Video Article

Isolation and Culture of Cells from the Nephrogenic Zone of the Embryonic Mouse Kidney

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Abstract

Embryonic development of the kidney has been extensively studied both as a model for epithelial-mesenchymal interaction in organogenesis and to gain understanding of the origins of congenital kidney disease. More recently, the possibility of steering naïve embryonic stem cells toward nephrogenic fates has been explored in the emerging field of regenerative medicine. Genetic studies in the mouse have identified several pathways required for kidney development, and a global catalog of gene transcription in the organ has recently been generated http://www.gudmap.org/, providing numerous candidate regulators of essential developmental functions. Organogenesis of the rodent kidney can be studied in organ culture, and many reports have used this approach to analyze outcomes of either applying candidate proteins or knocking down the expression of candidate genes using siRNA or morpholinos. However, the applicability of organ culture to the study of signaling that regulates stem/progenitor cell differentiation versus renewal in the developing kidney is limited as cultured organs contain a compact extracellular matrix limiting diffusion of macromolecules and virus particles. To study the cell signaling events that influence the stem/progenitor cell niche in the kidney we have developed a primary cell system that establishes the nephrogenic zone or progenitor cell niche of the developing kidney *ex vivo* in isolation from the epithelial inducer of differentiation. Using limited enzymatic digestion, nephrogenic zone cells can be selectively liberated from developing kidneys at E17.5. Following filtration, these cells can be cultured as an irregular monolayer using optimized conditions. Marker gene analysis demonstrates that these cultures contain a distribution of cell types characteristic of the nephrogenic zone *in vivo*, and that they maintain appropriate marker gene expression during the culture period. These cells are highly accessible to small molecule and recombinant protein treatment, and importantly also to viral transduction, which greatly facilitates the study of candidate stem/progenitor cell regulator effects. Basic cell biological parameters such as proliferation and cell death as well as changes in expression of molecular markers characteristic of nephron stem/progenitor cells *in vivo* can be successfully used as experimental outcomes. Ongoing work in our laboratory using this novel primary cell technique aims to uncover basic mechanisms governing the regulation of self-renewal versus differentiation in nephron stem/progenitor cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/2555/

Protocol

1. Preparing Reagents for Kidney Digestion

1. 1.5 ml of 0.25 % Collagenase A (w/v) and 1 % Pancreatin digest (w/v) solution will be needed for extraction of nephrogenic zone cells (NZCs) from 8 E17.5 kidneys. Because Pancreatin takes approximately 2 hours to dissolve at room temperature, enough digest solution for the largest number of embryonic kidneys expected should be made prior to the experiment. Prepare digest solution by first adding 25 mg of Collagenase A to 10 ml Dulbecco's Phosphate Buffered Saline (DPBS) in a clear 15 ml, sterile centrifuge tube. It is critical that the DPBS does NOT contain calcium or magnesium as this will interfere with the enzymatic digest. Mix solution on a nutator for 10 minutes to dissolve Collagenase A. Add 100 mg of Pancreatin to dissolved Collagenase A solution and mix on a nutator for at least 2 hours. Alternatively, Collagenase A/Pancreatin digest solution can be mixed on a nutator overnight at 4° C. It is recommended that you wear a mask, as Collagenase A and Pancreatin disperse easily into the atmosphere during weighing and can irritate nasal passages.
2. Prepare a 5 % Fetal Bovine Serum (FBS) (v/v) solution in Hank's Buffered Saline solution (HBSS). Invert to mix. 1 ml for each tube of 8 kidneys will be necessary.
3. Prepare culture medium by supplementing Keratinocyte serum free medium with 1 % glutamine (v/v) and 1 % Penicillin-Streptomycin (PenStrep) (v/v).
4. Coat sterile flat bottom polystyrene tissue culture plates (96, 48, 24 or 6 well) with human fibronectin solution at a concentration of 5 mg per cm^2 of growth surface. 5 mg of fibronectin powder is resuspended in 5 ml of sterile H_2O. This is diluted 1:25 in sterile DPBS without calcium or magnesium and 250 ml is added to each well of a 24 well plate. Plates are then incubated with fibronectin solution at room temperature

for 1 hour followed by 1 hour at 4° C. Alternatively, plates can be incubated overnight at 4° C. The solution is then aspirated in a laminar flow hood prior to addition of medium/cells.

2. Dissecting Kidneys & Removing Capsules (Done At Room Temperature)

1. At E17.5, sacrifice the mother using a procedure approved by your Institutional Animal Care and Use Committee, remove the uterus, and place it in a Petri dish containing DPBS with calcium and magnesium. Under a dissecting microscope, use watchmakers' forceps to remove each embryo from the uterus and decapitate each embryo. Once the entire litter is out of the uterus, transfer the embryos to a clean Petri dish containing enough DPBS with calcium and magnesium to entirely cover the embryos leaving behind as much blood and tissue debris as possible.
2. Using forceps, make a circumferential incision in the abdominal wall of the embryo at the level of the umbilicus. Then place the embryo on its back, pin it down at the shoulders with one set of forceps, and remove the internal organs from the lungs down to the bladder using the other set of forceps. With practice this can be done in one motion. The kidneys should be attached to the back of the mass of organs. If the kidneys do not remain attached to the eviscerated organs, they can be carefully removed from the dorsal body wall. Kidneys that have been damaged during this dissection process should be discarded as they will contaminate the final culture with inappropriate cell types.
3. Gently tease away the kidneys from the rest of the organs, taking care to keep the kidneys connected to each other. Hold the tissue between the kidneys with one set of forceps and grab the adrenal gland attached to one of the kidneys with the other forceps. The adrenal gland should be attached to the kidney capsule. Gently peel the adrenal gland and capsule away from around the outside of the kidney, similar to peeling a banana. Take care not to split the kidney in half while peeling, and be sure not to damage the kidney tissue underneath the capsule. Carefully remove any remaining capsule as well as the ureter if it is still attached. Repeat with the remaining kidney.
4. Use a cut transfer pipet to place the kidneys into a 5 ml polystyrene tube of HBSS. Repeat steps 2.2-2.4 for each embryo. Kidneys may be pooled 8 per tube. With practice, dissection and capsule removal will take 2-3 minutes per embryo.

3. Enzymatic Digestion of Kidneys (All Steps At Room Temperature Unless Otherwise Indicated)

1. Remove HBSS solution from 5 ml tubes containing kidneys with a pipettor while carefully avoiding contact with kidneys. Immediately add 1.5 ml of Collagenase A/Pancreatin digest solution to the 5 ml tube containing kidneys before moving to the next sample. Repeat for each tube of kidneys.
2. Place tubes with enzyme/kidney digests on a nutator in a pre-warmed 37° C incubator for 15 minutes. 2 minutes prior to completion of the digest, add 3 µl of DNase (1 U/ml) to one fresh 5 ml polystyrene tube for each digest being performed.
3. Quickly remove digests from incubator, add 75 µl of FBS and invert 3 times to mix. Let stand on bench for 2 minutes.
4. Transfer 1.4 ml of the cell suspension to the 5 ml polystyrene tube containing DNase (1 U/ml) while leaving the remaining 0.2 ml cell suspension and kidneys behind. The extra 0.2 mL of solution left behind will contain impurities such as extracellular matrix.
5. Mix cell suspension/DNase on a nutator in a pre-warmed 37° C incubator for 10 minutes.
6. Quickly remove cell suspensions from incubator and transfer each to a 1.5 ml Eppendorf tube. Spin cell suspensions in microfuge at 325 g for 5 minutes.
7. Remove supernatant from cell pellet and resuspend pellet in 1 ml of 5% FBS/HBSS, pipetting up and down 5 to 6 times. Cap each tube before moving to the next sample.
8. Spin cell suspensions in a microcentrifuge at 325 g for 5 minutes.
9. Remove supernatant and add 0.5 ml of Keratinocyte serum free medium (KSFM) with additives to each tube and cap tube before moving to the next sample. After medium has been added to all tubes, gently resuspend each cell pellet by pipetting up and down 5 to 6 times with a 1ml micropipette. Combine all cell suspensions in a fresh 5 ml polystyrene tube.
10. Pre-wet two 40 micron cell-strainer caps placed on a 5 ml polystyrene round bottom tube with KSFM per sample. Pass cell suspensions through one cell strainer cap by transferring with a 1 ml micropipette. Avoid touching the filter with the pipette tip. Collect flow-through and repeat filtration through the second strainer cap. Remove the cell strainer cap and replace with a new cap from a normal 5 ml polystyrene tube.
11. Count cells with a hemocytometer or other cell counting device and transfer cells to wells of a fibronectin coated culture plate at a density of 100,000-200,000 cells per cm^2, diluting as required with KSFM with additives. Generally, we retrieve approximately $4.5x10^6$ cells per 10 embryo litter.
12. Incubate cells in a 37° C humidified incubator with 5% CO_2.
13. Allow cells to recover and attach for 2-4 hours in medium prior to stimulation with additional reagents. Stimulate cells with compound of interest for 1 to 48 hours. Purify RNA for PCR analysis using the Qiagen RNeasy Micro Kit following the manufacturer's instructions or follow the instructions below to fluorescently immunostain the cells.

4. Preparing Reagents for Fixation of Cells and Immunostaining

1. Prepare 4% PFA: Dissolve 4% paraformaldehyde in PBS (v/v) at 55° C with regular agitation. Cool to room temperature before use. This solution is toxic and should be discarded in designated waste containers.
2. Prepare permeabilization solution by adding Triton X-100 to PBS to obtain a 0.3% (v/v) solution.
3. Prepare a 5 % blocking solution in PBS with serum from the same species as was used to raise the secondary antibody to be used. For example, if you will detect a rabbit antibody against your target antigen with a donkey anti-Rabbit Alexa Fluor 568, prepare a blocking solution containing 5% donkey serum. Store at 4° C.
4. Prepare primary antibody solution by adding the appropriate concentration of antibody to 5% blocking solution.
5. Prepare secondary antibody solution immediately prior to use. Add fluorophore conjugated antibody at manufacturer's recommended concentration together with the nuclear counterstain DAPI and other organelle markers such as phalloidin to 5% blocking solution. If storage is required, keep solution in the dark at 4° C until use.

6. Prepare 50% glycerol/PBS solution (v/v) for storage of immunostained cells. Alternatively, Vectashield can be used.

5. Fixation of Cells and Immunostaining

1. The following protocol is designed for fixation and staining of NZCs in a 24 well plate. Volumes given are for a single well. Reagents should be gently pipetted during all steps to avoid detachment of cells, and plate agitation is not recommended in the incubation steps.
2. Gently remove medium with a 1 ml micropipette and add 0.5 ml of 4% PFA to attached cultured cells. Incubate for 15 minutes undisturbed at room temperature.
3. Remove 4% PFA and add 0.5 ml per well of permeabilization solution. Incubate for 10 minutes at room temperature.
4. Remove permeabilization solution and gently rinse twice with 0.5 ml of PBS per rinse.
5. Add 0.5 ml of blocking solution and incubate at room temperature for 60 minutes.
6. Remove blocking solution and add 0.225 ml primary antibody solution.
7. Incubate at room temperature for 2 hours or overnight at 4° C.
8. Remove primary antibody solution and gently rinse twice with 0.5 ml of PBS per rinse.
9. Add 0.225 ml secondary antibody solution. Incubate plate in the dark at room temperature for 60 minutes.
10. Remove secondary antibody solution and gently rinse twice with 0.5 ml of PBS per rinse.
11. Add 0.3 ml of 50% glycerol/PBS solution (v/v) or enough Vectashield to cover bottom of well surface.
12. Image cells with epifluorescence microscope or store wrapped in foil at 4° C.

6. Representative Results

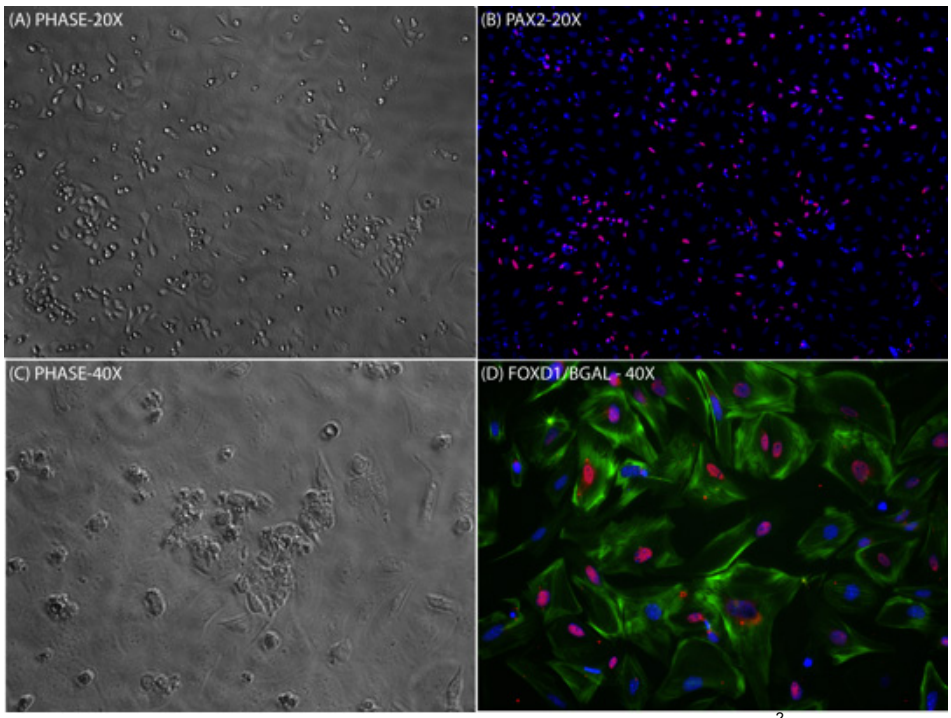

Figure 1. NZCs were purified from E17.5 embryos, plated at 200,000 cells per cm^2 and incubated at 37° C for a total of 24 hours. Cells were imaged by phase contrast with a Leica DM IRB inverted microscope prior to immunofluorescent staining. (A) 20X phase contrast view of NZCs after 24 hours in culture. (B) Cells were fixed and stained using a rabbit anti-PAX2 antibody specific for nephron progenitor cells (red) and a secondary Alexa Fluor 568 conjugated donkey anti-Rabbit. Note blue nuclei counterstained with DAPI. (C) 40X phase contrast view of a cluster of nephron progenitors (centered) after 24 hours in culture. (D) 40X image of NZCs isolated from LacZ+ embryos of the *Foxd1*^{LacZ} strain, which expresses β-galactosidase under the control of the *Foxd1* promoter. *Foxd1* is expressed in the stromal cell population within the nephrogenic zone of the developing kidney. Cells were stained with the F-actin marker phalloidin (Oregon green primary conjugate), the nuclear stain DAPI (Blue) and rabbit anti-β-galactosidase (secondary - Alexa Fluor donkey anti-Rabbit 568) for detection of *Foxd1*+ stromal cells (red).

Discussion

In this protocol we describe a method to isolate and culture cells from the nephrogenic zone of the embryonic kidney. It is the development of a method initially published as part of a study of the effects of BMP7 treatment on cells of the nephrogenic zone (Blank *et al.*, 2009). In the initial study, a series of experiments to characterize the cell types represented within the NZC population were conducted. Briefly, purification of NZCs from genetic reporters for the cortical stroma (*Foxd1*$^{+/lacZ}$) (Hatini *et al.*, 1996), collecting duct (*Hoxb7*$^{+/cre}$;*R26*^{rEYFP}) (Srinivas *et al.*, 2001; Yu *et al.*, 2002), and cap mesenchyme (*Bmp7*$^{+/lacZ}$)(Godin *et al.*, 1998) revealed that approximately 35% of harvested NZCs are cortical stroma, 52% are cap mesenchyme and that collecting duct contamination represents less than 0.04%. Lineage marking experiments using the *Bmp7*$^{+/cre}$;*R26*^{rEYFP} strain (Oxburgh *et al.*, 2004; Srinivas *et al.*, 2001) showed that approximately 10% of NZCs may be cells of the cap mesenchyme lineage that have differentiated and lost *Bmp7* expression. Viability studies with 7AAD showed a survival rate in isolated NZCs of approximately

95%. We have recently experienced success in separating cell subpopulations within NZCs through flow cytometric sorting. However, the paucity of membrane-associated markers specific to each of the subpopulations of the nephrogenic zone has limited these analyses, and currently only cells derived from mice carrying fluorescent marker genes is feasible.

The efficacy of this protocol depends greatly on speed in the dissecting step. Both cell survival and responsiveness to growth factor stimulation increase when the kidneys remain in HBSS for shorter periods of time before the enzymatic digestion. A shorter preparation time thus results both in higher cell viability and a more robust response. Furthermore, care must be taken when removing the supernatant after spinning the cell suspension as the cell pellet is easily disturbed resulting in significant cell loss.

Since the initial work of Clifford Grobstein describing embryonic organ culture (Grobstein, 1953a; Grobstein, 1953b), the kidney has been a model system for organogenesis and the interaction between epithelial and mesenchymal cells in development. Recent microanatomic gene expression studies have revealed an unanticipated complexity of cell types within each of these cellular compartments (Brunskill *et al.*, 2008), and complementary methods such as NZC culture are required to understand the interrelationship between these cells during nephrogenesis. The ultimate goal of our primary cell investigations is to define conditions in which nephrogenic zone cells can be indefinitely expanded *in vitro* both for studies of nephrogenesis and engraftment in the adult. The NZC culture system is an important step toward this goal, and ongoing studies in our laboratory aim to define the mechanisms of growth control in these cells.

Disclosures

No conflicts of interest declared.

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