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Podocytes in culture: past, present, and future

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Human genetic and *in vivo* animal studies have helped to define the critical importance of podocytes for kidney function in health and disease. However, as in any other research area, by default these approaches do not allow for mechanistic studies. Such mechanistic studies require the availability of cells grown *ex vivo* (i.e., in culture) with the ability to directly study mechanistic events and control the environment such that specific hypotheses can be tested. A seminal breakthrough came about a decade ago with the documentation of differentiation in culture of primary rat and human podocytes and the subsequent development of conditionally immortalized differentiated podocyte cell lines that allow deciphering the decisive steps of differentiation and function of '*in vivo*' podocytes. Although this paper is not intended to provide a comprehensive review of podocyte biology, nor their role in proteinuric renal diseases or progressive glomerulosclerosis, it will focus specifically on several aspects of podocytes in culture. In particular, we will discuss the scientific and research rationale and need for cultured podocytes, how podocyte cell-culture evolved, and how cultured podocytes are currently being used to uncover novel functions of podocytes that can then be validated *in vivo* in animal or human studies. In addition, we provide a detailed description of how to properly culture and characterize podocytes to avoid potential pitfalls.

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RATIONALE FOR THE NEED OF PODOCYTES IN CULTURE

The study of kidney disease is complex because the onset is often undetected, diseases may be acute or chronic in nature, the genetic makeup of the host leads to variable clinical syndromes, and multiple organs are often involved simultaneously. To study disease susceptibility, mechanisms, prognosis, and potential therapies, most authorities, including the authors, would say that studying humans is ideal and, in most instances, this is the gold standard. However, this is not always possible and we would argue that this is an exception rather than a rule. Moreover, many diseases, in particular kidney diseases, are not that common in the general population, thus increasing the challenge of limiting studies to man. There is no doubt that the research questions should primarily stem from patients with kidney disease. However, studies verifying proof of principle are limited, if not impossible in man.

Thus, in renal and nonrenal research, the use of experimental models in animals has proved invaluable. Moreover, the genome era with the development of null or transgenic mice, and more recently, with the ability to restrict or delete expression to a specific cell, has significantly advanced our understanding of many aspects of kidney disease. Nevertheless, animal models are often limited because they do not always fully replicate their human counterpart. For example, the current mouse models of diabetic nephropathy do not typically demonstrate the features of the human disease such as Kimmelstiel–Wilson nodules. Moreover, there are no mouse models of other podocyte diseases such as membranous nephropathy and the background mouse strain plays a critical role in disease initiation and progression.[1] Similarly, while positional cloning has proven invaluable and has uncovered mutations such as those in phospholipase C epsilon (PLCE1) underlying nephrotic syndrome, PLCE1-null mice do not have any overt phenotype,[2] suggesting that animal models too may be limited. Yet, well-characterized animal models do provide insights into disease, albeit often descriptive, and in many instances, provide a good starting point for the decision to try a particular therapy in human disease. Most significantly, however, despite the many merits of transgenic/null mice and animal models, they do not, by default, allow for mechanistic studies, calling for the use of cells grown *in vitro*.

WHERE DID CELL-CULTURE STUDIES BEGIN?

The initial studies using cells from either man or animals grown in culture date back to the 1950,[3–5] including cells derived from the kidney.[6] Although cells grown *ex vivo* (i.e., in culture) do not fully replicate the *in vivo* environment, they have several major advantages. These include the ability to directly study mechanistic events, to control the environment such that specific hypotheses can be tested, and that multiple experiments can be performed to validate the initial observations. Although perhaps obvious, it is critical to use podocytes in culture to study events related to podocytes. Thus, although informative, the use of other cells such as human embryonic kidney cell line, COS, and mouse embryonic fibroblasts (MEFs) to study podocytes is not fully representative and thus has the potential to be erroneous. Although these nonpodocyte cells might be useful to initially characterize novel protein:protein interactions, they are not podocytes and therefore the functional relevance of these novel interactions need to be confirmed in podocytes to generalize these findings to podocytes.

There is a large body of literature using cells in culture in cardiac, central and peripheral nervous system, liver, blood, immune, and other organ-system research. Each cell-culture system has unique properties and characteristics that differ from others and podocytes are no exception. In this review we will provide comprehensive and compelling support that the study of podocytes in culture is a valid and invaluable model system, when used together with animal models and human studies, to uncover novel and mechanistic events in these important cells. It is our belief that with each model system (man, animal, cells), validation of the results need to be confirmed with a different model system.

THE PAST: HISTORY OF PODOCYTES IN CULTURE

Cultivation of podocytes *in vitro* was first introduced in the mid-1970s[7–9] and glomerular epithelial cells in culture from a variety of species have since been described in man,[7,8,10–12] pigs,[13] rats,[14–16] and mice.[17,18] The first step in culturing podocytes is based on the isolation of encapsulated or decapsulated glomeruli from kidney cortex.[9] A good preparation is considered when 95% of the cellular constituents are glomeruli, with the remainder comprising tubules. Thus, to further reduce tubular contaminations (and enhance the percentage of glomeruli in the preparation), a Ficoll-gradient centrifugation step may be included.[19] After 4 or 5 days of primary culture, a monolayer of cobblestone-like epithelial cells is observed. However, since the beginning of these pioneering experiments substantial doubts about the origin and validity of the cells growing out from the isolated glomeruli have been raised.[20–22] Subsequent studies showed that this uncertainty could be ruled out by starting the generation of primary cultures specifically in manually selected decapsulated glomeruli that are devoid of parietal epithelium.[11,16] The presence of podocytes in culture was further supported by the observation that differentiated podocytes can leave their position on the glomerular basement membrane and migrate onto the surface of cultured kidney slices.[23]

After the isolation of glomeruli, two alternative protocols have been used that ultimately lead to similar results, the generation of primary cultures of podocytes. In the first protocol, isolated glomeruli are generally cultured for 4 or 5 days before they are subcultured by passing trypsinized glomerular outgrowths over sieves with 25 μm pore size to remove the remaining glomerular cores consisting mainly of mesangial and endothelial cells.[24] In the second protocol, isolated glomeruli are digested with collagenase for 30 min, passed over a sieve with 25 μm pore size and the cells that pass through the sieve are used for cultivation.[25] In both protocols, cells are routinely plated on type I collagen-coated dishes to promote cell proliferation. The proliferating cells form a monolayer of cobblestone appearance. As they reach confluence, the cells are subcultured after trypsinization or collagenase treatment. The media used varied from Dulbecco's modified Eagle's medium or RPMI 1640 supplemented with 5–20% fetal calf serum[13] or 3T3-fibroblast-conditioned medium[24] to hormonally defined serum-free media.[26] The cells obtained under these conditions have mostly been used as primary cultures or as early subcultures of primary outgrowths,[14,27,28] but in addition permanent and nonpermanent cell lines have been established.[10,11,15,17,18] Despite advancing the field, these cells were still not ideal and the quest for the cell that replicates the *in vivo* counterpart was only just beginning.

CLUES TO UNLOCKING THE TRUE *IN VITRO* PODOCYTE PHENOTYPE: THE TALE OF TWO DIFFERENT PHENOTYPES OF CULTURED PODOCYTES

As discussed above, early on the cobblestone-type cells were the first to be used to investigate podocyte function in culture because these cells were able to survive under cell culture conditions. However, in these early studies the unspecific cobblestone-appearance of the cells was used as the sole characteristic to prove that the glomerular outgrowths were podocyte-derived.[9] A breakthrough emerged in that the identification of podocytes was advanced by the demonstration of podocyte 'markers' including TN10[29] and podocalyxin.[10,11] Of note, podocalyxin is not specific for podocytes because it is also expressed by endothelial cells.[30] Thus, podocycalyxin alone is not sufficient to prove that cells in culture are podocytes. Expression of intermediate filament proteins (e.g., cytokeratin, vimentin, or desmin) and cell junction proteins (e.g., ZO-1) have also been used, but they too do not allow definitive proof of origin of cultured podocytes, since they are also expressed in other cell types. Thus, their presence or absence in cultured podocytes should rather be seen in the light of mimicking *in vitro* the developmental modulations of these proteins observed during podocyte maturation *in vivo*.[31–35]

Another major problem associated with culturing podocytes had been the historical rapid dedifferentiation *in vitro* that accompanies the loss of the specific cell architecture and

results in the cobblestone morphology of 'standard' podocytes in culture. The latter share many features with immature podocyte precursor cells during early glomerular development, including the lack of synaptopodin expression (see below). Their low degree of differentiation is also reflected by the reappearance of lymphohaemopoietic marker antigens[36] expressed *in vivo* only transiently during the early stages of podocyte development.[37]

A second type of epithelial cells growing out from isolated glomeruli shows a different phenotype (Figure 1b). These are large (up to 500 μm) arborized, often binucleated cells that exhibited no proliferative activity. This obviously limited propagation and the ability to establish a cell line *in vitro*. A novel discovery was that in contrast to cobblestone cells, these cells expressed synaptopodin.[16,38] Synaptopodin is a key marker of a differentiated podocyte phenotype, because *in vivo* the expression of synaptopodin is specific to postmitotic differentiated podocytes.[39] Arborized podocytes had been reported to contribute between 10% of rat[16] and almost 100% of cultured porcine glomerular epithelial cell,[13] both after roughly 10 days of culture.

THE PRESENT: INDUCTION OF DIFFERENTIATION IN CULTURED RAT AND HUMAN PODOCYTES

In the mid-1990s, serious efforts were undertaken to develop a podocyte culture model that would demonstrate *in vitro* expression of synaptopodin, a key marker of differentiated podocytes *in vivo*.[39] This effort was motivated by the observation discussed above that the cultivation of podocytes under then standard conditions leads to rapid dedifferentiation, including the loss of processes and synaptopodin expression.[39] It was further motivated by the fact that synaptopodin is not expressed in other cells such as fibroblasts, suggesting that results obtained from studying synaptopodin in fibroblasts or other model cell lines would not necessary reflect its true function in podocytes. To optimize the *in vitro* characteristics, we modified the culture conditions for rat and human podocytes by simply avoiding repeated subcultivation.[38] This led to profound phenotypic changes in podocytes *in vitro*, including the conversion of cells with the cobblestone phenotype into cells with the characteristic arborized phenotype that more closely resemble *in vivo* podocytes. Both cobblestone and arborized cells originate from podocytes, as evidenced by the expression of a podocyte-specific O-acetylated ganglioside[40] and WT-1.[41] The differentiation into arborized cells leads to growth arrest and is reflected by the formation of processes and the expression of synaptopodin, which was never detected in cobblestones. Taken together, we achieved (partial) differentiation of cultured podocytes by avoiding repeated subcultivation, resulting in a phenotype more closely reflecting *in vivo* podocytes.[38] Although these results were indeed satisfying, there was still room for improvement.

A MAJOR BREAKTHROUGH: CONDITIONALLY IMMORTALIZED MURINE PODOCYTES RETAIN A DIFFERENTIATION POTENTIAL SIMILAR TO THEIR *IN VIVO* COUNTERPARTS

The differentiation of primary human and rat podocytes results in rapid growth arrest.[38] Although this reflects the mature *in vivo* counterpart, it limits cell culture abilities because passaging cells that do not increase in number is technically problematic. To circumvent this problem, we took advantage of the Immortomouse[42] and established conditionally immortalized mouse podocyte cell lines, which are highly proliferative when cultured under permissive condi-

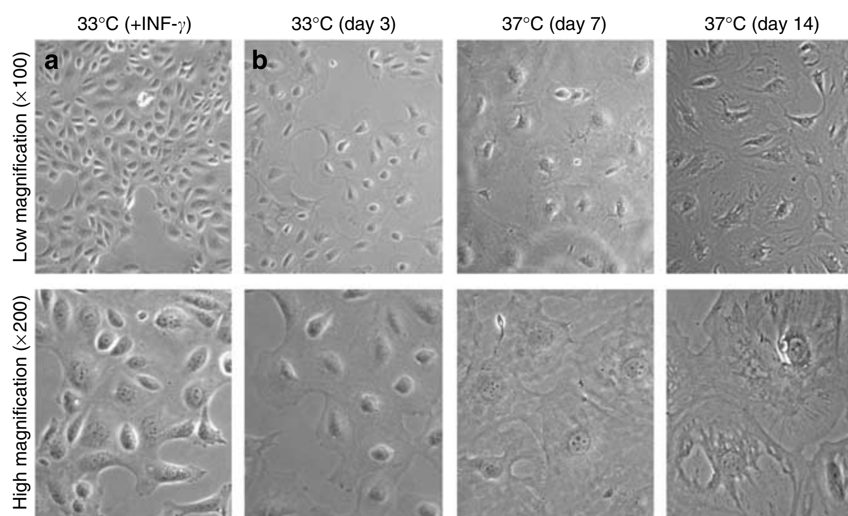

Figure 1 | Light microscopic morphology of cultured podocyte. (**a**) Podcytes grown under permissive conditions (at 33°C with 20 U/ml INF-γ) display a characteristic cobblestone morphology. The cells form an epithelial monolayer as they reach confluence. (**b**) Differentiating (3, 7) and differentiated (14) podocytes grown under nonpermissive conditions (days at 37°C, no INF-γ). Under nonpermissive conditions, the cells develop interdigitating processes, which are only connected at sites of process interdigitations. On day 14, large, flat arborized cells with well-developed prominent processes can be seen.

Table 1 | Similarities between podocytes *in vitro* and *in vivo*: if they look, smell, and act like a podocyte, they are probably podocytes

Anti-cell culture view	Pro-cell culture view	Refs.
Cultured podocytes lack slit diaphragms	They express a slit diaphragm-like structure	Reiser *et al.*[44] and Gao *et al.*[45]
Cultured podocytes lack expression of podocyte-specific proteins	They stably express the vast majority, if not all, of the podocyte-specific proteins described *in vivo*	See Table 2
They represent a nonquiescent phenotype	PCNA, Brdu, cyclins E, A, and M are absent in differentiated podocytes grown at 37°C, consistent with a quiescent phenotype FACS analysis further supports cell-cycle exit	Mundel *et al.*,[43] Griffin *et al.*,[70,71] and Hiromura *et al.*[95]
Cultured podocytes are not exposed to hemodynamic effects	Mechanical stretch is available if deemed necessary	Endlich *et al.*[51]
	Full differentiation in metanephric kidney culture is possible in the absence of hemodynamic effects	Nagata *et al.*[96]

FACS, fluorescence-activated cell sorting; PCNA, proliferating cell nuclear antigen.

tions. The details are described elsewhere.[43] In brief, nonpermissive conditions render the majority of podocytes growth arrested within 6 days and induce many characteristics of differentiated podocytes (Table 1). Both proliferating and differentiating podocytes express WT-1. During differentiation, an ordered array of actin fibers and microtubules started to extend into the forming cellular processes, reminiscent of podocyte processes *in vivo*.[43] Similar to primary cultures,[38] the cytoskeletal rearrangement and process formation were accompanied by the onset of synaptopodin expression. Moreover, electrophysiological studies showed that differentiated murine podocytes respond to bradykinin by changes in intracellular calcium concentration.

Taken together, these studies established for the first time that conditionally immortalized murine podocytes *in vitro* retain a differentiation potential similar to podocytes *in vivo*,[43] including the formation of slit-diaphragm-like cell–cell contacts[44,45] (Figure 2). This breakthrough has since made it possible to explore the molecular mechanisms underlying podocyte differentiation and function using an inducible *in vitro* model.[43] Of note, despite published[46,47] and unpublished claims (Table 1), when properly cultured (see below), these cells express virtually all proteins described in podocytes to date, including among others: $\alpha 3\beta 1$ integrin,[48] α-actinin-4,[49–51] angiotensin 2 receptor[52], B7-1,[53] CD2AP,[54,55] ILK,[56] myosin II,[51] Nck1/2,[57] nephrin,[50,53] P-cadherin,[44] Pod1,[58] podocalyxin,[58] podocin,[50,53] synaptopodin,[43] TRPC6,[59] transforming growth factor-β,[60] vascular endothelial growth factor,[61,62] and WT-1[58] (Table 2).

HOW CULTURED PODOCYTES HAVE ADVANCED THE FIELD IN HEALTH AND DISEASE BEYOND *IN VIVO* STUDIES

Podocytes in culture are now being widely used to study virtually all aspects of podocyte biology in health and disease (Table 3). These innovative studies by laboratories throughout the world include the regulation of the cytoskeleton,[49,51,63–68] cell cycle,[69–71] survival,[72,73] signaling[56,58,74–79] cell:cell,[53,80] and cell:matrix adhesion[48,81] as well as podocyte channel physiology.[68,82–85] Table 3 also shows how podocytes in culture have been successfully used to gain mechanistic insight into the role of podocytes in the pathogenesis of diabetic[62,86] and human immunodeficiency virus-associated nephropathy.[87–89] Here, we will highlight two recent examples because they exemplify how podocytes in culture have helped to explore the function of proteins in podocytes, which could have not come from *in vivo* studies, because the protein is not expressed in healthy podocytes (B7-1[53]) or because the protein is dispensable for healthy podocytes but has an important protective role under pathologic (stressed) conditions (cyclin I[71]).

What we have learned from the B7-1 experience and cultured podocytes: for a variety of reasons beyond the scope of this review, we developed cultured podocytes from $\alpha 3$ integrin-deficient ($\alpha 3^{-/-}$) mice that bear a strong morphological resemblance to podocytes in congenital nephrotic syndrome and other pathological conditions with foot process (FP) effacement.[90,91] These $\alpha 3^{-/-}$ podocytes allowed us to uncover an unanticipated novel role for costimulatory molecule B7-1 in podocytes as an inducible modifier of glomerular permselectivity.[53] In particular, this study was the first to demonstrate that B7-1 is expressed in podocytes under a variety of stressed conditions, in culture, and *in vivo*, both in experimental and human proteinuric kidney diseases. We specifically showed that podocyte B7-1 reorganizes the actin cytoskeleton of podocytes and modulates slit diaphragm (SD) organization. Most significantly, the effects of B7-1 in podocytes were found independent of T and B cells and represent a novel unique function of B7-1.[53] As discussed above, these result most probably would have not come from *in vivo* studies, because B7-1 is not expressed in healthy podocytes.[53] Similarly, cyclin I is constitutively expressed in normal podocytes but its function remained unclear. Cyclin $I^{-/-}$ mice develop normally and have no renal phenotype. Surprisingly, cyclin $I^{-/-}$ podocytes in culture revealed a novel role for this enigmatic cyclin by enhancing podocyte survival following injury. Once a role for cyclin I in podocyte survival was uncovered in culture, its role was confirmed in a disease model *in vivo*.[71]

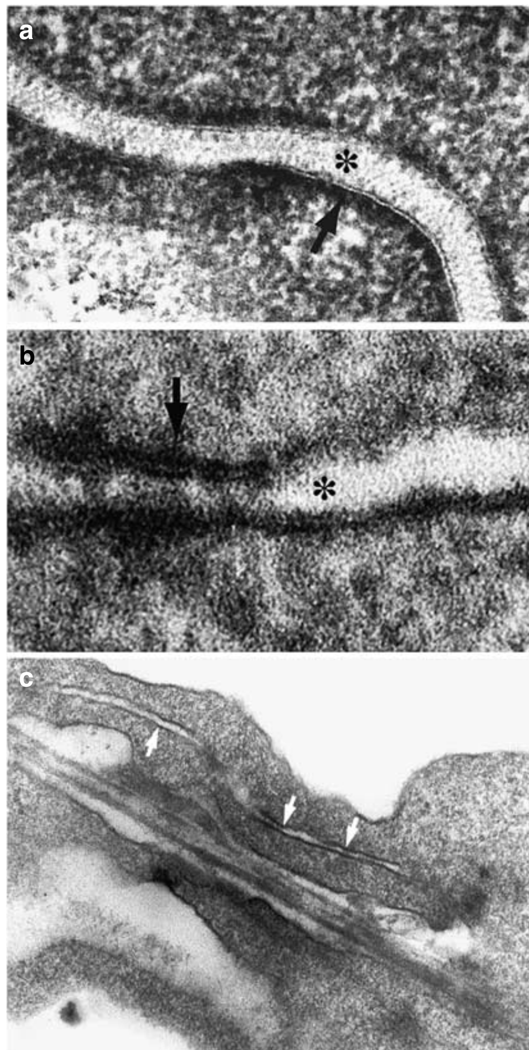

Figure 2 | Morphologic comparison of the slit diaphragm complex *in vivo* and *in vitro*. (**a**) Tangential section of the slit diaphragm *in situ* reveals a zipper-like structure with a central linear bar (asterisk). At the cytoplasmic insertion site, electron-dense material is observed (arrow). (**b**) A similar junction is observed between processes of cultured podocytes. As *in vivo*, the intercellular space (asterisk) is 25–40 nm wide and electron-dense material is found at the cytoplasmic insertion site (arrow). (**c**) Low-power micrograph showing the longitudinal orientation of the junction between foot process-like processes of two adjacent podocytes (arrows). Original magnification in (**a**) × 65 000, (**b**) × 70 000, and (**c**) × 9500. Reprinted from Reiser *et al.* (2000)[44] (with permission).

Table 2 | Taking advantage of the expression of podocyte proteins by podocytes *in vivo* to use as markers of cell specificity *in vitro*

Podocyte protein	Refs.
$\alpha3\beta1$ integrin	Reiser *et al.*[48]
α-Actinin-4	Asanuma *et al.*,[49] Fan *et al.*,[50] and Endlich *et al.*[51]
Angiotensin 2 receptor	Durvasula *et al.*[52]
B7-1	Reiser *et al.*[53]
CD2AP	Shih *et al.*,[54] and Huber *et al.*[55]
ILK	de Paulo *et al.*[56]
Myosin II	Endlich *et al.*[51]
Nck1/2	Verma *et al.*[57]
Nephrin	Fan *et al.*,[50] and Reiser *et al.*[53]
P-cadherin	Reiser *et al.*[44]
Pod1	Davidson *et al.*[58]
Podocalyxin	Davidson *et al.*[58]
Podocin	Fan *et al.*,[50] and Reiser *et al.*[53]
Synaptopodin	Mundel *et al.*,[43] and Davidson *et al.*[58]
TRPC6	Reiser *et al.*[59]
TGF-β	Wu *et al.*[60]
VEGF	Kretzler *et al.*,[61] and Iglesias-De La Cruz *et al.*[62]
WT-1	Mundel *et al.*,[43] and Davidson *et al.*[58]

TGF, transforming growth factor; VEGF, vascular endothelial growth factor; TRPC6, transient potential receptor 6.

Table 3 | Examples of biological mechanisms and kidneys diseases that are being studied using podocytes in culture

Biological mechanism/ disease	Refs.
Cytoskeleton	Asanuma *et al.*,[49,63] Endlich *et al.*,[51] Kobayashi *et al.*,[64–67] and Moller *et al.*[68]
Cell cycle	Pippin *et al.*,[69] and Griffin *et al.*[70,71]
Podocyte survival	Schiffer *et al.*[72,73]
Signaling	de Paulo *et al.*,[56] Davidson *et al.*,[58] Tossidou *et al.*,[74] Peters *et al.*,[75] Huber *et al.*,[76] Jung *et al.*,[77] Guan *et al.*,[78] and Yang *et al.*[79]
Cell:cell adhesion	Reiser *et al.*,[53] and Hunt *et al.*[80]
Cell:matrix adhesion	Reiser *et al.*[48,81]
Channel physiology	Endlich *et al.*,[68] Huber *et al.*,[82] Bek *et al.*,[83,84] and Henger *et al.*[85]
Diabetic nephropathy	Iglesias-De La Cruz *et al.*,[62] and Susztak *et al.*[86]
HIV associated nephropathy	He *et al.*,[87] Husain *et al.*,[88] and Schwartz *et al.*[89]

HIV, human immunodeficiency virus.

HOW TO PROPERLY CULTURE PODOCYTES: SHORTCUTS WILL LEAVE YOU FLATFOOTED

As discussed earlier, podocytes *in vivo* typically do not proliferate.[92] Thus, to replicate this nonproliferative terminally differentiated and highly specialized phenotype *in vitro*, special cell-culture conditions are required. To generate large numbers of cells, cells are first grown under growth-permissive conditions, where immortalized podocytes replicate. Growth media consists of RPMI 1640 (or Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum supplemented with 20–100 U/ml of mouse interferon gamma (INF-γ) to drive T-antigen expression.[43] The culture dishes are usually coated with type I collagen to promote podocyte proliferation. As the tsA58 mutant T antigen is temperature sensitive,[42] a culture temperature of 33°C is required, which must be carefully monitored in the incubator (see below). To silence the tsA58 T antigen, thereby inducing cell cycle exit and differentiation, the cultures are then switched to medium lacking INF-γ and transferred to an incubator set at 37°C. The removal of INF-γ from the culture media results in the loss of IFN-γ-driven H-2Kb-promotor activity[42] and the higher temperature of 37°C causes the degradation of the remaining tsA58 T antigen.[43] Again, the

proper temperature of the incubator is paramount. If the actual temperature is too low, the T antigen will be only partially degraded, thereby preventing the cell from cell-cycle exit. It is not recommended to expose the podocytes to temperatures higher than 37°C to avoid the induction of heat shock, which in turn causes cell stress.

To generate podocyte lines from the H-2Kb-tsA58 transgenic Immortomouse[42] or from knockout mice after crossing with the Immortomouse,[49,53] kidneys are removed and glomerular isolation is performed by sterile dissociation of minced kidney cortex on a series of differential sieves.[43] Glomeruli are captured on the smallest sieve, resuspended in media supplemented with INF-γ (100 U/ml), placed on type I collagen-coated plates, and incubated at 33°C. Alternatively, to promote initial cell outgrowth, one group (PM) routinely now grows the isolated glomeruli for 3–5 day at 37°C in high-dose INF-γ (100 U/ml) containing medium before shifting them to 33°C and 50 U/ml of INF-γ. Once stable clonal cell lines have been established, the concentration of INF-γ should be gradually decreased to a maintenance level of 10–20 U/ml.[43] As soon as islets of cobblestone-shaped podocyte outgrowing from isolated glomeruli become visible (usually after 4–7 days), the separation from glomerular cores and contaminating tubular fragments can be conducted. Here, two approaches have been used. In the classical approach, the outgrowths are trypsinized and passed over a 25-μm sieve to remove glomerular cores and tubular fragments.[43] The cells are then replated, expanded, and stocks of this pool are frozen as an initial backup. To generate monoclonal cell lines, the pool undergoes limiting dilution cloning and individually growing clones are then identified as podocytes by immunofluorescence microscopy for WT-1[43] and podocin, which is also expressed under permissive conditions. WT-1- and podocin-expressing clones are then transferred to 37°C in the absence of INF-γ to induce growth arrest and differentiation. After 14 days at 37°C, the T antigen should be completely degraded (which should be verified by Western blot and immunofluorescence microscopy) and the cells should have exited the cell cycle and have completely stopped proliferating.[43] At this stage, differentiation must be confirmed by immunolabeling for synaptopodin.[43] With this approach, we initially identified several wild-type podocyte cells lines, termed MPC cells.[43] As discussed above, the subsequent and still ongoing analysis of these cells confirmed the expression of virtually all proteins described in podocytes *in vivo* to date (Table 2).

Alternatively, outgrowths from decapsulated glomeruli missing Bowman's capsule can be isolated using cloning cylinders. After replating at a low density (1.8 cells/cm^2) to generate colonies from single cells, colonies with characteristic podocyte morphology are identified and reisolated using cloning cylinders. Again, the potential podocyte lines are then ready for further phenotypical and functional characterization as described above.

Similar to undifferentiated primary podocytes in culture,[38] the actively proliferating conditionally immortalized podocytes growing under permissive conditions display an epithelial morphology.[43] They are small in size, exhibit a polygonal or 'cobblestone' appearance, and have a relatively small cytoplasmic volume (Figure 1a). When podocytes are placed in growth restrictive conditions (37°C without INF-γ), again similar to primary differentiated podocytes,[38] they substantially increase in size, stop replicating, and take on a more complex arborized morphology (Figure 1b)[43] including the formation of cellular structures comparable with filtration slits *in vivo*[44] (Figure 2). Podocytes are typically plated in growth-restrictive conditions at a concentration of 5000–10 000 cells/cm^2.

A word of caution about cultured podocytes: like most cultured cells, the longer podocytes are maintained in culture, the greater the chance for spontaneous mutations leading to transformation. This is particularly important because immortalized podocytes carry a conditional T antigen. Therefore, a large bank of low passage stocks should be cryopreserved and individual aliquots should not be used for more than 10 to 15 passages. In addition, the responsiveness to INF-γ withdrawal under permissive conditions, the effective growth arrest after 14 days at 37°C as well as the expression of podocyte makers (Table 2) should be confirmed on a regular basis, that is for every fresh passage used. In addition, maintaining cells at high density can induce tumor-like transformation resulting in foci of cells with reduced contact inhibition or aberrant growth (Figure 3a). On replating, the transformed cells will initially

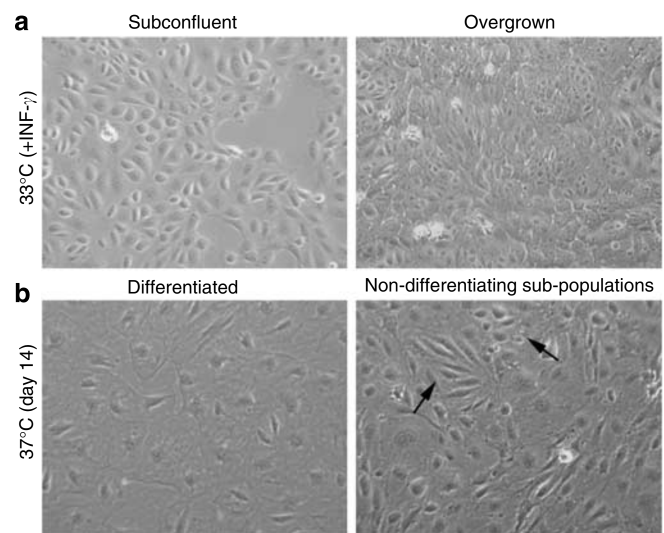

Figure 3 | Comparison of properly and nonproperly cultured podocytes. (**a**) Left panel: subconfluent proliferating podocytes with typical cobblestone morphology ready for subcultivation. Right panel: overgrown cells displaying dome formation and tumor-like irregular morphologies. These cells need to be discarded. (**b**) Left panel: homogenous population of well differentiated arborized podocytes after 14 days at 37°C. Right panel: in addition to differentiated podocytes, subpopulations of dividing, nondifferentiating cells can be found (arrows). The presence of such nondifferentiating cells can, among others, result from the replating of overgrown cells shown in (**a**) or from insufficient use of INF-γ under permissive conditions.

appear normal, but they have acquired a growth advantage and therefore eventually will ‘overtake and overrun’ the culture. Because of their growth advantage, these cells do not respond to the change in culture temperature or removal of INF-γ and appear as foci of proliferating cells in growth-restrictive conditions (Figure 3b). Once transformed populations are present, it is very difficult, if not impossible, to eliminate them. Thus, transformed cultures should therefore always be discarded.

ALTERING PODOCYTE GENE EXPRESSION IN CULTURE: APPLYING STATE OF THE ART MOLECULAR TOOLS

Gene expression and gene silencing are invaluable tools in understanding podocyte biology. However, like other post-mitotic cells such as neurons or cardiac myocytes, podocytes show relatively low transfection efficiency, typical ranging between 10 and 20 % for cells grown under permissive conditions. Over the past few years, an array of new transfection reagents have been introduced that allowed the successful podocyte transfection of podocytes, in particular of green fluorescent protein or otherwise tagged proteins to visualize transfected cells.[49,53,63,68] It is critical that one carefully quantitates transfection efficiency. A simple way to determine transfection efficiency is to transfect the cells with a green fluorescent protein vector, visualize the nuclei of all cells with 4,6-diamidino-2-phenylindole and determine the percentage of green fluorescent protein-expressing cells. Under optimized conditions,[49] we routinely achieve 10–20% transfection efficiency for proliferation cells grow at 33°C. Transient transfection dose not provide incorporation of DNA into the genome and is lost on mitosis. Owing to loss of expression in daughter cells following mitosis, transient transfection is not ideal for podocytes in growth-permissive conditions. However, transient transfection is simpler to perform and less time consuming than stable transfection or viral transduction.

Stable transfection has several advantages over transient transfection in that it does result in gene incorporation into the genome. This allows for expression to be transmitted to several generations of daughter cells before being lost. In addition, stable transfection utilizes cotransfection with a growth selection advantage, which results in cultures with virtually all cells expressing the transgene. One disadvantage of stable transfection is that because the gene must still be introduced to the cell and incorporated into the genome, efficiency is usually low, which results in a small number of cells surviving selection. Because of this, selection and expansion of stable transfectants is somewhat time-consuming, taking typically between 2 and 3 months for the generation of the stable cell lines. Nevertheless, we have successfully used this approach for the stable overexpression[53] or knockdown[49] of proteins in podocytes.

WHERE IS THE FIELD HEADING?

As with other postmitotic cells, viral transduction is emerging as the ideal method for the alteration of gene expression in podocytes in culture. Viral transduction is accomplished by transfecting an expression vector into a viral particles producing cell line. Viral particles are shed into the medium, which is transferred onto podocytes for infection. Viral transduction incorporates the advantages of stable transfection including the continuous expression and selection capacity with better introduction into the cell than transient transfection. In addition, viral particles containing media can be frozen for later use. Most significantly, viral transduction of cDNAs or shRNAs, for example through retroviral,[87] adonoviral,[72] or lentiviral vectors (PM, unpublished results), can also be achieved in growth-arrested, differentiated podocytes maintained under nonpermissive conditions. We are currently developing optimized protocols for the lentiviral infection of differentiated podocytes, both for protein overexpression and RNAi knockdown studies. Together with an updated detailed protocol for the cultivation of podocytes, we will make them available on the homepage of the Mundel Lab (www.mssm.edu/labs/mundel/), after they have been validated.

PITFALLS AND TROUBLESHOOTING: THE ACHILLES HEEL OF CELL CULTURE

It is critical that maticulate attention be paid to all details of podocyte culture to maximize the benefits of this model system and to prevent false-positive and false-negatives. Like any cell system, devoted care is paramount for consistency. An incomplete list of typical pitfalls with podocyte cultures is compiled in Table 4.

Pitfall no 1: Not enough care: similar to hippocampal neurons in cultures,[93] podocytes in culture require very careful handling, thus ensuring highly reproducible results with respect to differentiation and expression of podocyte-specific molecules. The cells are conditionally immortalized and thus very sensitive to minute changes in temperature as low as 1°C.

Pitfall no 2: Loss of sterile conditions: the cultures should always be maintained under sterile conditions and mycoplasma-free reagents must be used to prevent mycoplasma infection. Contamination with mycoplasma leads to decreased proliferation and the formation of long, thin, highly branched spine bearing processes, and should be tested for on a regular basis.

Table 4 | Typical pitfalls in culturing podocytes

Overcrowding of proliferating cells over the weekend causing second hit
Inadequate concentration of IFN-γ at 33°C
Impaired proliferation at 33°C owing to omission of type 1 collagen
Inadequate dispersion after splitting before replating
Loss of IFN-γ-specific activity owing to repeated freeze–thawing
Variable plating density at replating
Not long enough differentiated at 37°C
Presence of nondifferentiating subcolonies at 37°C
Incorrect incubator temperature, both at 33°C or 37°C
Mycoplasma infection
Too high number of passages

IFN, interferon.

Pitfall no 3: Loss of proliferative stimuli under permissive conditions: although type IV collagen is the major component of the glomerular basement membrane, under permissive conditions, the cultured podocytes grow faster and better on type I collagen and overall the cells appear more healthy. Type I collagen has to be coated to the cell-culture dish for at least 1 h before removal and rinsed twice with phosphate-buffered salines. Within 12 h of seeding under permissive conditions, mitotic cells should become obvious in most fields. The use of other matrices or plastic may be desired depending on the experimental protocol but changes in proliferation and differentiation have to be anticipated and accounted for in the interpretation of the data. Uneven cell growth may occur and confluent patches may develop if the cells are not thoroughly dispersed after splitting before replating. To preserve the activity of INF-γ, working aliquots should be kept frozen and thawed only once when ready for use. As a general rule, cultures should not be become more than 80–95% confluent to prevent irreversible loss of contact inhibition and multilayering (Figure 3a).

Pitfall no 4: (the biggest one of all) The 'Friday-to-Monday gap': overgrowing permissive cultures is one, if not the major, reasons for podocyte culture failure later on. This is a key point. In contrast to many other cells (e.g., mesangial cells, fibroblasts, human embryonic kidney cell line 293, Madin–Darby canine kidney cells, COS7 cells, etc), podocytes do not tolerate the typical 'Friday-to-Monday gap'. If podocytes overgrow during this or any time period, they will suffer a 'second hit-like insult' that will lead subsequently to a loss of differentiation. This insult is irreversible and cannot be rescued by simply replating the overgrown cells at a lower density, even if the cells appear morphologically normal after replating. Instead, they need to be discarded and replaced with a fresh aliquot. This is probably the single most frequent mistake that is made while culturing these podocyte cell lines, thereby causing the above-discussed claims (Table 1) including the loss of nephrin expression in culture. When the desired confluency is reached (80–95%), the podocytes need to be subcultured.

Pitfall no 5: Inconsistent replating/passaging: for the detachment of the cells for the purpose of replating/passaging cells, the culture medium is removed and the dish is rinsed with phosphate-buffered saline, followed by incubation with prewarmed fresh 0.05% trypsin/ethylenediaminetetraacetic acid solution (5ml per T75 flask). This is time sensitive (no more than 5–7min) to avoid cell death. While preparing the next passage of cells, T75 flasks precoated with type I collagen, should be inoculated with 150 000–200 000 cells at 37°C. Podocytes growing at 33°C can be inoculated as low as 50 000 cells per T75 flask because the cells proliferate well in the presence of 10–20 U/ml of IFN-γ. It is essential to allow the cells to become quiescent and differentiate for at least 14 days before they express the entire panel of marker proteins described above (Table 2). Finally, as a general rule it is also important to recognize that if on day 14 after thermoshift to 37°C and removal of IFN-γ, the cells continue to proliferate (Figure 2b) and do not express the podocyte-marker proteins listed in Table 3, they are most probably constitutively immortalized and should be discarded.

Table 5 | Currently available podocyte cell lines *in vitro* based on published literature (at the time of submitting the manuscript)

Podocyte cell line	Refs.
Wild-type mouse	Mundel *et al.*[43] and Schiwek *et al.*[46]
$\alpha3$ Integrin$^{-/-}$	Reiser *et al.*[53]
CD2AP$^{-/-}$	Schiffer *et al.*[73]
FGF2$^{-/-}$	Davidson *et al.*[58]
Cyclin I$^{-/-}$	Griffin *et al.*[71]
p21$^{-/-}$	Wada *et al.*[97] and Petermann *et al.*[98]
p27$^{-/-}$	Petermann *et al.*[98]
Synaptopodin$^{-/-}$	Asanuma *et al.*[49]
HIV transgenic	Schwartz *et al.*[89]
Wild-type human	Saleem *et al.*[99]
DDS mutant human	Viney *et al.*[100]

HIV, human immunodeficiency virus.

Table 6 | Proposed criteria required to classify a cell in culture as a podocyte

Feature	Details
Morphology	Arborized appearance Processes extend from cell body Large cytoplasmic to nuclear volume ratio Stress fiber formation
Protein expression	WT-1 Synaptopodin Nephrin Podocin CD2AP Ezrin VEGF production
Cell cycle	Absence of DNA synthesis (negative for Brdu, PCNA, cyclin E, and A; no cdk2 activity) Absence of mitosis (negative for cyclin B, no cdc2 activity; no mitotic figures) Expression of p27 and p57 Do not require cell–cell contact to exit cell cycle
Functional responses	Apoptosis induced by TGF-β, PAN Motile Processes induced by ATRA Dexamethasone induces shape changes and enhanced survival

ATRA, all-trans retinoic acid; PAN, puromycin aminonucleoside; PCNA, proliferating cell nuclear antigen; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

THE FUTURE: AN OUTLOOK

With the rapid and exciting pace of discovery of novel podocyte genes, several genetically altered mice have been generated. It therefore comes as little surprise that there is a growing number of wild-type and knockout murine as well as wild-type and genetically altered human podocyte lines available for cell-culture studies (Table 5). There are now several other new podocyte cell cultures available too. These include coculture studies of podocytes and glomerular endothelial cells in an attempt to better study permeability, growing podocytes in matrigel for a three-dimensional effect,

or growing podocytes that were isolated from the urine.[94] To hasten the process, we (SJS and JP) have recently developed a system whereby we utilize a biotinylated antibody against mouse podocytes that is linked to a magnetic bead. Following the incubation of the glomerular preparation to the antibody, a magnet is used to separate the podocytes from the other cells, thus circumventing the use of cell-specific cloning.

In summary, the availability of conditionally immortalized differentiated podocytes in culture has developed into an indispensable tool for the mechanistic analysis of podocyte function. In combination with animal models and human studies to confirm the *in vitro* results, the study of podocytes in culture is a valid and invaluable model system that will continue providing novel insight into the function of these unique cells in health and disease. Finally, what are the criteria that should be used to characterize a cell in culture as a podocyte? In keeping with well-defined criteria for other cells such as neurons and cardiomyocytes, we have proposed a set of strict criteria that is essential to define a cell *in vitro* as a true podocyte. These are described in Table 6.

In conclusion, we would like to indicate that requests for freely available 'quality-controlled' podocytes and requests for 'hands-on' introduction to podocyte cell culture in our laboratories are welcome and even encouraged. Enquiries may be addressed to PM (peter.mundel@mssm.edu), JR (jreiser@partners.org), or SJS (stuartjs@u.washington.edu).

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Appendix: A detailed protocol for the cultivation of conditionally immortalized podocytes

Morphology

Epithelial under permissive conditions.
Mesenchymal, process bearing under nonpermissive conditions.

Description

Conditionally immortalized mouse podocyte cell lines were established from the Immortomouse, which carries a thermosensitive (ts58A) variant of the SV 40 T antigen as a transgene. T-antigen expression can be stimulated by mouse IFN-γ. Proliferating cells express WT-1 and podocin. They differentiate within 14 days when grown under nonpermissive conditions in the absence of IFN-γ. Differentiated cells develop prominent processes and express synaptopodin.

Handling of frozen cells

If on arrival, continued storage of the frozen cells is necessary, the vials should be stored in liquid nitrogen and not at -80°C. Storage at -80°C will result in loss of viability.

Thawing of cells

1. Prepare a collagen-coated 75 cm^2 tissue culture flasks.
2. Thaw cells rapidly (within 2 min) in a 37°C water bath.
3. Spin down at 600 *g* for 5 min.
4. Resuspend pellet and transfer to flask.
5. Add 25 ml medium.
6. Add 50 U/ml IFN-γ (can be reduced to 10–20 U/ml later on).
7. Place the cells in an incubator set at 33°C.
8. Verify correct temperature of incubator with thermometer.

Medium renewal

1. Change medium every second day (we mean it!).
2. Avoid 'Friday-to-Monday gap'.
3. Always add IFN-γ when growing cells under permissive conditions.

Subcultivation

1. Never allow the cultures to become 100% confluent.
2. Use cells at 80–95 % confluence.
3. Remove and discard culture medium.
4. Briefly rinse the cell layer with phosphate-buffered saline.
5. Add 5 ml trypsin–ethylenediaminetetraacetic acid solution to flask.
6. Incubate at room temperature for 5–6 min.
7. Monitor detachment of cells under microscope.
8. Shake flask carefully to completely detach cells completely.
9. Put content in 50 ml Falcon tube (A).
10. Add 35 ml medium to inactivate trypsin to a total volume of 40 ml.
11. Transfer 16 ml from tube A in new Falcon tube (B).
12. Spin cells at 2300 r.p.m. (600 *g*) for 5 min, discard supernatant.
13. Respuspend pellet of tube B in 3 ml freezing medium.
14. Freeze two aliquots of 1.5 ml each.
15. Resuspend pellet from tube A in 4 ml medium.
16. Transfer 1 ml of cell suspension into each new 75-cm^2 flask.
17. Add 25 ml medium and IFN-γ.

The cells should be grown for the first two passages with 50 or 100 U/ml IFN-γ, then for two passages with 20 U/ml IFN-γ and finally with 10 U/ml IFN-γ for continuing culture

Thermoshift to 37°C. Always subculture cells before thermoshift.

Verify correct temperature of incubator with thermometer.

Plate a 1:10 to dilution from a 95% confluent layer.

Complete growth medium. RPMI 1640 medium with glutamine (Gibco).

10 % fetal bovine serum.

100 units/ml Pen/Strep (Gibco).

Cryoprotectant medium. Complete culture medium supplemented with 8% (v/v) dimethylsulfoxide.

Collagen coating.

1. All cell culture dishes should be coated with 0.1 mg/ml type I collagen.
2. Incubate for 1 h at 37°C with collagen solution.
3. Remove collagen solution.
4. Rinse once with phosphate-buffered saline.

References. Mundel P, Reiser J, Borja AZ, *et al.* Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp Cell Res* 1997; **236**: 248–258.

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