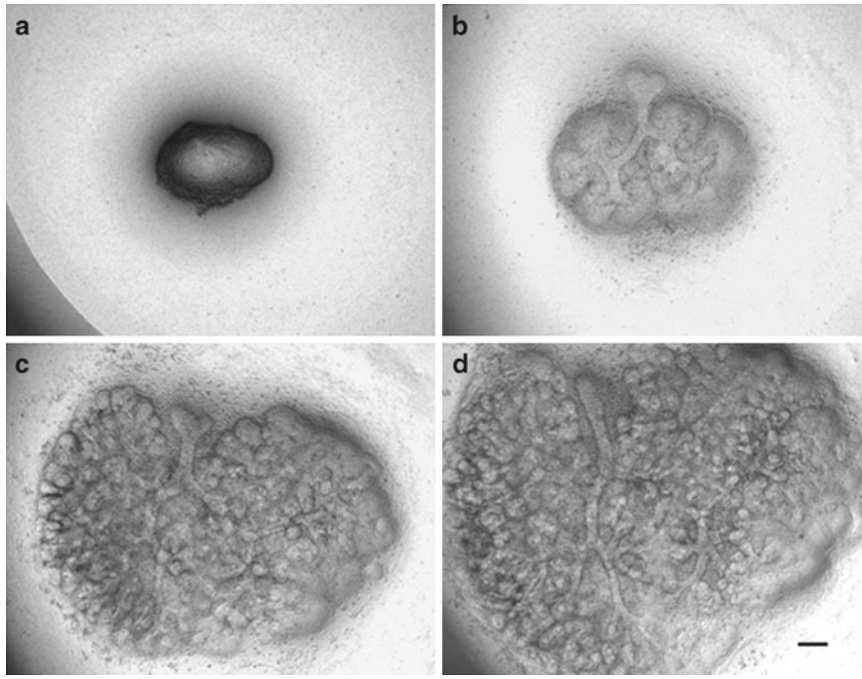

Fig. 1. Two-Dimensional (2D) in vitro rat embryonic kidney rudiments (E13) cultured on Transwell filters for different times: (**a**) control at day 0, (**b**) 2 days, (**c**) 5 days, (**d**) 7 days. All samples were cultured with DMEM/F12 medium supplemented with 10% FBS and 1× Antibiotic-antimycotic solution. Scale bar = 200 μm.

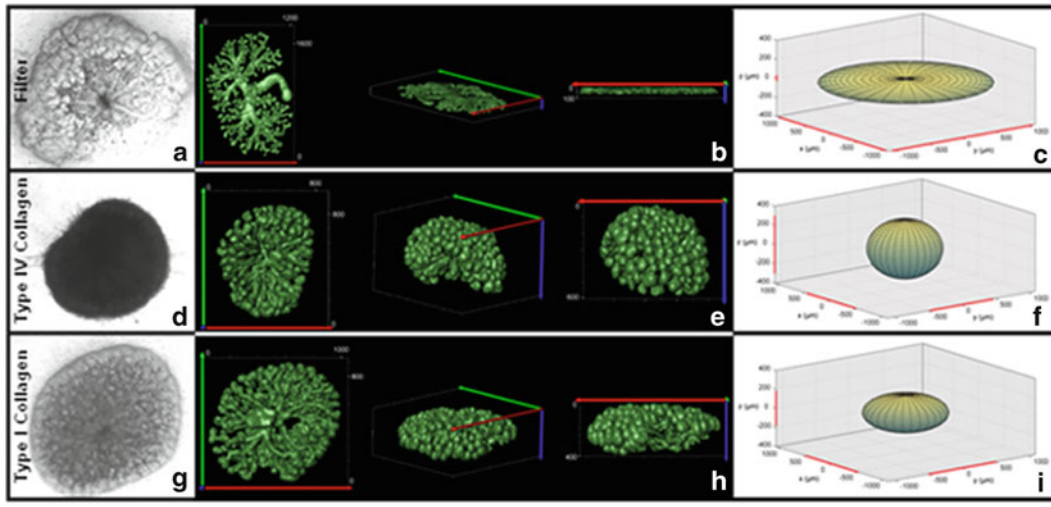

Fig. 2. 3D projection of the branching ureteric bud of E12 HoxB7-GFP mouse kidneys cultured for 7 days. Kidneys in the traditional filter culture grew flat (2D) and along the filter (**a**–**c**), while kidneys cultured in type IV collagen (**d**–**f**) or type I collagen (**g**–**i**) grew much thicker and in a more 3D manner (units, μm). A part of this figure is from ref. 15.

pins, carefully tease the metanephric mesenchyme away from the ureteric bud.

5. In a biological safety cabinet, prepare the 50% Matrigel solution as described above (see Note 5). Place Transwell into a separate tissue culture dish. For these studies, a 24-well tissue culture dish is typically used.
6. Pipette ~80 μL of 50% Matrigel solution directly into a Transwell filter. Using a 50-μL Wiretrol micropipette, transfer 1–2 clean iUBs with a minimum of L-15 medium directly into the Matrigel solution.
7. Under the dissection microscope, use the insert pin with the angled tip to position and suspend each iUB within the Matrigel; this must be done until the Matrigel gels, as the buds tend to sink to the bottom of the insert (see Note 6). Repeat these steps for each iUB to be cultured in 3D extracellular matrix gels.
8. Prepare the growth medium as described below. Pipette 400 μL of this BSN-conditioned medium (see Subheading 3.5 for preparation of BSN-conditioned medium) (see Note 9) into the wells of a separate 24-multiwell tissue culture plate and add 125 ng/ml each of GDNF and FGF1. The BSN conditioned media should also be supplemented with 10% FCS and 1× antibiotic/antimycotic. Transfer the Transwell tissue culture inserts into the prepared wells. Make sure that there are no air bubbles beneath the filter.
9. Place the entire setup into a CO_2 incubator and culture for 7–10 days (without medium changes) at 37°C with ~95% humidity.
10. Examine and photograph the growth and branching of the iUBs using an inverted microscope equipped with phase-contrast (Fig. 3).

3.5. BSN-Conditioned Medium

1. Culture BSN cells to confluence in 100-mm tissue culture dishes (at least 20) containing 10 mL of DMEM/F12 with 10% FBS and 1× antibiotic-antimycotic solution at 37°C in a 5% CO_2 incubator.
2. Remove the growth medium, wash the monolayers at least 3× with PBS and aspirate PBS.
3. Pipette 10 mL of serum-free DMEM/F12 to the culture dishes and maintain the culture at 37°C in a 5% CO_2 incubator.
4. Collect the serum-free medium after 2–3 days of incubation and pool the medium in a clean, sterile flask or bottle.
5. Apply the ~200 mL of BSN-conditioned medium to a 0.22-μm membrane filter to remove cellular debris and further concentrate the medium 30-fold with a Centricon (Millipore) filter with an 8-kDa nominal molecular mass cutoff.

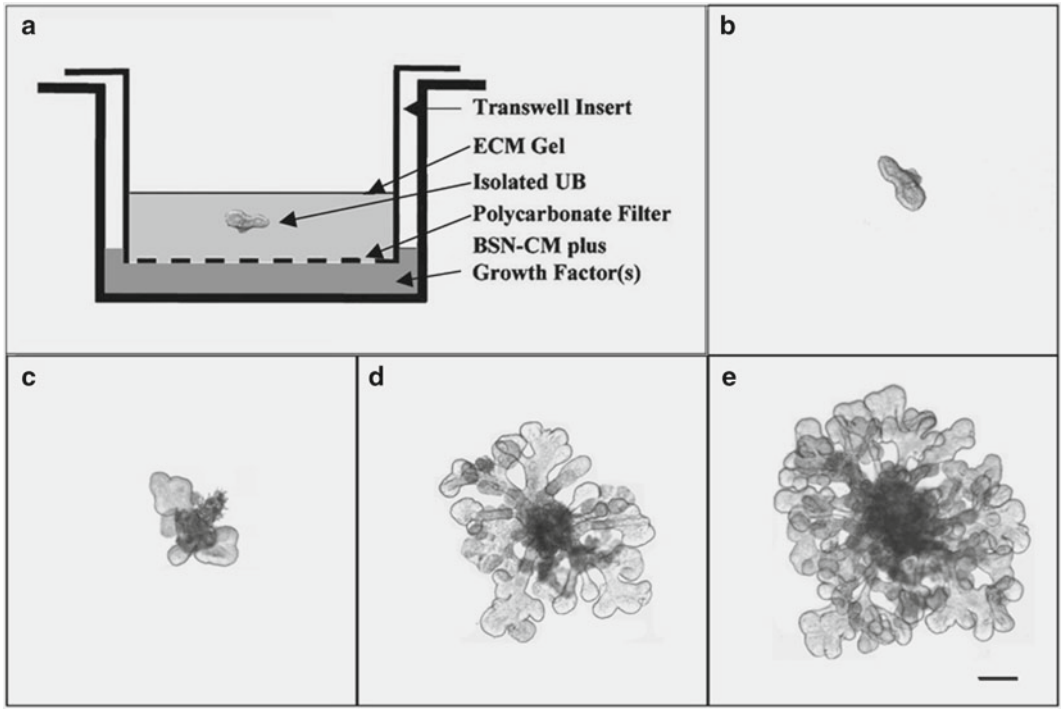

Fig. 3. Schematic drawing of in vitro culture of iUB in Matrigel (**a**), and phase contrast images of iUB culture for different time: (**b**) control at day 0, (**c**) 2 days, (**d**) 5 days, (**e**) 7 days. Isolated UB samples were cultured with BSN-conditioned medium supplemented with 10% FBS and 1× Antibiotic-antimycotic solution, and 125 ng/mL FGF1 and 125 ng/mL GDNF. Scale bar = 200 μm. A part of this figure is from Ref. 14.

4. Notes

1. Do not forget to add the FBS or DNase I solution during separation of UB and MM. Without these supplements added to the L-15 medium, the kidneys will become extremely sticky and will adhere to each other as well as the bottom of the tissue culture dish.
2. After placing the kidneys/UBs on the Transwell filters, the inserts must remain moist throughout the remaining steps of the protocol; otherwise, the applied organs/tissues may dry to the filter during the following transfer and positioning steps.
3. The tissues/organs are cultured on the top of the filter at the air–media interface. If excess media/moisture accumulates on the filter, it should be removed. The growth of the tissue/organs is adversely affected if they are completely covered by liquid.
4. For preparation of type I or IV collagen solutions, keep all reagents on ice during the process. First, mix all other reagents, and then add type I or IV collagen to the above mixture. Gently pipette to obtain a homogeneous solution (avoid bubbles).

5. Store the Matrigel at −80°C prior to thawing. Thaw the Matrigel by gently shaking it under hot running tap water. Prior to complete thawing, plunge the Matrigel into an ice-bath and continue to shake until it is completely thawed. The Matrigel should be highly viscous, but not solidified. Store the thawed Matrigel on ice in the refrigerator. Matrigel should be thawed at least 24 h prior to use, as the shaking will generate numerous air bubbles which need time to dissipate. When in use, always keep the thawed Matrigel on ice.
6. Until proficiency with the technique is acquired, it is suggested that each well be completed before the next is begun. In other words, do not pipette Matrigel into all of the wells and then transfer the kidneys. There is the potential for the Matrigel to gel before all the wells have been completed.
7. For 3D cultures, ensure that kidneys/UBs are suspended within the extracellular matrix gel; if they are at the bottom or top of the gel, the growth pattern will be altered. Finally, sandwiching the kidneys/UBs between two layers of gels is to be avoided, as this will also result in altered growth.
8. By comparison of 2D and 3D culture of embryonic kidney rudiments (Fig. 2), we found that type IV collagen supports the deepest tissue growth and the largest kidney volume, followed by type I collagen culture and the filter culture system. Furthermore, only kidneys in type IV collagen exhibited a 3D umbrella-like branching pattern characteristic as in vivo kidney development.
9. If BSN-conditioned media is unavailable growth and branching of the isolated UB can be achieved by supplementation of DMEM/F12 with purified growth factors. For example, pleiotrophin (17) or heregulin (18) can be added to the media along with GDNF and FGF1.

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References

1. Shah MM, Sampogna RV, Sakurai H, Bush KT, Nigam SK (2004) Branching morphogenesis and kidney disease. Development 131: 1449–1462
2. Dressler GR (1995) The genetic control of renal development. Curr Opin Nephrol Hypertens 4:253–257
3. Sampogna RV, Nigam SK (2004) Implications of gene networks for understanding resilience and vulnerability in the kidney branching program. Physiology 19:339–347
4. Pohl M, Stuart RO, Sakurai H, Nigam SK (2000) Branching morphogenesis during kidney development. Annu Rev Physiol 62:595–620
5. Costantini F, Kopan R (2010) Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. Dev Cell 18:698–712
6. Lelongt B, Ronco P (2003) Role of extracellular matrix in kidney development and repair. Pediatr Nephrol 18:731–742
7. Lechner MS, Dressler GR (1997) The molecular basis of embryonic kidney development. Mech Dev 62:105–120
8. Bard JB, Ross AS (1991) LIF, the ES-cell inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. Development 113:193–198
9. Davies J (1994) Control of calbindin-D28K expression in developing mouse kidney. Dev Dyn 199:45–51
10. Michos O, Gonçalves A, Lopez-Rios J et al (2007) Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. Development 134: 2397–2405
11. Thesleff I, Ekblom P (1984) Role of transferrin in branching morphogenesis, growth and differentiation of the embryonic kidney. J Embryol Exp Morphol 82:147–161
12. Sebinger DD, Unbekandt M, Ganeva VV et al (2010) A novel, low-volume method for organ culture of embryonic kidneys that allows development of cortico-medullary anatomical organization. PLoS One 5:e10550
13. Steer DL, Bush KT, Meyer TN, Schwesinger C, Nigam SK (2002) A strategy for in vitro propagation of rat nephrons. Kidney Int 62:1958–1965
14. Qiao J, Sakurai H, Nigam SK (1999) Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. Proc Natl Acad Sci USA 96:7330–7335
15. Rosines E, Schmidt HJ, Nigam SK (2007) The effect of hyaluronic acid size and concentration on branching morphogenesis and tubule differentiation in developing kidney culture systems: potential applications to engineering of renal tissues. Biomaterials 28:4806–4817
16. Rosines E, Johkura K, Zhang X et al (2010) Constructing kidney-like tissues from cells based on programs for organ development: toward a method of in vitro tissue engineering of the kidney. Tissue Eng Part A 16:2441–2455
17. Sakurai H, Bush KT, Nigam SK (2001) Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogeneisis. Development 128:3283–3293
18. Sakurai H, Bush KT, Nigam SK (2005) Heregulin induces glial cell line-derived neurotrophic growth factor-independent, non-branching growth and differentiation of ureteric bud epithelia. J Biol Chem 280:42181–42187

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