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Culture Methods of Glomerular Podocytes

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Abstract
Podocytes (glomerular visceral epithelial cells) cover the exterior surface of the glomerular capillaries and contribute to the glomerular filtration membrane. Failure of podocyte function is involved in the progression of chronic glomerular disease; accordingly, research interest into podocyte biology is driven by the need for better protection and perhaps recovery of these cells in renal diseases. This review aims at summarizing available techniques for podocyte cell cultures from both the past and present, with special attention to the currently used methods. The establishment of classical primary cultures is based on isolation of glomeruli by differential sieving. Plating of glomeruli onto a collagen surface is followed by an outgrowth of cobblestone-like cells that, after replating, differentiate into arborized, mature podocytes. Currently, the majority of research studies use immortalized podocytic cell lines most often derived from transgenic mice bearing a conditional immortalizing gene. The podocytes can also be collected and cultured from healthy or diseased animal or patient urine. The urinary podocytes obtained from subjects with active glomerulopathies display higher proliferation potential and viability in vitro, perhaps due to disease-induced transdifferentiation. Finally, a list of phenotypic markers useful for identification and characterization of the cultured podocytic elements is provided.

Introduction

Podocytes, also called glomerular visceral epithelial cells, are terminally differentiated, highly specialized cells with a unique location, architecture, and relevance. They cover the exterior surface of the basal membranes of the glomerular capillaries with multiple interdigitating foot-like processes [1]. The processes, with filtration slits in between, are the most prominent features of podocytic cells. The slit diaphragm plays a crucial role in establishment of the size- and charge-selective permeability of the glomerular filtration barrier, thus preventing proteinuria. The podocytes are often the prominent targets in many types of renal injury; failure of the podocyte function decisively contributes to the development of glomerulosclerosis, seen in the progression of many renal diseases [2].

Since the mid-90s, when initial groundbreaking reports on the possible involvement of podocytes in the progression of chronic glomerular disease appeared [e.g., 3, 4], these cells have become a favorite subject of research in experimental nephrology. As any other cell type, podocytes can be studied by a variety of in vivo and in vitro approaches. For the latter, cell culture technology is invaluable for studying the (patho)physiological properties of podocytes.

The last review article that was devoted solely to culture methods of podocytes appeared in 1996 [5]. Since that time, a plethora of new information and novel technological advances have been generated in this field. Our review aims at summarizing the techniques available for

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podocyte cultures from both the past and the present, with special attention paid to the more currently used methods. We discuss first the establishment of primary cultures and then the variety of immortalized podocytic cell lines. We also review the potential to cultivate podocytes from urine as a new and promising method for monitoring the activity of some glomerulopathies, such as idiopathic nephrotic syndrome, membranous nephropathy, lupus nephritis, and diabetic nephropathy [6].

Finally, we provide a list of phenotypic markers useful for proper identification and characterization of the cultured podocytic elements. This comprehensive review will help investigators choose a culture method best suited for a particular research goal and will perhaps also encourage much-needed research on podocyte biology in general.

Primary Cultures of Podocytes

Krakower and Greenspon [7] in 1954 were probably the first who described a method for isolation and primary culture of renal podocytes. However, it was not until the 70s and 80s that modifications of the described practice allowed the technique to become more widely used. The methods for culturing glomerular visceral epithelial cells, as well as other types of glomerular cells, represented a significant scientific advancement, as they allowed a more specific investigation into the role(s) of individual glomerular cell types in both renal physiology and renal pathology. Some of the experimental techniques developed during the 70s are still used today, albeit in modified form.

The majority of experimental procedures used for the preparation of podocytic cultures are based on the original work of Krakower and Greenspon [7], later modified by Burlington and Cronkite [8]. Briefly, under aseptic conditions, decapsulated rat cortical slices are pressed through a series of stainless steel sieves (sieving method) with decreasing pore sizes of 250 and 150 μm; as a final step, the glomeruli are then collected on a 75-μm sieve. The original procedure described by Krakower and Greenspon [7] used only two screens of 250 and 150 μm. Glomeruli from other species can be harvested using different size combinations of screens [9]. For example, for human glomeruli, Striker et al. [10] used a series of sieves of 190, 140, 120, and 104 μm (retention). With the sieving technique, it has been reported that, for the rat kidney, about 86% of the glomeruli were not encapsulated [11].

After collection, the glomeruli can be plated either directly onto a culture dish for an outgrowth of cells or subjected to enzymatic dissociation. There are several protocols for the digestion of glomeruli. Striker et al. [10] treated human glomeruli with collagenase (750 U/ml) at 37°C for 10–60 min. Harper et al. [12] digested rat glomeruli with 0.2% trypsin for 20 min, followed by an incubation in 0.1% collagenase for 40 min at 37°C. Kreisberg et al. [11] incubated rat glomeruli in Hanks' balanced salt solution containing collagenase (0.1%), trypsin (0.2%), and deoxyribonuclease (0.01%) at 37°C for 20 min. Enzymatic dissociation is currently not the preferred method, however, as it is difficult to prevent overdigestion of glomeruli, which may then impair cell viability and lead to the liberation of mesangial cells, thus resulting in a mixed cell population in vitro. Enzymatic dissociation of the entire glomeruli can be justified in some cases, e.g., if coupled to cloning of the particular cell types; the efficiency of this approach, however, is rather low.

Nonenzymatic Preparation of Glomeruli

Currently, glomeruli needed for the establishment of both primary and conditionally immortalized podocytic cultures are typically harvested without any additional enzymatic treatment, as described originally by Quigg et al. [13] and later modified by Johnson et al. [14]. The isolated glomeruli are first seeded onto commercially available bovine dermal collagen matrix (Vitrogen; Collagen Corp., Palo Alto, Calif., USA) plates and cultured in K1-3T3 media. Colony outgrowths of glomerular epithelial cells are replated onto a collagen type I surface and subsequently maintained in K1 media with NuSerum; alternatively, serum-free medium consisting of either K1 medium or CS-2 medium with supplements (epidermal growth factor, hydrocortisone, and nonessential amino acids) can also be used. The glomerular epithelial cells, putatively the podocytes, appear polygonal in shape under a phase-contrast microscope; they can be passaged several times, as long as they continue to proliferate. When grown on type I collagen, they appear slightly larger than when cultured on an uncoated plastic surface. Using immunofluorescence staining, cultured cells are negative for factor VIII and Thy 1.1, but positive for Fx1A, cytokeratin, and podocalyxin.

The sieving technique is believed to remove the parietal sheet of glomerular cells, thus exposing the visceral glomerular cells (podocytes) and allowing their outgrowth. However, some authors have questioned the homogeneity of the glomerular cell cultures prepared in this classical way. For instance, Weinstein et al. [15] isolated

glomeruli from young 50-gram rats by differential sieving (250, 105, and 62 μm); the glomeruli were then maintained on collagen in a 1:1 mixture of K1 media containing 5% NuSerum and conditioned media from Swiss mouse 3T3 fibroblasts containing 10% fetal bovine serum (FBS). From 5 to 15% of all harvested glomeruli were encapsulated, but independently of the presence of Bowman's capsule, the vast majority of the cell outgrowth displayed polygonal cobblestone morphology and expressed known in vivo markers for parietal cells (positive for cytokeratin, negative for vimentin and desmin). However, a few cells appeared larger, were irregularly shaped, and they did not proliferate. If, alternatively, the kidney was perfused with collagenase before the isolation of glomeruli, no cell outgrowth could be obtained [15].

In an attempt to avoid podocyte damage during the sieving process, Yaoita et al. [16] harvested glomeruli using only two screens, with pore sizes of 250 and 75 μm. This method was performed without any mechanical pressure on the glomeruli. Decapsulated and encapsulated glomeruli were manually separated under a microscope and then cultured separately on collagen type I in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 5% FBS. The authors observed a higher rate of cell outgrowth from the decapsulated glomeruli (mean 25% of them) as compared with isolation by conventional sieving methods described previously [14]. They also described different types of cells dependent on whether or not they were harvested from decapsulated or encapsulated glomeruli. Decapsulated glomeruli generated large arborized cells with a marker profile typical of podocytes in vivo (positive for WT-1, synaptopodin, and podocalyxin). On the other hand, cells from encapsulated glomeruli fell morphologicaly into two distinct cellular types: polygonal cobblestone-like cells localized in the colony centers and large irregularly shaped cells occupying the colony peripheries. Using immunostaining, the cobblestones stained positive for WT-1, but the stain was weaker than that for the arborized cells; they were also faintly positive for synaptopodin, yet no podocalyxin was detected. The large irregularly shaped cells were very weakly positive for synaptopodin and negative for podocalyxin. It has been suggested then that the cells growing out from the decapsulated glomeruli are podocytes, while the polygonal cells from encapsulated glomeruli are derived from parietal epithelial cells, and part of them (the large irregularly shaped elements) modify their phenotype to resemble podocytes in vitro.

In their landmark study, Mundel et al. [17] modified standard culture conditions so that remarkable differentiation of podocytes was achieved in the first subculture and that further passages could be avoided. They observed a conversion of cobblestones (undifferentiated and proliferating podocytes) into arborized cells that more closely resembled podocytes in vivo; moreover, some intermediate phenotypes were documented. In the procedure of Mundel et al. [17], primary cellular outgrowth (fig. 1a) is separated from the glomerular cores with 25-μm sieves in the first passage; the cells are then allowed to differentiate on type I collagen. Within 2–3 weeks, the subcultured cobblestones transform into individual arborized cells (fig. 1b). The podocyte origin of both cell types was confirmed by positive staining for WT-1, as well as podocyte-specific O-acetylated ganglioside; furthermore, the arborized cells expressed synaptopodin (formerly pp44), a prominent marker of differentiated podocytes. The process of conversion was independent of most culture condition variables (e.g., type of culture medium, serum contents in medium, type of coating). Only a high plating density delayed the conversion, but failed to prevent it.

The work of Mundel et al. [17] showed for the first time that podocytes can effectively differentiate in vitro. In addition, this study has brought to light evidence that podocytic cells can appear in culture in two different forms and that there is a direct conversion of dedifferentiated cobblestone cells through intermediate phenotypes into the arborized cells of a more advanced differentiation state (but not terminally differentiated because of lack of podocalyxin and a '51-kDa slit-membrane-associated protein'). It is with this point that the work by Mundel et al. [17] disagrees with the findings published by Weinstein et al. [15] and Yaoita et al. [16] discussed above. Finally, according to Mundel et al. [17], the conversion to arborized cells is inducible by avoiding subcultivation of the cobblestones. This observation is useful, as it facilitates preparation of mature podocytic cultures for in vitro experiments.

Primary Cultures of Human Fetal Podocytes

Primary cultures of human fetal podocytes have also been derived [18]. Using the differential sieving technique (149- and 70-μm sieves), glomeruli were harvested from fetal kidneys at 8–18 weeks of gestation (from therapeutic abortions). In order to obtain glomerular outgrowths, the glomeruli were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS). After the first passage, the cells were cultured in medium with the FCS content decreased to 1% for 14 days, with the aim to promote exit of the cells

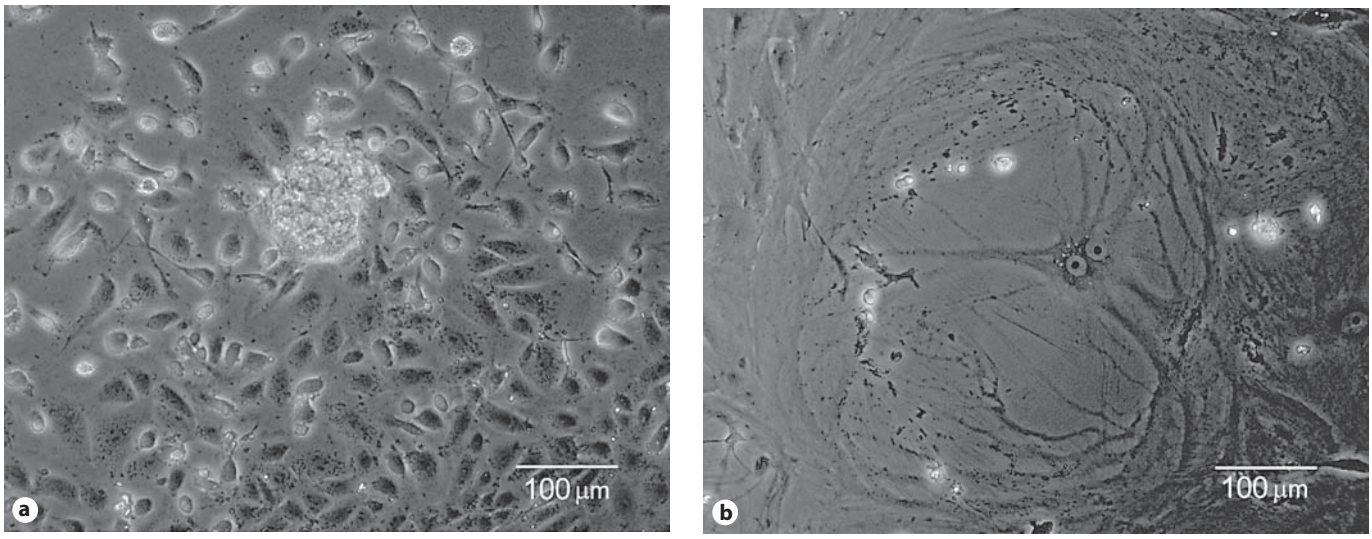

Fig. 1. Primary podocyte cultures prepared according to Mundel et al. [17] in various stages of development in vitro. **a** Cellular outgrowth from a decapsulated glomerulus 5 days in vitro after plating onto collagen type I in RPMI 1640 plus 10% FCS. Mostly undifferentiated cobblestone-like cells are seen. ×200. **b** Fully differentiated arborized podocytes on day 10 of the first subculture under identical culture conditions. ×200.

from the cell cycle and differentiation. After this period, the cellular phenotype was confirmed, and the cells were further subcultured and then used for experiments with the second to the fifth passage. After the sixth passage, some phenotypic changes and limited viability were observed. Although the cells displayed positive phenotypic markers of mature podocytes, such as WT-1, synaptopodin, C3b complement receptor-1, and, in some recent work [19], nephrin, it is in our opinion that their differentiation state should be considered with caution, because the cells were derived from highly immature fetal kidneys and were kept in a proliferating state through several subcultures.

The same isolation methods for glomeruli, used for the establishment of primary podocytic cultures, also serve to prepare glomeruli as a source of immortalized podocytic cell lines, as discussed below.

Immortalized Podocytes

The majority of terminally differentiated cells display only restricted proliferation capability; initially, only tumor cells or spontaneously transformed, immortalized nontumor cells could give rise to continuous cell lines. In order to provide the in vitro cultured cells with an ability to divide indefinitely, immortalized cell lines have been established from various tissues [e.g., 20–22]. Several methods for the production of continuously (constitutively) immortalized cells have been developed. One of them is based on the insertion of an immortalizing gene encoding the simian virus 40 (SV40) large tumor antigen (TAg) into in vitro cultured cells by transfection or retroviral infection. Alternatively, the cells for culture are isolated from transgenic mice harboring the SV40 TAg immortalizing gene.

The main concern with the cells continuously expressing an immortalizing gene is that the cellular physiology is altered due to continuous proliferation [23–25]. Moreover, particular methods have other disadvantages. In order to obtain sufficient amounts of transfected cells, a large number of cells for treatment is required; in the viral-mediated infection, only dividing cells are sensitive to gene incorporation. Also, for this type of an approach, the immortalizing gene is distributed randomly in cell populations. Another concern is that transgenic mice bearing an immortalizing gene appear to suffer from tumors and aberrant development [25]. Therefore, in order to overcome these problems, conditional immortalizing genes are inserted into cultured cells to switch from the proliferation stage to differentiation following inactivation of the immortalizing gene or its product. For example, the SV40 temperature-sensitive mutant strain tsA58 encoding a thermolabile TAg that is capable to immortalize only

at the permissive temperature has been used in many cell lines [e.g., 26–28]. However, this method did not remove the general difficulties associated with generating cell lines by means of transfection or infection. A more advanced approach has been developed using transgenic mice harboring a 'double' conditional immortalizing gene. The animals were generated by the insertion of a hybrid construct consisting of the mouse major histocompatibility complex *H-2K*^{b} class I promoter, induced by interferons, and the tsA58 early region encoding TAg [29, 30]. Cells isolated from this transgenic mouse, or 'immortomouse', are allowed to multiply only under a permissive temperature and the simultaneous presence of interferon gamma (IFN-γ), whereas a nonpermissive temperature without any IFN-γ shifts the cells to differentiation.

Exposure to IFN-γ induces activation of the *H-2K*^{b} promoter, driving expression of the immortalizing protein tsA58 TAg which remains stable and is functional at the permissive temperature (33°C). If IFN-γ is removed and the cells are placed at a nonpermissive temperature (37–39.5°C), the tsA58 TAg protein synthesis stops, the previously expressed tsA58 TAg is rapidly degraded, and the cells shift from proliferation to differentiation [26, 30, 31]. The cells generated by this method are genetically homogeneous, can be harvested in large numbers, and can terminally differentiate [25]. However, they are still not identical to native cells, because they carry an immortalization gene that is abnormally expressed during cell propagation, and the prolonged effects of this abnormal expression on the cell physiology are not known [32].

As primary cultured podocytes derived from rat or human kidney display partial in vitro differentiation associated with growth arrest, there has been the specific aim among podocyte researchers to create a podocyte cell line retaining the potential for both proliferation and differentiation. The first long-term cultured immortalized cell line of glomerular epithelial cells – putative podocytes – was established together with lines of mesangial and glomerular endothelial cells from mice transgenic for the early region of SV40 (encoding the TAg) [33]. Major problems were associated with this line, as well as other irreversibly immortalized podocyte cell lines derived later [34, 35], where the cells would proliferate but without any significant tendency to differentiate.

More recently, in their cornerstone work, Mundel et al. [36] described a generation of conditionally immortalized murine podocyte lines. From kidneys of adult (10-week-old) transgenic *H-2K*^{b}-tsA58 mice created by Jat et al. [25], the glomeruli were isolated by the standard three-step sieving method and grown in RPMI 1640 medium with 10% FCS. The outgrowths of the cells were separated from the remaining glomerular cores using a sieve with a pore size of 33 μm; the cells were then further propagated on collagen type I at 33°C in the same medium with the addition of 100 U/ml IFN-γ (generating permissive conditions). To induce differentiation, the cells were shifted to 37°C and maintained in the medium without IFN-γ (nonpermissive conditions). Under the nonpermissive conditions, most of the cells became growth arrested within 6–14 days. The podocyte phenotype was confirmed by positive WT-1 staining in both proliferating and differentiating cells; the differentiated cells were also positive for synaptopodin. The IFN-γ concentration could be gradually reduced to 10 U/ml without noticeable effects on cellular proliferation or morphology. Spontaneous transformation of cells was occasionally observed in cultures exceeding 30 passages. As a standard nonpermissive temperature, Mundel et al. [36] chose 37°C, because culturing at 39°C, originally used by Jat et al. [25], resulted in a higher rate of cell death. Interestingly, the growth arrest was reversible, if the cells were returned back to permissive conditions.

The immortalized podocytes used in recent studies are usually derived from murine or human kidney tissue. Most authors have established murine podocyte lines as described by Mundel et al. [36] in the above-mentioned work, with some small modifications. The murine immortalized podocytes are exclusively derived from transgenic animals (the 'immortomouse') rather than by ex vivo transfection or infection. In these studies, both proliferating and differentiating podocytes were maintained in RPMI 1640 medium supplemented with 10% FCS. In order to propagate the cells, a temperature of 33°C and the presence of IFN-γ in the culture medium (permissive conditions) are required. IFN-γ is typically added at a concentration of 10 U/ml, yet Schiffer et al. [37] and Wada et al. [38] used concentrations as high as 20 and 50 U/ml, respectively, without any reference to an effect on cell division. Cell differentiation was then induced by the omission of IFN-γ from the complete medium and by raising the temperature to 37–39°C. The cells were allowed to mature on collagen type I for 7–14 days, most often for at least 10–14 days [e.g., 38–41].

It is unclear whether an IFN-γ concentration >10 U/ml is beneficial, as Mundel et al. [36] showed that there is no increase in cell proliferation at higher concentrations. Further potential controversy exists about what should be recommended as the nonpermissive temperature. As mentioned above, some investigators use 38°C or 39°C instead of the standard accepted 37°C, with an

aim to halt proliferation and enhance differentiation. The higher temperature matches the body temperature of the mouse (38–39°C). However, as also suggested by Mundel et al. [36], increasing the nonpermissive temperature to 39°C increases cell death. Thus, it appears that the influence of a higher temperature on the survival of differentiating cells has yet to be rigorously tested. More detailed examination of the dependence of cell maturation status on the length of culturing under nonpermissive conditions would be also quite useful, as this sort of information seems to be lacking in the available literature.

Attachment Factors

Another important aspect in the propagation and differentiation of immortalized podocytes represents the treatment of the culture dish surface with attachment factors. In principle, most investigators have maintained podocytes in culture dishes coated with collagen type I, simply because of its commercial availability. However, the main structural protein of the glomerular basement membrane is collagen type IV [42].

Cybulsky et al. [43] investigated the effects of various extracellular matrices, including collagens I and IV, on both adhesion and proliferation of extensively subcultured (spontaneously immortalized?) rat glomerular epithelial cells. No significant difference in cell adhesion to various substrates was found. Collagen type I in the form of a gel supported the cell proliferation most effectively. Thin films of both collagen type I and collagen type IV in equivalent amounts were also able to support proliferation, to a similar extent.

Mundel et al. [17] also tested the influence of the provided substrate (type I and IV collagens, uncoated plastic, and positively charged Falcon Primaria dishes) on growth and differentiation of primary cultured rat and human podocytes. The choice of the plating substrate had no qualitative influence on the phenotypic conversion of primary cultured podocytes from cobblestones into arborized cells. Bijian et al. [44] documented that both collagen type I and collagen type IV serve as attachment factors for primary cultured rat podocytes more effectively than laminin or uncoated plastic and also enhance cell survival after puromycin-induced damage.

Currently, a comprehensive comparison of the influence of type I and IV (canine NC1 hexamers) collagens on podocytes and bone marrow stromal cell (BMSC) differentiation has been documented by Perry et al. [42]. Murine conditionally immortalized podocytes differentiated into cells with a similar appearance regardless of the collagen matrix used. Of the podocyte-specific proteins, WT-1 and myosin IIA were expressed in both undifferentiated and differentiated cells, while synaptopodin, actin, and α-actinin were expressed in differentiated cells only, all of them regardless of the collagen matrix used. However, CD2AP staining and its pattern were dependent on the type of matrix: while on collagen type I, only mature podocytes expressed CD2AP, on type IV collagen, both undifferentiated and differentiated podocytes were positive for CD2AP, but the positivity changed from diffuse cytoplasmic to cell peripheral localization which better corresponds to an in vivo association with the slit diaphragm complex. Surprisingly, no cells on any matrix synthesized podocin.

This same study [42] also showed canine BMSCs differentiating into podocyte-like cells when cultured on type I and type IV collagens. These cells expressed the podocyte-specific proteins such as WT-1, synaptopodin, actin, α-actinin, and myosin IIA on both matrices, but the number of cells expressing these proteins was much higher on type IV collagen as compared with collagen type I. Furthermore, the expression of CD2AP in the BMSC-derived podocytes was localized to the cell periphery on collagen type IV, but was localized as weak and diffuse cytoplasmic staining on collagen type I. As in podocytes, no BMSC-derived cells were indicative of podocin. The BMSCs cultured on uncoated plastic tended to resemble undifferentiated podocyte cell lines.

Taken together, accumulated evidence suggests that although collagen type IV should be a more physiological attachment substrate than collagen type I, there is practically little difference between these two substrates in terms of their effects on podocyte adhesion, differentiation, and growth in vitro. On the other hand, collagen type IV can enhance the differentiation of mesenchymal precursors into podocytes much more so than collagen type I.

Immortalized Podocyte Models Mimicking Dynamic Environment in Renal Glomeruli

Podocytes in vivo are exposed to mechanical forces, arising from glomerular capillary pressure and glomerular filtration [45]. In order to imitate physiological and pathophysiological intraglomerular conditions, immortalized mouse podocytes have recently been tested in conjunction with various mechanical and fluid shear stress models [46–48]. In order to create a stretch stress model [46], differentiated conditionally immortalized mouse podocytes, prepared as reported by Mundel et al. [36], were seeded onto flexible silicone membranes coated with collagen type I and covered with culture medium.

After cell attachment, the membrane was mounted on a Plexiglas manifold connected to a custom-built apparatus that produced cyclic pressure variations. The pressure variations caused upward and downward motion of the silicone membranes that transmitted biaxial cyclic mechanical stress to podocytes.

Fluid shear stress experiments can also be performed using a model described by Friedrich et al. [48]. Before an experiment, the conditionally immortalized differentiated murine podocytes were allowed to seed onto collagen type IV-coated glass coverslips and kept for 3–5 days until they formed a confluent monolayer. Thereafter, the coverslips were fixed in a flow chamber, where an appropriate flow of culture media was generated by a connected pump. All these experiments with the flow chamber were performed in the incubator at 37°C.

Human Immortalized Podocytes

In contrast to the amount of work on murine podocytic lines, a smaller number of studies have appeared to date that used immortalized podocytes of human origin. One of the first human podocyte lines that has been established is from a 1-month-old baby kidney, described by Delarue et al. [34]. The glomerular visceral epithelial cells were obtained by collagenase digestion of glomeruli isolated from a normal human baby kidney (judged to be unsuitable for transplantation); subsequently, the cells were transfected in vitro. As the transforming (immortalizing) genes, both the oncogenes T-SV40 and Ha-ras were introduced into the cells by the calcium phosphate procedure [49]. Immortalized cells were maintained in 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium supplemented with dexamethasone, insulin, transferrin, sodium selenate, and 1% FCS at a temperature of 37°C. Incorporation and expression of both transforming genes were then tested. Only T-SV40 was detected at the protein level; in contrast, Ha-ras failed to be transcribed, although it was integrated into the cell genome. As compared with parental nontransfected cells, the established cell line, marked as 56/10A1, displayed some variations in morphology (smaller cells with leaky junctions and large intercellular spaces). However, the majority of these features were also reported in podocyte lines derived from SV40 transgenic mice [33]. Despite some morphological abnormalities and enhanced proliferation capability, the immortalized cells expressed several determinants currently considered specific for differentiated glomerular visceral epithelial cells, i.e., PHM5, CALLA (common acute lymphoblastic leukemia antigen), and cytokeratin [50, 51], and retained the phenotype for over 50 successive passages. However, cytokeratin was expressed with no evident pattern of filaments, in contrast to differentiated epithelial cells. Importantly, in order to confirm the podocyte phenotype, some later published studies reported expression of WT-1, the main universal podocyte marker, in this cell line [52]. This specific cell line retains the ability to proliferate due to the presence of the T-SV40 gene and/or its origin from a 1-month-old kidney, and, simultaneously, its potential to differentiate seems to be somewhat improved, although still limited. Perhaps the presence of Ha-ras in the genome, although not expressed, can positively influence differentiation, as reported by Amsterdam et al. [53] for similarly transformed ovarian granulosa cells.

Another conditionally immortalized human podocyte cell line, created by Saleem et al. [54], is more widely used. Unlike the establishment of the cell line 56/10A1 described above, in this study the glomeruli were harvested from a nephrectomy specimen of a 3-year-old child, taken due to a unilateral antenatal obstructive/reflux nephropathy. The cultures of primary human podocytes were infected by a retrovirus carrying a construct consisting of a SV40 TAg gene containing both the tsA58 and U19 mutations. In a manner similar to the murine cell lines, the cells were propagated at 33°C, and their differentiation was induced by maintenance on collagen type I at the nonpermissive temperature of 37°C for 7–14 days. Throughout this period, the podocyte-specific markers WT-1, synaptopodin, and nephrin were detected in the cultured cells [54, 55].

During 2005 alone, approximately twenty-seven studies were published, where immortalized podocyte cell lines were used, in comparison to merely one paper that was published on the use of primary podocyte cultures and yet one more paper, where the authors used both the immortalized murine podocyte cell line as well as murine and rat primary cultures [56]. In contrast, in 1997, two papers with experiments on primary cultured podocytes and one paper describing the use of an immortalized cell line were published. Studies using immortalized podocyte cultures appear to prevail nowadays. Work with these lines is certainly more convenient as compared with primary culture experiments. However, one should always keep in mind that these cells in general represent an experimental model with a high degree of artificiality, as they have been derived by means of unnatural gene insertion into the cellular genome. Accordingly, the experimental data obtained with these cells should be interpreted with a certain caution and, at best, should be reproduced in a different model.

Podocyte Cultures from Urine

In the urinary sediment from some patients with kidney disease, Pascual et al. [57] and Hara et al. [58] detected elements positive for the C3b complement receptor 1 and podocalyxin, respectively. This suggests that some podocyte fragments or complete podocyte bodies can be shed into urine, although both markers are not fully specific for podocytes. In further studies, other authors [59, 60] have confirmed the presence of podocalyxin-positive cells in the urine in a variety of other experimental and human glomerular diseases. This is in line with the idea that during certain glomerulopathies injuried podocytes can detach from the glomerular basement membrane and be released into the urine. However, there was a lack of direct evidence that the podocalyxin-positive cells were genuine podocytes. Moreover, the viability of these cells was not tested. Vogelmann et al. [61] were the first who succesfully cultured human urinary cells that were identified as podocytes. Freshly voided urine, both from patients with glomerulopathies and healthy control subjects, was repeatedly centrifuged and the pellet resuspended in sterile human diploid fibroblast solution; finally, the pellet was resuspended in DMEM/Ham's F-12 with 10% FBS supplemented with antibiotics. The cells were routinely plated on plastic, because it was found that various attachment factors such as collagen, fibronectin, and laminin had no apparent influence on cell growth. This is somewhat surprising, because these attachment factors, collagen in particular, are considered important stimuli for proper adhesion, growth, and survival of the nonurinary podocytes [e.g., 43, 44, but see 17]. The results of Vogelmann et al. [61] showed that podocytes exist in urine, independently of whether the urine was obtained from healthy subjects or from patients with both active or inactive glomerulopathies; all cells were viable and able to grow in vitro.

The growth pattern of urinary podocytes seems to be very similar to that of primary cells migrating out from explanted glomeruli or to that of immortalized human podocyte lines, as within a few days, colonies made of cobblestone-like cells appear. After 1–2 weeks of growth, many of the cells underwent apoptosis, while the remaining elements differentiated, as indicated by the development of cell processes. Interestingly, the urinary cells from patients with active glomerular diseases showed rapid proliferation, until a subpopulation of them underwent apoptosis, while the rest of them developed processes. After several trypsinization treatments and replating, they again grew to confluence, indicating a high proliferation potential. In contrast, the urinary cultures from both healthy persons and subjects with inactive glomerulopathies displayed smaller cell colonies; also the cells proliferated shorter, died sooner in culture, and did not survive replating. Vogelmann et al. [61] speculated that the podocytes from controls or from patients with inactive diseases, unlike those from patients with active glomerulopathies, are shed because they are senescent, resulting in the limited proliferation potential. Another idea we can offer, inspired by some alternate in vivo studies, is that podocytes in active glomerular disease undergo dedifferentiation in response to some intraglomerular damaging factors which can give them a higher proliferation capability. As far as the identification of the cells cultured by Vogelmann et al. [61] is considered, these cells expressed some podocyte-specific markers such as podocalyxin, podocin, WT-1, synaptopodin, and P-cadherin. However, there was no evidence for GLEPP-1, another podocytic marker. Interestingly, the nuclear-localized universal podocytic marker WT-1 tended to disappear within a few weeks which is never seen with nonurinary podocytes. Some cells positive for podocytic markers (WT-1 and podocin) coexpressed markers of other cellular types such as cytokeratin-8 (tubular epithelial cells) or α-smooth muscle actin (myofibroblasts, mesangial cells) which may indeed suggest dedifferentiation and/or transdifferentiation of cultured podocytes.

Petermann et al. [62, 63] and Yu et al. [64] studied rat urinary podocytes in culture. In addition to healthy control rats, they used rats with passive Heymann nephritis (model of experimental membranous nephropathy) [62], streptozotocin-induced diabetic nephropathy [63], as well as puromycin-aminonucleoside-induced nephrosis (model of minimal-change nephropathy), anti-Thy 1.1 nephritis (model of mesangioproliferative nephritis), and five-sixths nephrectomy (model of hypertensive progressive renal damage) [all in 64]. In these studies, rat urine was collected for 12 or 24 h or obtained by means of bladder puncture, following diuresis forced by intravenous injection of saline. The urine samples were partially neutralized by combining with Hanks' balanced salt solution; the cells were collected by centrifugation, resuspended in the culture medium (DMEM, 10% FBS supplemented with antibiotics), and plated onto a collagen type I substrate. The adherent cells were subsequently grown in the same culture medium, but containing 5% FBS. While no viable podocytes were found in the urine from healthy control rats, in the urine

of rats with passive Heymann nephritis, viable cells were present that proliferated ex vivo, until a subset of them underwent apoptosis after day 4 in culture. Such an apoptotic crisis of podocytic cultures is consistent with the observation of Vogelmann et al. [61] on human urinary podocytes.

Petermann et al. [62] also attempted to confirm the expression of both mRNA and protein for the podocyte-specific markers. The adherent cells strongly expressed nephrin, podocin, and also GLEPP-1 that was not expressed in human urine podocytes [61]. Surprisingly, no mRNA for nephrin was detected. Synaptopodin showed only weak positivity, and no WT-1 protein was expressed, although WT-1 mRNA was present in the cells; these findings correspond to the results obtained by Vogelmann et al. [61]. The weak or missing expression of these proteins controlling and/or indicating the differentiation status of podocytes can perhaps be explained by certain dedifferentiation associated with proliferation of podocytes. No coexpression of the podocyte markers with any markers of other cell types (sign of transdifferentiation) was detected. Another interesting piece of information presented in the study of Petermann et al. [62] is that the results were the same independently of whether either fresh or several-hour-old urine was used. This is indicative that podocytic elements are not damaged by a prolonged stay in the urine. Further studies done by Petermann et al. [63] and Yu et al. [64] using other models of experimental rat nephropathies yielded identical or very similar results.

Importantly, detection of podocytes in the urine is suggested by numerous studies [reviewed in 65] as a promising marker of the activity of glomerular diseases. Obviously, for routine detection of urinary podocytes, techniques like immunocytochemistry or fluorescence-activated cell sorting of the urinary sediment would be more suitable than cultures of urinary podocytes which, in turn, are more eligible for basic research.

Identification of Cultured Podocytes

The proper identification of cultured cells and confirmation of their phenotype nowadays represent an integral part of any in vitro cell culture technique. Originally, the main and single criterion for identification of cultured podocytic cells was the cellular morphology, as seen under the phase-contrast microscope. From the current point of view, identification based on cellular morphology is quite uncertain, since the observed shape of both primary cultured cells and immortalized cell lines depends on many variables, including culture conditions; additionally, the cellular form can grossly differ from that observed in vivo. Currently, morphology is only a subsidiary criterion for cell identification. It is nevertheless generally accepted that the small, polygonal cells with a cobblestone-like appearance are dedifferentiated proliferating podocytes, while the large, branched, and often binucleated arborized cells are mature podocytes [17]. It should be noted here that many studies in the past were actually performed on immature podocytes.

Another obsolete method for phenotype confirmation of cultured podocytes was based on a selectively toxic effect of puromycin aminonucleoside on this cellular type [reviewed in 51]. However, results of this selection were rather inconsistent; additionally, the positively marked cells – podocytes – could not be utilized for further experiments, because they were damaged by puromycin.

In current research practice, cultured cells are most properly identified by means of immunochemical detection (immunocytochemistry, immunofluorescence) of a protein that should be expressed exclusively by podocytes. The choice of proteins as podocyte markers has been somewhat changing over time, as it was found that some of these markers are not specific for podocytes, or their expression was found variable. For example, in the past, the C3b complement receptor-1 [51, 66] and the common acute lymphoblastic leukemia antigen [67] were used widely as podocytic markers. However, for both proteins, some discrepancies between the expression in vivo and that in vitro have been found, and reports on their expression in cultured podocytes have been rather contradictory [68]. For these reasons, they have been used with decreasing frequency.

The proteins most often used as podocytic markers in current research efforts are summarized in table 1 [69–96]. WT-1 is considered a universal marker for both undifferentiated and differentiated podocytes; however, it should be noted that the protein has been also found in parietal epithelial cells [97] and parietal podocytes [72] in vivo. In addition, some researchers use yet other molecules for identification of both podocyte types, such as a cell membrane localized O-acetyl GD3 ganglioside [98] or the secreted vascular permeability factor/vascular endothelial growth factor [99].

In order to confirm the podocyte phenotype, the majority of working groups use a palette of three to five podocyte-specific markers. It is our opinion that the op-

Table 1. Summary of the recently most often used markers of podocytic phenotype in vitro

Marker	Characterization	Localization in cultured podocytes	Presence in other cells/organs	Function in podocyte/ glomerulus/kidney	Ref.
Wilms' tumor protein (WT-1)	52–65 kDa, four zinc finger transcription factor	*Immature and mature podocytes* Nuclear localization	Parietal podocytes in vivo Ovaries, testes (developing, mature) Bone marrow cells Neurons	Regulation of nephrogenesis Induction and maintenance of mesenchymal-epithelial transition during glomerulogenesis Maintenance of podocyte mature phenotype	69–72
Glomerular epithelial protein-1 (GLEPP-1)	200–205 kDa, receptor-like membrane protein, tyrosine phosphatase	*Mature podocytes* Apical membrane, especially the surface of foot processes	Neurons in developing brain – mRNA detected only	Regulation of structure and function of foot processes	73–76
Podocalyxin (also called PHM-5 antigen)	140 kDa, membrane sialoprotein	*Mature podocytes* Apical membrane	Glomerular endothelium Vascular endothelium Erythrocytes Platelets Megakaryocytes	Role in maintaining podocyte separation through charge repulsion	77–81
Synaptopodin (also called pp 44)	100, 110 kDa, proline-rich actin-associated protein	*Mature podocytes* Cytoskeleton in major and foot processes	Neurons	Role in maintenance of actin-based shape Role in maintenance and motility of foot processes	82, 83
α-Actinin-4	100 kDa, member of the superfamily of actin-binding proteins	*Mature podocytes* Cytoskeleton in foot processes	Renal capillaries Blood vessels Uterus Colon Pancreas	Formation of foot processes Stabilization of cell adhesion Role in cell motility	84–87
Nephrin (also called 5-1-6 antigen)	180 kDa, transmembrane protein of the immunoglobulin superfamily of cell adhesion molecules	*Immature and mature podocytes* Slit diaphragm	Brain Pancreas	Maintenance of structure of foot processes and slit diaphragm Interaction with podocin and CD2AP	54, 88–91
Podocin	42 kDa, transmembrane protein of the immunoglobulin superfamily	*Mature podocytes* Slit diaphragm	No	Stabilization of slit diaphragm Interaction with nephrin and CD2AP	54, 92–94
CD2-associated protein (CD2AP)	80 kDa, transmembrane protein of the immunoglobulin superfamily	*Mature podocytes* Slit diaphragm	Renal tubular cells Cells of collecting ducts	Formation and stabilization of slit diaphragm Role as connector protein between nephrin, podocin, and cytoskeleton	95, 96

Table 2. Summary of benefits and limits of each type of culture method

	Primary cultures	Immortalized cell lines	Urine podocytes
Benefits	Natural genome more compatible with in situ cells	Continuous maintenance of cells due to unlimited proliferation Controlled switch to differentiation	Cells damaged in situ by appropriate pathogenetic factors
Limits	Limited proliferation of cells Time- and number-restricted maintenance of cultured cells	Insertion of unnatural immortalizing gene into cell genome may cause phenotype alterations	Yielded as artificially dedifferentiated/ transdifferentiated cells

timal approach is the detection of several mature podocyte markers from different cell compartments (i.e., a protein of apical membrane, foot process/slit diaphragm, cytoskeleton, a secreted protein) in combination with WT-1 as a marker for both immature/mature podocytes.

Conclusions

For research into podocyte function under normal or pathogenic conditions, a growing array of in vitro systems is available nowadays, ranging from classical primary cul-

tures, which now seem to be underestimated by some investigators, through convenient but rather artificial immortalized cell lines to quite unconventional cultivations of podocytes from animal or human urine. Each of these alternatives has its own advantages and limitations (table 2). Certainly, a combination of several different in vitro systems together, with obtainable evidence in vivo, can best assure the relevance of experimental data to the biology of podocytes. This considerable research interest has been driven mainly by the need for better protection and recovery of these cells in renal diseases. Any further research efforts leading us to a better understanding of podocyte biology and pathophysiology are most welcome.

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