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Review Article

# Podocyte culture: Tricks of the trade

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**SUMMARY AT A GLANCE**

Podocyte cell lines are becoming widely used in renal research. This article gives a detailed description of the production and culture of immortalized podocyte cell lines. Furthermore, these methods can be applied to essentially any cell type, providing a practical approach to study the interactions of renal cell types *in vitro*.

**ABSTRACT:**

**Podocytes (glomerular epithelial cells) lie on the urinary aspect of the glomerular capillary and play a key role in the selective filter that underlies kidney function. They are injured in various forms of renal disease: the extents of this injury and its reversibility have major implications for treatment and prognosis. Until recently, podocytes were difficult to study *in vitro* because of a previous lack of techniques for obtaining differentiated cells in quantities adequate for research. In recent years, this problem has been solved for rodent and human podocytes and there has been an explosion of research using cultured cells. These authors have led the development and characterization of human podocyte cell lines and in this article describe the methods that have allowed them to do this.**

In recent years, one of the fastest moving areas of research progress in nephrology has been the appreciation of the importance of the visceral glomerular epithelial cell, hereinafter referred to as the podocyte, in health and disease. Podocytes play a key role in the prevention of proteinuria in the healthy situation, are important targets of injury in a variety of renal diseases and are important determinants of outcome.[1,2] Improved understanding of podocyte biology has come from two main arenas: first, molecular genetics of single gene disorders which lead to rare forms of congenital nephrotic syndrome; and second, focused study of this specialized cell type *in vivo* and *in vitro*. The purpose of this article is to review the current state of knowledge in relation to the *in vitro* study of podocytes. The authors have most experience of human podocyte culture, but where relevant we will also discuss study of podocytes from other species. Our aim is to help new investigators to join this exciting field.

## (a) Human podocytes

When cells are directly separated from tissue and propagated *in vitro* they are referred to as 'primary culture cells'. For podocytes, this typically requires isolation of glomeruli by differential sieving, plating of glomeruli onto a collagen surface (use of collagen surface is optional, currently we use tissue culture treated surface instead) and outgrowth of cobblestone-like cells (further details will be given later). Some of the early work on rat[3] and human[4] podocytes used primary culture podocytes, but the problem was that these cells did not develop the features of differentiated cells and they continued to proliferate, whereas differentiated podocytes are quiescent cells that do not proliferate. When specific markers of differentiated podocytes (such as nephrin and podocin) became known in the early 1990s, it was clear that podocytes suitable for *in vitro* study needed to

demonstrate expression of these markers. It is our impression that workers rarely use primary culture podocytes now because they do not express these markers and because superior alternatives are available, but there is a recent review on this aspect of the subject.[5]

One technique to increase the number of cells available and to develop clonal populations of cells which should in theory be homogeneous and stable is to transform the cells with an oncogene. The transforming gene usually used is SV40, a monkey-derived gene which promotes unregulated proliferation of the cells into which it is transfected. Sraer and colleagues in Paris produced an SV40-transformed human podocyte cell line[6,7] and they generously shared this reagent with other workers including us. We found that this cell line was easy to propagate and we rapidly accumulated large numbers of cells for *in vitro* experiments. However, again the cells did not develop the phenotype of differentiated podocytes and we felt that newer more representative cell lines were needed. In 1997, Peter Mundel and colleagues reported[8] the characterization of a mouse podocyte cell line derived from the 'Immortomouse' whose cells all express SV40 transforming gene under the control of a gamma-interferon response element. Thus, cells from this mouse can be induced to express higher levels of SV40 by treatment *in vitro* with gamma-interferon. The original mouse podocyte cell line, which in time came to be known colloquially as 'Mundelocytes', was shown to express markers of mature podocytes and was generously shared with other researchers, becoming very widely used for understanding podocyte biology. In collaboration with Peter Mundel, we[9] applied a similar principle to the development of a human podocyte cell line: this time the SV40 had to be supplied to the cells *in vitro* after isolation of the cells of interest. The SV40 construct that we used is temperature-sensitive, giving us control of its expression *in vitro*: at 33°C the transgene is expressed, allowing the cells to be transformed and to proliferate vigorously. When the cells are moved to a culture temperature of 37°C, akin to the normal physiological body temperature, the transgene is silenced and the cells become differentiated, ceasing to proliferate. This approach had been previously used by our collaborator Mike O'Hare in other cell types[10] and the original normal human podocyte cell line, known colloquially as 'Saleemocytes', has now been widely shared and studied by numerous groups worldwide. The next section gives more details of the techniques required for the generation of these cells.

The intention of this article is to focus on human cells, but we should briefly mention that similar methods have been applied to podocyte culture from various species:

### (b) Mouse podocytes

In addition to the 'Mundelocytes' described earlier, other mouse podocyte cell lines have been derived[11] and more recently Shankland and colleagues have derived mouse parietal epithelial cell lines to study the biology of this less well-characterized cell type.[12]

### (c) Rat podocytes

Many of the best characterized experimental models of glomerular disease *in vivo* have been in rats, which seem to be generally more susceptible than mice. It was therefore natural for researchers to wish to have rat podocyte cell lines with which to conduct parallel studies *in vitro*. Primary culture[13] and transformed[14] rat podocytes have been described.

### (d) Other species

Insects provide a powerful research tool because of their rapid rate of reproduction and comparatively simple organ structure. The analogous cell to the podocyte in *Drosophila* (fruit fly) is the nephrocyte[15] but as yet we are not aware of the development of cell lines derived from these.

## CONDITIONALLY IMMORTALIZED HUMAN PODOCYTE CELL LINES

Conditionally immortalized human podocyte cell lines have been developed by transfection using both the temperature-sensitive mutant U19tsA58 of the SV40 large T antigen (SV40) and the essential catalytic subunit of the hTERT telomerase gene.[9,10] The hTERT vector expresses telomerase activity to maintain telomere length, preventing the occurrence of replicative senescence.[16]

Transfection of cells with SV40T allows cells to proliferate at the 'permissive' temperature of 33°C. Transfer to the 'non-permissive' temperature of 37°C results in the inactivation of large T antigen with minor changes in gene expression.[17] Podocytes then enter growth arrest (Fig. 1) and express markers of differentiated *in vivo* podocytes, including the novel podocyte proteins, nephrin, podocin, CD2AP, and synaptopodin, and known molecules of the slit diaphragm ZO-1, alpha-, beta-, and gamma-catenin and P-cadherin.[18]

### How to isolate and immortalize human podocytes

#### Materials and transportation

The donated human kidney (or portion of kidney) is packed in saline, on ice, and transferred by courier to the laboratory. The kidney is kept in a cool condition (kidney in separate container surrounded with wet ice bags/packs) during transportation at all times. Cells can be successfully cultured up to 24 h post nephrectomy. We believe that children's kidney tissue is most productive, but we have successfully generated cell lines from adult kidney too.

#### Primary human podocyte culture

Set up the laminar flow hood before proceeding. Place sieves in order from top to bottom: 425 μM, 180 μM, 125 μM,

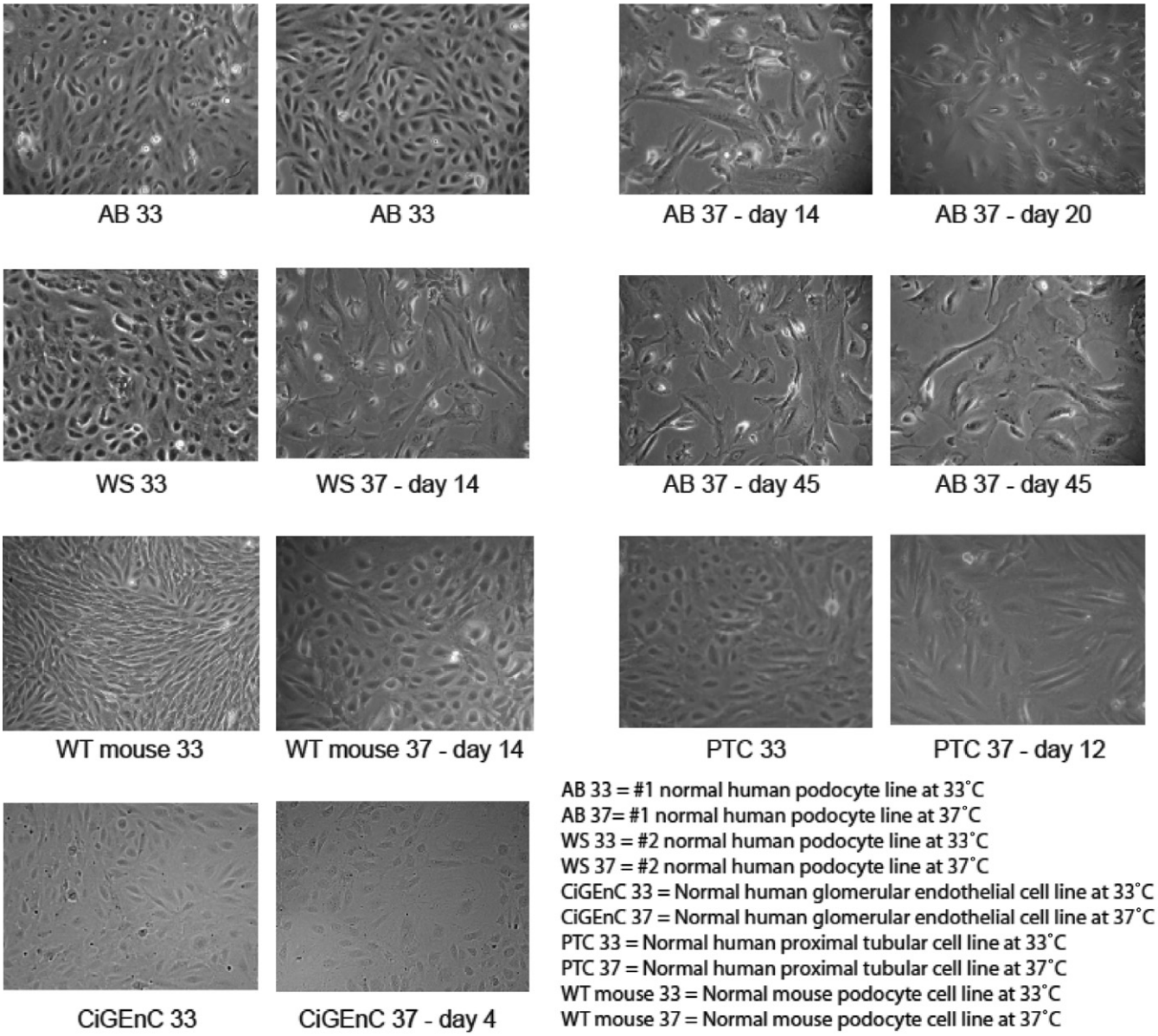


**Fig. 1** Some of the cell lines generated in our laboratory (code at lower right of figure).

90 μM (the smallest size is needed only for a kidney from a young child) sieves (Endecotts limited, London) and below them all a sterile container to collect the sieved material. Remove the outer membrane/capsule of the kidney and isolate the cortex with sterile disposable scalpels into small pieces from the medulla into a Petri dish. Chop up the cortex into small pieces then transfer to the sieve in a laminar flow hood and cut up more finely. Use a sterile plunger from a 50 mL or 100 mL syringe to push the small pieces through the top sieve (425 μM) while thoroughly washing the sieve with RPMI-1640 medium (without additives) or sterile phosphate-buffered saline (PBS). Repeat this until little is left on the top sieve. Sieving is achieved by fluid flushing and not washing the plunger for the 180 μM sieve onwards. From this point onwards culture medium is used throughout (detailed below). Pipette up glomeruli by lifting the sieves and washing down glomeruli to one side of the wall of the 125 μM sieve (for an adult kidney) or 125 μM and 90 μM sieves (for a young child's kidney). Transfer glomeruli to culture treated flasks or Petri dishes (IWAKI 3123-75 or 4020-010) and place into 37°C incubator. Only change the medium when some of the glomeruli are firmly attached (3–5 days). Usually cellular outgrowth starts in 7–10 days, at which time the majority of cells are podocytes. At this stage podocytes grow rapidly and predominate; after 2 weeks other cells such as mesangial cells may appear and would eventually take over, so it is important to harvest podocytes within 2 weeks to avoid contamination with other cell

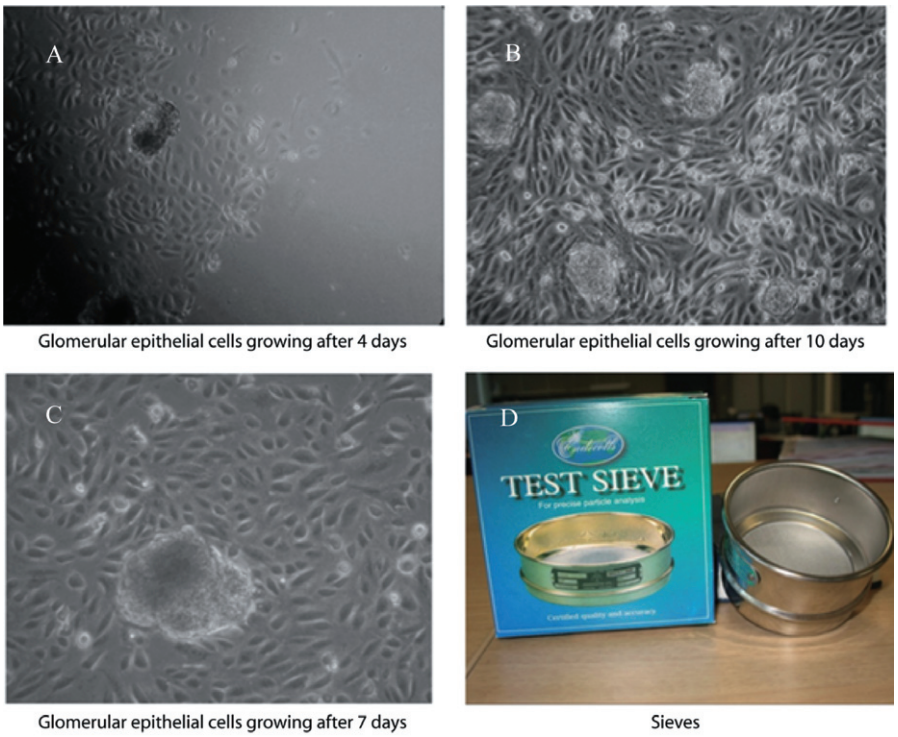


**Fig. 2** (A–C) Podocytes emerging; (D) example of sieve.

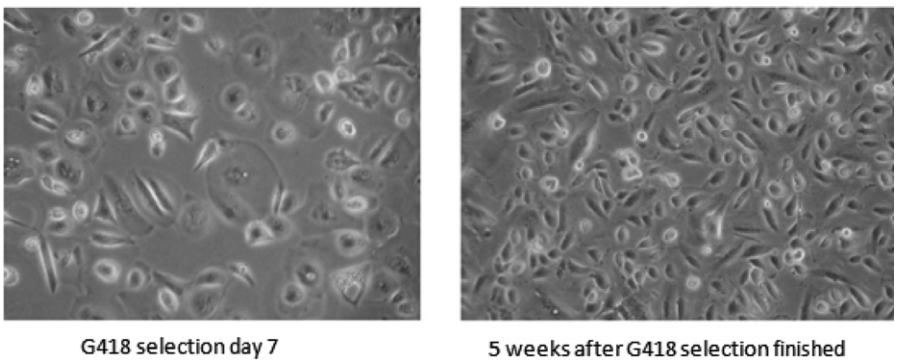


**Fig. 3** Cells after transducing with vectors and selection with G418.

types. Occasionally, contamination with non-podocytes may necessitate subcloning (see Subcloning of immortalized podocytes). Trypsinize cells (Sigma T3924 which is 0.05% trypsin; Sigma-Aldrich, Dorset, UK) and separate single cells away from the glomeruli using a 40 µM cell strainer when patches of podocytes reach confluence. Re-plate cells in T75 or T25 culture treated flask with less than 40% density overnight. These are primary culture podocytes, ready to be transduced with the immortalizing transgene on the following day (Fig. 2).

## Immortalization and selection

Primary cells are infected with tsSV40T and hTERT vectors[9] containing respectively G418 and hygromycin resistance genes, over 18 h with Polybrene 10 µg/mL (Sigma H-9268). Then subconfluent cells are transferred from 37°C to 33°C for selection using G418 (400 µg/mL; Sigma-Aldrich) and hygromycin (25 µg/mL; Sigma-Aldrich) for 2 weeks (Fig. 3). Currently we use a bicistronic vector containing tsSV40T and hTERT, which has a single resistance cassette to G418.

Keep in culture until new immortalized cells grow, taking at least 1 month (Fig. 3).

## Subcloning of immortalized podocytes

To obtain a homogenous cell culture derived from single cell clones, cells are subcloned using treated NIH 3T3 fibroblasts as non-dividing feeder cells. Grow NIH 3T3 fibroblast cells at 37°C till confluent then treat with 0.25 µg/mL mitomycin C overnight. Change the medium after treatment and trypsinize cells on the following day and reseed NIH 3T3

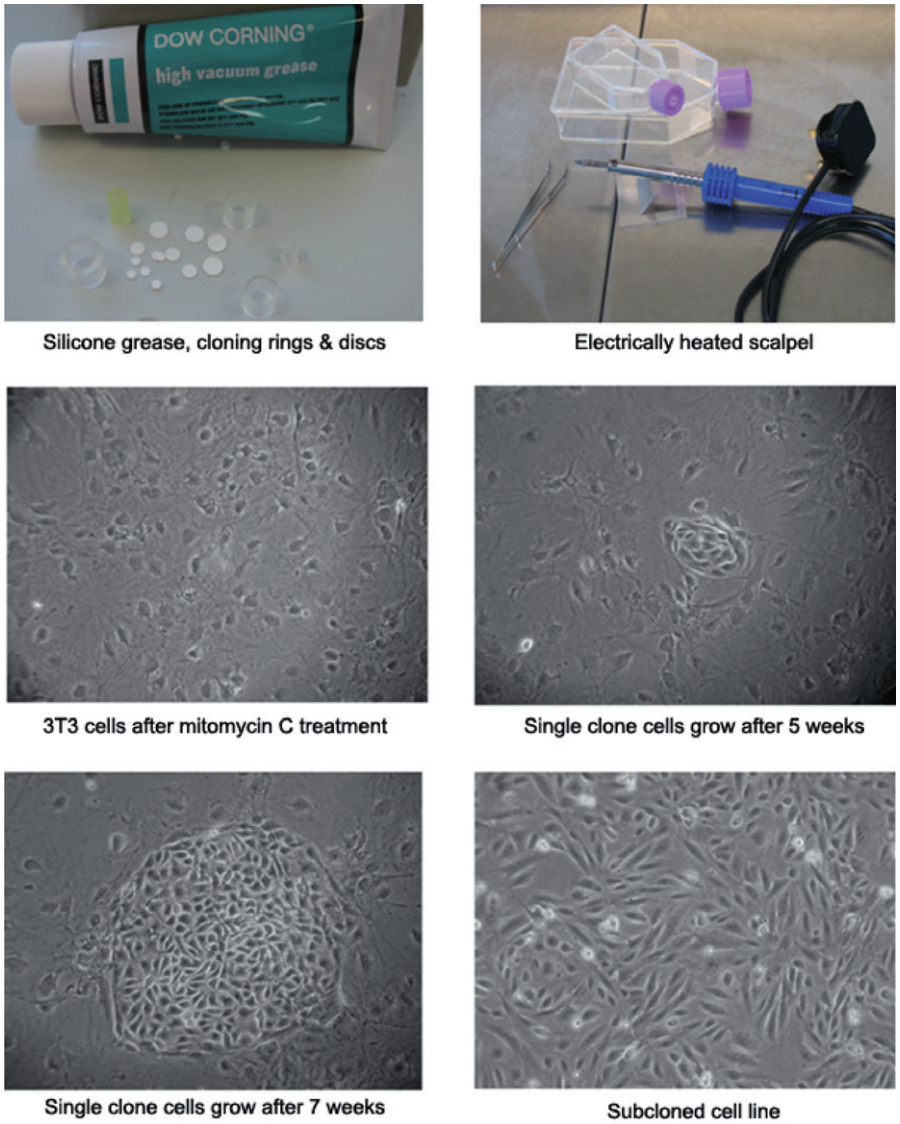


**Fig. 4** Cloning and subcloning.

cells in $4 \times 75$ cm$^2$ flasks or 5–6 Petri dishes containing ~$10^5$ cells or ~$5 \times 10^4$ cells in each dish. Count podocytes before trypsinizing, then dilute the cell suspension to the desired seeding concentration into each NIH 3T3 flask or Petri dish, for example 100 cells, 300 cells, 500 cells and 1000 cells. Leave cells at 33°C for another 5–7 days and then change the medium as necessary.

After about 5 weeks, single clonal cells grow out visibly which are picked by cloning rings or cloning discs (both from Sigma-Aldrich). Cut off the top of a flask with an electrically heated scalpel, and using sterile forceps dab cloning rings with silicone grease (Fisher scientific laboratory – autoclave before use) or discs with 0.25% trypsin-EDTA. Then remove medium and rinse with PBS, place rings or discs to a clone. According to the size of clone, cloning rings are usually used to pick larger size clones. When the cloning ring is sealed firmly on a clone, add trypsin/EDTA into rings as per normal trypsinization of cells. Trypsin needs 5 min at 37°C. Then transfer cloning cells or discs into individual flasks or culture plate at 33°C. Leave discs in for at least 48 h. Keep culturing cells until they are confluent and then freeze cells, make sure there are plenty of stocks all the time (Fig. 4).

## Characterization of cell lines

Experimental procedures are performed on the clonally selected cells by growing cells at 40% confluence on cover slips in Petri dishes at 33°C followed by differentiation for 10–14 days at 37°C. Fix cells before staining with 2% paraformaldehyde solution adding 2% sucrose. Immunofluorescence staining for podocyte markers, protein extraction from culture flasks or plates is performed after differentiation for 14 days at 37°C. We detect podocyte proteins, such as nephrin, podocin, CD2AP, and synaptopodin,

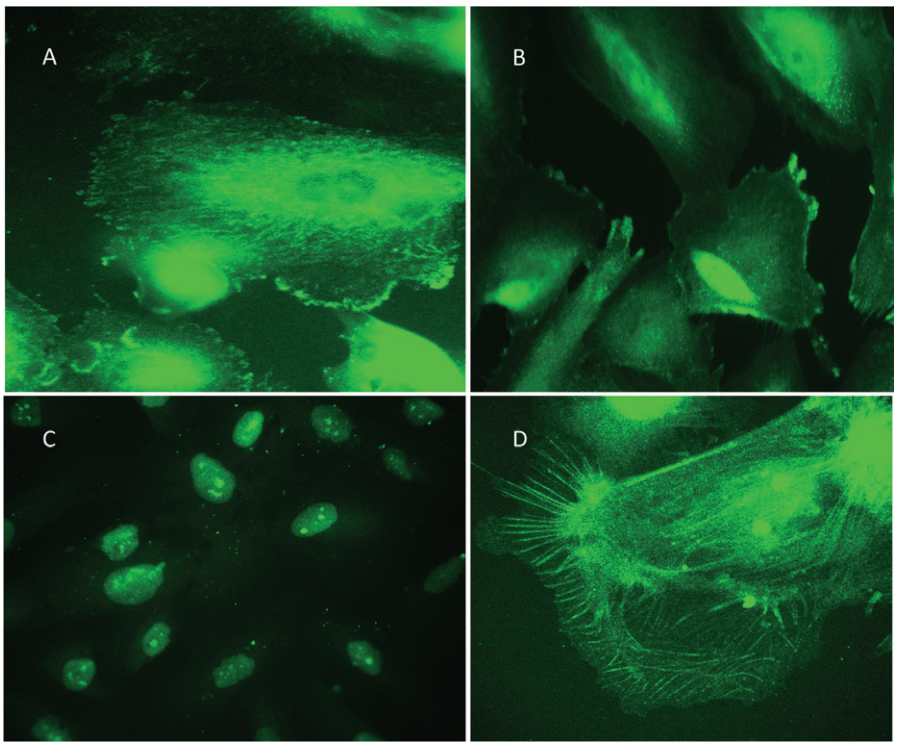


**Fig. 5** Immunofluorescence images of differentiated human podocytes labelled for (A) Nephrin (monoclonal antibody 50A9, kind gift of Prof K Tryggvasson, Stockholm, Sweden); (B) Podocin (rabbit polyclonal antibody, Sigma P0372); (C) WT1 (mouse monoclonal F6 antibody, Santa Cruz – sc-7385); (D) Synaptopodin (mouse monoclonal antibody, Progen 65194).

and known molecules of the slit diaphragm ZO-1, alpha-, beta-, and gamma-catenin and P-cadherin (Fig. 5).

### Continuous culture of immortalized podocyte cell lines

Incubators kept at 33°C and 37°C, 5% $CO_2$,

### Medium

- RPMI-1640 Sigma R-8758
- Insulin-Transferrin-Selenium Invitrogen 41400045
- Foetal Bovine Serum (10% v/v) Sigma F7524
- Pen/Strep Sigma P4333

Use of antibiotics (Pen/Strep) is optional for cell lines.

Use standard tissue culture-treated flasks or plates. We do not use special coatings such as collagen routinely as we have concluded that they do not offer any further benefit to cell culture. We do not specially treat flasks or plates ourselves.

### Passage and long-term storage

Let immortalized podocytes grow at 33°C to 100% confluence, then freeze 40% and split the rest 1:3. For subsequent passages, split cells 1:3 to 1:5 when at 80% confluence. Use low concentrations of trypsin/EDTA (Sigma T3924 or equivalent with trypsin 0.05%) and expose the cells for as short a time as possible.

Ensure freezing of at least 30% of each passage for long-term storage (liquid nitrogen) and availability of low passage numbers for the future.

### Thermoswitching

Move cells from 33°C to 37°C when cells are 40–60% confluent. Change medium three times per week. Usually it takes 14 days for full differentiation. They proliferate abundantly at 33°C, and after thermoswitching to 37°C, usually take 1–3 days before cell division fully ceases. The transgene is actually designed to inactivate fully at 39.5°C but we normally see complete quiescence at 37°C for most human podocytes (sometimes with mouse podocytes it is necessary to go up to 38.5°C or above for full differentiation).

## A WORD ABOUT CO-CULTURE

We would like to finish with a word about cell co-culture. We restate the view[2] that the glomerular capillary wall should be seen as a tripartite structure in which the three components (podocytes, glomerular basement membrane and glomerular endothelial cells) are interdependent and each of crucial significance, such that a focus on any one component of that structure might be inappropriately simplistic. The advantage of being able to grow stable differentiated podocytes *in vitro* is that we can study their interactions with other pure renal cell types:

1 Glomerular endothelial cells. We have used similar conditional immortalization techniques to generate human glom-

erular endothelial cell lines.[19] We are particularly interested to study the interactions of these cells with normal and mutant podocytes, also to develop three-dimensional culture systems including flow, so as to mimic as closely as possible the *in vivo* situation.[20]

**2** Mesangial cells. The third cell type in the glomerulus is the mesangial cell: as with podocytes, much if the early work used either primary culture or transformed mesangial cells: we have developed and characterized[21] a conditionally transformed normal human mesangial cell line and we anticipate developing complex co-culture systems with all three glomerular cell types.

**3** Tubular cells. We have reported the development of a conditionally immortalized human proximal tubular cell line[22] and we firmly believe that similar techniques can in principle be used to transform any cell type.

## CONCLUSIONS

Human podocyte cell lines can now be reliably propagated for study *in vitro*. We believe that conditional immortalization provides the most reliable and representative cell lines: we are proud of the fact that podocyte cell lines originally developed in Bristol are now in widespread use across the world and we would like to encourage other workers to reproduce our results.

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