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REVIEW

Renal proximal tubular epithelial cells: review of isolation, characterization, and culturing techniques

Matic Mihevc[1] · Tadej Petreski[1,2] · Uroš Maver[2,3] · Sebastjan Bevc[1,3]

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Abstract

The kidney is a complex organ, comprised primarily of glomerular, tubular, mesangial, and endothelial cells, and podocytes. The fact that renal cells are terminally differentiated at 34 weeks of gestation is the main obstacle in regeneration and treatment of acute kidney injury or chronic kidney disease. Furthermore, the number of chronic kidney disease patients is ever increasing and with it the medical community should aim to improve existing and develop new methods of renal replacement therapy. On the other hand, as polypharmacy is on the rise, thought should be given into developing new ways of testing drug safety. A possible way to tackle these issues is with isolation and culture of renal cells. Several protocols are currently described to isolate the desired cells, of which the most isolated are the proximal tubular epithelial cells. They play a major role in water homeostasis, acid–base control, reabsorption of compounds, and secretion of xenobiotics and endogenous metabolites. When exposed to ischemic, toxic, septic, or obstructive conditions their death results in what we clinically perceive as acute kidney injury. Additionally, due to renal cells' limited regenerative potential, the profibrotic environment inevitably leads to chronic kidney disease. In this review we will focus on human proximal tubular epithelial cells. We will cover human kidney culture models, cell sources, isolation, culture, immortalization, and characterization subdivided into morphological, phenotypical, and functional characterization.

Keywords Proximal renal tubule · Acute kidney injury · Chronic kidney disease · Cell morphology · Cell isolation · Cultured cells · Lab-on-a-chip device · Stem cells · Pharmacokinetics

Abbreviations

AAP	Alanine aminopeptidase
ABC	ATP-binding cassette
ACE	Angiotensin-converting enzyme
AEC	Adenylate energy charge
AKI	Acute kidney injury
Albumin-FITC	Albumin-fluorescein isothiocyanate conjugate protein bovine
ALP	Alkaline phosphatase
AQP	Aquaporin
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AVP	Arginine vasopressin
BAK	Bioartificial kidney
BCRP	Breast cancer resistance protein
CD	Cluster of differentiation
ciPTEC	Conditionally immortalized proximal tubular epithelial cells
ciPTEC-T	Conditionally immortalized proximal tubular epithelial cells isolated from tissue

✉ Uroš Maver
uros.maver@um.si

✉ Sebastjan Bevc
sebastjan.bevc@ukc-mb.si; sebastjan.bevc@um.si

Matic Mihevc
matic.mihevc@gmail.com

Tadej Petreski
tadej.petreski@gmail.com

[1] Department of Nephrology, Clinic for Internal Medicine, University Medical Centre Maribor, Ljubljanska ulica 5, 2000 Maribor, Slovenia

[2] Faculty of Medicine, Institute of Biomedical Sciences, University of Maribor, Taborska ulica 8, 2000 Maribor, Slovenia

[3] Department of Pharmacology, Faculty of Medicine, University of Maribor, Taborska ulica 8, 2000 Maribor, Slovenia

ciPTEC-U	Conditionally immortalized proximal tubular epithelial cells isolated from urine
CKD	Chronic kidney disease
CLDN	Claudin
CYP	Cytochrome
DPP	Dipeptidyl peptidase
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBP	Fructose-1,6-bisphosphatase
FMO	Flavin monooxygenase
G6PD	Glucose-6-phosphate dehydrogenase
GGT	γ-Glutamyl transpeptidase
GLUT	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
HD	Hemodialysis
HK-2	Human kidney 2
HKC	Human kidney tubule cell
HPLC	High-performance liquid chromatography
hTERT	Human telomerase reverse transcriptase
LAP	Leucine aminopeptidase
LDH	Lactate dehydrogenase
LSC	Liquid scintillation counting
LC–MS	Liquid chromatography-mass spectrometry
MATE	Multidrug and toxin extrusion
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
NADH	Nicotine adenine dinucleotide
NCAM	Neural cell adhesion molecule
NEP	Neutral endopeptidase
NP	Nephron progenitors
OAT	Organic anion transporter
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
OSR	Odd-skipped related
PBMC	Peripheral blood mononuclear cells
P-gp	P-glycoprotein
PT	Proximal tubule
PTEC	Proximal tubular epithelial cells
PTH	Parathyroid hormone
RNAi	Interference RNA
RRT	Renal replacement therapy
SGLT	Sodium-glucose cotransporter
SLC	Solute carrier
SV40T	SV40 large T antigen
TEER	Transepithelial/transendothelial electrical resistance
UB	Ureteric bud
UGT	UDP-glucuronosyltransferase
ZO	Zonula occludens

Introduction

Kidney diseases can generally be classified as acute and chronic. Chronic kidney diseases (CKDs) are related to a spectrum of different pathophysiological mechanisms that result in worsening kidney function. On the other side, an important part of clinical manifestations of kidney disease are acute kidney injuries (AKIs), which are represented by nephrotoxicity and are most often related to (over)use of medication or exposure to other substances. In recent years both entities have been recognized as interlinked, meaning AKI often leads to progressive CKD, while CKD presents a major risk factor for acute worsening of patient condition (i.e. AKI) [1, 2].

The global prevalence of all-stage CKD in 2017 was 697.5 million or 9.1% [3]. Despite the clearly rising prevalence of CKD in the last century (29.3% increase since 1990), which even established CKD as one of the most common chronic diseases, the choice of renal replacement therapy (RRT) still remains limited [2, 3]. Currently, patients have to decide whether to undergo hemodialysis (HD) (at-home or in-center), peritoneal dialysis (PD), or kidney transplantation (from a living or deceased donor), whilst keeping in mind, that not all options are readily available around the world [4]. In Europe in 2017, 57% of CKD patients were treated with hemodialysis, 5% with peritoneal dialysis, and 37% with kidney transplantation [5]. Researchers are desperately trying to develop novel RRT that would improve the treatment of end-stage kidney disease patients. One goal is the construction of a bioartificial kidney (BAK) that could be implanted into the patient, while another approach is to improve the regular HD process by creating a living membrane device used instead of a dialyzer to filter blood more efficiently [6–8].

Another important segment in current basic and clinical research related to nephrology is the study of nephrotoxicity. Compared to CKD, this research is focusing more on AKI. About 1% of hospitalized adults above 60 years of age develop AKI due to their medication, such as non-steroidal anti-inflammatory drugs or antibiotics [7, 9, 10]. One of the problems in studying drug nephrotoxicity is the lack of appropriate models that could envision the related pathophysiology in high enough complexity to enable improvements in drug choice, especially in patients taking several drugs simultaneously. In the past, most nephrotoxicity studies were based on animal experiments, which are often difficult to translate into human use. At the same time, they are related to considerable costs and pose numerous ethical issues [10–14]. Metanalyses have shown that human

responses to different medications are predicted correctly only in about 10–50% of times [7]. This figure is not only alarming because it is low, but also due to the huge interval, showing that current testing methods are far from reliable. One way to overcome these issues was to implement studies on 2D kidney cell cultures [15–25]. Furthermore, tissue-engineered kidney models were built [6, 26–35]. Their construction is based on appropriate cell lines that can be used in two ways. Individual cell types can be forced into establishing an architecture reliably simulating the functionality of in vivo tissues or parts of organs (i.e. already showing the appropriate structure) can be cut out and used for testing [12].

The following chapters will focus on various aspects of in vitro model formation based on the proximal tubular epithelial cells (PTEC). Systematically we deal with their isolation, characterization, model formation (i.e. culturing conditions), and testing. We briefly also introduce a "specific" part of the model formation, namely cell immortalization with its advantages and drawbacks.

Human kidney cell culture models

Human kidney cell culture models exist in a range of complexities (Fig. 1) [36]. The simplest designs are cultures based on a single cell type. When specific kidney cells are in question, one can isolate glomerular, tubular, and mesangial cells, podocytes, and others [37]. A problem with freshly isolated cells is that they are most useful for studying acute effects of nephrotoxins, whereas for long-term effects primary cell cultures should be established [10, 38].

A simple design can be further upgraded to sealed monolayers on filters with select permeability (for simulating "controlled" membrane transport), which can be exposed to different mediums on both cell sides. A 2D cell culture of human cells is simple, cost-efficient, and compatible with standard laboratory equipment. It can be even considered as potentially high throughput, since it enables testing of a variety of conditions and/or treatments. However, 2D cell cultures lack the structure of in vivo models, fail to interact with surrounding cells similarly to native tissues, and do not express kidney function as observed in vivo. As drug candidates strongly rely on results from early-stage in vitro assays to represent in vivo pharmacodynamics and -kinetics, further work had to be done [36, 39].

This led to novel tissue engineering strategies that employ kidney-on-a-chip technologies or bioprinted 3D structures using a biological cell source that is drafted on a scaffolding material and exposed to specific mediums that regulate growth, differentiation, and function [10–14, 40]. These modern built cell structures offer a much more complex simulation of the in vivo environment, and hence enable the study of physiology and pathophysiology of tissues. As such, these models provide a better framework for drug efficacy and toxicity studies compared to traditional methods [41].

Although many novel sophisticated technological culture systems (e.g. such that copy the cellular environment including native fluid flow, pressure, composition, and 3D extracellular matrices (ECM)) are available these days, the main aim of all models remains to form and maintain living cells that resemble the in vivo phenotype as closely as possible. The nephron, the kidney's main structural and functional unit, consists of different segments, from which each performs a specific physiological function. Ideally, all cell types, as well as the vasculature system, should be incorporated into the design of a kidney-on-a-chip device. Nevertheless, the current state-of-the-art shows that combining two or more kidney cell types in a single model is still rather rare. Most of existing models are based on a single cell type. PTEC, which mimic secretion of the waste products and reabsorption of vital compounds from the glomerular filtrate, are the most common described cell [12, 13, 36, 40, 42].

Human proximal tubular epithelial cells sources

Primary human proximal tubular epithelial cells

Freshly isolated human PTEC from the cortex of the kidney are currently the gold standard for the development of tissue-engineered models [12, 29, 30, 32]. Considering genetic (and phenotypic) changes that can result from the techniques of cell immortalization and long-term cell passaging, these offer various advantages, as well as commonly yield far more representative results [43].

Viable human PTEC can be obtained directly from a kidney from the opposite pole of a cancerous kidney after nephrectomy, or they can be isolated using a kidney biopsy of a non-cancerous patient [12, 13]. Rarely, non-transplantable fresh post-mortem kidneys are available (e.g. due to excessive glomerulosclerosis, arterial plaques or other factors), which present a much more abundant source of fresh cells, although the time until their isolation, presents a significant limiting factor [15].

Basic protocols of human PTEC isolation consist of micro-dissection (mechanical pre-processing) of a tissue sample to obtain ~ 1 mm^3 fragments, enzymatic dissociation with collagenase type I or hyaluronidase, filtration through a sieve (e.g. nylon-based, with mesh diameters in the range from 300 to 45 µm), density-gradient centrifugation, and use of a selective culture media. The culture media are protocol-specific but are usually based on the use of Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F-12), supplemented with fetal bovine

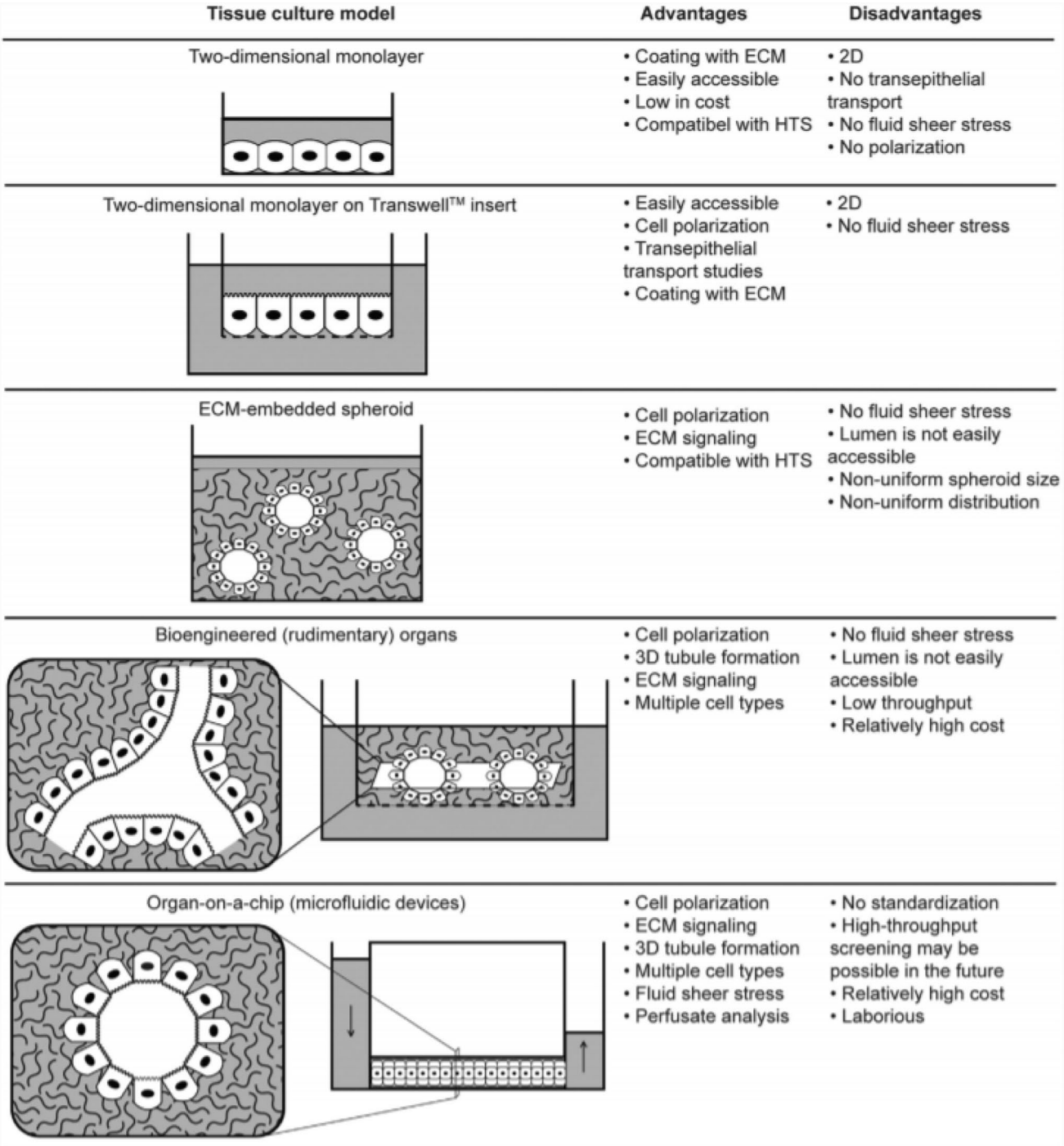

Fig. 1 Evolution of 2D and 3D tissue culture models, including advantages and disadvantages [36]. *ECM* extracellular matrix, *HTS* high throughput screening

serum, penicillin/streptomycin, human transferrin, selenium, hydrocortisone, insulin, and epidermal growth factor. This process results in PTEC that are > 95% of proximal tubular origin [13, 25, 28, 42, 44, 45]. Additional purification of PTEC can be performed with trypsinization and flow-cytometric sorting for the proximal tubular marker leucine aminopeptidase (LAP) [44]. When the isolated cells are plated and cultured as described, they reach confluence approximately 10–13 days after seeding. After that they can be subcultured or frozen. The subcultured cells exhibit doubling times of 24–48 h and reach confluence in 3–5 days [25, 45]. Van der Hauwaert et al. described PTEC isolation by

fluorescence-activated cell sorting (FACS) using antibodies to CD 10 (neutral endopeptidase, NEP) and CD13 (aminopeptidase-N, APN), two proximal tubular epithelial cell markers, which led to establishment of a pure, functional, and stable culture [28].

Purified PTEC can be cultured on semi-permeable membranes where one can access the apical and basal domains used in bi-directional flux assays (to be used for example in renal clearance studies) [29]. When seeded on Transwell™ or metal inserts, PTEC form a monolayer with an electrical resistance similar to the native tissue, expression of metabolic enzymes, formation of transport channels, and functional drug transporters, for up to 5 days [46]. Accordingly, primary PTEC have been mostly used to identify the routes and extent by which specific small molecules enter and leave PTEC. However, their use is limited to a week or two (a maximum of 12 doublings), since cells quickly lose their phenotypic properties during passaging [13, 25, 44]. New isolates can be obtained in case of longer experiments, but at the cost of potential differences in the phenotypic expression and transporter activity of the cells from respective sources. Therefore, primary PTEC are not suitable for long term exposures which are required to study kidney repair [12, 46].

Studies have shown that limited longevity and loss of functional differentiation seen in 2D monolayer cultures might be partly associated with the lack of a fluidic mechanosensory input found in the proximal tubule (PT) in vivo [29, 32]. When compared to static 2D models, PTEC in a microfluidic organ-on-a-chip device exhibited enhanced epithelial cell polarization with twofold longer microvilli, significantly greater functional characteristics (Na^+/K^+ ATPase activity, glucose and urea transport, albumin uptake, ammonia production, vitamin D biotransformation), their viability was extended up to 4 weeks [29–32]. Furthermore, toxicity studies have shown greater cisplatin-induced damage recovery of PTEC and injury-reversibility with organic cation transporter (OCT)-2 inhibitor cimetidine in microfluidic device compared to 2D models [29, 33].

Lastly, it is important to note that besides human, animal kidney cell lines (most commonly Madin-Darby canine kidney cells) are utilized in kidney tissue engineering as well but will not be discussed herein, since this review focuses on human tissue-derived cells [47].

Immortalized Cell Lines And Their Limitations

One way of dealing with the limited "expiration date" of PTEC is to develop immortal cell lines at the cost of losing (some) protein expression and differentiation [48]. First PTEC immortalization was performed with transduction of human papilloma virus 16 E6/E7 genes, which resulted in the so-called human kidney 2 (HK-2) cell line. They maintained proliferation of immortalized and sub-cloned cells for up to 30 passages [15]. HK-2 cells have some renal characteristics such as brush border enzymatic activity (i.e. activity related to alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), LAP and are positive for proximal tubular epithelial marker APN, as well as show some functional characteristics (Na^+ dependent glucose transport, adenylate cyclase responsiveness to parathyroid hormone (PTH)) [15]. However, Tang et al. found that HK-2 cells expressed aquaporin (AQP)-3 which is also expressed in cells isolated from the early distal tubule [16, 49]. Jenkinson et al. compared HK-2 cells to human PTEC and found that both expressed similar ATP-binding cassette (ABC) transporters, however no uptake transporters such as organic anion transporter (OAT)-1, OAT3, OCTs, nor breast cancer resistance protein (BCRP) were found in the HK-2 line [18]. Toxicity studies have shown that HK-2 do not show a dose–response relationship to nephrotoxins (cisplatin, acetaminophen, gentamicin, 5-fluorouracil) that would resemble in vivo. This was concluded based on results, showing that these cells require extremely high doses to achieve a toxic response or even no response could be observed [17].

Another method of cell immortalization was tried using suppression of cell-cycle related genes using antisense oligonucleotides (ASOs) or small interference RNA (siRNA). Sanechika et al. transfected siRNA to a tumor suppressor protein p53 and cyclin-dependent kinase inhibitor protein $p16^{INK4a}$ and extended the viability of PTEC by 33 and 63 doublings, respectively. The lifespan of the transfected cells was also controllable since cell division ceased within 2 weeks after the transfection was ended. PTEC expressed the glucose transporter (GLUT)-1 and GGT equally throughout the doublings. In BAK, reabsorption of water, Na^+, glucose, as well as metabolism of β-2 microglobulin and pentosidine were found. Nevertheless, the authors concluded that more studies will be necessary to fully evaluate the extend of phenotype preservation [27].

Recently, Li et al. reported the development of pseudo-immortalized SA7K cell line via a so-called zinc finger nuclease-mediated knockout of a cell cycle protein. SA7K cells exhibited normal epithelial morphology and phenotype. Compared to HK-2 cell line, SA7K maintained similar expression of uptake and efflux transporters as well as activity of CYP3A4 at early passages. However, no OAT1 and OAT3 mRNA expression was found, whereas uptake of OAT1 substrate p-aminohippuric acid (PAH) was demonstrated. The authors concluded that the reason for this disparity remained to be clarified [50].

Conditionally immortalized cell lines

Transduction of oncogenes into a human-derived cell line poses a risk of oncogene expression and hence potential complications in growth of such cells [48]. One approach

to limit the risk is to transfect cells with an immortalizing construct that activates their replication under "permissive" conditions (low temperature, addition of doxycycline) and terminate it under "nonpermissive" conditions (high temperature) [12, 13, 46]. A successful technique to achieve conditional immortalization is transfection of PTECs with the temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) [19]. When cultured at permissive temperatures of 33 °C, the SV40T conditionally immortalized PTEC (ciPTEC) are maintained up to 50 passages [46]. Racusen et al. first constructed the so-called human proximal kidney tubule cell (HKC) lines immortalized with the SV40T. They described three HKC lines that formed apical microvilli, expressed brush border enzymes, and showed normal transporter activities. However, no well-formed tight junctions were found [19]. Recently, Nugraha et al. used the HKC-8 cell line to form a 3D model of kidney fibrosis. PTEC cell injury was found when triggered by cyclosporin A and aristolochic acid. Injured PTEC even induced differentiation of fibroblasts into myofibroblast [26].

Another method of conditional immortalization is transfection of PTECs with human telomerase reverse transcriptase (hTERT), which limits replicative senescence by maintaining telomere length [23]. Wieser et al. constructed the so-called RPTEC/TERT1 line, which formed tight junctions and densely packed microvilli, and could be stimulated by PTH but not with arginine vasopressin (AVP). The developed cell line reacted also with an enhanced ammonia genesis in lower pH, and showed functional Na^+ dependent phosphate uptake, as well as activity of the megalin/cubilin transporter system [20]. Toxicity studies have shown that the RPTEC/TERT1 cell line responds to sub-cytotoxic doses of several xenobiotics and heavy metals [21, 22]. Long-term repeated dose exposure to non-cytotoxic concentrations of selected nephrotoxic compounds affected cellular regulation, activated specific stress responses (oxidative stress, DNA damage-induced stress, metal-induced stress), and decreased expression of differentiation markers, among them the tight junction protein claudin 2 (CLDN2), the Na^+/K^+ ATPase α1 subunit (ATP1A1), and parathyroid hormone receptor 1 (PTH1R) [22]. Additionally, several potentially clinically useful biomarkers of kidney injury (IL-19, DEFB4, CRYAB, SPINK1, LCN2, PI3, CEACAM6) were identified in toxicity studies on the RPTEC/TERT1 cell line [22].

Furthermore, several studies reported successful double transfection with SV40T and hTERT in PTEC exfoliated in urine [6, 23, 24]. ciPTEC isolated from urine (ciPTEC-U) were well characterized, expressed transporter proteins (multidrug resistance protein 4 (MRP4), P-glycoprotein (P-gp), OCT2, BCRP) and maintained drug transporter functionality (Na^+ dependent phosphate uptake, albumin endocytosis), as well as metabolic activity for more than 45 passages [23, 24]. Theoretically, this should be enough for the development of BAK or a kidney-on-a-chip for most of applications. Jansen et al. compared ciPTEC-U to ciPTEC derived from kidney tissue (ciPTEC-T) and found similar functional as well as epithelial characteristics. However, expression of ECM proteins was higher in ciPTEC-T [24]. Mihajlovic et al. found that both ciPTEC lines showed absence of allostimulatory effects on peripheral blood mononuclear cells (PBMC) proliferation nor were these cell lines able to work as non-professional antigen presenting cells through the expression of human leukocyte antigen (HLA) and co-stimulatory molecules. Accordingly, ciPTEC could represent a "safe" option for BAK engineering application [6].

To conclude, as for all renal cell lines, the major limitation of ciPTEC is a rapid decrease in OATs expression upon culturing [46]. OATs functionality is essential for studying drug induced nephrotoxicity [51]. One approach to solve this issue is transduction of lentiviral particles containing genes encoding human OAT constructs. Nieskens et al. transduced OAT1 and OAT3 proteins in ciPTEC and constructed cell line with stable expression of OAT1 and OAT3 transporters for up to 29 passages [51]. On the contrary, two other studies, in which they transduced the OAT1 protein to ciPTEC, failed to demonstrate extended OAT1 gene expression [34, 35].

Pluripotent stem cell derived kidney cells

Stem cells emerged as an interesting option in the development of in vitro kidney models [52]. The nephron, the main functional unit of the kidney consist of glomeruli and renal tubules. It develops relatively early during fetal development from metanephric mesenchyme, which includes nephron progenitors (NP) along with stromal progenitors, and the ureteric bud (UB) [53–56]. The UB undergoes development and branching to form collecting ducts and signals the NP to begin differentiation into epithelial cells which connect to ductal cells [54]. A small subset of undifferentiated NP promotes UB proliferation, while the stromal progenitors support ureteric branching. Furthermore, endothelial progenitors take care of joining everything with the endothelial network. This complex interaction enables production of nephrons with systemic connection [55].

One of the main causes of the irreversibility of CKD is also the fact that NP in the metanephric mesenchyme cease propagation and are terminally differentiated at 34 weeks of gestation. This results in the inability of the kidney to regenerate itself with forming additional nephrons [53, 56]. With the rise in numbers of kidney patients and shortage of transplantable organs, researchers have tried culturing NP with the idea of being able to grow glomeruli and tubules on a differentiation medium, which could pose as a potential therapeutic application and represent a step toward kidney

regenerative medicine and kidney organogenesis [57]. Additionally, it would also aid in drug and toxicity testing, as well as disease modelling, and could pose an innovative step towards renal replacement therapy [57–59]. Some results already show that adult kidney progenitor cells enhance the process of regeneration after acute kidney injury in rats [52].

Currently, NP cells can be generated from embryonic stem cells or induced pluripotent stem cells using directed differentiation. Researchers identified during embryonic stem cell research that the UB precursors differentiate from the T^+ immature mesoderm, which becomes the anterior intermediate mesoderm, and migrate caudally to form the Wolffian duct. On the other hand, the NP remain in the T^+ immature mesoderm longer and differentiate into the posterior intermediate mesoderm (Fig. 2). As embryonic cells are difficult to attain, a lot of effort has been put into creating pluripotent stem cells from adult renal cells. Researchers have developed protocols for reprogramming primary normal human mesangial cells using retroviral delivery to develop induced pluripotent stem cell colonies capable of differentiation into renal progenitor cells. Additionally, they attempted generation of induced pluripotent stem cells from urine, as renal tubular cells are shed into urine each day. The idea is noteworthy as it does not require invasive procedures, but the reprogramming efficiency is currently quite low, up to 4%. Furthermore, several protocols are also described to obtain pluripotent stem cells from other tissues such the foreskin or cells such as keratinocytes and dermal fibroblast cells [55, 58].

Several surface markers have been identified for NP cells, namely Neural Cell Adhesion Molecule 1 (NCAM1) and frizzled 7 protein (FZD7). NCAM1 is a glycoprotein that is expressed in condensed mesenchyme, nephrogenic zone, and Wilms' tumor progenitor blastema and is not expressed in mature kidney epithelia. Additionally, NP cells can be identified by the expression of several transcriptional factors like SIX2 and odd-skipped related 1 (OSR1). These NP cells are multipotent and can differentiate into multi-segment nephron structures with characteristics of podocytes, proximal tubules, loops of Henle, and distal tubules [52, 55, 58].

Recently, researchers have used NP cells derived from urine to construct a 3D model, which could be used for nephrotoxicity tests, pharmacokinetics, nephrogenesis and kidney disease studies [60]. Additionally, a protocol has been described for Glomerulus Chip formation using directed differentiation of human induced pluripotent stem cells glomerular podocytes [61]. Another possible use of renal progenitor cells is repopulation of decellularized kidney scaffolds, which showed to be functional using a simulated bio-reactor set up [62]. Hiratsuka et al. have further reported a protocol for kidney tissue generation from human pluripotent stem cells (hPSC) using synthetic messenger ribonucleic

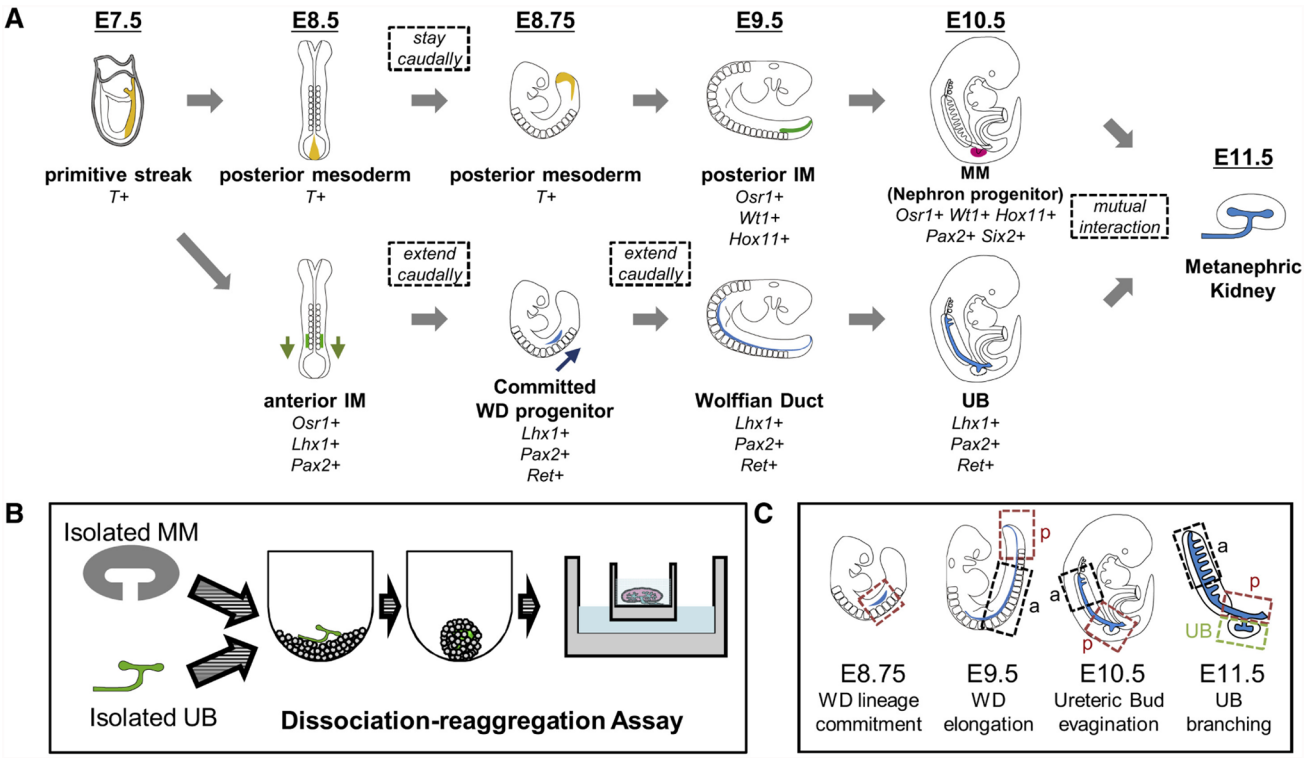

Fig. 2 Proposed model for kidney lineage specification (**a**), schematic of the dissociation/reaggregation assay (**b**), and schematic of the WD developmental process [55]

acid (mRNA) encoding transcription factors. The first set (FIGLA, PITX2, ASCL1, and TFAP2C) induced NP cells from hPSC, and the second set (HNF1A, GATA3, GATA1, and EMX2) induced nephron epithelial cells [63].

Proximal tubular epithelial cells (PTEC) and their characterization

The PT is a segment of the nephron that lies between the Bowman's capsule and the loop of Henley. PTEC are the most abundant cell type in the PT and play a major role in water homeostasis, acid–base control, reabsorption of compounds, as well as in secretion of xenobiotics and endogenous metabolites [64, 65].

Ideally, three segments of PTEC characteristics are evaluated when performing validation of any isolated PTEC. Phenotypical and morphological characterization are essential and independent from study design, whereas functional characterization depends on the study's focus, which can be (various) transport mechanisms, intracellular metabolism, drug nephrotoxicity, hormone responses, etc.

A review of the latest most common techniques in each of the mentioned three characterization segments is described below.

Morphological characterization

PTEC are tall cuboidal epithelial cells with a polarized structure. Their apical and basolateral membrane domains are separated by a junctional complex (Fig. 3). The latter works like a seal and enables that molecules from the tubular lumen to travel transcellularly instead of paracellularly [66]. Any alterations in this tissue organization, as found for example in polycystic kidney disease or AKI, result in kidney dysfunction [28, 66].

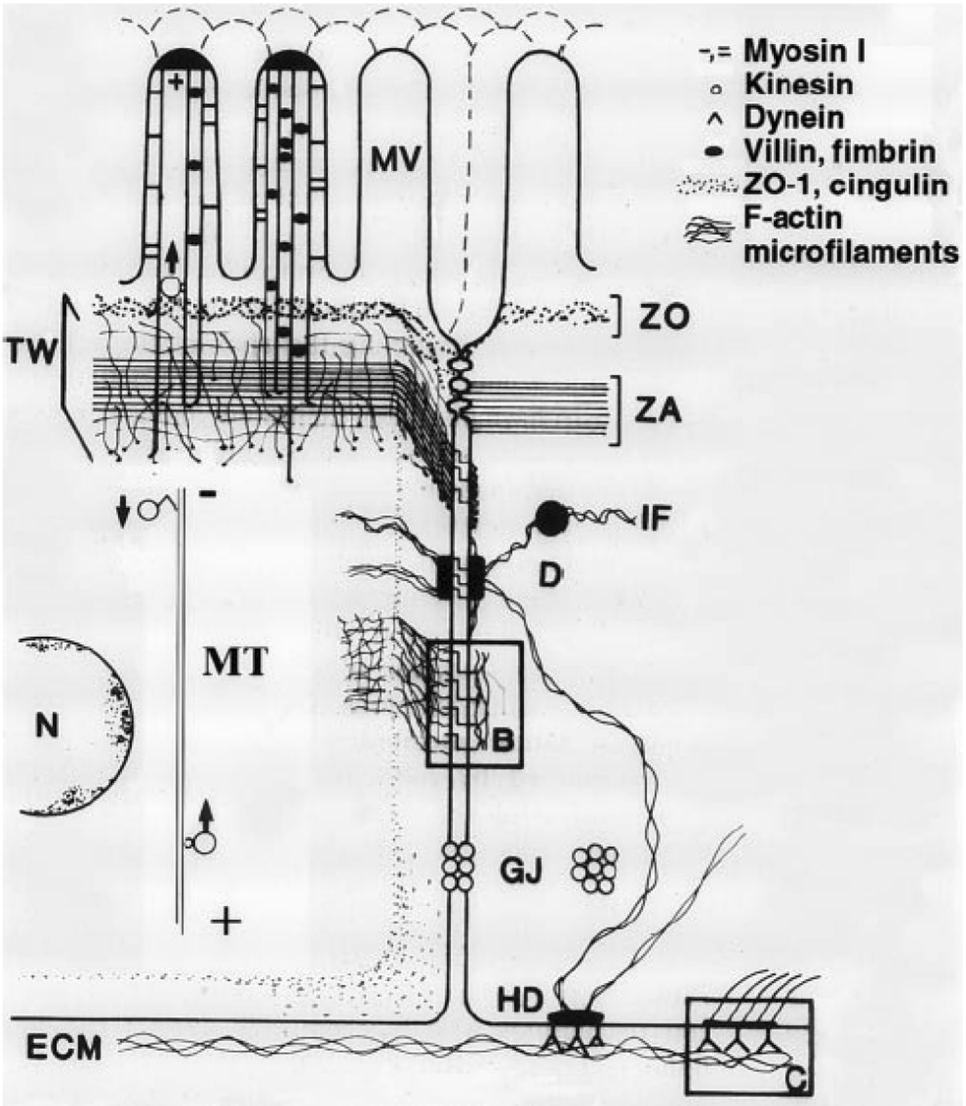

Fig. 3 Polarized structure of the proximal tubular epithelial cell [66]. The apical membrane domain has a long brushborder with microvilli (MV) and a terminal web (TW), which make connections with the lateral junctions. The lateral junctional complex separates the apical and basal domains. It consists of four types of junctions: tight junctions (zonula occludens (ZO)), anchoring junctions (zonula adherens (ZA) and desmosomes (D)), and lastly communicating (gap) junctions (GJ). The basal membrane domain is attached to the extracellular matrix (ECM) via hemidesmosomes (HD)

The apical (luminal, urine) membrane domain has a long brush border with microvilli, that increase the luminal surface, and a terminal web, which makes connections with the lateral junctions. Additionally, the apical membrane contains numerous endocytic vesicles and a prominent lysosomal compartment [25, 28, 66–68].

The basolateral (blood) membrane has deep infoldings, which enable extensive lateral cell processes to interdigitate with neighboring cells. Extensive interdigitations are also the site of numerous mitochondria, which provide energy for active transport. More detailed, the basolateral membrane is divided into a lateral and basal region. The lateral region is separated from the apical by the junctional complex. Four types of junctions are known (zonula occludens (ZO), zonula adherens, desmosomes, gap junctions), where especially expression of ZO-1 protein is among techniques for a quick scan of epithelial character [28, 66, 69]. The basal region is attached to the ECM via hemidesmosomes mediated by integrins [66, 68].

Morphological characterization is usually performed using transmission electron microscopy, which enables a "clear" observation of the above-mentioned characteristics (Fig. 4). When using light microscopy, epithelial cobblestone appearance and formation of hemicysts or "domes" (subepithelial collections of fluid with upward displacement of portions of the monolayer) are indicative of the proximal tubule character [19, 20, 24, 28–33, 42, 44, 64].

Polarization and membrane integrity determination

Polarization of PTEC is quickly determined using a combination of electron microscopy (brush border, cobblestone morphology, tight junctions) (Fig. 4), immunocytochemistry (ZO-1 protein expression), and western blotting/immunofluorescence (apical location of brush border enzymes APN and GGT, basolateral location of the Na^+/K^+ ATPase) [42, 46].

Transepithelial/transendothelial electrical resistance (TEER) is a widely used method to measure the integrity of tight junction dynamics in PTEC monolayers as well as their polarity. TEER values are strong indicators of the integrity of cellular barriers before they are evaluated for transport of drugs and chemicals [28, 70]. Higher TEER values represent an increased tightening of the cell–cell

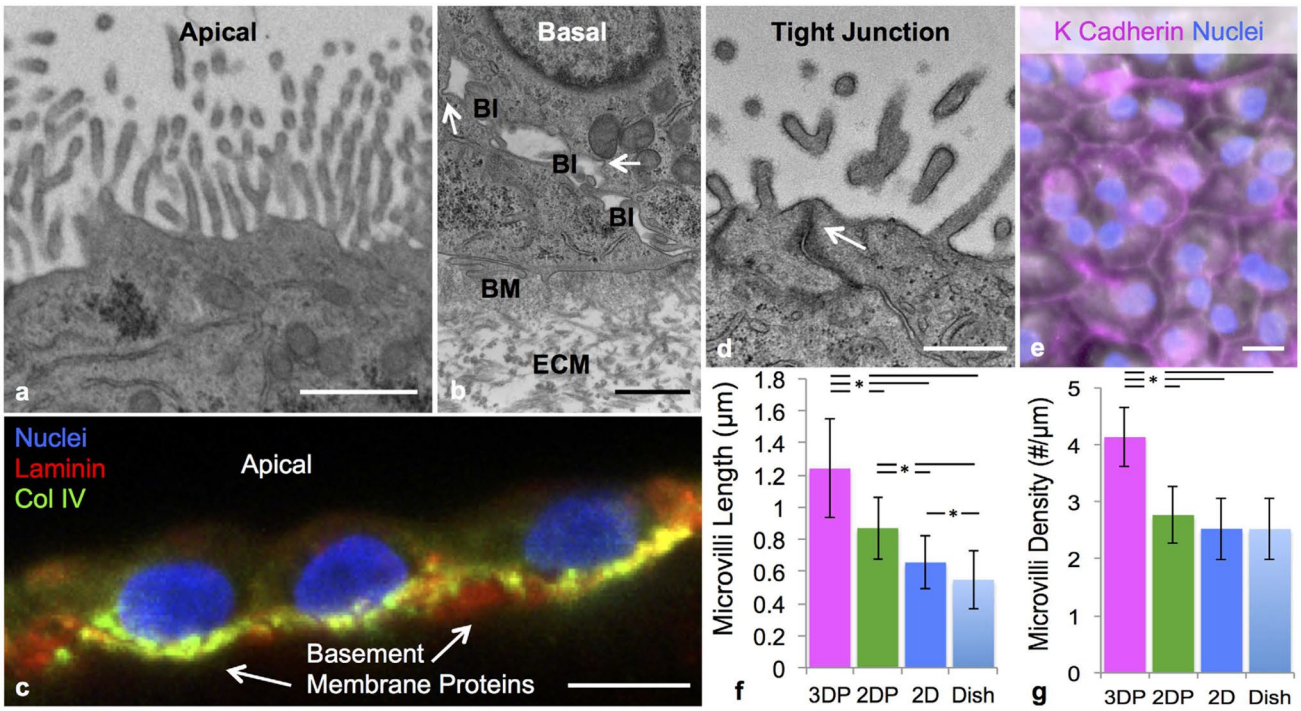

Fig. 4 Transmission electron microscopy of the proximal tubular epithelial cells at 6 weeks of growth in 3D bioprinted tubule [31]. **a** The apical membrane with long microvilli, indicative of brush border, scale bar = 1 µm, **b** the basolateral membrane with basolateral interdigitations (BI), circular interdigitations marked with white arrows, basement membrane (BM) proteins secreted by cells, and extracellular matrix (ECM) underneath, scale bar = 1 µm, **c** basement membrane proteins secreted by cells, laminin in red, collagen IV in green, scale bar = 10 µm, **d** tight junction between two proximal tubular epithelial cells (white arrow), scale bar = 500 nm, **e** stained cell junction protein K Cadherin, scale bar = 10 µm, **f** a comparison of microvilli length and **g** density in PTEC growth in 3D bioprinted proximal tubule (3DP) compared to PTEC growth on ECM in 2D model with perfusion (2DP), PTEC growth in 2D model without perfusion (2D), and PTEC growth on bare tissue culture dish without perfusion (Dish). (Color figure online)

contacts and thus reduced leakiness of the monolayer, while decreased TEER values suggest increased leakiness and therefore increased weakening of the tight junctions or even cell death [71].

Phenotypical characterization

To confirm the epithelial origin of PTEC, several epithelial markers of the PT have been described (AQP1, N-cadherin, neutral endopeptidase (NEP), APN, lotus lectin, beta catenin, pan-cytokeratin). Furthermore, markers of distal tubule and collecting duct (AQP 2/3, prominin 2, uromodulin, mucin 1, E-cadherin), mesenchymal (vimentin), and myofibroblast markers can be used as a control to ensure that the correct phenotype has been obtained (Fig. 5) [20, 24, 28–33, 42, 44–46, 64, 72, 73].

Functional characterization

PTEC have a variety of functional characteristics, which can be sorted into four groups: brush border enzyme expression and activity, drug transporter expression and activity, drug metabolism enzyme expression and activity, and cellular energetics with redox status.

Brush border enzyme expression and activity

The PT has a dominant role in the excretion of different enzymes in the urine. As enzymes of the brush border are location specific, they can be used as markers of differentiated PTEC function (Table 1) [29, 74]. The physiological role of many of these enzymes is not well understood. GGT, alanine aminopeptidase (AAP), and lactate dehydrogenase (LDH) are normally present in urine because of tubular

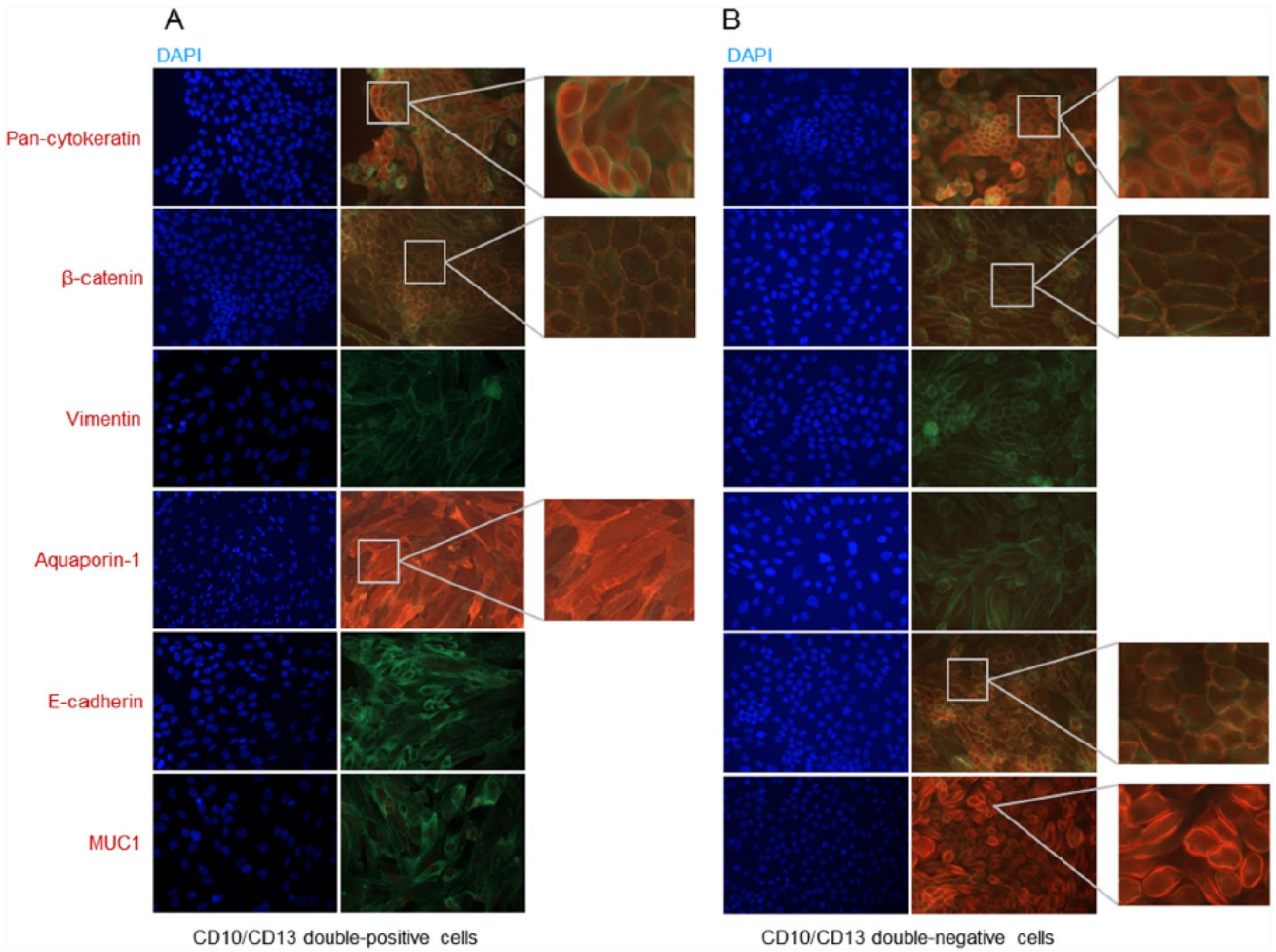

Fig. 5 Immunofluorescence labelling for selected epithelial markers in **a** CD10/CD13 double-positive cells and **b** CD10/CD13 double-negative cells [28]. CD10/CD13 double-positive cells were positive for pan-cytokeratin and β-catenin (two epithelial markers), aquaporin-1 (proximal tubular marker), and were negative for vimentin (mesenchymal marker) as well as MUC1 and E-cadherin (distal tubular markers)

Table 1 Summary of brush border enzymes characterization techniques

Target	Method	Description	References
Brush-border enzymes characterization			
GGT	Selective immunohistochemistry	Apical location	[19, 42, 72]
APN (CD13)	Western blotting/immunofluorescence	Apical location	[23, 28, 67, 72]
NEP (CD10)	Western blotting/immunofluorescence	Apical location	[28]
LAP	Western blotting/immunofluorescence	Apical location	[42, 44]
DPPIV	Western blotting/immunofluorescence	Apical location	[72]
Brush-border enzymatic activity assays			
GGT	LC–MS	Measurement of the efflux of GSSG in the presence of GGT inhibitor acivin	[32]
GGT	Spectrophotometry	Measurement of the pNA release after the GPNA enzymatic cleavage	[20, 33, 50, 72]
ALP	Spectrophotometry	Measurement of the pNP release after the pNPP enzymatic cleavage	[28, 29, 78]
DPPIV	Spectrophotometry	Measurement of the p-nitroaniline release after the GGpN enzymatic cleavage	[72]
ACE	ELISA	Measurement of the angiotensin II release after the angiotensin I enzymatic cleavage	[33]

GGT γ-glutamyl transpeptidase, *APN* aminopeptidase N, *NEP* neutral endopeptidase, *LAP* leucine aminopeptidase, *DPPIV* dipeptidyl peptidase IV, *CD* cluster of differentiation, *LC–MS* liquid chromatography–mass spectrometry, *GSSG* oxidized form of glutathione, *ALP* alkaline phosphatase, *pNA* para-nitroanilide, *pNP* p-nitrophenol, *pNPP* p-nitrophenyl phosphate, *GGpN* γ-glutamyl-p-nitroanilide, *ACE* angiotensin-converting enzyme, *ELISA* enzyme-linked immunosorbent assay

cell shedding. Their activity is increased in the setting of AKI, which causes up-regulation of genes encoding urinary enzymes or increased leakage of enzymes due to membrane disruption [74]. Decreased activity of ALP is often indicative of PTEC dedifferentiation [29]. GGT plays an important role in the γ-glutamyl cycle which is a glutathione synthesis and degradation pathway [32]. APN is important in salt handling as it reduces basolateral Na^+/K^+ ATPase activity [75]. Dipeptidyl peptidase IV (DPPIV) is a marker of cellular polarization and brush border formation as it is associated with the membrane of the microvilli and apical invaginations [76]. NEP has a major role in the degradation of vasoactive peptides including angiotensin II and bradykinin, as well as generation of angiotensin-(1–7) [77].

Drug transporter expression and activity

Intact drug transporter expression and activity is very important when evaluating cell culture kidney models. PTEC are involved in the reabsorption of vital compounds from the glomerular filtrate and secretion of waste products and xenobiotics [36].

Reabsorption of vital compounds is taking place at the apical membrane. Freely filtered low molecular weight proteins are reabsorbed via megalin-cubilin receptor-mediated endocytosis. Next to transporter-mediated endocytosis, transporters of the solute carrier family (SLC) are responsible for the transporter-mediated reabsorption of phosphate (sodium-phosphate cotransporters NaPi-IIa and NaPi-IIc), amino acids (SLC1, SLC7, SLC36, SLC38, SLC43), and glucose (GLUT1, GLUT2, sodium-glucose cotransporter (SGLT)-1, SGLT2) [36, 51, 64, 65].

Many small molecules of clinical interests (uremic toxins, xenobiotics) are albumin-bound or are too large to be freely filtered by the glomerulus. Their secretion instead depends on tubular secretion which is a two-step process [51, 65]. The first step is their uptake from blood followed by their excretion into tubular lumen across the apical membrane [28, 44, 65].

Active uptake from blood is facilitated by uptake transporters at the basolateral membrane, which are members of the SLC family. SLC transporters transport substances down or against their concentration gradient, which is maintained by the basolateral Na^+/K^+ ATPase. Based on their substrate, they can be divided into two groups: organic cation transporters (major OCT isoform expressed in the PT is OCT2) and organic anion transporters (OAT 1, OAT3, anion transporter polypeptide 4C1 (OATP4C1)) (Fig. 6) [46, 65].

Furthermore, intracellular accumulation of substrates is prevented by active efflux at the apical membrane. Efflux is mediated by SLC family transporters or ABC transporters. Compared to SLC transporters, ABC transporters use energy generated by hydrolyzation of adenosine triphosphate (ATP) to transport molecules across the cell membrane. The SLC transporters group includes the organic cation/carnitine transporters 1 and 2 (OCTN1/OCTN2), OAT4, urate transporter 1 (URAT1), and multidrug and toxin extrusion 1 (MATE1). The most studied ABC transporter is P-gp also known as multidrug resistance protein 1

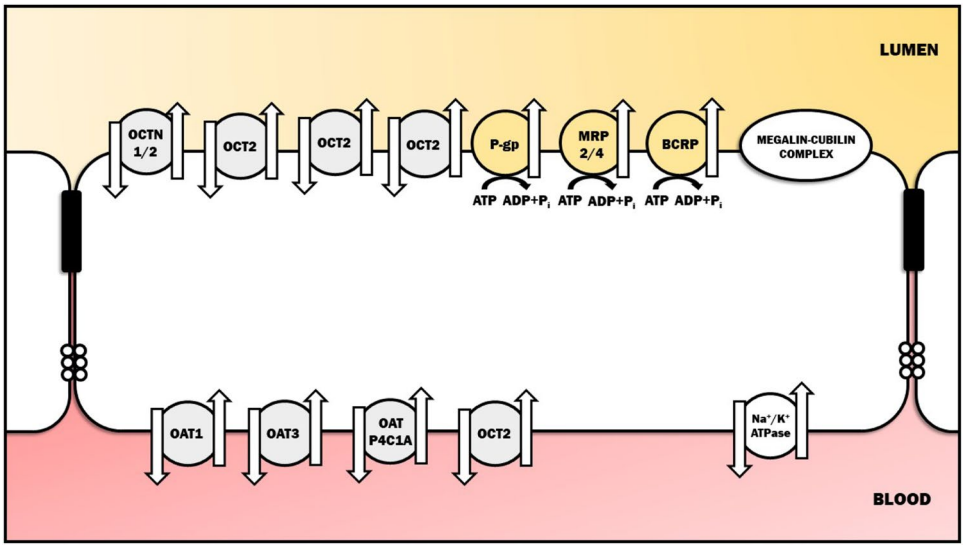

Fig. 6 Transport system in the proximal tubular epithelial cell. *OAT* organic anion transporter, *OCT* organic cation transporter, *OCTN* organic cation/carnitine transporter, *URAT* urate transporter, *MATE* multidrug and toxin extrusion, *P-gp* P-glycoprotein, *MRP* multidrug resistance-associated protein, *BCRP* breast cancer resistance protein

(MDR1). Additionally, multidrug resistance-associated protein (MRP)-2/4 and BCRP are commonly studied (Fig. 6) [36, 46, 65].

When evaluating drug transport activity, PTEC need to be grown on a matrix-like Transwell™ filter plates. The matrix serves as a cell growth substrate and permits compounds to diffuse through the filter pores. Importantly, the polarized structure of cells is preserved. The apical membrane facing upwards mimics the tubular lumen, whereas the basolateral membrane facing downwards mimics renal interstitial space [79, 80].

Generally, drug transport activity assays are based on the principle of drug transport inhibition (Table 2). Inhibition of basolateral uptake (e.g. OATs, OCTs) reduces the intracellular accumulation of drugs and has a nephron protective effect. Contrary, inhibition of apical efflux transporters (e.g. P-gp, MRP2, BCRP, OCTN) reduces drug exit from PTEC, which can lead to intracellular drug accumulation and nephrotoxicity [81]. Special care should be given to basolateral transporters as they might be internalized into vesicles and become dysfunctional. Therefore, it is important to demonstrate both, expression as well as functionality of basolateral transporters. Similarly, functionality of apical membrane transporters needs to be demonstrated so that complete drug handling is intact [80].

Drug metabolism enzyme expression and activity

The general belief in terms of kidney's drugs metabolizing capacity has been that the metabolic clearance of drugs is determined exclusively by the liver while the kidneys play a minor role. However, multiple in vitro and in vivo studies demonstrated that kidneys have also a significant drug-metabolizing capacity [87].

Currently, immortalized or ciPTEC are used to understand the pathophysiology of kidney diseases, therapeutic efficacy of drugs, nephrotoxicity of compounds, and for studying therapeutic interventions for kidney repair. Understanding the metabolic enzymes present in PTEC cell lines is important for prediction of probable in vitro behavior of drugs being tested. Accordingly, determination of expression (both mRNA and protein), as well as activity of relevant enzymes, is essential [46, 80, 88].

Enzymes involved in drug metabolism are divided into two classes. Phase I metabolizing enzymes are members of the cytochrome (CYP) P450 system or they belong to the group of flavin-containing monooxygenases (FMO). They are responsible for oxidation, reduction, and hydrolyzation of xenobiotics. Compared to animal PTEC, only few CYP enzymes (CYP3A4/5, CYP4A11, CYP1B1, CYP2B6, CYP2D6, CYP4F2) were found to be constantly expressed in human PTEC. Among them, especially CYP3A4/5 and CYP4A11 are expressed at relatively high levels in the PT [46, 87, 89]. Summary of CYP enzymes activity assays is presented in Table 3.

Phase II metabolizing enzymes are members of glutathione S-transferases (GSTA1/2/4/5, GSTM1/2/3/4/5, GSTP1, GSTT1/2, FSTZ), UDP-glucuronosyltransferases (UGT1A1/6/9, UGT2B7, UGT8), and sulfontransferases (SULT1A1/3, SULT1B1/2). They are responsible for glucuronidation, sulfation, methylation, acetylation, and glutathione or amino acids conjugation. Conjugated products are more hydrophilic and therefore water-soluble, which makes them easily excretable and reduces their toxicity potential [46, 87, 88].

Table 2 Summary of drug transporters activity assays at the apical and basolateral membrane

Target	Method	Description	References
Apical membrane transporters			
SGLT2	Immunofluorescence, spectrophotometry	Measurement of the glucose analogue 2-NBDG uptake in the presence of SGLT2 inhibitors dapagliflozin, apigenin, and canagliflozin	[32, 33, 82]
MRP 2/4	LSC	Measurement of the ^{14}C-PAH efflux in the presence of MRP2/4 inhibitor probenecid	[44]
MRP2	Fluorimetry	Measurement of the calcein efflux in the presence of MRP2 inhibitors cyclosporin A or indomethacin	[44, 83, 84]
BCRP/MDR1	Fluorimetry	Measurement of the Hoechst 33,342 dye efflux in the presence of MDR1 modulators cyclosporin A, verapamil and nelfinavir or BCRP inhibitor fumetrimorgin C	[18, 44, 83]
BCRP/MRP4	LSC	Measurement of the kynurenic acid uptake in the presence of KO143 (BCRP inhibitor) and MK571 (MRP inhibitor)	[24, 35, 50, 83]
P-gp	Fluorimetry	Measurement of the calcein uptake in the presence of P-gp inhibitors PSC-833 or verapamil	[23, 29, 34, 35, 50, 72, 83]
Megalin-cubilin complex	FACS	Measurement of the albumin-FITC uptake at 4 °C to inhibit endocytosis	[23, 24, 29, 31, 50, 67, 85]
Na/P_i II	LSC	Measurement of the $^{32}PO_4$ uptake in the absence of sodium by replacing NaCl with N-methyl-D-glucamine	[20, 23, 24]
Basolateral membrane transporters			
OAT 1/3	LSC (^{14}C-PAH), LC–MS (indoxyl sulphate)	Measurement of the ^{14}C-PAH or uremic toxin indoxyl sulphate efflux in the presence of OAT 1/3 inhibitor probenecid	[32, 50, 72]
OAT 1/3	FACS	Measurement of the fluorescein uptake in the presence of OAT 1/3 inhibitors PAH, estrone sulphate, probenecid, furosemide, and cimetidine	[34, 51, 86]
OCT2	LSC	Measurement of the radiolabelled creatinine efflux in the presence OCT2 inhibitor Decynium-22	[44]
OCT2	Fluorimetry	Measurement of the fluorescent cationic OCT2 substrate uptake in the presence of OCT2 inhibitor cimetidine	[86]

SGLT2 sodium glucose cotransporter 2, *2-NBDG* fluorescent glucose analogue, *LSC* liquid scintillation counting *LC–MS* liquid chromatography-mass spectrometry, *FACS*, fluorescence-activated cell sorting, *OAT* organic anion transporter, *PAH* para-aminohippuric acid, *^{14}C-PAH* radiolabelled PAH, *MRP* multidrug resistance-associated protein, *BCRP* breast cancer resistance protein, *MDR* multidrug resistance, *OCT* organic cation transporter, *P-gp* P-glycoprotein, *PSC-833* valspodar, *albumin-FITC* albumin-fluorescein isothiocyanate conjugate protein bovine, *Na/P_i II* Na/P_i cotransporter type II

Table 3 Summary of CYP enzymes activity assays

Target	Method	Description	References
CYP1 (A/B)	Fluorimetry	Measurement of the resorufin release after EROD enzymatic cleavage	[21, 89]
CYP1A2	LC–MS	Measurement of the 4-acetamidophenol and D_4-paracetamol release after phenacetin hydroxylation	[89]
CYP2B6	Fluorimetry	Measurement of the resorufin release after pentoxyresorufin depentylation	[89]
CYP2C9	LC–MS	Measurement of the 4′-hydroxydiclofenac and $^{13}C_6$-4′-hydroxydiclofenac release after diclofenac hydroxylation	[89]
CYP2C19	LC–MS	Measurement of the 4′-hydroxymephenytoin and 3-acetamidophenol release after mephenytoin hydroxylation	[89]
CYP2D6	Fluorimetry	Measurement of the fluorescent product release after AMMC hydroxylation	[89]
CYP27B1	LC–MS	Measurement of the calcitriol release after calcidiol enzymatic cleavage	[32, 90]
CYP3A4	LC–MS	Measurement of the dehydrofelodepine and D_3-dehydrofelodepine release after felodipine hydroxylation	[89]
CYP3A4	Fluorimetry	Measurement of the fluorescent product release after BOMC hydroxylation	[89]

CYP cytochrome, *EROD* ethoxyresorufin-O-deethylase, *LC–MS* liquid chromatography-mass spectrometry, *AMMC* 3-(2-N,N-diethyl-N-methylamino)ethyl-7-methoxy-4methylcoumarin, *BOMC* butyloxymethylcoumarin

Cellular energetics and redox status

PTEC cellular energetics validation consists of mitochondrial function, glycolysis rate, gluconeogenesis rate, and adenine nucleotide charge evaluation. Redox status is evaluated with the glutathione (GSH)/glutathione disulphide (GSSG) ratio and rate of lipid peroxidation (Table 4) [80].

Mitochondria produce energy for the Na^+/K^+ ATPase to drive ion transport across the cellular membrane. Furthermore, they supply energy for cellular repair and regeneration [91]. Their function is evaluated with mitochondrial oxygen consumption and intracellular ATP concentration both indicating intact aerobic respiration [92]. Another technique is measurement of the mitochondrial membrane potential ($\Delta\Psi_M$), which plays a central role in processes such as ATP production, reactive oxygen species generation, and Ca^{2+} uptake. Decreased values of $\Delta\Psi_M$ are associated with the opening of mitochondrial permeability pores and loss of electrochemical gradient indicating PTEC apoptosis [93, 94].

The glycolysis rate is evaluated with pyruvate and lactate production as well as LDH and hexokinase activity. Increased pyruvate and lactate concentrations or increased hexokinase and lactate dehydrogenase activity are indicators of cells hypoxia and their dysfunction [95].

Gluconeogenesis rate is evaluated with fructose-1,6-bisphosphatase activity and detection of net production of glucose from precursors lactate and glutamine [96, 97].

The adenylate energy charge (AEC) is a simple index which measures the energy status of the cell. AEC is defined as the ratio between ([ATP] + 0.5 [ADP]) and ([ATP] + [ADP] + [AMP]). When all energy poll is in the form of AMP, the energy charge is zero. Contrary, when

Table 4 Summary of cellular metabolism and redox status assays

Target	Method	Description	References
Mitochondrial function			
Mitochondrial oxygen consumption	Polarimetry	Measurement of the succinate-stimulated oxygen consumption after incubation with DCVCS	[92, 94]
Mitochondrial membrane potential	Confocal microscopy	Detection of mitochondrial fluorescent signals after incubation with the JC-1 fluorescent agent	[94]
Intracellular ATP	Luminescence detection	Detection of the luminescent signal produced in the reaction of beetle luciferin with luciferase	[94, 102]
Glycolysis rate			
Lactate production	Fluorimetry	Detection of the oxidized form of lactate produced in the lactate dehydrogenase reaction	[17, 33, 71, 95, 102]
LDH activity	Fluorimetry	Measurement of the change in NADH absorption	[71, 102]
Pyruvate production	Fluorimetry	Detection of the oxidized form of pyruvate produced in the pyruvate oxidase reaction	[95]
Hexokinase activity	Colorimetry	Detection of the colored product produced in the G6PD reaction	[95]
Gluconeogenesis rate			
Glucose production	Colorimetry	Detection of the net synthesis of glucose from precursors lactate and glutamine	[96]
Fructose-1,6-bisphosphatase activity	Colorimetry	Detection of the colored product produced in the FBP reaction	[97]
Adenylate energy charge			
Adenine nucleotides	HPLC	Measurement of the intracellular contents of adenine nucleotides after separation of ATP, ADP, and AMP by reversed phase HPLC	[103, 104]
Redox status			
GSH/GSSG status	Spectrophotometry/HPLC	Determination of the GSH/GSSG ratio and quantification of the GSSG in the reaction of GSH with Ellman's reagent	[105]
Lipid peroxidation	Colorimetry	Detection of the 4-HNE by-product of lipid peroxidation	[106]

DCVCS S-(1,2-dichlorovinyl)-L-cysteine sulfoxide, *JC-1* 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzoimidazolylcarbocyanine iodide, *ATP* adenosine triphosphate, *LDH* lactate dehydrogenase, *NADH* nicotine adenine dinucleotide, *G6PD* glucose-6-phosphate dehydrogenase, *FBP* fructose-1,6-bisphosphatase, *ADP* adenine diphosphate, *AMP* adenosine monophosphate, *HPLC* high-performance liquid chromatography, *GSH* glutathione, *GSSG* oxidized glutathione, *4-HNE* 4-hydroxynonenal

all energy pool is in the form of ATP, the energy charge is one. Optimal AEC ranges between 0.7 and 0.95 [98].

GSH/GSSG ratio is a useful indicator of the oxidative stress. Reduced glutathione (GSH) is an important redox buffer and main antioxidant, which prevents damage caused by free radicals and peroxides. Healthy cells store more than 90% of total glutathione in the reduced form. When cells are exposed to oxidative stress, the glutathione reductase catalyzes the reaction where oxidized glutathione (GSSG) is formed. Accumulation of GSSG decreases GSH/GSSG ratio and switches the redox potential towards a more oxidized state [99, 100].

Lipid peroxidation status is another indicator of oxidative stress. Among many secondary products formed during lipid peroxidation, 4-hydroxynonenal (4-HNE) is the most toxic and therefore a major bioactive marker of lipid peroxidation [101].

Conclusions

The field of renal cell studies is ever evolving. As more and more patients succumb to CKD and are in need for RRT, innovative approaches must be invented to tackle the increasing incidence, either in the form of new drug (therapy) development, as well as on the level of better understanding of the related disease pathophysiology, again leading to novel potential treatment strategies. Additionally, polypharmacy is on the rise and as the population grows older, potential toxic drug–drug interactions and changes in drug efficiency in patients with renal diseases must be studied even more vigorously. Some of the future goals that are already on the horizon are development of advanced in vitro kidney models, 3D bioprinting methods to generate bioengineered kidneys, using decellularized kidneys and later repopulating them with human renal cells, and using microfluidic organ-on-a-chip devices to study renal disease and drug toxicity in vitro. As we are more and more successful in isolating, growing, and differentiating renal cells individually, slowly we will begin to develop increasingly complex 3D models using several cells in an in vivo like structure. Until then, we must rely on older drug assays and observational studies, and existing RRT.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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